Bacterial Degradation of N-Lauroyl-L-valine[†]

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Studies were conducted on the degradation of N-lauroyl-L-valine by type cultured bacteria. Many strains could utilize sodium N-lauroyl-L-valinate as carbon and nitrogen sources for their growth. Metabolism of N-lauroyl-L-valine was investigated in detail using *Ps. aeruginosa* AJ2116. Lauric acid was identified by gas chromatography suggesting cleavage of N-acyl linkage in N-lauroyl-L-valine.

Lauric acid might be metabolized to capric acid (C₁₀) and caprylic acid (C₈) because the accumulated substances gave nearly identical peaks with those of authentic fatty acids on gas chromatograms. The experiment using N-lauroyl-L-valine (¹⁴C) indicated that ¹⁴CO₂ was produced as a final product. Valine was not detected because it might be metabolized very rapidly immediately after its release.

It was supposed that the enzymes or enzyme systems degrading N-lauroyl-L-valine might be constitutive from the experiment using two kinds of cells grown in the medium containing N-lauroyl-L-valine or nutrient broth.

Agents responsible to degradation of pesticides under natural environment are classified into two groups; physical ones such as light, temperature and pH, and biological ones such as soil microorganisms and plants. Among them the degradation by soil microorganisms is very important because the greater part of pesticide used on plants penetrates into the soil.

In the previous paper,¹⁾ it was reported that several amino acid derivatives, as new pesticides, showed some preventive effect against rice blast. Among them N-lauroyl-L-valine (hereafter referred to as No. 5) was most effective to the disease. In this paper, aerobic bacterial degradation of No. 5 in pure culture using type culture is investigated.

MATERIALS AND METHODS

Microorganisms. Eighty four kinds of aerobic bacteria belonging to 16 genera and 56 species which were obtained from Central Research Laboratories,

Ajinomoto Co., Inc. (Kawasaki, Japan) were used throughout this work.

Cultivation methods

1) *Test tube scale cultivation.* The medium for the test tube cultivation contained 0.1% KH₂PO₄, 0.04% MgSO₄·7H₂O, 1% sodium N-lauroyl-L-valinate (hereafter referred to as No. 5—Na) or Blastidicin S obtained from Kaken Chemical Co., Ltd. and 0.05% Bacto yeast extract (Difco), and the pH was adjusted to 7.0. The medium (10 ml) was added to a test tube (60 ml vol), and sterilized by autoclaving at 120°C for 20 min.

A loopful of cells grown on nutrient agar slant at 30°C for 24 hr was inoculated to the medium. The cultivation was performed at 28°C for 48 hr. The state of growth was recorded according to the following signs.

Sign	Optical density of the medium at 562 m μ
—	less than 0.003
±	less than 0.040
+	less than 0.310
++	less than 0.405
+++	less than 0.530
++++	less than 0.920

2) *Flask scale cultivation.* The medium contained 0.1% KH₂PO₄, 0.04% MgSO₄·7H₂O, 0.03% (NH₂)₂CO, 0.03% (NH₄)₂SO₄, 3% CaCO₃, 3% No. 5—Na, 0.05% Bacto yeast extract (Difco) and the pH was

[†] Control of Plant Diseases by Amino Acid Derivatives. Part II. A brief account of this paper was presented by T. Misato at the U.S.—Japan Seminar on environmental toxicology of pesticides, Oiso, Japan, October 28, 1971.

FIG. 1. Labeled N-Lauroyl-L-valine.

adjusted to 7.0. The medium (50 ml) was added to a shaking flask (500 ml) and sterilized by autoclaving at 120°C for 20 min. Cultivation was performed at 35°C. Cultures were diluted five times with 0.1 N HCl and optical density measured at 562 m μ by a Hitach/Model 101 Spectrophotometer.

Assay of $^{14}\text{CO}_2$ produced from N-lauroyl-L-valine. Structural formula of N-lauroyl-L-valine (^{14}C) synthesized chemically from L-valine and lauric acid- ^{14}C was indicated in Fig. 1. Cells which were cultivated in the medium containing 3% No. 5—Na, 0.1% KH_2PO_4 , 0.04% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% Bacto yeast extract (Difco) and 3% CaCO_3 , pH 7.0, in a shaking flask (500 ml vol), at 35°C for 72 hr were washed three times with M/15 phosphate buffer solution, and suspended in the same buffer solution. Optical density at 562 m μ of cell suspension was adjusted to 1.00. Cell suspension (2.5 ml) and 0.3 ml of N-lauroyl-L-valine (^{14}C) solution (93135 cpm) were mixed in a Warburg's vessel.

