Isolation and Identification of Two L-Azetidine-2-carboxylic Acid-Degrading Soil Microorganisms, *Enterobacter agglomerans* and *Enterobacter amnigenus*

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Soil samples collected at several times during the growing season and at different locations within *Convallaria majalis* beds in Ann Arbor, MI, were screened for their ability to grow with the cyclic amino acid, L-azetidine-2-carboxylic acid (L-A-2-C), as their sole nitrogen source (i.e., metabolize L-A-2-C). Two different soil microorganisms were isolated, characterized, and identified using fundamental selection methods, the standard battery of biochemical characterization tests, and scanning electron microscopy. The assignment of the identity of these organisms as *Enterobacter agglomerans* and *Enterobacter amnigenus* was further verified by comparison with authentic microbial samples obtained from ATCC that were able to utilize L-A-2-C as their sole nitrogen source.

L-Azetidine-2-carboxylic acid (**1**) (L-A-2-C)[1] is a four-member cyclic amino acid discovered by Fowden[2] and Virtanen and Linko[3] in *Convallaria majalis* and *Polygonatum officinale*, two members of the Liliaceae family. L-A-2-C occurs at high concentrations in a variety of other species.[4−7] L-A-2-C exhibits growth-inhibitory effects in many biological systems. It is speculated that the presence of L-A-2-C protects the producing species from vertebrate and/or animal predation because L-A-2-C is the four-membered ring analogue of the naturally occurring proteinaceous amino-acid analogue, L-proline. The many observed toxic effects of L-A-2-C may be due to its structural similarity to L-proline. L-A-2-C is incorporated into proteins by competing with L-proline in the formation of L-Pro-tRNA (L-A-2-C-tRNA). After incorporation into proteins, L-A-2-C causes the polypeptide chain to turn through an angle 15° smaller than would L-proline, leading to nonfunctional proteins and deleterious effects. Species that produce L-A-2-C, however, have L-proline-activating enzymes that can differentiate L-proline and L-A-2-C, so that only L-Pro-tRNA is formed. As a consequence, only L-proline is incorporated into proteins. Many species that do not produce L-A-2-C have also developed mechanisms that can resist the toxic effects of L-A-2-C. Most mechanisms of resistance involve alterations in L-proline metabolism leading to the overproduction of the amino acid. Accumulation of an excess amount of L-proline thus prevents the incorporation of L-A-2-C into proteins. A soil microorganism, however, has been reported that is not only resistant to the toxicity of L-A-2-C, but is apparently able to metabolize the unusual amino acid and utilize it as a sole nitrogen source. Although the biosynthesis of L-A-2-C has been well-documented in a variety of species,[8] little information is available concerning the mechanism(s) of its degradation. It is not known whether such L-A-2-C degrading ability is widespread, or whether it exists only in a limited number of organisms. Nevertheless, the existence of such organisms provides the means to study how L-A-2-C is recycled in nature.

The degradation of L-A-2-C in the plants producing it is extremely slow, making the study of such a process somewhat difficult. Although the degradation of L-A-2-C has been observed in *Escherichia coli* and *Cyanidium caldarium* and some degradation products have been identified (including alanine, methionine, and serine), the details concerning these degradation processes remain sketchy, and the mechanisms involved have not been determined. The most detailed study on the metabolism of L-A-2-C was carried out by Dunnill and Fowden[9] with a soil microorganism found to be able to utilize L-A-2-C as a sole nitrogen source. Isolating the organisms from the soil around *C. majalis* L. (lily-of-the-valley) and feeding L-A-2-C to the cell-free extract, the initial degradation product was tentatively identified as 4-amino-2-hydroxybutanoic acid (**2**) (isohomoserine) (see Scheme 1). The exact mechanism involved, however, was, again, not determined, and the microorganism, tentatively identified as an *Agrobacterium* sp., was unfortunately not deposited in any standard culture collection and is no longer available from the investigators.

