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Utilization of a Cyclic Dimer and Linear Oligomers of ε-Aminocaproic Acid by *Achromobacter guttatus* KI 72

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A microorganism, strain KI 72 capable of utilizing ε-aminocaproic acid cyclic dimer as sole carbon and nitrogen sources was isolated from sludge and identified as *Achromobacter guttatus*. This bacteria utilized 1% of the cyclic dimer in a day and was not inhibited by the higher concentration of the dimer. The growth rate was independent of the cyclic dimer concentration in the medium, but the maximum cell concentration increased with the increase of substrate concentration. The cell yield was 0.7 mg dry cell/mg ε-aminocaproic acid cyclic dimer. Bacterial growth with the cyclic dimer as substrate was significantly stimulated by the addition of yeast extract. Ferric chloride was also stimulatory. Maximal growth was obtained in cultures incubated at pH 6 and at 33°C. Synthesized nylon oligomers, ranging from ε-aminocaproic acid up to its linear hexamer, were found to be catabolized by this organism.

Waste water from nylon factories is likely to contain ε-caprolactam, ε-aminocaproic acid (ACA), and linear and cyclic nylon oligomers of various lengths. These substances are not produced biologically and are decomposed by few organisms. The enzymes capable of hydrolyzing these oligomers and degrading the monomers are interesting from a standpoint of microbial adaptation.

Fukumura[1,2] reported isolation of organisms which hydrolized linear and cyclic oligomers of ACA except ACA cyclic dimer (ACA-c-dimer). Maekawa *et al.*[3] showed that *Pseudomonas* sp. catabolized ACA-c-dimer. Recently we reported that *Achromobacter guttatus* KF71, an isolate from sludge, metabolized ε-caprolactam but not ACA-c-dimer[4] and that subcellular fractions prepared from the bacterial cells converted ε-caprolactam to ACA.[5]

In this paper, a newly isolated microorganism *Achromobacter guttatus* KI72, capable of utilizing ACA-c-dimer as the sole carbon and nitrogen sources is described. The general taxonomic characteristics of the bacterium and certain aspects of the metabolism of various ACA oligomers, including the cyclic dimer, are reported.

MATERIALS AND METHODS

Microorganism. The microorganism employed was isolated from sludge samples used as inocula for 3-ml lots of basal medium with 0.2% ε-aminocaproic acid cyclic dimer (ACA-c-dimer). The cultures were incubated at 30°C for 2 days on a reciprocal shaker, and those showing better growth were successively subcultured several times. From the most rapidly growing culture a bacterium designated KI 72 was obtained by single colony isolation, and used throughout this study.

Culture. The bacterium was grown in a 500-ml Erlenmeyer flask containing 100-ml basal medium at 30°C on a rotary shaker unless mentioned specially. The basal medium consisted of 1% ACA-c-dimer, 0.3% KH_2PO_4, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.05% yeast extract. For slant culture the basal medium was supplemented with 1.5% agar. The seed culture was prepared by incubating the bacteria from the slant culture for 2 days, and the seed was used for 2% inoculum in an experiment.

Assay methods. Cell concentration measured turbidimetrically at 600 nm using a Hitachi spectrophotometer 101 was correlated with dry cell weight to provide a reference curve. ACA and linear and cyclic oligomer were detected by paper or thin layer chromatography (TLC) by using *n*-butanol: acetic acid: water (4: 1: 2), or *n*-propanol: ethylacetate: 7% aqueous ammonia (6: 1: 4) as a solvent system. The cyclic dimer was detected by spraying with modified Dragendorff reagent,[6] or standing in iodine vapor. The amino acids

were developed by spraying with ninhydrin (1% in *n*-butanol). ACA and its linear oligomers were determined by the modified ninhydrin method[7] with heating in an autoclave at 0.8 atm. Infrared spectroscopy (IR) was carried out with a Hitachi 215, nuclear magnetic-resonance spectroscopy (NMR) with a JEOL PS 100, and mass spectra determinations with a Hitachi RMU-6E.

ε-Aminocaproic acid cyclic dimer. ACA-c-dimer was a gift from Dr. Kotani, the Institute of Toyo Boseki, Co., Ltd., Katada, Shiga.

