Production of Antibacterial Compounds Analogous to Chloramphenicol by a *n*-Paraffin-grown Bacterium

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Corynebacterium sp. KY 4339, when grown on *n*-paraffin (a mixture of C-12 to C-14 fractions) as the sole carbon source, produced three kinds of antibacterial compounds which were tentatively named Corynecins. These compounds were isolated by the extraction from the culture broth with ethyl acetate and by the chromatographies on silicic acid and alumina columns. Each component demonstrated some similarity to chloramphenicol on thin-layer chromatogram. Although their biological activities were not so remarkably as that of chloramphenicol, the patterns of antibacterial spectra against gram-positive and gram-negative bacteria resembled to it.

For the production of corynecins, *n*-paraffin was a preferable carbon source. By controlling the pH of the medium in the neutral range and keeping the aeration at a high level during the fermentation, approximately 3 g of corynecins per liter of the medium were produced after 72-hr incubation.

Although intensive studies have been carried out for the microbial production of useful materials from hydrocarbons, little information is available concerning the production of antibacterial materials by *n*-paraffin-grown bacteria. Previous works from this laboratory demonstrated that Fluopsins¹⁾ and rhamnolipids²⁾ were produced by *Pseudomonad* capable of utilizing *n*-paraffin as the sole carbon source.

Extending the screening studies, we have found that *Corynebacterium* sp. KY 4339, when grown on *n*-paraffin as the sole source of carbon, also produced some antibacterial materials in the aqueous phase of the culture medium, which were tentatively named corynecins.

The present paper deals with the isolation and purification of corynecins, and their biological activities. In addition, the culture conditions essential for corynecin production are described. The chemical structure of

the respective materials isolated will be presented in the succeeding paper.³⁾

MATERIALS AND METHODS

Microorganisms. *n*-Paraffin-utilizing *Corynebacterium* were from our laboratory stock. Among them, *Corynebacterium* sp. KY 4339 was studied extensively.

Media and culture conditions. Standard inoculum was prepared by the growth in a medium containing polypepton 1.0%, meat extract 1.0%, sorbitol 5.0% and NaCl 0.3%, which was adjusted to pH 7.0. For the production of the antibiotics, the medium used in the previous paper¹⁾ was slightly modified by the addition of CaCl₂·2aq. 0.02% and yeast extract 1.0%. The bacterium was grown at 30°C in 250-ml Erlenmeyer flasks or 5-liter jar fermentors according to the method as described in the previous works.^{4,5)}

*Determination of cell growth and *n*-paraffin remained.* Assays were performed by the methods described shown in the previous papers.^{4~6)}

Assay of corynecins. Corynecins were bioassayed against *Escherichia coli* KY 8201 by a paper disc-

agar diffusion method. The test organism (approximately 10^9 cells/ml) was preincubated in the medium (10 ml) consisting of peptone 1.0%, meat extract 0.5% and NaCl 0.25% at pH 7.0 for 3 hr at 30°C. The culture (0.6 ml) was poured into a fresh medium (10 ml) containing peptone 0.5%, meat extract 0.3% and agar 1.0%. The mixture was spread on agar (2.0%) plate containing the same components as in the pre-incubation medium. Samples taken out of the fermentation broth were centrifuged. The aqueous phase thus separated was quantitatively applied to 8-mm discs, dried at room temperature for 30 min and placed on the seeded plate. Incubation at 30°C for 22 hr gave clear growth-inhibited zone when the concentration of corynecins was higher than 200 $\mu\text{g/ml}$ in the medium. Purified corynecin II which had been isolated from the fermentation broth was used as the standard of antibacterial materials. A straight line was obtained when the zone-diameter was plotted against common logarithm of the corynecin concentration. Other tests for the biological activities are described in the text.

Thin-layer chromatography. Silicagel G (Merck) plates were used in this work. The following solvent system was employed, chloroform: methanol (93: 7). The detection of materials was done by exposing over an ultraviolet lamp or by spraying the Ehrlich reagent.⁷⁾ Rhodamine 6G (0.05% in ethanol) was also used.