Based on our interest in the biosynthesis, chemistry, and metabolic disposition of unusual cyclic amino acids, we have initiated a program to determine both the mechanism of the biosynthesis of L-A-2-C by *Actinoplanes ferrugineus*[8] as well as its degradation by various microorganisms. The purpose of the present study is to isolate a soil microorganism that is either the same as or similar to that utilized by Dunnill and Fowden[9] for their initial degradation studies of L-A-2-C, inasmuch as a sample of their organism is not available. The logical source for seeking such an organism would be the soil around the plant *C. majalis* from which the original organism was isolated. We wish to report herein the successful results of our search for a microorganism capable of utilizing L-A-2-C as a sole nitrogen source.

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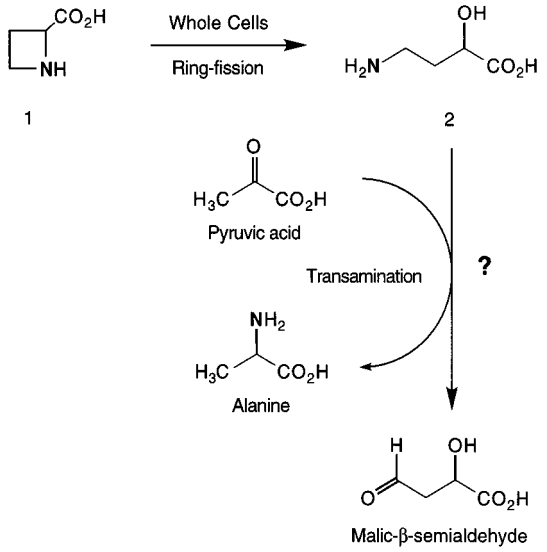
Scheme 1. Postulated Metabolic Pathway for the Degradation of L-A-2-C.



Results and Discussion

It has been shown by Dunnill and Fowden[9] that a microorganism isolated from the soil around the roots and rhizomes of *C. majalis* was able to degrade L-A-2-C. They suggested that the bacterium, by degrading L-A-2-C, played a role in recycling the nitrogen (or carbon) of the amino acid. The microorganism, which was loosely identified as an *Agrobacterium* sp. closely related to *A. radiobacter* is, however, no longer maintained (the organism was unfortunately never deposited in a standard collection) by those investigators, has never been reported since by other researchers, and is not available from any other sources. Therefore, to study the degradation of L-A-2-C in the *Agrobacterium* sp. or other microorganisms, it was necessary to search for a bacterium capable of carrying out such a process. A logical source for such bacterium was the soil around *C. majalis*.

Soil samples were collected around the plant *C. majalis* during different times of the growing year, from plots of various ages, at various distances from the plant, and at various depths, in and around Ann Arbor, MI. These samples were subjected to a selection process that gave microorganisms capable of growing with L-A-2-C as the sole nitrogen source. To ensure that any contaminating organisms (organisms that could not utilize L-A-2-C) that lay dormant were not carried through the selection process, the microorganisms that were able to grow on the L-A-2-C medium were transferred to an ammonium chloride-containing medium (medium B−ammonium chloride is the sole nitrogen source) from which single colonies were again transferred back to media containing only L-A-2-C as the sole nitrogen source. Any contaminating organisms would be revealed in the nonselective medium, and single colonies of these organisms would not grow when subsequently transferred back to an L-A-2-C-containing medium. Among the samples collected, only those obtained in areas immediately next to the roots yielded microorganism(s) capable of growing with L-A-2-C as the sole nitrogen source. Furthermore, the positive samples were all obtained from older plots (10+ years in age, based on the owners' estimation) when the leaves of the plants were just unfolding. This may indicate a natural selection process in which, over time, organisms capable of utilizing L-A-2-C were selected in an area close to the plants where a high concentration of L-A-2-C existed.

The isolated organisms that could utilize L-A-2-C as the sole source of nitrogen were Gram-negative. Light microscopy (400 ×) showed rod-shaped, motile organisms. Scanning electron microscopy (8000−46 000 ×) showed mostly single cells of approximately 0.5 μm in diameter and 1.3 μm in length (Figure 1). Some cells had a single polar flagellum, while the remaining ones did not appear to have any flagella. Some were present in pairs. Long string colonies of approximately 10 cells were also observed under electron microscopy. Those cells appearing in pairs or in long strings had no flagella (See Figure 1).

