ISOLATION OF L-CYCLOSERINE FROM *ERWINIA UREDOVORA*

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An antibiotic which seems to be a cell wall synthesis-inhibitor was isolated from a bacteria strain identified as *Erwinia uredovora*. The antibiotic was identified with L-cycloserine from its physico-chemical properties. This is the first example for isolation of L-cycloserine as a microbial product.

In the course of our screening work for new antibiotics with inhibitory activity to cell wall synthesis from soil bacteria, we found that a strain, No. 821, identified as *Erwinia uredovora* produces an antibiotic which causes spheroplast formation of *Escherichia coli* LS-1 (a supersensitive mutant to β-lactam antibiotics) in a hypertonic medium containing 12% sucrose. This observation strongly suggests the antibiotic to act as an inhibitor of cell wall synthesis. The antibiotic was isolated and identified with L-cycloserine.

D-Cycloserine is one of the oldest antibiotics and well known as an inhibitor of alanine racemase and D-Ala-D-Ala synthetase.[1] The antibiotic is produced from a variety of *Streptomyces* species.[1,2] Production from strains of *Pseudomonas fluorescens* has also been reported.[3] L-Cycloserine had been chemically synthesized and some of its antimicrobial property and mode of action had been studied,[4,5] but the production of L-cycloserine from any of microorganisms has not been reported.

This report provides the first example for the production of L-cycloserine as a secondary metabolite of microorganisms. The taxonomy of the producing organism, and the isolation and identification of L-cycloserine are presented.

Taxonomy

The bacterium, strain No. 821 was isolated from a water sample of rice-paddy in Osaka Prefecture, Japan.

Morphology and Staining of Cell Wall

1) Cell Shape and Size: Moderate size of straight rods with rounded ends at the early stage ($0.7 \times 1.5 \sim 3$ μm). Variability in length was observed at the late stage. Jointed forms of two cells were commonly observed.

2) Motility and Flagella: Motile with a few ($3 \sim 4$) peritrichous flagella.

3) Gram-staining: Negative.

4) Spore-formation: Not observed.

5) Poly-β-hydroxybutyrate Granules: Not detected by microscopic test for the cells grown on succinate-mineral salts medium.[6]

6) Capsule: Not formed.

Growth Characteristics

1) Colony on Agar: Circular, convex, entire colony with wet, smooth surface, cream color and semi-translucent density (nutrient agar, 28°C, 1~3 days).

2) Growth on Agar Stroke: Moderate, filiform growth with dull shining surface, soft to butyrous structure, cream color and semi-translucent density (nutrient agar).

3) Growth in Broth: Significant and uniform growth was observed. No pellicle or flocculents growth was observed (nutrient broth).

4) Visible and Fluorescent Pigments: No visible and fluorescent pigments were formed on KING's A and B media.[7]

5) Gliding and Spreading Activity: The strain did not glide or spread on peptonized milk agar for "Gliding Bacteria".[8]

Conditions for Growth

1) Oxygen Requirement: Weakly facultative anaerobic on soft-agar stab test. Fermentative result with acid production but no gas formation was observed by HUGH-LEIFSON's OF-test. The bacterium, however, did not grow in Gas-Pak system.

2) Temperature for Growth: Rapid growth occurred at 27~37°C. Weak growth was observed at 40°C but no growth was seen at 41°C or over.

3) Nutritional Requirement: No nutritional requirement was detected. The strain grew on STANIER's standard mineral base medium[6] supplemented with the following materials: fructose, ethanol, trehalose, Ca-2-ketogluconate, inositol, D-xylose, D-ribose, saccharic acid, glucose, sucrose, Na-acetate, Na-lactate, DL-alanine and DL-arginine, respectively. Negligible or no growth was seen on malonic acid, glycolic acid, *p*-hydroxybenzoate, L-valine, L-rhamnose, levulinic acid, mesaconic acid, Na, K-tartarate, adonitol, 2,3-butyleneglycol, hydroxybutyric acid and tyramine.