ε-Aminocaproic acid linear oligomers. ACA-oligomers were synthesized according to Zahn and Hildebrand.[8] *N*-Carbobenzoxy-ε-aminocaproic acid (Z·ACA) was synthesized from ACA and carbobenzoxychloride. *N*-Carbobenzoxy-ε-aminocaproic acid linear dimer (Z·ACA_2) was prepared from Z·ACA, ACA, triethylamine, and ethylchloroformate in toluene. *N*-Carbobenzoxy-ε-aminocaproic acid linear trimer (Z·ACA_3) or tetramer (Z·ACA_4) was prepared from Z·ACA_2, and ACA or ACA_2 by a similar method in tetrahydrofuran. Also the *N*-carbobenzoxy-ε-aminocaproic acid linear pentamer (Z·ACA_5) or hexamer (Z·ACA_6) was made from Z·ACA_3 and ACA_2 or ACA_3 by using dimethylformamide as solvent. ACA_2, ACA_3, ACA_4, ACA_5, and ACA_6 were prepared by decarbobenzoxylation of the corresponding *N*-carbobenzoxy derivatives by using hydrogenolysis on paladium charcoal in acetic acid. The identity of these synthesized samples was confirmed by melting point, IR spectram, and TLC.

Dimethylimino ether of ACA-c-dimer. ACA-c-dimer (10 g) was suspended in 30 ml of benzene, 8.5 ml of dimethyl sulfate were added dropwise, and the mixture was refluxed for 20 hr with stirring. To the reaction mixture, 100 ml of 15% Na_2CO_3 was added, and the mixture was kept at 50°C for 1 hr with stirring. After removal of insoluble crystals formed by the addition of 100 ml of ethylacetate, the organic layer was separated and dried over anhydrous sodium sulfate. By evaporating off the solvent, crystals of the crude product were obtained (yield 3 g). For further purification, the crystals were dissolved in ether. After removing the insoluble materials and concentrating the ether solution, pure white crystals of the dimethylimino ether were obtained. mp 85°C. IR $\nu_{max}^{Nujol}cm^{-1}$: 1680 (C=N), 1265, 1235, 1135. Mass spectra (*m/e*): 254 (M^+), 239 ($M^+ - CH_3$), 207, 126, 96, 55, 41. NMR (in $CDCl_3$) δ (ppm): 3.60 (6H, OCH_3, singlet), 3.30 (4H, $-CH_2-N=$, triplet), 1.10~1.75 (12H, multiplet).

Other chemicals were obtained commercially.

RESULTS

A strain KI72 was isolated from sludge by an enrichment culture technique, and the taxonomic study was carried out according to Bergey's Manual of Determinative Bacteriology.[9] The flagella were observed under an electron microscope (Hitachi MS70). Its characteristics and some other properties were similar to those of *Achromobacter guttatus* listed in Bergey's Manual. The isolate, KI72 was identified as *Achromobacter guttatus*, but different from *Achromobacter guttatus* KF71

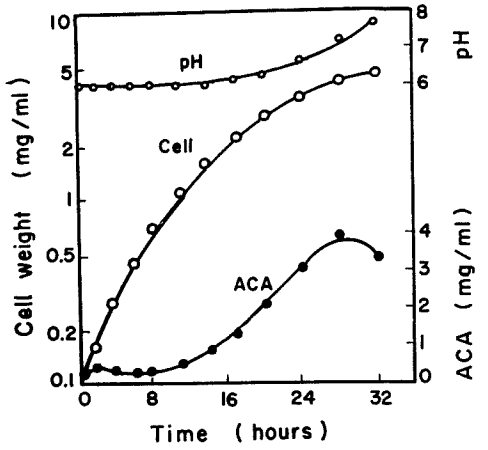

FIG. 1. Time Course of the Growth of *Achromobacter guttatus* KI 72 on ε-Aminocaproic Acid Cyclic Dimer. For details see the text.

