Lytic Enzyme toward Aniline-assimilating *Rhodococcus erythropolis* AN-13: Screening and Production[†]

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A bacterial strain, SH-548, that produces a lytic enzyme toward intact cells of aniline-assimilating *Rhodococcus erythropolis* AN-13, was isolated from soil. The isolated bacterium was identified as a *Flavobacterium* species. The growth conditions for the enzyme production by *Flavobacterium* sp. SH-548 were examined; organic nitrogen compounds, such as meat extract and Polypepton, were effective for its production. The lytic enzyme of this strain lysed intact cells of *Rhodococcus*, *Bacillus*, *Nocardia*, *Corynebacterium*, *Brevibacterium*, *Streptococcus*, *Micrococcus*, *Cellulomonas* and DAB (diaminobutyric acid)-type coryneform bacterial strains. However, it did not act on those of *Staphylococcus aureus* or gram-negative bacteria, *Enterobacter*, *Escherichia*, *Klebsiella*, *Proteus* or *Pseudomonas* strains. Bacterial strains having cell walls of the glycolyl type were readily lysed by this enzyme.

In the previous papers,^{1,2)} we reported the isolation of *Rhodococcus erythropolis* AN-13 that can grow on aniline as a sole carbon and nitrogen source. The metabolism of aniline by this strain has also been described.³⁾ Aniline is metabolized by the bacterium through catechol and β -ketoadipic acid to inorganic end products, ammonia, carbon dioxide and water.³⁾

For subsequent investigations on enzymes related to the aniline metabolism, a cell-free extract of *R. erythropolis* AN-13, that should be prepared under mild conditions, was required. In addition, lytic enzymes toward intact cells of this strain were essential for obtaining its protoplasts. Commercially available bacteriolytic enzymes, egg white lysozyme and Achromopeptidase (Wako Pure Chem. Ind., Osaka), were tested as to their ability to lyse intact cells of *R. erythropolis* AN-13 grown on aniline. However, these enzymes did not act on them.

R. erythropolis strains have cell walls of the

glycolyl type^{1,4,5)} that contain *N*-glycolyl-muramic acid. There is little information available on lytic enzymes toward intact cells of glycolyl-type bacteria. We thus performed screening tests on microorganisms producing lytic enzyme(s) toward aniline-grown intact cells of *R. erythropolis* AN-13; a bacterial strain, exhibiting a lytic activity strong enough to lyse these cells in its cultural fluid, was isolated from soil. In this report, we describe the isolation and identification of this lytic enzyme-producing bacterium, growth conditions for the enzyme production and spectra of the lytic activity toward various bacteria.

MATERIALS AND METHODS

Chemicals. The chemicals used in this work and their sources were as follows: Polypepton, meat extract, yeast extract and Polypepton S were from Daigo Eiyō, Osaka; casamino acids and beef extract from Difco Lab., Detroit; Miki from Ajinomoto Co., Tokyo; and malt extract from Nakarai Chem., Kyoto.

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Microorganisms. The strains used for lytic activity tests were: generous gifts from the Central Research Lab., Ajinomoto Co., Kawasaki; from the Institute for Fermentation, Osaka; stocked cultures in our laboratory; and cultures isolated in our series of studies.¹⁾ The sources of the strains are given in Table III.

Media. For the isolation of microorganisms, a two layer solid medium was used: the lower medium contained 59 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 26 g of KH_2PO_4 , 10 g (dry weight) of aniline-grown intact cells of *R. erythropolis* AN-13 and 8 g of agar in 1 liter of water (pH 6.8); the upper medium contained 5 g of Polypepton and NaCl, and 20 g of agar in 1 liter of water (pH 7.0). The lower medium (10 ml) without the cells was autoclaved at 121°C for 15 min and cooled to 45°C, and then 0.1 g of the cells was added with mixing. The mixture was then placed on a petri dish (95 mm diameter) and solidified. The upper medium after autoclaving was solidified on another petri dish (90 mm diameter). The solid medium was taken out of this plate and placed on the lower medium in the 95 mm diameter plate.