To identify the organism(s), a culture growing on an L-A-2-C slant (medium E) was analyzed by the Microbiology Laboratory at the University of Michigan Hospital Clinical Laboratories using the standard battery of biochemical tests for identification of microorganisms. These tests check for the ability of the microorganisms to carry out certain biochemical reactions, the production of certain characteristic chemicals, or the presence of certain enzymes. Using the results of these tests, a standard identification scheme was followed to identify the organism(s) isolated. The results of the tests are summarized in Table 1.

All test results were definite, except that the test for production of acid from the sugar melibiose (6-*O*-α-D-galactopyranosyl-D-glucose) gave ambiguous results. It was suspected, from these results and the results of the microscopy studies (flagella vs no flagella), that either two species or two strains of one single species were present in the culture. The original culture was subcultured for further testing. It was then determined that the original culture indeed contained two organisms, one which gave a positive test for acid from melibiose and the other one a negative test. The microorganisms were identified as *Enterobacter agglomerans* (positive melibiose) and *Enterobacter amnigenus* (negative melibiose), two closely related species of bacteria.

To confirm the accuracy of identification, reference samples of these two organisms were obtained from the American Type Culture Collection and examined by both scanning electron microscopy and biochemical characterization screening. The ATCC strains were ATCC 27155 and 33072. ATCC 27155 is actually *Erwinia herbiocola* subsp. *herbicola* Dye, which is the neotype of *E. agglomerans*. Biochemical screening gave identical results with the isolated organisms. Electron microscopy examination showed that both reference organisms had general morphology similar to the bacteria isolated from the soil (Figure 2). Thus, they both had the same size as the isolated organism. *E. agglomerans* had single, polar flagellum, while *E. amnigenus* had no flagella. Both were able to grow in pairs, but only *E. agglomerans* was found in long strings. For further comparison, the reference organisms were tested for their ability to grow on L-A-2-C. Both *E. agglomerans* and *E. amnigenus* were able to grow in media containing L-A-2-C as the sole nitrogen source. When

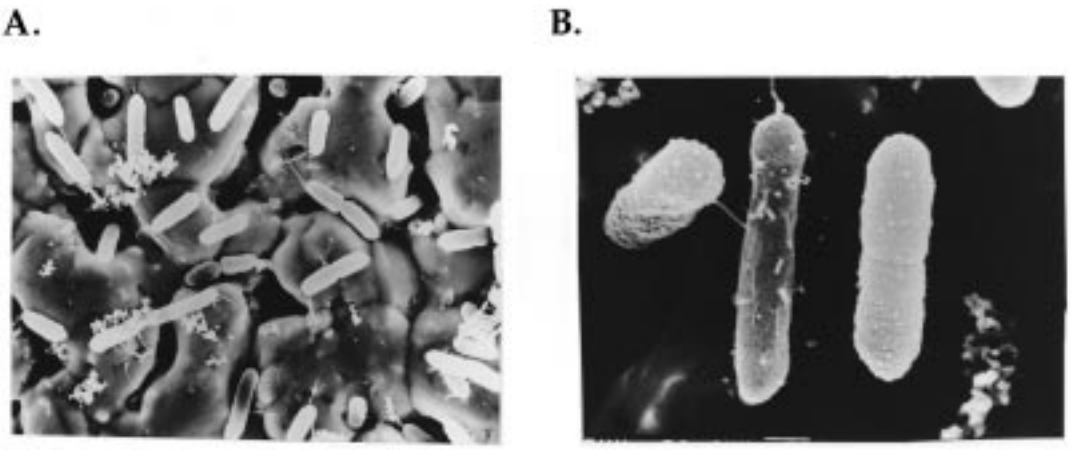


Figure 1. (**A**) Electron micrograph of soil microorganism(s) isolated from L-A-2-C-containing media (sole carbon source) at 8.05K×; (**B**) magnified view of representative microorganism(s) at 30.6 kV.

