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Microbial Transformation of Squalene: Terminal Methyl Group Oxidation by *Corynebacterium* sp.

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Corynebacterium sp. strain SY-79 was isolated from soil, using squalene as a carbon source. Microbiological properties of this bacterium are described. The metabolic product of this bacterium from squalene was identified as 2,6,10,15,19, 23-hexamethyl-2,6,10,14,18,22-tetracosahexaenedioic acid (squalenedioic acid).

Squalene is an important precursor in the biosynthesis of steroids and is ubiquitous as a natural product. It is an acyclic triterpene whose structure is symmetric with regard to its C12-C13 bonding (see Fig. 2, part I). It has 6 double bonds and 10 methylene and 8 methyl groups which show almost identical reactivities to chemical reagents such as selenium dioxide. Therefore, squalene is a suitable model compound for the screening of regioselective microbial transformation of acyclic olefins and, thus, will be useful for production of starting materials for organic synthesis. The survey of metabolites in a culture broth of squalene-utilizing microbes is also interesting from the viewpoint of degradation pathways of branched olefinic compounds by microbes in nature.

In serial studies on the screening of squalene-utilizing bacteria from soil, we have isolated *Arthrobacter* sp. strain Y-11 (9) and *Corynebacterium* sp. strain S-401 (6), which catalyzed promising reactions on squalene and other acyclic hydrophobic compounds (5, 8). Prompted by these previous results, we continued the screening of squalene-utilizing microbes and isolated a new *Corynebacterium* sp. which oxidized squalene to squalenedioic acid. In this report, we describe the identification of this new isolate and its metabolic products.

MATERIALS AND METHODS

Organisms. A strain of *Corynebacterium* sp., SY-79, isolated from soil was used throughout this study.

Culture media. Three types of culture media were used in this study: medium A (NH_4NO_3, 0.04%; $MgSO_4 \cdot 7H_2O$, 0.01%; K_2HPO_4, 0.1%; KH_2PO_4, 0.1%; yeast extract, 0.02%; squalene, 0.03% [wt/vol]; medium B (NH_4NO_3, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.05%; K_2HPO_4, 0.1%; KH_2PO_4, 0.1%; yeast extract, 0.1%; Polypepton (Wako Pure Chemical, Ind., LTD., Osaka, Japan), 2%; squalene, 0.5% [wt/vol]; and medium C (medium B without squalene, supplemented with 1% glucose). Squalene and glucose were sterilized separately and mixed before inoculation.

Isolation of microorganisms from soil. Soil samples were incubated in medium A (3 ml per test tube) on a reciprocal shaker at 30°C for 3 days. After three successive transfers, the culture broths were extracted with dichloromethane, and metabolites were analyzed on thin-layer chromatography (TLC) plates, using *n*-hexane–ether (10:1) as the solvent system. Squalene ascended on TLC with the solvent front line, and oxidized metabolites of squalene were detected at lower R_f values as polar compounds. The metabolites were located on the TLC plate by $KMnO_4$ solution. Single colonies were isolated by plate culture from culture broths that accumulated degradation products.

Oxidation of squalene by *Corynebacterium* sp. strain SY-79. A seed culture of *Corynebacterium* sp. strain SY-79 (1 ml) was inoculated into medium B (50 ml) in a 500-ml conical flask. Cultivation was carried out on a rotary shaker at 30°C for 4 days. After the cultivation, the pH of the broth was adjusted to 3 with 3 N HCl solution, and the broth was extracted three times with 100 ml of dichloromethane. The extracts were combined and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure at 30°C, and the residual oil was purified by column chromatography, using *n*-hexane–isopropanol (0.5%) as the solvent system. When the crude oil was left to stand for a few days before purification by column chromatography, it slowly crystallized.

Cell growth and production of squalenedioic acid. Medium B (50 ml) was placed in a 500-ml conical flask, and 0.5 ml of seed culture was inoculated. Twelve culture flasks were incubated on a rotary shaker at 30°C, and two flasks were used to determine pH, recovered squalene, squalenedioic acid, and cell growth at 24-h intervals. To determine the products, we treated each culture broth as described above and separated it into recovered squalene and squalenedioic acid by column chromatography. Solvents were removed completely by evaporation, and the products were weighed. Cells in the broth were collected by centrifugation (14,000 × *g*) after the dichloromethane extraction and suspended in an appropriate amount of water for measurement of optical density at 600 nm. At the same time, 12 identical flasks containing medium B without squalene were incubated as a control experiment. Their pHs, quantities of dichloromethane-soluble fraction, and cell growth were also determined by the same methods as described above.