Chemicals. *n*-Paraffin containing mainly C-12 to C-14 fractions was obtained from Nikko Petrochemical Co. (Japan). Fractionated samples of C-11 to C-18 were purchased from Kanto Koatsu Chemical Co. (Japan). An authentic chloramphenicol was obtained from the Fuji Plant of this company. All other chemicals were the best reagent grades available.

RESULTS

Production of antibacterial substances in the culture medium

When *Corynebacterium* sp. KY 4339 was incubated with *n*-paraffin (a mixture of C-12 to C-14 fractions) as the sole source of carbon in 250-ml Erlenmeyer flasks, the cell growth was observed after overnight incubation and continued for about 3 days. After 4-day incubation, antibacterial activity of the culture broth was detectable apparently as a growth-

inhibited zone on the plate seeded with *E. coli*. It is more clearly observed when *Bacillus subtilis* was employed in place of *E. coli* as the test organism. The active compounds were extracted with *n*-butanol or ethyl acetate from the culture broth after it had been acidified to pH 4.0. The solvent was evaporated off and the residue was treated with a small volume of methanol. After centrifugation, the supernatant was subjected to thin-layer chromatography. The result is presented in Fig. 1. Three spots of lower R_f 's

FIG. 1. Thin-layer Chromatogram of the Extract of the Culture Broth of *Corynebacterium* sp. KY 4339.

Detection of spots was performed by exposing over a UV lamp (UV), by spraying with 0.05% Rhodamine 6G ethanol solution (Rd) or by spraying with the Ehrlich reagent (Er). Biological activity (Ac) was assayed by the method as described in the text. Chloramphenicol (CM) was used as a marker.

were detected by exposing the plate over a UV lamp or by spraying with Rhodamine 6G in sun light. Five spots were apparently visualized on Rhodamine-sprayed plate exposed over a UV lamp. Each spot was scraped out to small tubes and extracted with methanol in order to assay their biological activities against *E. coli*. Three spots (I, II

and III) gave clear growth-inhibited zones on the agar plate. Chloramphenicol used as a marker in this experiment demonstrated some similarities to Substance I, II and III on thin-layer chromatogram with regard to the mobilities, the colorations with the detecting reagents and the bioautographies, as shown in Fig. 1.

Then, in an attempt to isolate these compounds, large-scale incubation was performed using 30-liter fermentor containing 18 liters of the medium, under agitation (450 rpm) and aeration (15 liters/min) at 30°C.

Isolation and purification of the biologically active substances

A typical isolation procedure is given below for starting volume of 15 liters of the culture broth after incubation for 72 hr. The broth was acidified to pH 4.0 with hydrochloric acid and centrifuged at 10,000 rpm for 20 min. The aqueous layer (9.5 liters) thus obtained was extracted repeatedly with ethyl acetate. The extract (11 liters) was concentrated at 40°C *in vacuo*. The viscous residue (100 ml) was washed with an excessive amount of *n*-hexane and extracted again with ethyl acetate. After drying over sodium sulfate, the extract was concentrated to a small volume and subjected to chromatography on a 22 cm² × 20 cm column of silicic acid (Kokusan Chemical Work Limited). The column was first washed with chloroform to elute non-polar compounds. The compounds giving Spot IV and V on thin-layer chromatogram were eluted with a mixture of chloroform and methanol (97:3). Biologically active three compounds were separately eluted with chloroform-methanol (95:5) in the order of III, II and I. Each fraction (III 720 ml, II 850 ml or I 1200 ml) was concentrated and the residue was purified on an alumina column (13 cm² × 12 cm) using the same solvent system as above. Concomitant fluorescent materials were com-

pletely eliminated through this chromatography. Each effluent was concentrated to syrup, and it was dissolved by boiling with ethanol and the solution was filtered. White needles came out after evaporation of the solvent. They were recrystallized from dichloroethane. Purified material I (470 mg), II (70 mg) and III (120 mg) were obtained.