Table 1. Biochemical Characteristics of the L-A-2-C-Utilizing Soil Bacteria

β-galactosidase (ONPG)	+
arginine dihydrolase	−
lysine decarboxylase	−
ornithine decarboxylase	−
citrate	−
hydrogen sulfide	−
urease	−
amylase	+
tryptophan deaminase	−
indole	−
Voges−Proskauer	+
gelatinase	−
glucose utilization	+
acid from:	
mannitol	+
inositol	−
D-sorbitol	+
L-rhamnose	+
salicin	+
melibiose	+/−
arabinose	+

the mature cells were suspended in a buffer containing L-A-2-C, both organisms were able to metabolize the amino acid as followed by TLC. These results are consistent with the identification of the isolated bacteria as a mixture of *E. agglomerans* and *E. amnigenus*.

Although the two "different" organisms isolated in this study were able to grow with L-A-2-C as the sole nitrogen source, only one degradation product of L-A-2-C, namely isohomoserine, was obtained from both organisms. This finding is not surprising considering the similarities of the organisms in their biochemical characteristics; however, the identification of these organisms as species entirely different from the one reported previously in the literature was unexpected and somewhat disappointing.

Dunnill and Fowden studied the degradation of L-A-2-C in a microorganism isolated from the soil around *C. majalis* and isolated a product of degradation that was consistent with the one observed in our study.[9] The organism they obtained, however, was identified as an *Agrobacterium* sp., a member of the Rhizobiaceae family, instead of an *Enterobacter*, which belongs to the Enterobacteriaceae family.[9] One could simply accept the fact that the species obtained by Dunnill and Fowden[9] is actually different from those obtained in our study and that the ability to grow with L-A-2-C as the sole nitrogen source can be found in at least two families and possibly in a wide variety of organisms. A closer look at the classification of bacteria and the characteristics of *E. agglomerans*, however, may give a somewhat different view.

First, defining bacterial species is not as precise as one might desire. The taxonomic hierarchy is a conventional arrangement, and the concept of a bacterial species is less definitive than for higher organisms. Even though the organisms isolated in our study are classified in a genus different from the organism isolated by Dunnill and Fowden,[9] the actual difference between them may not be as large as the taxonomy suggests. Close examination of the classification of these organisms revealed that there is an indirect link between the species *E. agglomerans* and the *Agrobacterium* species. According to *Bergey's Manual of Systematic Bacteriology*,[10] *E. agglomerans* is actually a heterogeneous species considered synonymous with *Erwinia herbiocola*, *Erwinia uredovora*, and *Erwinia stewartii*. A list of synonyms of *E. agglomerans* has been reported by Ewing and Fife.[11] It is, in fact, difficult to define the groups now referred to as *E. agglomerans* and *Erwinia* species. The important thing to note is that some strains of *E. herbiocola* are synonymous with an *Agrobacterium* species, *A. gypsophilae*. This means that members of the *Erwinia* (or *Enterobacter*) genus and members of the *Agrobacterium* genus may actually resemble each another. So it is possible that the organism used to study the degradation of L-A-2-C by Dunnill and Fowden[9] and identified as an *Agrobacterium* sp. is related to or similar to the organisms isolated in our study. Because no authentic sample of the species studied by Dunnill and Fowden[9] is available, whether these species are indeed similar will never be determined.[12] Regardless, studies are presently underway in our laboratory to isolate, purify the enzyme(s) responsible for the degradation of L-A-2-C to isohomoserine, and determine further metabolic steps in the degradation−transformation pathway. In addition, once the purified enzyme is in hand, the mechanism of this unusual hydrolytic ring opening of L-A-2-C will be determined.

