

## Biosynthesis of Bile Acids in a Variety of Marine Bacterial Taxa

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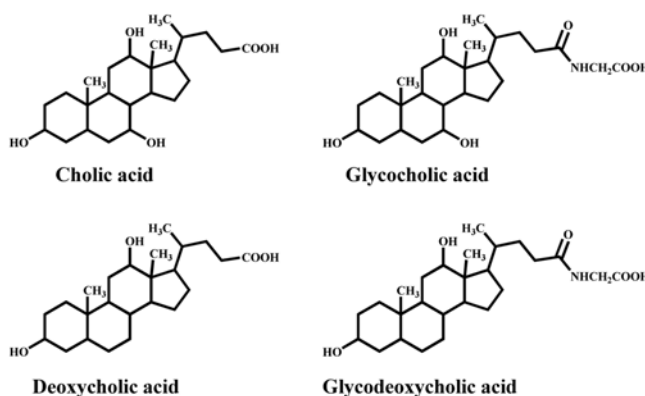
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**Abstract** Several marine bacterial strains, which were isolated from seawater off the island Dokdo, Korea, were screened to find new bioactive compounds such as antibiotics. Among them, *Donghaeana dokdonensis* strain DSW-6 was found to produce antibacterial agents, and the agents were then purified and analyzed by LC-MS/MS and 1D- and 2D-NMR spectrometries. The bioactive compounds were successfully identified as cholic acid and glycine-conjugated glycocholic acid, the 7 $\alpha$ -dehydroxylated derivatives (deoxycholic acid and glycodeoxycholic acid) of which were also detected in relatively small amounts. Other marine isolates, taxonomically different from DSW-6, were also able to produce the compounds in a quite different production ratio from DSW-6. As far as we are aware of, these bile acids are produced by specific members of the genus *Streptomyces* and *Myroides*, and thought to be general secondary metabolites produced by a variety of bacterial taxa that are widely distributed in the sea.

**Keywords:** Cholic acid, glycocholic acid, bile acid, 7 $\alpha$ -dehydroxylation

Bile acids are principal ingredients of bile synthesized in the liver of vertebrates such as mammals and fish, and secreted into the duodenum to play an important role in lipid metabolism. They are hydroxylated carboxylic acids, having a steroid skeleton and carboxylic side chain. A variety of natural bile acids have been reported that are different in the number and substituted position of the hydroxyl group and the side chain length [1, 2].

Some of the hydroxylated bile acids such as C24 cholic acid (CA), chenocholic acid (CCA), and deoxycholic acid (DCA) are widely distributed within mammals, including



**Fig. 1.** Chemical structure of bile acids and 7 $\alpha$ -dehydroxylated derivatives.

human, dog, rat, and guinea pig, and thought to be emulsifiers that help to digest and absorb water-insoluble fat [12]. CA, CCA, and DCA are conjugated to glycine, producing glycocholic acid (GCA), glycochenocholic acid (GCCA), and glycodeoxycholic acid (GDCA) (Fig. 1), respectively, which are required for the proper digestion and absorption of cholesterol, lipids, and other lipid-soluble compounds [18].

Although many bioactive compounds other than bile acid and its derivatives have been reported in prokaryotes [7, 8, 10, 14], few reports are available for bile acids and its derivatives. As the first report on bile acid biosynthesis, a facultative anaerobic *Streptomyces faecium* AN-21 has been reported to produce 3,7,12-trihydroxy-24-choleic acid methylester (methylcholate), which exhibits antibacterial activity against Gram-positive and Gram-negative bacteria [15]. The marine bacterium *Myroides* sp. strain SM1 produces surface-active bile acids (CA, DCA, GCA, and GDCA), using a biosynthetic conversion of cholesterol to CA [12]. Interestingly, the human intestinal anaerobic bacteria *Eubacterium* sp. strain VPI 12708 [4] and *Clostridium* sp.