Incubation was performed at 35°C for 18 hr and $^{14}\text{CO}_2$ produced was trapped in 0.5 ml of ethanolamine in a center well of the vessel. Radioactive carbon dioxide dissolved in the buffer solution was gassified by adding 0.5 ml of 1 N H_2SO_4 in a side arm to the buffer solution after incubation. Radioactivity of $^{14}\text{CO}_2$ in ethanolamine was counted in a liquid scintillation counter. Quantitative determination of No. 5—Na was made colorimetrically by pinacyanol chloride- HCl_3 method modified by Kamimura.³⁾

After incubation cells and residual N-lauroyl-L-valine (^{14}C) mixture in the vessel were oxidized according to the method of Baba *et al.*,²⁾ and then the radioactivity of $^{14}\text{CO}_2$ produced was measured by a liquid scintillation counter.

Quantitative determination of L-valine. The amino acid was determined quantitatively by microbial bioassay method.

*Experiment of consumption of N-lauroyl-L-valine by *Ps. aeruginosa* AJ2116 during incubation.* Cells which were grown in the nutrient broth at 35°C for 42 hr or in the medium containing 1% No. 5—Na, 0.1% KH_2PO_4 , 0.04% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% Bacto yeast extract (Difco) and 3% CaCO_3 , pH 7.0 in a shaking flask (500 ml vol) at 35°C for 72 hr were suspended in 10 ml of M/15 phosphate buffer (pH 7.0) after washing three times by the same buffer. Optical density at 562 m μ of the cell suspension in five fold incubation broth was adjusted to 0.450. Incubation was performed

at 35°C with shaking. After incubation cells were removed by centrifugation, and amount of No. 5 remained in the supernatant was determined quantitatively.

Identification of No. 5 by gas chromatography. Trimethylsilylation was carried out for the preparation of samples for gas chromatography. In a test tube 0.5 ml of incubation medium, 1 ml of pyridine, 1 ml of N,O-bis-(trimethylsilyl) acetamide and one or two drops of trimethyl-chlorosilane were mixed. The tube was heated in a mantle heater for about 10 min till pyridine boiled. An aliquot (3 μl) of the mixture was injected into gas chromatograph. Peaks were tentatively identified by comparison of the retention time. Conditions of gas chromatography; apparatus, F and M Model 402 gas chromatography, glass column, 4% SE-30 on Diatoport S (1/4 $\times$ 4 inches), column temperature, 180°C, carrier gas, Helium, flow rate, 60 ml/min and detection method, hydrogen flame ionization.

RESULTS

1) Utilization of No. 5—Na as carbon and nitrogen sources by aerobic bacteria

Utilization of No. 5—Na by aerobic bacteria was tested in the test tube scale. As shown in Tables I, II and III, many strains of aerobic bacteria utilized No. 5—Na for their growth. Generally, gram negative bacteria belonging to *Pseudomonas*, *Achromobacter* and *Enterobacterium* utilized sodium N-lauroyl-L-valinate more actively than gram positive ones. But No. 5—Na utilizing bacteria are not limited to specific genera or species, but distributed widely in the natural world.

In case of experiments using Blasticidin S instead of No. 5—Na, most strains could not utilize the antibiotic by this method. These data indicate that No. 5—Na can be degraded very rapidly by many bacterial strains.

2) Decomposition of No. 5—Na by *Pseudomonas aeruginosa* AJ2116

As stated above, many strains of bacteria utilized actively No. 5—Na. Metabolism of No. 5—Na by *Ps. aeruginosa* AJ2116 was investigated in the flask scale. Table IV shows growth of *Ps. aeruginosa* AJ2116 in the medium containing No. 5—Na and consumption of

TABLE I. DEGRADATIVE SPECTRUMS OF AEROBIC BACTERIA AGAINST N-LAUROYL-L-VALINE (No. 5)