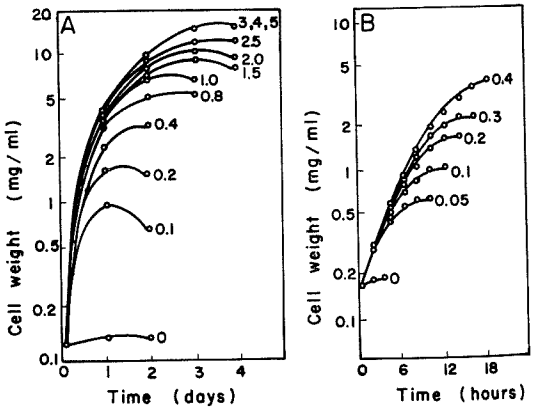

FIG. 2. Time Course of the Bacterial Growth on Different Concentrations of Substrate.

A, Higher concentration ACA-c-dimer
B, Lower concentration of ACA-c-dimer
The numbers indicate the initial concentration of the substrate (%). 0.1% of the seed culture was added at 0 time.

which had been reported in our previous paper[4] in ability to utilize ε-caprolactam. Iizuka *et al.*[10] isolated *A. cycloclastus* as an ε-caprolactam utilizing bacteria, but the strain KI72 was different from it in acid production from glucose.

Effect of culture condition

Figure 1 shows the time course of the culture in 600 ml of the basal medium in a 3-liter Erlenmeyer flask inoculated with 1 ml of the seed culture and incubated on a reciprocal shaker. The cell mass increased for 27 hr and ACA was formed with the increase in the cell mass. Though several kinds of amino acids were detectable in trace amounts by paper chromatography, all ninhydrin positive materials were calculated to be ACA in this figure. The pH increased gradually from 6.2 to 7.2 after remaining constant for 16 hr. On examination by paper chromatography, the culture filtrate yielded two large spots and small spots representing natural amino acids, identified as alanine aspartic acid, glutamic acid, glycine, isoleucine, methionine, phenylalanine, threonine, and valine when analyzed by amino acid analyzer (Hitachi KLA–3B).

The increase in cell mass in the basal medium containing various concentrations of ACA-c-dimer are shown in Fig. 2. Differences in the growth rate were not observed in media containing more than 0.4% substrate. The time course of growth in media with less than 0.4% substrate was measured, but no remarkable difference was detected. These results were attributed in part to the poor solubility of the substrate (0.2% in water), and to the high affinity of the substrate for the cell. When the initial substrate concentration and the maximum cell dry weight obtained were plotted, a linear relation was obtained up to 1.5%. The cell yield constant from this results was calculated to be 0.7 mg dry cell/mg substrate provided.

The bacteria grew on the basal medium without added organic nitrogen source except ACA-c-dimer though slowly. This meant that

Table I. Effect of Various Nitrogen Sources on Growth

Nitrogen source[a]	Cell dry weight (mg/ml)	
	24 hr	48 hr
Control	0.84	1.62
NH_4NO_3	0.85	1.57
$(NH_4)_2SO_4$	0.81	1.52
Yeast extract	4.34	5.34
Casamino acids	3.09	4.12
Polypeptone	2.77	4.27

[a] 0.05% of each nitrogen source was added to the basal medium prepared without yeast extract and 10^{-4} M $FeCl_3$ was added.

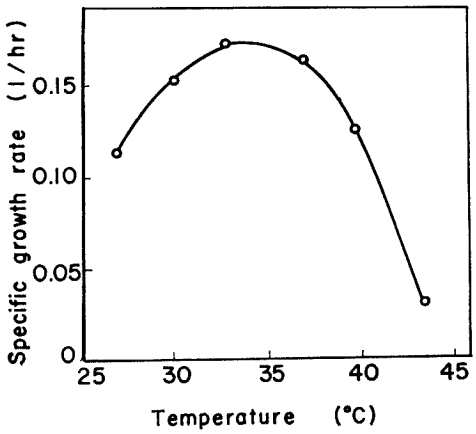

Fig. 3. Effect of Incubation Temperature on the Specific Growth Rate.
The initial cell concentration was adjusted to O.D. 0.4 and incubated in a water bath with reciprocal shaking.