The compound I, II and III thus obtained were subjected to thin-layer chromatography and identified with the respective compounds observed initially in the culture broth. In comparison with chloramphenicol by thin-layer chromatography, the isolated compound (corynecin III) gave an identical *R_f* with it. All of the isolated compounds gave also the same coloration with the Ehrlich reagent and UV-absorptin spots on thin-layer chromatogram as chloramphenicol. On the basis of these findings and the results of the further characterization of these compounds, the following structures were proposed for them; N-acetyl (I), N-propionyl (II) and N-isobutyryl (III) derivatives of D-(—)-threo-*p*-nitrophenyl serinol. The detail structural study will be presented in the succeeding paper.³⁾

Biological activities of corynecins

Assays for the minimal inhibitory concentration of the respective compounds isolated were performed according to the conventional agar-dilution method. The results are listed in Table I. Corynecin III, which gave an identical *R_f* with chloramphenicol on thin-layer chromatogram, had a greater antibacterial activity than other two compounds, but much weaker than chloramphenicol. However, it should be noticed that the patterns of all the corynecins demonstrated some similarities to that of chloramphenicol.

Culture conditions essential for corynecin production

Since the culture media and the conditions

TABLE I. ANTIBACTERIAL ACTIVITIES OF ISOLATED COMPOUNDS
Activities were expressed as the minimal inhibitory concentration (MIC).

Test organisms	MIC $\mu\text{g/ml}$			CM
	I	II	III	
<i>Streptococcus faecalis</i> ATCC 10541	83	20	20	3.0
<i>Staphylococcus aureus</i> ATCC 6538	83	20	20	1.3
<i>Bacillus subtilis</i> ATCC 10707	20	10	10	0.6
<i>Escherichia coli</i> ATCC 26	83	20	20	1.3
<i>E. coli</i> (multiple drug resistant)	83	83	83	42
<i>Pseudomonas aeruginosa</i> BMH 1	83	83	83	21
<i>Proteus vulgaris</i> ATCC 6897	20	5.2	2.6	0.2
<i>Shigella sonnei</i> ATCC 9290	5.2	5.2	5.2	0.6
<i>Salmonella typhosa</i> ATCC 9992	42	20	10	1.3
<i>Klebsiella pneumoniae</i> ATCC 10031	42	10	3.0	0.3

were primarily designed for glutamic acid fermentation,⁶⁾ effects of various components in the medium and the culture conditions were studied for the production of corynecins.

Carbon sources

The production of corynecins was discovered firstly in the medium containing *n*-paraffin as the sole carbon source. Therefore, the other carbon sources and fractionated samples of *n*-paraffin were examined. As shown in Table II, the *n*-paraffin mixture was a more preferable carbon source for corynecin production.

Nitrogen sources

Both ammonium sulfate and phosphate were available as nitrogen source for glutamic acid fermentation⁸⁾ but the latter depressed significantly the corynecin production despite of no effect on the cell growth. This result suggests that an excessive amount of phosphate ions affects depressively on corynecin biosynthesis. Carbonate and nitrate did not serve for any increase in the production. Although casamino acid, polypepton and corn steep liquor gave no significant effects, yeast extract and meat extract apparently stimulated the production.

TABLE II. EFFECT OF CARBON SOURCES ON CORYNECIN PRODUCTION

Bacterium was incubated in 250-ml flasks containing the medium as described in the text, in which carbon source was replaced by each compound as shown below, for 4 days at 30°C with shaking. The cell growth was semi-quantitatively estimated by measuring the packed volume of the cells after centrifugation at 3000 rpm for 20 min.

Carbon source	Growth after 1 day	Corynecins produced mg/ml
Glucose	+	—
Sorbitol	+	—
Mannitol	+	—
Fructose	++	0.12
Sucrose	++	0.36
<i>n</i> -Paraffin, Mix C ₁₂ -C ₁₄	+++	0.48
C ₁₁	+	±
C ₁₂	++	0.12
C ₁₃	+++	0.35
C ₁₄	+++	0.29
C ₁₅	+++	0.25
C ₁₆	+++	0.20
C ₁₇	+++	±
C ₁₈	+++	±
C ₁₉	+++	—
C ₂₀	++	—

Aeration

It has been known that a relatively larger quantity of oxygen is required for hydrocarbon

fermentation. Therefore, the effects of aeration on the growth as well as the corynecine production were tested. Various volumes of the culture medium (10 to 90 ml) in 250-ml Erlenmeyer flasks were incubated at 30°C for 4 days on a rotary shaker at 200 rpm. The result is presented in Fig. 2. As expected,

FIG. 2. Effect of Aeration on Bacterial Growth and Corynecine Production.