Microorganisms	AJ No.	Other No.	The state of growth No. 5 BCS	
<i>Pseudomonas aeruginosa</i>	2116	ATCC 10145	++++	—
<i>Pseudomonas ovalis</i>	2011	IAM 1002	+++	—
<i>Pseudomonas desmolytica</i>	2003	IAM 1089	++	—
<i>Pseudomonas syncyanea</i>	2029	IFO 3750	++	—
<i>Pseudomonas fluorescens</i>	2035	IAM 1154	+++	—
<i>Pseudomonas synxantha</i>	2038	IFO 3913	++	—
<i>Pseudomonas stutzeri</i>	2056	IAM 1504, ATCC 9114	++	—
<i>Pseudomonas putrefaciens</i>	2059	IAM 1510, ATCC 12099	—	—
<i>Pseudomonas putrefaciens</i>	2060		—	—
<i>Pseudomonas mildenbergii</i>	2063	IAM 1505, ATCC 795	+++	—
<i>Pseudomonas chlororaphis</i>	2068	IAM 1515, ATCC 9447	+++	—
<i>Pseudomonas taetrolens</i>	2069	IAM 1653, IFO 3460	++	—
<i>Pseudomonas perolens</i>	2077	ATCC 10757	++	—
<i>Pseudomonas maltophila</i>	2082	ATCC 13637	++	—
<i>Pseudomonas maltophila</i>	2090	M-126	++	—
<i>Pseudomonas maltophila</i>	2276	IAM 1438	++	—
<i>Pseudomonas maltophila</i>	2311		++	—
<i>Pseudomonas maltophila</i>	2313		++	—
<i>Pseudomonas straminea</i>	2122	CB-12	+	—
<i>Pseudomonas straminea</i>	2124	IAM 1598	+	—
<i>Pseudomonas lacunogenes</i>	2197	IAM 1568	—	—
<i>Pseudomonas lacunogenes</i>	2199	IAM 1590	—	—
<i>Pseudomonas lacunogenes</i>	2217	IAM 1532	—	—
<i>Pseudomonas azotoformans</i>	2241	IAM 1487	+++	—
<i>Pseudomonas nitroreducens</i>	2242	IAM 1408	+++	+
<i>Pseudomonas nitroreducens</i>	2244	IAM 1430	+++	—
<i>Pseudomonas nitroreducens</i>	2245	IAM 1443	+++	—

this compound. The consumption of No. 5—Na was low in the absence of $(\text{NH}_4)_2\text{SO}_4$ and $(\text{NH}_2)_2\text{CO}$.

3) Investigation whether the decomposition of No. 5—Na by *Ps. aeruginosa* AJ2116 was performed by constitutive enzyme systems or inductive ones

Generally the decomposition of pesticides is performed by inductive enzyme systems, that is, the decomposition is antidotal. It was investigated whether the decomposition of No. 5—Na by *Ps. aeruginosa* AJ2116 was performed antidotally or not. Two kinds of cells grown in the medium containing No. 5—Na and in the nutrient broth were used. Figure 2 shows residual curves of No. 5—Na in the incubation medium by two kinds of cells. The tendency of residual curves of No. 5—Na was almost same between two kinds of cells.

FIG. 2. Consumption of N-Lauroyl-L-valine by *Ps. aeruginosa* AJ2116 during Incubation in Phosphate Buffer.

×—× by cells grown in the nutrient broth
○—○ by cells grown in the medium containing N-lauroyl-L-valine

From these results the enzymes or enzyme systems involved in the degradation of No. 5—Na by this strain may be recognized as constitutive.

TABLE II. DEGRADATIVE SPECTRUMS OF AEROBIC BACTERIA AGAINST N-LAUROYL-L-VALINE (No. 5)

Microorganisms	AJ No.	Other No.	The state of growth	
			No. 5	BcS
<i>Achromobacter liquidum</i>	2385	IAM 1667, IFO 3084	—	—
<i>Achromobacter xerosis</i>	2398	CCM 71-A	+	—
<i>Achromobacter delmarvae</i>	2405	M-13	—	—
<i>Achromobacter delmarvae</i>	2390	IAM 2390	—	—
<i>Achromobacter pestifer</i>	2412	N-10-3	++	—
<i>Achromobacter winogratski</i>	2427	NCIB 9017	±	—
<i>Achromobacter lwoffii</i>	2428	NCIB 9020	±	—
<i>Achromobacter delicatulus</i>	2429	NCIB 9302	+	—
<i>Flavobacterium citreum</i>	2452	IAM 1158	+	—
<i>Flavobacterium gasogenes</i>	2454		+	—
<i>Flavobacterium rigense</i>	2455	IAM 1238	—	—
<i>Flavobacterium arborescens</i>	2489	ATCC 4358	++	—
<i>Flavobacterium aquatile</i>	2513	ATCC 11947	±	—
<i>Enterobacter aerogenes</i>	2631	24PDI	—	—
<i>Enterobacter aerobacter</i>	2646	IAM 1133, ATCC 8329	++	—
<i>Enterobacter cloacae</i>	2655	IAM 1064	++	±
<i>Enterobacter cloacae</i>	2659	25HPI	—	—
<i>Escherichia coli</i>	2632		++	—
<i>Escherichia coli</i>	2617	ATCC 11775	++	—
<i>Erwinia herbicola</i>	2669	K-59-1	++	—
<i>Erwinia herbicola</i>	2670	K-59-2-1	—	—
<i>Proteus vulgaris</i>	2764	IAM 1025	—	—
<i>Proteus vulgaris</i>	2766		—	—
<i>Proteus vulgaris</i>	2773		—	—
<i>Agrobacterium radiobacter</i>	2781	IAM 1526, ATCC 4718	++	—