4) Growth on NORRIS's Glucose, Nitrogen-free Medium: No growth.

Physiological Characters

1) Gelatin Liquefaction: Weakly positive.

2) Starch Hydrolysis: Negative.

3) Hydrolysis of Carboxymethylcellulose[9]: Positive.

4) Chitin Hydrolysis[9]: Negative.

5) Hydrolysis of Agar: Negative.

6) Esculin Hydrolysis: Positive.

7) Litmus Milk: Peptonized with acid production.

8) 3-Ketolactose Formation from Lactose[10,11]: Negative.

9) 2-Ketogluconate Formation from Ca-gluconate[12]: Positive.

10) Arginine Dihydrolase Activity[6]: Negative (STANIER, PALLERONI and DOUDOROFF's method).[6]

11) Lysine Decarboxylase: Negative.

12) Ornithine Decarboxylase: Negative.

13) Glutamic Acid Decarboxylase: Negative.

14) Denitrification[13]: Negative (Gas-Pak method and SELLER's medium).

15) Nitrite Formation from Nitrate: Positive.

16) Indole Formation: Positive.

17) Methyl Red Test: Negative.

18) Voges-Proskauer Reaction: Positive.

19) H_2S Formation: Negative (TSI agar SULKIN and WILLET, 1940).[13]

20) Deoxyribonuclease Activity (JEFFRIE, HOLTMAN and COUSE, 1957)[14]: Positive.

21) KOVAC's Oxidase: Positive.

22) Catalase: Positive.

23) Sensitivity to 0/129 (DAVIS and PARK, 1962)[15]: Sensitive.

24) Growth on KCN Broth[16]: Did not grow

on 0.01% and 0.05% KCN broth.

25) Acid Formation from Ethanol[17]: No acid was formed on 0.5% ethanol agar medium[17].

26) Acid Formation from Carbohydrates: Acid production was observed from the followings: glucose, fructose, galactose, D-xylose, sucrose, maltose, salicin, mannitol, lactose, melibiose, trehalose, mannose and L-arabinose. Weak acid formation was observed from glycerol, cellobiose, inositol and raffinose, but no acid was produced from α-methylglucoside, dextrin, rhamnose and adonitol.

The bacterium, strain No. 821 is Gram-negative, weakly facultative anaerobic, ordinary size of straight rods with a few peritrichous flagella and does not show N_2-fixation and gliding and/or spreading activity. The bacterium has no nutritional requirement and does not form acid from ethanol. These results clearly indicate the bacterium belongs to Family *Enterobacteriaceae*.

The strain produces 2-ketogluconate from Ca-gluconate but does not form 3-ketolactose. The growth on KCN broth is negative and the glutamic acid, arginine, ornithine and lysine decarboxylase activity are also negative in common. The strain produces indole from peptone and acid from lactose. These evidences support that the bacterium should not be placed under *Agrobacterium* or *Serratia* but under the genus *Erwinia*.

Detailed comparison of the all taxonomic characters of the strain with those of 13 species of *Erwinia*[18] showed that the present strain closely resembles *Erwinia uredovora*. The only differences between the strain No. 821 and *E. uredovora* are that *E. uredovora* produces acid from sorbitol, adonitol, rhamnose and dextrin but the strain No. 821 does not. Although we could not test the parasitism of the strain to uredia of *Puccinia graminis*, we concluded that the present bacterium is closely related to *E. uredovora*.

Fermentation and Isolation

The bacterial suspension of one slant of the strain No. 821 was suspended in 10 ml of sterilized saline, and 0.2 ml of the suspension was inoculated into 500-ml Sakaguchi flasks each containing 100 ml of a medium composed of glycerol 3.0%, Polypeptone 0.5%, meat extract 0.5% and sodium chloride 0.5%, pH 5.5. The flasks were shake-cultured at 27°C for 24 hours.