Experimental Section

General Experimental Procedures. TLC was performed on precoated Si gel plates utilizing a mobile

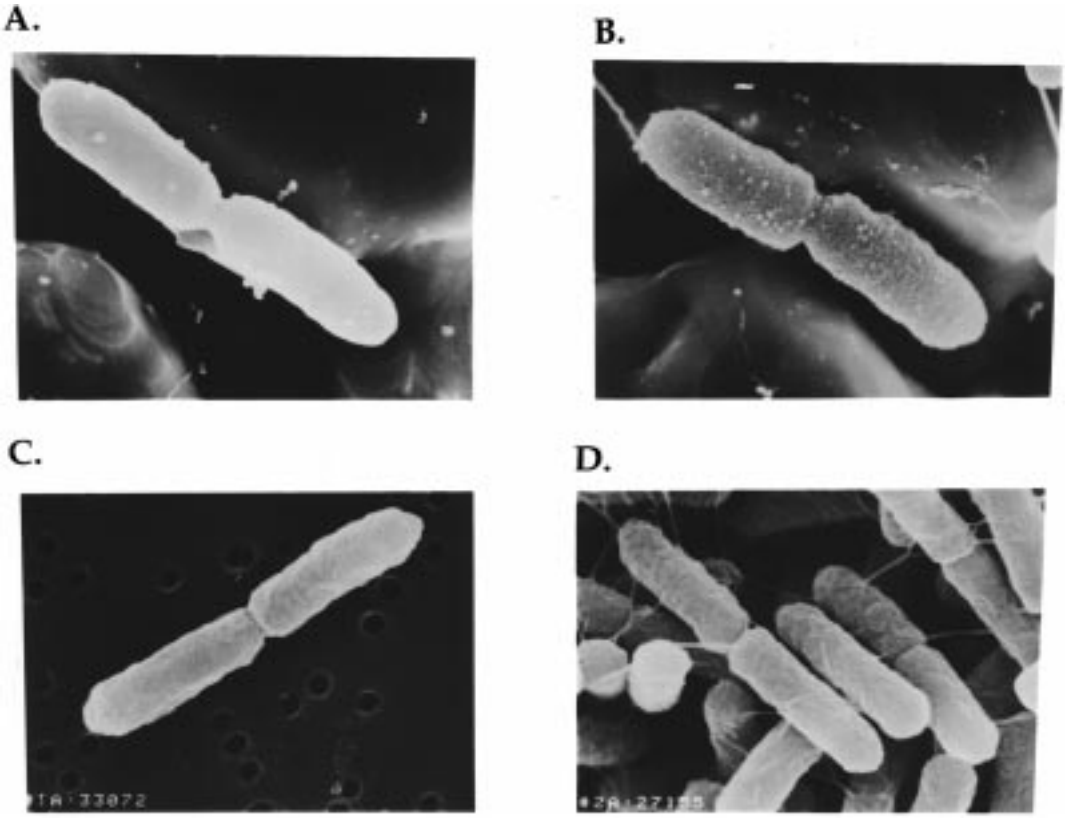


Figure 2. (**A**) Reisolated L-A-2-C-utilizing, melibiose-negative bacteria, with no polar flagella; (**B**) reisolated L-A-2-C-utilizing melibiose-positive bacteria, with a single polar flagellum; (**C**) ATCC 33072 *E. amnigenus*; (**D**) ATCC 27155 *Erwinia herbiocola* subsp. *herbicola* Dye.

phase of either solvent A, *n*-propanol−H_2O (9:5), or solvent B, EtOH−28% NH_3 (2:1). Compounds were visualized with UV light and/or by spraying with a modified ninhydrin solution (50 mL of 0.1% ninhydrin in EtOH plus 2 mL of *technical* grade collidine).[13] L-Azetidine-2-carboxylic acid, R_f 0.39 (A) and 0.36 (B), gave a crimson-scarlet color upon development (100 °C for 5 min) whereas L-isohomoserine, R_f 0.52 (A) and 0.62 (B), gave a bluish purple color upon development. Gram staining was performed by standard methods.