FIG. 3. Gas Chromatograms of Incubation Broth Containing Sodium N-Lauroyl-L-valinate by *Ps. aeruginosa* AJ 2116.

A: Broth before incubation.

B: Broth after incubation for 14 hr.

C: Authentic caprylic acid (C_8), capric acid (C_{10}), and lauric acid (C_{12})

Column: 4% SE-30 on Diatoport S 1/4 Inch tem. 180°C.

TABLE III. DEGRADATIVE SPECTRUMS OF AEROBIC BACTERIA AGAINST N-LAUROYL-L-VALINE (NO. 5)

Microorganisms	AJ No.	Other No.	The state of growth No. 5 BcS	
<i>Micrococcus lysodeikticus</i>	1002	IAM 1050, IFO 3333	+	—
<i>Micrococcus lysodeikticus</i>	1009	IAM 1313, ATCC 4698	+	—
<i>Micrococcus varians</i>	1005	IAM 1314, ATCC 399	+	—
<i>Micrococcus conglomeratus</i>	1015	IAM 1448	—	—
<i>Micrococcus flavus</i>	1021	ATCC 400	+	±
<i>Micrococcus luteus</i>	1074	CCM 132	+	—
<i>Micrococcus luteus</i>	1003	ATCC 398	+	—
<i>Serratia marcescens</i>	2682	IAM 1021	++	—
<i>Serratia marcescens</i>	2689	IAM 1223	++	—
<i>Staphylococcus aureus</i>	1056	IAM 1011, ATCC 6538	+	—
<i>Staphylococcus aureus</i>	1057	IAM 1058	+	—
<i>Sarcina lutea</i>	1217	ATCC 272	+	—
<i>Sarcina lutea sub Sp. flava</i>	1026	IAM 1491	+	—
<i>Bacillus subtilis</i>	1235	IAM 1069, ATCC 6633	—	—
<i>Bacillus subtilis</i>	1240	IAM 1214, IFO 3027	—	—
<i>Bacillus subtilis</i>	1246	IAM 1108	—	—
<i>Bacillus subtilis</i>	1308	IAM 1225	—	—
<i>Bacillus subtilis</i>	1311	IAM 1521	—	—
<i>Bacillus subtilis</i>	1315	ATCC 6051	—	—
<i>Bacillus cereus</i>	1264	IAM 1029	+	—
<i>Bacillus megatherium</i>	1277	IAM 1166	—	—
<i>Bacillus megatherium</i>	1273	IAM 1073	—	—
<i>Bacillus polymyxa</i>	1278	IAM 1210	—	—
<i>Corynebacterium</i>	1379	IAM 1399	+	—
<i>hydrocarboclastus</i>				
<i>Corynebacterium</i>	1381	IAM 1428	+	—
<i>hydrocarboclastus</i>				
<i>Corynebacterium</i>	1389	IAM 1400	+	±
<i>hydrocarboclastus</i>				
<i>Corynebacterium equi</i>	1376		+	±
<i>Arthrobacter ureafaciens</i>	1436	IAM 1390	—	—
<i>Arthrobacter globiformis</i>	1422	ATCC 8010	—	—
<i>Brevibacterium acetylicum</i>	1441	IAM 1651, ATCC 953	±	—
<i>Brevibacterium ammoniagenes</i>	1443	IAM 1641, ATCC 6871	+	—
<i>Brevibacterium lypolyticum</i>	1450	IAM 1398	+	—