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Dimethyl ester of squalenedioic acid. Squalenedioic acid (0.379 g; 0.806 mmol) was dissolved in 100 ml of ether and treated with excess diazomethane-ether solution at 5°C for 1 h. Acetic acid was added to decompose the remaining diazomethane. After the ether was evaporated, the residue was dissolved in dichloromethane and washed with 10% sodium bicarbonate solution. The solvent layer was dried over anhydrous sodium sulfate, and the solvent was removed by evaporation. The residual oil (0.4 g) was purified by silica gel (10 g) column chromatography, using *n*-hexane and *n*-hexane–ethyl acetate (0.5 to 1.0%, yield, 0.375 g).

Analytical methods. Infrared absorption spectra were recorded on a Hitachi model 215 Grating Infrared Spectrometer, mass spectra with a Hitachi RMU-6E, and nuclear magnetic resonance (NMR) spectra with a Hitachi R-24B (60 Mz) or JEOL PS100 (100 MZ).

Chemicals. Squalene was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo) and used without further purification. Silica gel type G (E. Merck AG, Darmstadt, Germany) was used for TLC, and Merck silica gel 60 (70 to 230 mesh) was used for column chromatography.

RESULTS

Identification of *Corynebacterium* sp. strain SY-79. Morphological results were obtained from electron micrographs; using cells grown on medium C after 18 h and 7 days of cultivation. Gram staining was carried out with *Rhodotolura rubra* as a control. Staining was confirmed by both 18-h and 7-day cells.

Strain SY-79 is a gram-positive, nonsporulating, nonmotile bacterium. The size of cells was 0.5 to 0.6 by 0.8 to 1.2 μm. They were club shaped and showed neither a spherical nor a chain form. Agar colonies appeared as circular, convex colonies with a smooth, glistening, orange surface. Strain SY-79 is an aerobe, because it showed surface growth and nonliquefaction by gelatin stab culture.

Other characteristics of this strain are as follows: growth in 4% NaCl; no acid and gas from glucose, lactose, sucrose, or maltose; starch, cellulose, and casein not hydrolyzed; acetylmethylcarbitol, indole, and hydrogen sulfide not produced; methyl red test, negative; citrates not utilized; nitrites produced from nitrates; catalase, positive, and urease not produced; growth factors not required. The guanine plus cytosine content was determined with the cells grown in medium C. The value of the guanine plus cytosine content was 63%, which was calculated by the melting temperature of 95.2°C with maximum hyperchromicity of 1.34.

Since this strain SY-79 is a gram-positive, non-sporulating, and club-shaped bacterium, it was presumed to belong to the coryneform bacteria. This strain did not belong to the family *Actinomycetales*, because it did not show filamentous forms at any culture stage. The cell walls of strain SY-79 were hydrolyzed in 6 N HCl solution at 105°C for 8 h. DL-Diaminopimelic acid was detected by paper chromatography of this hydrolysate (7). As the cell walls of *Arthrobacter* sp. do not contain DL-diaminopimelic acid, we identified strain SY-79 as a *Corynebacterium* species (1).

Purification and identification of squalenedioic acid. *Corynebacterium* sp. strain SY-79 was cultured at 30°C for 4 days in 50 ml of medium B which contained 0.5% (vol/vol) squalene. Dichloromethane extraction of the culture broth gave crude crystals of product (86 mg) after evaporation of the solvent. The crude crystals were dissolved in 1 ml of dichloromethane and placed on a silica gel column (30 g of silica gel 60 packed by *n*-hexane). After elution of the recovered squalene (10 mg) with *n*-hexane, squalenedioic acid was eluted with *n*-hexane–isopropanol (0.5%). The yield was 64 mg, and the melting point was 86°C. The chemical composition found analytically was: C, 76.35%; H, 9.87%. The calculated composition for $C_{30}H_{46}O_4$ was C, 76.55%; H, 9.85%. The mass spectrum (*m/z*) was: M^+ 470. The infrared spectrum (in Nujol per centimeter) was: 1,680 (C = O), 1,640 (C = C). The NMR spectrum (in $CDCl_3$ and tetramethylsilane) gave δ values of: 1.60 (12H, s, (*Z*)-CH_3C=), 1.82 (6H, s, *C*H_3(HOOC)C=), 5.16 (4H, t, HC=), 6.90 (2H, t, HC=), 11.25 (2H, broad, COOH). Infrared absorption bands at 1,680, 1,640, and 2,600 to 3,200 cm^{-1} indicated that the product has α,β-unsaturated carboxyl groups. The presence of two carboxyl groups was also suggested by an NMR signal at δ 11.25 ppm (2H) which disappeared upon addition of D_2O. The terminal position of the carboxyl groups on the squalene skeleton was determined by the NMR signal of two groups of methyl protons which appeared at δ 1.82 ppm, owing to the deshielding effect of a vicinal carboxyl group. The geometrical isomerism of both carboxyl groups was determined to be *Z* on the basis of the downfield shift of the olefinic protons (δ 6.90 ppm) on C3 and C22. This geometrical isomerism was also confirmed by comparison of the NMR signals of the metabolite with those of squalene. Thus, we identified the structure of the metabolite as 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaenedioic acid (squalenedioic acid) (see Fig. 2, part II).