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strain TO-931 [17] are known to dehydroxylate CA on the 7 $\alpha$  position, resulting in DCA, which is more toxic to the host and has been implicated in colon carcinogenesis, although CA is also provided by the human host. Coleman and coworkers initially proposed a new pathway for 7 $\alpha$ -dehydroxylation in *Eubacterium* VPI 12708 [3]. The above description reveals that the bile acid biosynthesis of prokaryotic cells is presently limited to specific members of the genera *Streptomyces* and *Myroides*.

## MATERIALS AND METHODS

### Structural Identification of Antibiotic Metabolites

Marine bacterium *Donghaeana dokdonensis* strain DSW-6, grown on Difco Marine Broth 2216 (MB) agar plate [21], was cultured in 50 ml of MB liquid for 48 h at 25°C. The culture (50 ml) was transferred to 2 l of fresh MB liquid in a 5-l baffled flask, and further incubated for 48 h at 25°C with vigorous shaking. The total of 12-l orange-colored supernatant was concentrated to 2 l with a rotary evaporator (55°C), and then extracted once with ethyl acetate to remove organic solvent-extractable metabolites. The remaining cell culture was extracted with water-saturated *n*-butanol, and the organic extract was evaporated. The dried metabolites (2.7 g) were dissolved in methanol, and separated through a Solid-Phase Extraction Kit (Waters Sep-Pak Vac C18 cartridge). A 80% methanol fraction (0.36 g), showing antibacterial activity against *E. coli* strain KCTC 1924, was collected, and further analysis of the fraction by LC-MS/MS, 1D-NMR (<sup>1</sup>H and <sup>13</sup>C), and 2D-NMR (<sup>1</sup>H-<sup>1</sup>H COSY, HSQC, and HMBC) techniques was carried out.

### Bile Acid Production by Other Marine Bacteria

Bacterial strains used in the present investigation are listed in Table 1. The strains grown on MB agar plate were inoculated in 50 ml of MB liquid, and cultured for 48 h at

each strain's optimal temperature: 25°C for DSW-5 and DSW-6, and 30°C for DSW-1, DSW-8, KCTC 2396, DSM 43752, and DK17. A fraction of the culture (1.25 ml) was transferred to 400 ml of fresh MB liquid in a 1-l baffled flask, further cultivated for 48 h at the proper temperature with vigorous shaking, and centrifuged at 10,000  $\times g$  for 20 min. The culture supernatants were acidified to pH 2.0 with 6 N HCl, extracted with an equal volume of ethyl acetate, and evaporated [12]. The dried extracts (23.0–90.6 mg) from the tested strains were dissolved in 50% methanol (a final concentration of 5 mg/ml), and a 10- $\mu$ l aliquot was analyzed by LC-MS/MS technique. To quantify bile acids in the methanol extracts by using mass spectroscopy, each reference bile acid [products of Sigma-Aldrich (U.S.A.)] at different concentrations was used for plotting the standard graph versus peak area at *m/z* 466 ([M+H]<sup>+</sup>; molecular weight of GCA, 465.6), *m/z* 450 ([M+H]<sup>+</sup>; GDCA, 449.6), *m/z* 409 ([M+H]<sup>+</sup>; CA, 408.5), or *m/z* 393 ([M+H]<sup>+</sup>; DCA, 392.5) in LC-ESI-MS. The quantity of each bile acid was determined from the corresponding standard graph.

### LC-MS/MS Analysis

Liquid chromatography mass spectrometry (LC-MS) was performed using a Finnigan LCQ Advantage MAX ion trap mass spectrometer (Thermo Electron Co., U.S.A.) equipped with an electrospray ionization (ESI) source. Separation by HPLC was performed on a Finnigan Surveyor Modular HPLC Systems (Thermo Electron Co.), using a XTerra MS C18 column (5  $\mu$ m, 2.1  $\times$  150 mm, Waters, Ireland). Mobile phase A was water and mobile phase B was acetonitrile, and both contained 0.1% formic acid. Gradient elution at a flow rate of 0.2 ml/min was carried out as follows: 0–30 min with 10–100% B (linear gradient) and 30–50 min with 100% B (isocratic). Full-scan mass spectra were obtained in the positive ion mode at range *m/z* 50–700. Data-dependent tandem mass spectrometry (MS/MS) experiments were