The culture broth (3.0 liters) was filtered after admixed with 1-butanol (1.5 liters) and methanol (1.5 liters). The filtrate was evaporated under reduced pressure to be separated into two layers. The aqueous layer was adjusted to pH 3.0 and passed through a column (240 ml) of a Dowex 50X2 (NH_4^+) (Dow Chemical). The column was washed with water and eluted with 0.3 N NH_4OH. Active eluate fractions on an assay plate of *E. coli* LS-1 were collected and then passed through a column (300 ml) of a Dowex 1X4 (Cl^-) (Dow Chemical). After washing with water, the column was eluted with 2% acetic acid. An active eluate (*ca.* 60 ml) was diluted with water to 120 ml and again adsorbed on a Dowex 50X2 (NH_4^+) column (50 ml) and eluted with 0.3 N NH_4OH. The active eluate was evaporated and then freeze-dried to afford a crude powder (390 mg).

The crude powder was applied to a column (2.2×29 cm) of a microcrystalline cellulose, Avicel (Asahikasei), and developed with 75% acetonitrile. Active eluate fractions were collected, concentrated and after adjusting to pH 7.5 by dilute NaOH, freeze-dried to give a powder (80 mg). The powder was then subjected to preparative TLC on cellulose plates, Avicel SF (Asahikasei), with chloroform-ethanol-14% ammoniacal water (4: 7: 2). A zone of the antibiotic (Rf 0.15~0.20) was extracted with water and the extract was freeze-dried to give a powder (14 mg). It was again chromatographed on the same plates with chloroform - ethanol - water (4: 7: 2). A zone of the antibiotic (Rf 0.20~0.25) was extracted and

Fig. 1. IR spectrum of No. 821 (KBr).

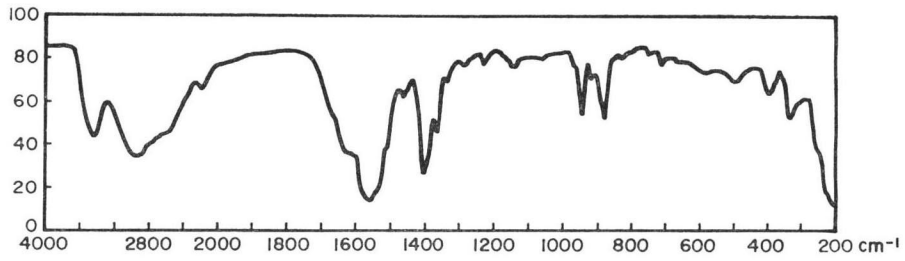


Fig. 2. ^1H NMR spectrum of No. 821 (200 MHz, D_2O, DSS internal ref).

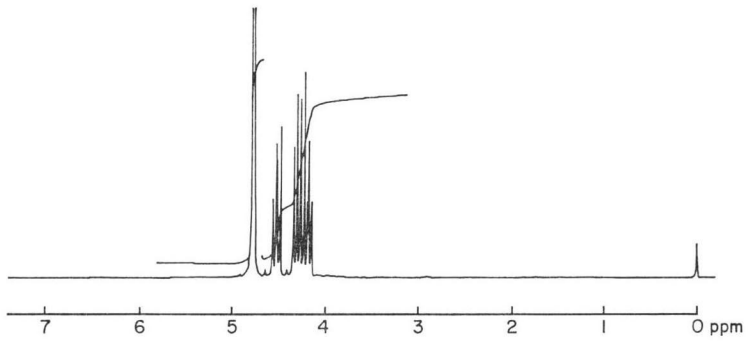


Table 1. Thin-layer chromatography of No. 821 and D-cycloserine.

Plate: Pre-coated cellulose plate, Avicel SF.
Detection: Ninhydrin reaction.