TABLE IV. GROWTH OF *Ps. aeruginosa* AJ 2116 IN THE MEDIUM CONTAINING SODIUM N-LAUROYL-L-VALINATE AND ITS CONSUMPTION

No. of Experiment	hr	Growth (O.D. at 562 m μ)	Residual No. 5 ^{b)} (mg/dl)	No. 5 consumed (%)
1	0	0	2820	0
	96	1.900	680	75.9
2 ^{a)}	0	0	2664	0
	96	1.500	1246	53.3

^{a)} (NH₄)₂SO₄ and (NH₂)₂CO were omitted from the medium^{b)} sodium N-Lauroyl-L-valinate

4) Identification of products of No. 5

Quantitative analysis of substances formed during cultivation of *Ps. aeruginosa* AJ2116

with No. 5 was investigated by gas chromatography. As shown in Fig. 3, lauric acid (C₁₂) was detected in the medium after incubation

TABLE V. PRODUCTION OF $^{14}\text{CO}_2$ FROM N-LAUROYL-L-VALINE(^{14}C) BY *Ps. aeruginosa* AJ 2116

N-Lauroyl-L-valine (^{14}C) Cells	+	+	+	—
	+	—	+	—
cpm	33409 (as $^{14}\text{CO}_2$)	93135 (as substrate)	61623 (except $^{14}\text{CO}_2$)	40 (background)

$$^{14}\text{CO}_2 \text{ produced} = \frac{33409 - 40}{93135 - 40} \times 100 = 35.8\%$$

$$\text{Recovery} = \frac{61623 - 40 + 33409 - 40}{93135 - 40} \times 100 = 101.8\%$$

(peak 3 in graph B), and two peaks (peaks 1 and 2 in graph A) were detected at the identical position with the retention times of authentic caprylic acid (C_8) and capric acid (C_{10}). Other peaks on the chromatogram A corresponded with those of the chromatogram B.

On the other hand valine was not detected in the incubation medium by microbial bioassay. Valine might be metabolized very rapidly by this strain. It seems that No. 5 was split to lauric acid and valine, and lauric acid was degraded to caprylic acid passing through capric acid by β -oxidation.

5) Production of $^{14}\text{CO}_2$ from No. 5— ^{14}C

Cells of *Ps. aeruginosa* AJ2116 grown in the medium containing No. 5—Na were incubated with No. 5— ^{14}C in a Warburg's manometer vessel. Radioactive carbon dioxide produced during the incubation was absorbed in 0.5 ml of ethanolamine in a center well. Radioactivity of $^{14}\text{CO}_2$ was measured by a liquid scintillation counter. Results are shown in Table V. Radioactivity of $^{14}\text{CO}_2$ produced during incubation was 33409 cpm and radioactivity except $^{14}\text{CO}_2$ was 61623 cpm. Therefore, $^{14}\text{CO}_2$ produced was 35.8% against initial radio activity, and recovery was 101.8%.

$$^{14}\text{CO}_2 \text{ produced} = \frac{33409 - 40}{93135 - 40} \times 100 = 35.8\%$$

$$\text{Recovery} = \frac{(61623 - 40 + 33409 - 40)}{93135 - 40} \times 100 = 101.8\%$$

From these data it seems that N-acyl linkage in No. 5 was cleft and lauric acid produced was degraded to CO_2 .

DISCUSSION

As shown in Tables I, II and III, No. 5—Na is degraded easily by many strains. It is suggested that No. 5 may not need any specific bacterial strains for its degradation under natural environments. Table IV indicates that the degradation of No. 5 was accelerated by the addition of $(\text{NH}_4)_2\text{SO}_4$ and $(\text{NH}_2)_2\text{CO}$. From this result, it is supposed that $(\text{NH}_4)_2\text{SO}_4$ or $(\text{NH}_2)_2\text{CO}$ added to soil as fertilizers may accelerate decomposition of No. 5 under natural conditions. Generally, it is said that the addition of fertilizers to the soil accelerates degradation of pesticides. As shown in Table V and Fig. 3, C_{10} and C_8 compounds were detected and the production of CO_2 was detected as an end product. Valine might be very rapidly metabolized and incorporated into protein synthesis systems or deaminated as soon as produced. It is supposed that lauric acid was metabolized to capric and caprylic acids by β -oxidation.

Conventional pesticides are generally antidotal. However, as shown in Fig. 2, the enzymes or enzyme systems decomposing No. 5 may be recognized as constitutive. Therefore, it is suggested that the degradation of No. 5 is not performed antidotally. This is one of the important characters of No. 5. From all above-mentioned data, it is supposed that No. 5 may be rapidly degraded in soil, and the use of this compound will not bring about the possibility of environmental pollution.

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