Materials. L-Azetidine-2-carboxylic acid was purchased from Calbiochem, Inc. (San Diego, CA). The bacterial strains ATCC 27155 (*E. herbiocola* subsp. *herbicola* Dye) and ATCC 33072 (*E. amnigenus*) were obtained in the lyophilized form from the American Type Culture Collection and reconstituted according to standard methods. Bacto-Agar and dehydrated skim milk were obtained from Difco (Detroit, MI) and prepared according to the enclosed instructions. Pre-scored TLC Si gel chromatography plates (GF_{254}, 0.25 mm × 2.5 cm × 10 cm) were obtained from Analtech, Inc. (Newark, DE). Other reagents were of the highest grade available and purchased from the standard commercial sources. Isohomoserine (L-4-amino-2-hydroxybutanoic acid) was prepared by the method of Woo et al.[14]

Sample Collection. The soil samples were collected from *C. majalis* planting beds at three different times of the year, based on the various stages of plant development: (A) after the appearance of shoots but before the unfolding of leaves; (B) after the unfolding of leaves but before the plants blossomed; and (C) during the time of full blossom (white flowers). Twenty plots of land, in and around Ann Arbor, MI, were selected where *C. majalis* had been growing for different lengths of time. The range was from less than 1 year to more than 20 years. For each time of the year and on each plot, samples were collected from three different sites: one from the outer edge of the plot, one from the center, and one from the remaining areas. At each site, three samples were collected that varied in the distance from the plant and the depth in the ground. The distance and depth were as follows: (A) 3−4 in. from the plant and on ground surface; (B) 3−4 in. from the plant but deep at root level; and (C) next to the plant and at root level.

Microorganism Isolation. The soil samples (540 total samples) were stirred in sterile distilled H_2O for several hours, and the H_2O was then used to inoculate a liquid medium (medium A). Degradation of L-A-2-C in the media was followed qualitatively with TLC. Inocula were taken from the media that showed degradation of L-A-2-C to inoculate fresh medium A. Degradation of L-A-2-C in the medium was again monitored with TLC. Inocula taken from this second medium, which continued to degraded L-A-2-C, were used to streak onto agar plates (medium C). Single colonies were collected, grown in liquid media (medium A, 50 mL), and subsequently maintained on agar slants (medium E). Medium A (1 L), KH_2PO_4, 4.08 g; glucose, 10.00 g; NaCl, 1.00 g; $MgSO_4$, 0.10 g; L-A-2-C, 1.40 g; $FeSO_4$, 10 mg; 1 N NaOH soln, 21 mL, final pH 7.2. Medium B (1 L), same as medium A with the following modifications: replace the L-A-2-C with NH_4Cl, 2.00 g. Medium C (1 L), same as medium B with the following modification, agar 1.5%. Medium D (1 L), same as

medium A with the following modifications, L-A-2-C, 28 mg; NH_4Cl, 150 mg. Medium E (1 L), same as medium A with the following modification, agar, 1%. All media were sterilized at 120 °C and 15 psi for 15−20 min.

Light Microscopy. The electron micrographs were obtained with the assistance of Dr. Bruce W. Donohoe, Director of the Cell Biology Laboratory, at the University of Michigan on an ISI model DS-130 high-resolution scanning electron microscope operating at 10 kV.

Biochemical Characteristics of the Bacteria. The biochemical characterization of the isolated microorganism(s) was performed in the laboratories of Dr. Carl L. Pierson, Director of the Clinical Microbiology Laboratory at the University of Michigan, utilizing the Micro-ID Enteric Identification System from Organon-Teknika (purchased from Curtin Matheson Scientific, Inc., Houston, TX). The characterization was performed on the initial mixture of organisms, on the reisolated single organisms, and on both reference microorganisms ATCC 27155 and ATCC 33072.

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References and Notes

(1) L-Azetidine-2-carboxylic acid (L-A-2-C), like all the natural amino acids except cysteine, possesses the *S* configuration at C-2 and is the only stereoisomer degraded by the organisms characterized here. A manuscript detailing this stereoselectivity as well as other stereochemical aspects of the mechanism of the unusual hydrolytic ring opening is in preparation.
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