Solvent system	Rf*
$CHCl_3$ - EtOH - 14% NH_4OH (4: 7: 2)	0.15
$CHCl_3$ - EtOH - H_2O (4: 7: 2)	0.25
$CHCl_3$ - EtOH - 10% AcOH (4: 7: 2)	0.40
1-BuOH - AcOH - H_2O (3: 1: 1)	0.42
1-PrOH - H_2O (4: 1)	0.24
CH_3CN - H_2O (3: 1)	0.30

* The identical Rf values were obtained with the both compounds.

Table 2. Antimicrobial activities of No. 821 and D-cycloserine.

Pulp disks of 6 mm diameter were used in the usual agar diffusion method. Inhibitory activity was expressed by diameters (mm) of inhibitory zones.

Test organism	No. 821 (500 μg/ml)	D-Cycloserine (500 μg/ml)
Escherichia coli LS-1	18.0	22.0
E. coli NIHJ JC-2	18.0	20.0
Staphylococcus aureus 209P JC-1	0	13.0
Bacillus subtilis PCI 219	(12.0)*	24.0
Candida albicans M-9	0	0

* Hazy zone.

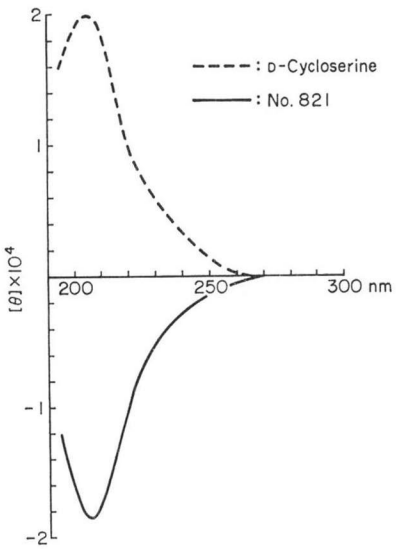
freeze-dried to give a purified preparation of the antibiotic No. 821 as a colorless amorphous powder (4 mg).

Characterization and Identification of L-Cycloserine

The antibiotic No. 821 is freely soluble in water. It shows amphoteric nature when examined by paper electrophoresis using buffer solutions of pH 4.0 and 9.3. The antibiotic gives positive reactions with ninhydrin, sodium nitroprusside and ferric chloride. A maximum in the UV spectrum measured in water is shown at 226 nm. The IR spectrum is illustrated in Fig. 1. These properties are quite identical with the reported data for D-cycloserine.[1,2] The ^1H NMR spectrum of this antibiotic (Fig. 2) is identical with that of an authentic specimen of D-cycloserine.

Chromatographic data also show identity of the antibiotic No. 821 with D-cycloserine. On a cellulose plate (Avicel SF) with several solvent systems, both the compounds exhibited the same Rf values as shown in Table 1. Moreover, both the compounds gave the same retention time (60.09 minutes), when analyzed by an automatic amino acid analyzer, Hitachi 835, in normal conditions directed for the instrument.

Fig. 3. CD spectra of No. 821 and D-cycloserine (in H_2O).



However, when antimicrobial activities of the antibiotic No. 821 and D-cycloserine were compared by pulp disk agar diffusion method, different antimicrobial properties were observed as shown in Table 2. D-Cycloserin is active against both of Gram-positive and Gram-negative bacteria.[1] The antibiotic No. 821 seems to have activity against Gram-negative bacteria but none or decreased activity against Gram-positive bacteria, though our experimental data covered only a limited range of the antimicrobial spectrum. Quite evident difference was shown in CD spectra of the both compounds (Fig. 3). The antibiotic No. 821 showed a negative Cotton effect at 205 nm, whereas D-cycloserine gave a positive Cotton effect at the same wave length.

From the above data, we concluded that the antibiotic No. 821 is the L-stereoisomer of D-cycloserine, which should be called L-cycloserine or L-4-amino-3-isoxazolidinone.

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