○---○, Bacterial growth; ●—●, Corynecine production.

Bacterium was incubated for 4 days at 30°C in shaking flasks.

the bacterial growth was more vigorous in the smaller volume of the medium, namely with higher aeration. The corynecine production was stimulated, associating with the cell growth, with higher aeration. This finding was also ascertained by the experiment using 5-liter fermentors, as shown in Table III.

Medium pH

Since the optimal pH for this fermentation had been found to be in the range of 6.5 to 7.3 in the small-scale experiment, it was examined again using fermentors equipped with an automatic pH-controller. As summarized in Table III, the cell growth was slightly depressed at the neutral pH with either high or low stirring, but it was essential for the corynecine

TABLE III. EFFECTS OF AGITATION AND pH ON CORYNECINE PRODUCTION

Cultivation was carried out using a 5-liter jar fermentor containing 3 liters of the culture medium, in which 0.3 liter of the seed culture preincubated for 30 hr in sorbitol-bouillon in a 2-liter flask was inoculated, under the aeration of 3 liters/ml at 30°C. pH was automatically controlled with 28% ammonium hydroxide.

Agitation	pH	Growth after 17 hr	Corynecins produced	
rpm			mg/ml	
450	6.0	0.76	0.60	0.78
	6.8	0.60	0.47	0.81
800	6.0	0.90	0.68	0.93
	6.8	0.86	0.78	1.40

production to control the pH of the medium—near the neutral.

Time course of the corynecine production

Under the optimal conditions according to the above data, the fermentation was carried out using 5-liter fermentor. During the incubation, 30 g of *n*-paraffin was supplemented twice at the time presented in Fig. 3, in addition to 300 g added initially into 3 liters of the culture medium. The progress of a typical fermentation is plotted in Fig. 3. The rapid cell growth was observed associating with the consumption of *n*-paraffin after the initial lag-phase for about 10 hr, and almost all of the carbon source was exhausted in 80-hr incubation. The corynecine production was observed from about 24 hr later than the cell growth occurred and proceeded steadily until the maximum concentration was reached at 72 hr after the inoculation. In this fermentation, the maximal amounts of corynecins produced were approximately 3.0 g/liter.

However, it is questionable if such a rapid growth as demonstrated under these conditions is preferable for the production of corynecins,

FIG. 3. Time Course of the Production of Corynecins by *n*-Paraffin-grown *Corynebacterium* sp.

Cultivation was performed using a 5-liter jar fermentor containing 3 liters of the medium in which yeast extract 1.5% and $\text{CaCl}_2 \cdot 2\text{aq}$. 0.02% were supplemented to the same components as described previously. An additional amount of *n*-paraffin was fed as described in the text. Culture conditions were as follows; agitation 800 rpm, pH 6.8 and aeration 3 liters/min.

● Cell growth, ○ corynecins estimated as corynecin II, △ *n*-paraffin remained.

because it is generally referred that a sluggish growth or slower consumption of carbon source is suitable for antibiotic production⁹⁾ If the latter finding is true in corynecin fermentation as well, it is necessary for improving the production yield to study further regulation of the bacterial growth during the fermentation.

DISCUSSION

In this paper, we first demonstrated that considerable amounts of chloramphenicol analogues, corynecin I, II and III, were produced by a bacterium grown on *n*-paraffin as the sole source of carbon. Although these compounds were known to be synthesized accompanying with chloramphenicol by *Streptomyces venezuelae*¹⁰⁾ or by the chemical

conversion of chloramphenicol, it has not attracted an industrial notice on account of their lower fermentation yields or insignificant biological activities. Even though this paper is concerned only with the isolation and the preliminary data of the culture conditions essential for the production of corynecins, these findings may open another possible process for chloramphenicol production, presumably because of the ease of the chemical or biological conversion of *p*-nitrophenyl serinol which is easily derived from corynecins.

The metabolic relationships underlying the biosynthesis of corynecins are currently under investigation. The question of the correlation in synthetic mechanism between chloramphenicol and corynecins is of great interest.

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