Dimethyl ester of squalenedioic acid. To confirm the molecular weight and structure of squalenedioic acid, we converted it to the dimethyl ester with diazomethane. Data of elementary analysis and spectrometry of the dimethyl ester are as follows. The chemical composition found analytically was: C, 77.30%; H, 10.28%. The calculated composition for $C_{32}H_{50}O_4$ was: C, 77.06%; H, 10.11%. The mass spectrum (*m/z*)

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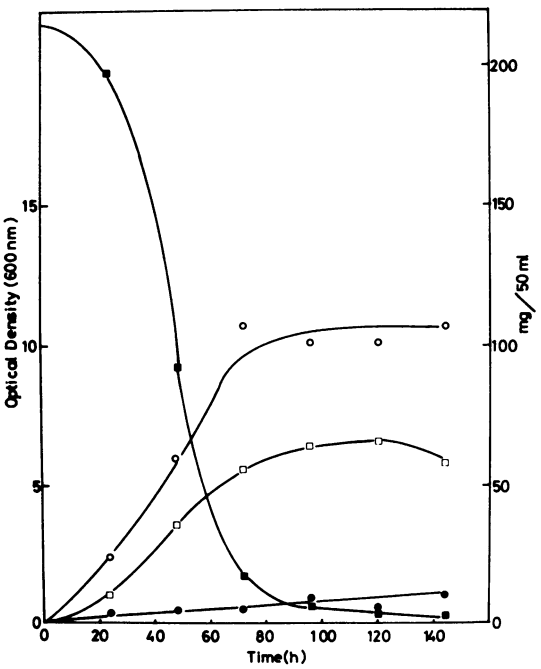

FIG. 1. Growth of *Corynebacterium* sp. strain SY-79 in medium B with (○) or without (●) squalene. Also shown are the time-course of squalene consumption (■) and the production of squalenedioic acid (□).

was: M^+ 498. The infrared spectrum (film) per centimeter was: 1,720, 1,650. The NMR spectrum in $CDCl_3$ and tetramethylsilane gave δ values of: 6.72 (2H, t, HC=), 5.12 (4H, t, HC=), 3.69 (6H, s, CH_3O), 1.90 to 2.40 (2OH, m, $—CH_2=$), 1.84 (6H, s, $CH_3(MeOOC)C=$), 1.58 (12H, s, (*Z*)-$CH_3C=$) (Me, methyl). All of these data supported the structure of squalenedioic acid (see Fig. 2, part II).

Cell growth and accumulation of squalenedioic acid. Growth of *Corynebacterium* sp. strain SY-79, accumulation of squalenedioic acid, and consumption of squalene are shown in Fig. 1. In medium B, *Corynebacterium* sp. strain SY-79 showed a slow growth rate ($\mu = 0.14\ h^{-1}$) and a long exponential phase (ca. 40 to 50 h) before the stationary phase. The accumulation of squalenedioic acid proceeded in parallel with growth and reached its maximum level (65 mg/50 ml) at the stationary phase. The chemical yield of squalenedioic acid calculated on the basis of squalene in the starting medium was 26%. The pH of the broth decreased gradually to pH 6.5 (80 to 100 h) with accumulation of squalenedioic acid and then increased again to pH 7.0 at the end of the cultivation (140 h).

Corynebacterium sp. strain SY-79 showed very poor growth in medium B in the absence of squalene and did not produce squalenedioic acid. In this case, the pH of the broth was constant throughout the cultivation.