Isolation of microorganisms. About 1 g of soil was suspended in 9 ml of 0.8% NaCl. The suspension was stirred vigorously for 1 min and then stood for 1 hr. One drop of the supernatant was applied to the upper medium in the petri dish. The plate was kept at 30°C for 3 to 14 days. Clear zones of lysed cells of *R. erythropolis* AN-13 were observed around some microbial colonies. These organisms were transferred to agar slants containing the upper medium. The cultures were incubated again in 7 ml of this medium without agar for 0.5 to 14 days at 30°C with shaking; and then the lytic activity toward intact cells of *R. erythropolis* AN-13 of the cultural supernatant was assayed. Strains showing high enzymatic activity were selected. Cultures passing this test were purified by streaking the colonies twice on the isolation medium on a plate. The pure cultures were stored at 8°C on a medium containing 5 g of Polypepton, meat extract and NaCl, and 15 g of agar in 1 liter of water (pH 6.8).

Identification tests. These were carried out by the methods reported previously¹⁾ and described in the 8th edition of Bergey's Manual.⁶⁾

Enzyme assay. The enzymatic activity was determined spectrophotometrically by measuring the decrease in optical density at 660 nm. The reaction mixture contained 3 ml of an intact cell suspension (optical density of 0.6 to 0.9) of *R. erythropolis* AN-13 in 25 mM potassium-sodium (K-Na) phosphate buffer (pH 7.1) and 0.1 to 1 ml of the enzyme solution; the cells for the enzyme assay were harvested at the late-exponential growth phase in aniline medium at pH 6.0⁷⁾ and were used within 1 week after harvesting. The mixture was incubated at 35°C with shaking, and then after 10 to 60 min incubation, the optical density at 660 nm

was measured with a Hitachi 139 spectrophotometer (Hitachi, Tokyo). The control sample contained 0.1 to 1 ml of the buffer used for the enzyme preparation instead of the enzyme. One unit of enzymatic activity was defined as the amount of enzyme that decreased the optical density of a 1 ml reaction mixture at 660 nm by 0.001 per min.

Cultural conditions. Strain SH-548 was preincubated at 30°C for 15 hr with shaking in 7 ml of medium containing 10 g of Polypepton, and 5 g of meat extract and NaCl in 1 liter of water (pH 6.8). The culture was transferred to 80 ml of medium of various compositions in 500 ml shaking flasks followed by cultivation at 30°C on a reciprocating shaker. At various times, 5 ml of each culture was taken; the turbidity of the sample was read at 660 nm, and then it was centrifuged at $40,000 \times g$ for 10 min and the supernatant was subjected to the enzyme assay.

Lytic tests toward various bacteria. Intact cells of various bacteria that were harvested at the late-exponential growth phase were suspended in 25 mM K-Na phosphate buffer (pH 7.1); the optical density of each suspension was adjusted to 0.62 at 660 nm. The lytic enzyme from strain SH-548 was prepared as follows: to 100 ml of the cultural supernatant of this strain was added $(\text{NH}_4)_2\text{SO}_4$ to 60% saturation; the precipitate was dissolved in 50 ml of 0.1 M K-Na phosphate buffer (pH 7.1), and the sample was dialyzed against the buffer overnight. To 3 ml of the cell suspension was added 0.1 ml of the dialyzed enzyme solution. After 30 min incubation, the decrease in optical density at 660 nm was determined.

RESULTS

Isolation of microorganisms

From 800 soil samples, 98 strains showed clear zones around their colonies, owing to the lysis of intact cells of *R. erythropolis* AN-13. The isolated strains were subjected to liquid cultivation in order to confirm their enzyme production. The cultural supernatants of most strains tested showed lytic activity that decreased the optical density of the reaction mixture at 660 nm by more than 0.2 after 1 hr incubation at 35°C. Of the isolates, strain SH-548 showed the strongest lytic activity toward intact cells of *R. erythropolis* AN-13. Therefore, strain SH-548 was used in subsequent experiments.