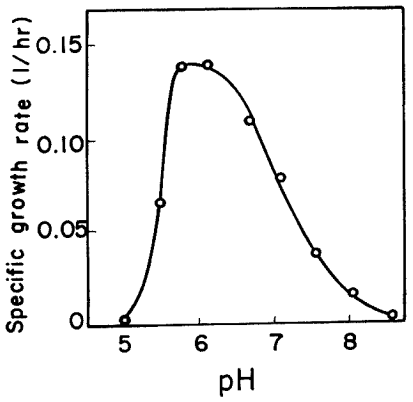

Fig. 4. Effect of pH of the Medium on the Specific Growth Rate.
The initial cell concentration was adjusted to O.D. 0.4. The initial pH was adjusted by adding KOH in medium containing 20 ml of 0.5 M KH_2PO_4.

the strain utilized ACA-c-dimer as sole carbon and nitrogen sources. Addition of inorganic nitrogen such as ammonium or nitrate to the above medium was not stimulatory, which suggested that the nitrogen source of the bacteria was sufficiently supplied from ACA-c-dimer. Addition of organic nitrogen source such as yeast extract, casamino acids or peptone improved the growth remarkably (Table I).

Effect of metal ions on the growth of the bacterium was examined. Ferric chloride (10^{-4} M) stimulated the growth twice as much as that in the control medium when tested in the basal medium without yeast extract, but in the basal medium containing yeast extract stimulation was not so much. Ca, Zn, and Mn had no effect on the growth. Cu, Ni, Co, and Hg ions were inhibitory.

The temperature providing maximal growth was 33°C (Fig. 3). The bacteria did not grow when incubated at temperatures higher than 45°C. The bacteria were grown in media of various pH values adjusted with high concentration of phosphate, and maximal growth rate was obtained at pH 6.0. The change of the medium pH was less than 0.1 throughout this experiment. This results are shown in Fig. 4.

Substrate specificity of nylon oligomers for growth

When isolate KI72 was incubated in medium containing various lengths of nylon oligomers, ACA and its linear oligomers supported the bacterial growth to various degrees, which decreased with increase in the degree of polimerization (Table II). This may result from the decreasing solubility of the oligomers: trimer, tetramer, and pentamer; 1.8, 0.8, and 0.01% in water,[11] respectively. The hexamer is almost insoluble. Dimethylimino ether of ACA-c-dimer, which was prepared to give high solubility, was metabolized but no more than effective than ACA-c-dimer. The isolate KI72 grew on ε-caprolactam as well as the cyclic dimer.

TABLE II. EFFECT OF NYLON OLIGOMERS AND THEIR DERIVATIVES ON THE GROWTH

Addition[a]	Cell dry weight[b] (mg/ml)
ACA	0.70
ACA_2	0.38
ACA_3	0.50
ACA_4	0.44
ACA_5[c]	0.27
ACA_6[c]	0.24
ε-Caprolactam	0.65
ACA-c-dimer	0.56
ACA-c-dimer dimethylimino ether	0.16
Control	0.07

[a] Each substrate (2 mg/ml) was added to the basal medium. Abbreviations were given in MATERIALS AND METHODS.
[b] The cell weight was measured at 16 hr culture.
[c] Dimethylformamide was added at measurement of turbidity.

DISCUSSION

Achromobacter guttatus KI72, isolated from sludge, was found to utilize ε-caprolactam, ACA, its cyclic dimer, linear dimer, trimer, tetramer, pentamer, and hexamer of ACA, and dimethylimino ether of the cyclic dimer at various metabolic rates. Fukumura showed[1,2] that a strain of *Corynebacterium aurantiacum* metabolized ACA, ACA_2, ACA_3, and ACA_4, ACA-c-trimer. Another strain, *Achromobacter guttatus* KF71[4] could grow on ε-caprolactam but not the cyclic dimer. The cyclic dimer seems to have a stable configuration with strong intramolecular hydrogen bonds and this is a reason to be resistant to biological attack. The crude extract of *C. aurantiacum*[12] had two enzymes which hydrolyzed cyclic and linear oligomers of ACA. Also our recent results showed that one enzyme hydrolyzed ACA-c-dimer to ACA_2, and the other ACA-linear-oligomers after extensive purification.[13] In the experiment on carbobenzoxy derivatives of ACA oligomers, the monomer and the dimer were not utilized but longer oligomers were metabolized slowly. Acrylated ACA monomer and dimer were not utilized.

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