DISCUSSION

Corynebacterium sp. strain SY-79 transforms squalene to squalenedioic acid with a 26% yield when it grows in Polypepton-rich medium with squalene (Fig. 2). Accumulation of squalenedioic acid as a metabolite of squalene by *Nocardia* sp. was recently reported by Nakajima et al. (3). The data from the spectrometry and melting point studies of our product coincided with those of Nakajima's squalenedioic acid. Nakajima et al. (3) reported that the yield of squalenedioic acid from squalene is 34% and that they obtained no other products. We detected trace amounts of one nonpolar product above the location of squalenedioic acid on TLC and recovered only about 5% of the squalene after 4 days of cultivation. *Corynebacterium* sp. strain SY-79 and *Nocardia* (*Actinomycete*) sp. are closely related taxonomically (4), and the fact that both of them catalyzed the same biotransformation suggests a common degradation pathway for acyclic terpenoids in these lipophilic bacteria. Attempts to improve the yield of squalenedioic acid above 26% were unsuccessful. The rather low yield of squalenedioic acid and poor recovery of squalene suggest that the site of first oxidation of squalene by *Corynebacterium* sp. strain SY-79 is not highly regiospecific and that the first oxidation site directs further degradation of the molecule. The terminal oxidation product, squalenedioic acid, resists further metabolism and remained in the culture broth. On the other hand, other oxidation sites lead to metabolites which are soon consumed by the bacterium. Consequently, even in rather low yield, the resulting squalenedioic acid is pure enough to be isolated in crystalline form, and further purification is very easy.

Therefore, this microbial transformation is very useful for introduction of functional groups and synthons into the terminal position of squalene. Squalene derivatives which have a func-

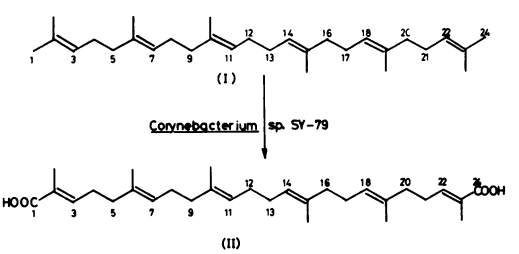

FIG. 2. Oxidation of squalene to squalenedioic acid by *Corynebacterium* sp. strain SY-79.

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tional group at their terminus are used as starting materials for the organic synthesis of other terpenoids. As an example, we synthesized (*S*)-(−)-squalene-2,3-epoxide, an important steroid precursor, from squalenedioic acid (10) by a process which involved asymmetric epoxidation (2). Our screening method with squalene as the carbon source has resulted in isolation of *Arthrobacter* sp. strain Y-11 (9), *Corynebacterium* sp. strain S-401 (6), and *Corynebacterium* sp. strain SY-79. All of these strains are taxonomically related lipophilic bacteria (4) and catalyze some useful reactions of hydrophobic compounds. Studies of the utilization of squalenedioic acid for organic syntheses and the application of strain SY-79 to other substrates to develop new bioconversions are in progress in our laboratory.

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LITERATURE CITED

1. **Cummins, C. S., R. A. Lelliott, and M. Rogosa.** 1974. Genus I. *Corynebacterium* Lehmann and Newmann 1896, 350, p. 602–617. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
2. **Katsuki, T., and K. B. Sharpless.** 1980. The first practical method for asymmetric epoxidation. J. Am. Chem. Soc. **102**:5974–5976.
3. **Nakajima, K., A. Sato, T. Misono, and T. Iida.** 1981. Microbial oxidation of the isoprenoid hydrocarbon squalene. Nippon Nogei Kagaku Kaishi **55**:1187–1195.
4. **Rogosa, M., C. S. Cummins, R. A. Lelliott, and R. M. Keddie.** 1974. Coryneform group of bacteria, p. 599–602. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
5. **Seo, C., Y. Yamada, and H. Okada.** 1981. Synthesis of fatty acid esters by *Corynebacterium* sp. Agric. Biol. Chem. **46**:405–409.
6. **Seo, C., Y. Yamada, N. Takada, and H. Okada.** 1981. Hydration of squalene and oleic acid by *Corynebacterium* sp. Agric. Biol. Chem. **45**:2025–2030.
7. **Yamada, K., and K. Komagata.** 1970. Taxonomic studies on coryneform bacteria. II. Principal amino acids in the cell wall and their taxonomic significance. J. Gen. Appl. Microbiol. **16**:103–113.
8. **Yamada, Y., N. Kusuhara, and H. Okada.** 1977. Oxidation of linear terpenes and squalene variants by *Arthrobacter* sp. Appl. Environ. Microbiol. **33**:771–776.
9. **Yamada, Y., H. Motoi, S. Kinoshita, N. Takada, and H. Okada.** 1975. Oxidative degradation of squalene by *Arthrobacter* species. Appl. Microbiol. **29**:400–404.
10. **Yamada, Y., C. Seo, and H. Okada.** 1981. Asymmetric synthesis of (s)-(−)-squalene-2,3-epoxide. Agric. Biol. Chem. **45**:1741–1742.

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