Identification of strain SH-548

The strain was an aerobic, gram-negative, non-flagellated and nonsporeforming rod

($0.5 \sim 0.8 \times 2 \sim 2.5 \mu\text{m}$). A gliding motion was not seen. Colonies were circular, convex, entire, smooth, glistening, mucoid, translucent and yellow on nutrient agar. Growth was abundant on the nutrient agar; swarming growth did not occur. The yellow pigments were insoluble in the nutrient agar. The bacterium grew at 10 and 30°C, but not at 35°C. Strain SH-548 hydrolyzed gelatin and casein, but it did not decompose cellulose, chitin or agar. Starch was slightly hydrolyzed on nutrient agar. The bacterium did not produce acid or gas in peptone medium from glucose, lactose, sucrose or maltose. These morphological and physiological data show that strain SH-548 is a *Flavobacterium* species.

Growth conditions for enzyme production

The growth conditions of *Flavobacterium* sp. SH-548 were examined to develop a good medium for the enzyme production. Table I shows the effects of sugars on the cell growth

TABLE I. EFFECTS OF SUGARS ON GROWTH AND ENZYME PRODUCTION

Flavobacterium sp. SH-548 was incubated at 30°C in medium containing 10 g of sugars and Polypepton, 5 g of meat extract and NaCl in 1 liter of water (pH 6.8). After 19 hr incubation, the cell growth and enzymatic activity were measured.

Sugar	Growth (OD ₆₆₀)	Lytic activity (units/ml)
None	4.2	242
Glycerol	4.9	227
L-Arabinose	4.2	224
Xylose	4.4	222
Fructose	6.8	249
Galactose	3.3	210
Glucose	8.3	258
Mannose	5.9	259
Sorbose	4.6	226
Mannitol	4.6	219
Sorbitol	4.4	224
Lactose	9.1	259
Maltose	10.9	258
Sucrose	6.7	245
Dextrin	7.3	258
Starch	7.8	228
Lyophilized cells of <i>R. erythropolis</i> AN-13	— ^a	167

^a Not measured.

and enzyme production by this strain. Markedly effective sugars for the enzyme production were not found; glucose, mannose, lactose, maltose and dextrin increased the enzymatic activity only a little. Of these sugars, dextrin was found to prevent or delay the autolysis of *Flavobacterium* sp. SH-548 during cultivation. Therefore, 1% of dextrin was added to the cultures in the following experiments. The addition of lyophilized cells of *R. erythropolis* AN-13 inhibited the enzyme production (Table I).

The effects of organic and inorganic nitrogen compounds on the cell growth and enzyme production are shown in Table II. Meat extract and Polypepton were the most effective of the nitrogen sources tested, although their effects were not pronounced. Then, the optimal concentration of meat extract for the enzyme production was investi-

TABLE II. EFFECTS OF NITROGEN COMPOUNDS ON GROWTH AND ENZYME PRODUCTION

Flavobacterium sp. SH-548 was incubated at 30°C in medium containing 10 g of nitrogen compounds and dextrin, 0.2 g of yeast extract, 1 g of KH_2PO_4 and $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.5 g of NaCl and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.5 mg of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and ZnCl_2 in 1 liter of water (pH 6.8). At various times, 5 ml of the culture was taken; cell growth and enzymatic activity were measured. The maximal lytic activity is listed with the incubation time taken for its production.

Nitrogen compound	Growth (OD ₆₆₀)	Maximal lytic activity (units/ml)	Incubation time (hr)
Meat extract	4.1	252	24
Beef extract	3.0	188	24
Yeast extract	5.3	220	24
Polypepton	8.1	245	24
Polypepton S	4.6	229	24
Casamino acids	6.7	202	36
Mieki ^a	5.9	104	72
KNO_3	1.6	4	72
NH_4NO_3	1.6	6	72
NH_4Cl	1.7	0	72
$(\text{NH}_4)_2\text{SO}_4$	1.7	4	72
$\text{NaNH}_4\text{HPO}_4$	1.7	178	48
Urea	1.3	98	48
Sodium glutamate	5.3	226	48
Asparagine	3.3	226	48

^a Mieki: 4.4 ml/liter was added.

gated in the range of 0.2 to 2% in combination with a concentration range of Polypepton of from 0.2 to 2%; the combination of 1% meat extract with 0.5% polypepton was most effective for the enzyme production (265 units/ml at 24 hr incubation).