**Table 1.** Lineage and isolation site of bacterial strains.

Strain	Lineage	Isolation Site	Reference
DSW-1	<i>Flexibacteraceae</i> ; <i>Dokdonia donghaensis</i> gen. nov., sp. nov.	Sea water off the island Dokdo, Korea	[18]
DSW-5	<i>Flavobacteriaceae</i> ; <i>Polaribacter dokdonensis</i> sp. nov.	Sea water off the island Dokdo, Korea	Unpublished
DSW-6	<i>Flavobacteriaceae</i> ; <i>Donghaeana dokdonensis</i> gen. nov., sp. nov.	Sea water off the island Dokdo, Korea	[19]
DSW-8	<i>Flavobacteriaceae</i> ; <i>Maribacter dokdonensis</i> sp. nov.	Sea water off the island Dokdo, Korea	[20]
KCTC 2396	<i>Hahellaceae</i> ; <i>Hahella chejuensis</i> gen. nov., sp. nov.	Marine sediment from the island Marado, Korea	[11]
DSM 43752	<i>Nocardiaceae</i> ; <i>Rhodococcus marinonascens</i> sp. nov.	Marine sediment (1,400 m) from northeastern Atlantic Ocean	[6]
DK17	<i>Nocardiaceae</i> ; <i>Rhodococcus</i> sp.	Oil-contaminated soil from Yeochon, Korea	[9]

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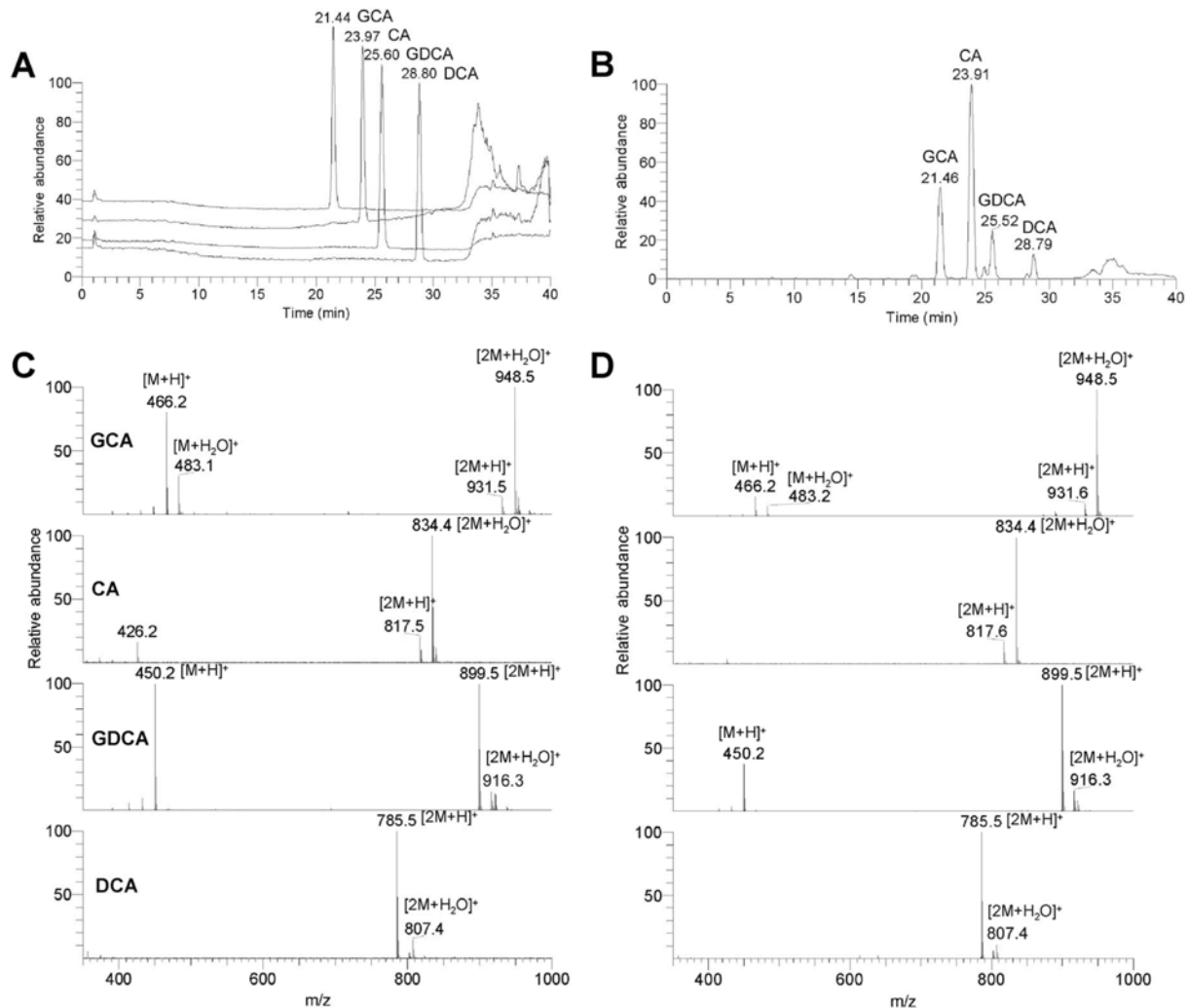
## RESULTS