NaCl (0.2%) and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.025%) were also effective for the enzyme production. The phosphate concentration had an influence on the enzyme production and autolysis of this strain: as the concentration of phosphate increased, the enzyme production decreased, but a low concentration of phosphate promoted autolysis of the strain. Then, 0.2% KH_2PO_4 and 0.8% $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ were added to the medium.

The original pH of the medium did not affect the enzyme production between pH 5.0 and 9.0. Aeration on cultivation has been reported to influence the production of lytic enzymes by a *Streptomyces globisporus* strain.⁸⁾ In *Flavobacterium* sp. SH-548, the effect of the oxygen supply on the enzyme production was investigated by altering the volume of medium in a 500 ml shaking flask; the production of enzyme was not affected by the volume of the medium in the range of 50 to 150 ml per flask.

These results show that the optimal medium for the enzyme production by *Flavobacterium* sp. SH-548 is as follows, per liter: dextrin, 10 g; meat extract, 10 g; Polypepton, 5 g; NaCl, 2 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g; KH_2PO_4 , 2 g; and $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 8 g; pH 6.8.

Figure 1 shows the time course of cell growth and lytic enzyme production by *Flavobacterium* sp. SH-548. The lytic activity toward *R. erythropolis* AN-13 cells appeared in the stationary growth phase and the first peak of activity was reached at 25 hr incubation. After the activity had decreased on further cultivation, it increased again and the second peak was reached at 65 hr incubation. As the second peak of lytic activity appeared, the optical density of the culture at 660 nm decreased and its viscosity increased owing to autolysis of the bacterium, indicating that the former lytic activity (first peak) was due to an

FIG. 1. Time Course of Cell Growth and Enzyme Production by *Flavobacterium* sp. SH-548.

The bacterium was incubated at 30°C in 100 ml of the optimal medium for enzyme production described in the text. At various times, 5 ml of the culture was taken; cell growth (○—○), lytic activity (●—●) and pH (□—□) of the sample were measured.

FIG. 2. Lysis of *R. erythropolis* AN-13 Cells by an Enzyme of *Flavobacterium* sp. SH-548.

The reaction mixture contained 3 ml of intact cell suspension (optical density of 0.87 at 660 nm) of *R. erythropolis* AN-13 and 0.025 to 1 ml of cultural supernatant of *Flavobacterium* sp. SH-548 in a final volume of 4 ml. Cultural supernatant: ○—○, 0 ml; ●—●, 0.025 ml; △—△, 0.05 ml; ▲—▲, 0.1 ml; □—□, 0.25 ml; ■—■, 0.5 ml; ◎—◎, 1 ml.

extracellular enzyme of this strain and the latter activity (second peak) to an intracellular enzyme released on autolysis. We probably detected the former lytic activity in the tests on the growth conditions, in view of the cultivation time. The former lytic enzyme was also used in the following experiments.

Lysis of *R. erythropolis* AN-13

The crude lytic enzyme in the cultural fluid of *Flavobacterium* sp. SH-548 readily lysed intact cells of *R. erythropolis* AN-13 grown on aniline (Fig. 2). When 0.5 to 1 ml of the enzyme solution was used, the cells of *R. erythropolis* AN-13 were solubilized almost

TABLE III. LYTIC ACTIVITY TOWARD INTACT CELLS OF VARIOUS BACTERIA

Incubation was performed at 35°C for 30 min in a mixture containing 3 ml of cell suspension (optical density of 0.62 at 660 nm) and 0.1 ml of enzyme ((NH₄)₂SO₄-precipitated fraction) from *Flavobacterium* sp. SH-548.

Strain	Medium ^a	Lytic activity (relative value)
Gram-positive bacteria:		
<i>Arthrobacter simplex</i> AJ 1420 (ATCC 6946) ^b	A	23
<i>Arthrobacter ureafaciens</i> AJ 1421 (ATCC 7562) ^b	A	12
<i>Bacillus circulans</i> IFO 13626 ^c	B	93
<i>Bacillus licheniformis</i> IFO 12200 ^c	B	138
<i>Bacillus megaterium</i> ^d	B	61
<i>Bacillus subtilis</i> IFO 3009 ^c	B	268
<i>Brevibacterium linens</i> AJ 1520 (ATCC 8377) ^b	A	70
<i>Brevibacterium testaceum</i> AJ 1464 (IAM 1537) ^b	A	39
<i>Cellulomonas flavigena</i> AJ 1571 (ATCC 484) ^b	A	45
<i>Corynebacterium diphtheriae</i> AJ 1414 (ATCC 11913) ^b	A	19
<i>Corynebacterium equi</i> AJ 1402 (ATCC 6939) ^b	A	54
DAB-type coryneform bacteria AN-3 ^c	B	5
AN-6 ^c	B	31
AN-9 ^c	B	18
AN-11 ^c	B	45
AN-14 ^c	B	53
AN-15 ^c	B	5
AN-20 ^c	B	25
<i>Micrococcus lysodeikticus</i> AJ 1053 (ATCC 4698) ^b	A	100
<i>Micromonospora chalybeata</i> AJ 9116 (NARI 2517) ^b	C	33
<i>Nocardia asteroides</i> AJ 9112 (IFO 3384) ^b	A	98
<i>Nocardia erythropolis</i> IFO 12682 ^c	A	24
<i>Nocardia erythropolis</i> AJ 9126 (ATCC 4277) ^b	A	27
<i>Rhodococcus erythropolis</i> AN-1 ^c	D	62
AN-2 ^c	D	52
AN-5 ^c	D	118
AN-12 ^c	D	72
AN-13 ^c	D	129
AN-17 ^c	D	32
AN-18 ^c	D	55
AN-19 ^c	D	45
<i>Staphylococcus aureus</i> ^d	B	0
<i>Streptococcus faecalis</i> AJ 3263 (ATCC 12984) ^b	E	43
<i>Streptomyces rimosus</i> AJ 9042 (IAM 0014) ^b	C	0
<i>Streptomyces venezuelae</i> AJ 9154 (IFO 13096) ^b	C	35
Gram-negative bacteria:		
<i>Enterobacter aerogenes</i> IFO 13534 ^c	B	0
<i>Enterobacter cloacae</i> IFO 12935 ^c	B	0
<i>Escherichia coli</i> IFO 3301 ^c	B	0
<i>Klebsiella pneumoniae</i> IFO 13541 ^c	B	0
<i>Proteus mirabilis</i> IFO 3849 ^c	B	0
<i>Proteus vulgaris</i> ^d	B	0
<i>Pseudomonas maltophilia</i> AN-4 ^c	B	0
AN-7 ^c	B	0
AN-8 ^c	B	0
AN-10 ^c	B	1
AN-16 ^c	B	0
<i>Serratia liquefaciens</i> IFO 12979 ^c	B	4

^a A: Polypepton, 1% (w/v); casamino acids, 0.5%; yeast extract, 0.5%; and Na₂HPO₄·12H₂O, 0.1%; pH 7.3.

B: Nutrient broth (pH 7.3).

C: Yeast extract, 0.4%; malt extract, 1%; and glucose, 0.4%; pH 7.3.

D: Aniline medium (pH 6.0).⁷⁾

E: Polypepton, 1%; casamino acids, 0.5%; yeast extract, 0.5%; malt extract, 0.5%; glucose, 0.5%; and Na₂HPO₄·12H₂O, 0.1%; pH 7.3.

^b From Ajinomoto Co.

^c From the Institute for Fermentation.

^d Stocked cultures in our laboratory.

^e Isolated in our series of studies.¹⁾

completely after 60 min incubation.

Glycolic acid production by lysis

R. erythropolis AN-13 has been reported to have cell walls of the glycolyl type.¹⁾ It was examined as to whether the lytic enzyme of *Flavobacterium* sp. SH-548 hydrolyzed the *N*-glycolyl bond of *N*-glycolylmuramic acid in the cell walls of this strain or not. When lyophilized aniline-grown cells of *R. erythropolis* AN-13 were hydrolyzed with 6 N HCl at 100°C for 3 hr, 83 nmol of glycolic acid per mg of cell was released from the cell walls. The lytic enzyme (50 units), on the other hand, lysed the lyophilized cells and produced 50 nmol of glycolic acid per mg of cell on 2 hr incubation at 35°C. These results suggest that this lytic enzyme has activity toward the *N*-glycolyl bond of *N*-glycolylmuramic acid in the cell walls of *R. erythropolis* AN-13.