### Structural Identification of Antibiotic Metabolites

*n*-Butanol extract of *D. dokdonensis* DSW-6 culture showed antibacterial activity against *E. coli* KCTC 1924. A 80% methanol fraction with antibiotic activity, obtained through a solid-phase extraction column, was analyzed by LC-MS/MS. Two major peaks with molecular weights of  $m/z$  466 ( $[M+H]^+$ ) and 409 ( $[M+H]^+$ ) appeared at 21.46 and 23.91 min, respectively, in the total ion chromatogram. Their fragmentation pattern and retention time were identical to those of authentic bile acids GCA and CA,

respectively (Figs. 2B and 2D), which had previously been reported to be produced in the marine bacterium *Myroides* sp. strain SM1 [12]. The two metabolites, which were purified through an HPLC C18 column, were further confirmed to be GCA and CA, respectively, by  $^{13}\text{C}$ -NMR analysis: GCA from DSW-6, [13.0, 17.8, 23.2, 24.2, 27.9, 28.7, 29.6, 31.2, 33.1, 34.1, 35.9, 36.5, 37.0, 40.5, 41.0, 43.0, 43.2, 47.5, 48.0, 69.0, 72.9, 74.0]; CA from DSW-6, [13.0, 17.6, 23.1, 24.2, 27.9, 28.6, 29.6, 31.2, 32.3, 35.8, 35.9, 36.5, 36.8, 40.4, 41.0, 43.0, 43.2, 47.5, 48.0, 69.0, 72.9, 74.0].

Additionally, in an initial LC-MS/MS analysis of the 80% methanol fraction containing GCA and CA, two other peaks with parent ion  $m/z$  values of 450 and 393 were concurrently detected at retention times slightly different than those of GCA and CA (25.52 and 28.79 min,



**Fig. 2.** MS/MS spectral analysis of bile acids produced by *Donghaeana dokdonensis* strain DSW-6.

Total ion chromatogram and mass spectrum of authentic bile acids (A and C) and four DSW-6-produced bile acids (B and D) resolved by HPLC at indicated retention time: GCA, glycocholic acid at 21.46 min; CA, cholic acid at 23.91 min; GDCA, glycodeoxycholic acid at 25.52 min; DCA, deoxycholic acid at 28.79 min.