Lytic activity spectra

Table III shows the lytic activity of the enzyme of *Flavobacterium* sp. SH-548 toward intact cells of various bacteria. This enzyme lysed intact cells of all the gram-positive bacterial strains tested, except for *Staphylococcus aureus* and *Streptomyces rimosis* AJ 9042, but not those of the gram-negative bacteria. Intact cells of *Bacillus* and *Rhodococcus* strains, *Micrococcus lysodeikticus* AJ 1053, *Nocardia asteroides* AJ 9112 and *Brevibacterium linens* AJ 1520 were readily solubilized by the enzyme. *Brevibacterium testaceum* AJ 1464,⁵⁾ *Corynebacterium equi* AJ 1402,⁵⁾ *N. asteroides* AJ 9112,⁹⁾ *Rhodococcus* strains^{1,5)} and DAB-type coryneform bacteria AN-3, 6, 11, 14 and 20 (Aoki, unpublished data) have cell walls of the glycolyl type. Intact cells of these strains were also lysed by the enzyme.

DISCUSSION

In this report, we described the isolation and identification of a bacterium that produces a lytic enzyme toward intact cells of *R. erythropolis* AN-13 grown on aniline; the bacterium

was named *Flavobacterium* sp. SH-548. The enzyme production by this strain was increased by the addition of organic nitrogen compounds such as meat extract, Polypepton, yeast extract, asparagine and glutamate (Table II). The sugars tested increased its production only a little (Table I). The lytic enzyme almost completely lysed intact cells of *R. erythropolis* AN-13 grown on aniline (Fig. 2). Therefore, it will be a useful enzyme for the preparation of cell-free extracts and protoplasts of this bacterium.

Flavobacterium sp. SH-548 readily caused the autolysis of its own cells during incubation for the enzyme production. The addition of dextrin and phosphate delayed the lysis. Since the autolysis of cells will lead to contamination by intracellular components of the cultural fluid, which is undesirable for the subsequent purification procedure, 1% dextrin, 0.2% KH_2PO_4 and 0.8% $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ were added to the medium. In this medium, two peaks of lytic activity were seen on cultivating *Flavobacterium* sp. SH-548 (Fig. 1). The activity of the first peak seems to be due to an extracellular lytic enzyme and that of the second peak to an intracellular released on autolysis of the cells. Comparison of these two enzymes should be interesting as to investigations on the autolysis process of this strain.

Flavobacterium sp. SH-548 also lysed its own cells during storage at 8°C on agar slants containing meat extract and Polypepton, and died within about one month. Cells of this strain, therefore, were lyophilized and stored in a freezer.

Kato *et al.*¹⁰⁾ have reported a lytic enzyme produced by *Flavobacterium* sp. L-11. The enzyme seems to be different from that of *Flavobacterium* sp. SH-548: the former shows a marked lytic activity toward intact cells and isolated cell walls of *S. aureus*; on the other hand, the latter did not act on intact cells of this strain at all.

The enzyme of *Flavobacterium* sp. SH-548 readily lysed intact cells of glycolyl-type bacteria that are distributed in the coryneform bacteria and actinomycetes. This enzyme at-

tacked the *N*-glycolyl bond of *N*-glycolylmuramic acid in the glycolyl-type bacterium *R. erythropolis* AN-13, and released glycolic acid from the cell walls. The lytic enzyme system of *Flavobacterium* sp. SH-548 probably contains an enzyme that catalyzes the hydrolysis of the *N*-glycolyl bond of *N*-glycolylmuramic acid.

Little was previously known of lytic enzymes that lyse intact cells of glycolyl-type bacteria. In this work, we first found an enzyme that practically exhibits lytic activity toward intact cells of glycolyl-type bacteria. We are currently proceeding with the purification and characterization of this enzyme.

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