**Table 2.** Quantification of the bile acids produced by bacterial strains.

Strain	Concentration±SD (µg/ml) <sup>a</sup>			
	Cholic acid	Deoxycholic acid	Glycocholic acid	Glycodeoxycholic acid
DSW-1	52.0±0.8	11.6±0.7	5.5±0.6	N.D. <sup>b</sup>
DSW-5	83.6±2.1	13.6±0.4	50.7±1.4	130.5±0.7
DSW-6	64.6±0.4	13.0±0.8	23.5±0.7	76.9±0.1
DSW-8	114.2±0.7	53.6±0.1	10.9±3.1	5.1±0.9
KCTC 2396	13.0±0.4	1.1±0.9	52.9±0.7	34.7±1.0
DSM 43752	2.4±0.7	N.D.	3.9±0.7	6.2±1.0
DK17	N.D.	N.D.	N.D.	N.D.

<sup>a</sup>Each value represents mean (±standard deviation) of triplicate experiments.

<sup>b</sup>Not detected.

respectively) (Fig. 2B). Moreover, the *m/z* values of these parent ions were most likely derived by dehydroxylation reaction once on the steroid skeleton, having three hydroxyl groups: *m/z* 450 [M+H-O]<sup>+</sup> from *m/z* 466 [M+H]<sup>+</sup>; *m/z* 393 [M+H-O]<sup>+</sup> from *m/z* 409 [M+H]<sup>+</sup>. Indeed, their fragmentation pattern and retention time were identical to those of authentic bile acids GDCA and DCA, respectively (Figs. 2B and 2D), whose production in *Myroides* sp. strain SM1 has also been reported [12].

#### Production Profile of Bile Acids in Marine Bacteria

The productivity of bile acids by other marine bacteria, Korea isolates, was quantified by LC-ESI-MS spectrometry, and compared with each other. As shown in Table 2, although taxonomically different from DSW-6, all the marine bacteria tested (Table 1) produced at least three kinds of bile acids. In addition to the Korea isolates, *Rhodococcus marinonascens* DSM 43752, which was isolated from a marine sediment (1,400 m) in northeastern Atlantic Ocean, was also able to produce three kinds of bile acids. Interestingly, *Rhodococcus* sp. DK17, a negative control [9] and a Korea terrestrial isolate, was not able to produce these bile acids at all. Therefore, the present experimental data indicate that these bile acids are produced by a variety of bacterial strains that are widely distributed in seawaters around the world, including the Korean Peninsula. However, the type and the relative amount of bile acids varied among the strains (Table 2), indicating that the production is dependent on both bacterial strains and culture conditions such as growth medium and temperature.

#### DISCUSSION

Since the first report on the biosynthesis of bile acid in prokaryotic *Streptomyces faecium* AN-21 (3,7,12-trihydroxy-24-cholanic acid methylester) in 1995 [15], there has been only a single additional report that the marine bacterium *Myroides* sp. strain SM1 produces four kinds of bile acids

(CA, DCA, GCA, and GDCA) [12]. Although *Myroides* sp. SM1 has been investigated for the biosynthesis of bile acids from cholesterol, which was added into culture media as a precursor, the detailed biosynthetic route from cholesterol to bile acids has not yet been experimentally clarified.

Thus, there are a few questions raised about the biosynthetic pathway and ecological advantage of bile acid production in prokaryotic cells. Since mammals produce CA and CCA from cholesterol through two previously known routes [13], and *Myroides* sp. SM1 is also able to biosynthesize CA from cholesterol, the biosynthetic pathway in both eukaryotes and prokaryotes appears to be similar. Furthermore, some *M. odoratus* strains have been found in insect guts [16] and freshwater fishes [5], and therefore, bacterial bile acids might play a role in digestion and absorption of cholesterol, lipids, and other lipid-soluble compounds. Finally, the question of whether coexistence with these eukaryotes might be a symbiotic relationship remains to be clarified.

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