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Research paper

Search and discovery of actinobacteria capable of transforming deoxycholic and cholic acids

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ABSTRACT

The capability of 54 selected actinobacteria strains of different phyla to convert deoxycholic (DCA) and cholic (CA) acids under aerobic conditions was studied. Except for the two species, the strains did not grow on DCA (1 g/l) as a sole carbon source, but some of them effectively converted DCA performing 7β- and 9α- hydroxylation, 3α- and 12α-dehydrogenation, partial cleavage of the isoprenoic side chain and Δ^4-dehydrogenation. Ursocholic acid, 9α-hydroxy-3,12-dioxo-23,24-bisnorchol-4-ene-22-oic acid, 3-keto-DCA and other end metabolites had been firstly identified in the actinobacteria strains. The total yield of 12α-hydroxy-3-oxo-chol-4-ene-24-oic and 3,12-dioxochol-4-ene-24-oic acids from DCA with *Rhodococcus erythropolis* VKM Ac-1152 reached 95%. Almost 80% DCA were converted to 9α-hydroxy-3,12-dioxo-23,24-bisnorchol-4-en-22-oic acid by *Rhodococcus* sp. MTS-77. Unlike DCA, cholic acid (CA) was confirmed to be a growth substrate for majority of the examined strains, but only three *Rhodococcus* strains exhibited 7α- and/or 12α-HSDH activities thus forming 7-keto-DCA and 12-keto-chenodeoxycholic acid as major products from CA. Steroid metabolites were identified by TLC, GC, MS, ^{1}H- and ^{13}C NMR analyses.

The results may contribute to the knowledge of biocatalytic potential of diverse soil-dwelling actinobacteria towards bile acids, and could be applied at the development of novel bioprocesses for production of the valuable cholanic acids.

1. Introduction

Actinobacteria are known to possess diverse steroid-transforming activities and are capable of performing different modifications of steroid molecule, such as hydroxylation, introduction of double bonds, oxidation of carbonyl groups, reduction of steroid alcohols, de-esterification, partial, or full oxidation of the aliphatic side chains at C-17, and others [1]. Actinobacteria may transform broad spectrum of both natural and synthetic steroid substrates: 3-oxo- and 3β-hydroxy steroids of pregnane and androstane series, as well as sterols (phytosterol, cholesterol, ergosterol) and their derivatives [2–4].

Bile acids (BAs) differ from other steroids by saturated cyclopenthanoperhydrophenantrene nucleus showing *cis*-A/B ring juncture and C5-acyl side chain at C-17, being hydroxylated C_{24} 3α-hydroxy-5β-*H*-cholanoic acids. Due to their amphipathic features, BAs are the powerful surface-active compounds that are essential in the digestion and re-sorption of fats, fatty acids and lipid-soluble vitamins in the digestive tracts of vertebrates. BAs also participate in cholesterol balancing, apoptosis, colonic salvage, and serve other important physiological functions [5–7]. BAs may enter the environment in big amounts by extraction, and accumulate as multi-tons wastes during cattle and swine production [8]. Industrial wastes of ursodeoxycholic acid (UDCA) production from bile may also contain deoxycholic acid (DCA), which is a hazardous environmental pollutant [9].

The reports on microbial transformation of BAs mainly relate to commensal and pathogenic microorganisms in the human gastrointestinal tract [10]. Intestinal and soil anaerobic bacteria

Abbreviations: BA, Bile acid; DCA, deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid); CA, cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid); LCA, lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid); UCA, ursocholic acid (3α,7β,12α-trihydroxy-5β-cholan-24-oic acid); CDCA, chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid); 3-keto-DCA, (12α-hydroxy-3-oxo-5β-cholan-24-oic acid); 3α-HSDH, 3α-hydroxysteroid dehydrogenase; 3β-HSDH, 3β-hydroxysteroid dehydrogenase; 12α-HSDH, 12α-hydroxysteroid dehydrogenase.

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are capable of deconjugation, desulfation, oxidation and epimerization of hydroxyl groups at C-3, C-7 and C-12, as well as 7-dehydroxylation, and esterification of BAs [11,12]. In spite of very few bacteria can synthesize BAs [13], many aerobic bacterial species are able to grow on BAs as sole carbon and energy sources.

Cholate degradation has been best studied in *Pseudomonas* sp. strain Chol1 [14] and *Rhodococcus jostii* RH11 [15]. The well-known catabolic route includes steroid core degradation *via* the 9,10-secosteroid pathway releasing intermediates with a 3-keto-$\Delta^{1,4}$-diene structure of the steroid skeleton [16]. As shown by genomic analysis, the 9,10-secosteroid pathway for cholate degradation was conserved in members of *Rhodococcus* genus, while patchy distributed among *Proteobacteria* [17].

The existence of distinct cholate degradation pathway has been demonstrated for actinobacteria of *Dietzia* sp. strain Chol2 which degraded cholate via the intermediates with a 3-keto-$\Delta^{4,6}$-diene-7-deoxy structure of the steroid skeleton such as 3,12-dioxo-4,6-choldienoic acid [18].

Biotransformation of cholic acid (CA) and deoxycholic acid (DCA) may be a method of choice for production of valuable pharmaceuticals and their precursors [19], as well as for the obtaining of the compounds which are essential for cosmetics, material science and environmental cleansing [20]. The products of dehydroxylation, oxidation of hydroxyl groups, side chain degradation, as well as regio- and stereospecific hydroxylation are of potential practical importance. For instance, microbial 7β-hydroxylation of lithocholic acid (LCA) results in ursodeoxycholic acid (UDCA) which is widely used in medicine for treatment of cholestasis, sclerosing cholangitis, hepatitis and cirrhosis [21–23]. Recently, we have reported effective method for UDCA production that provides the yield of almost 90% [24]. Chemoenzymatic routes from CA to UDCA were recently reviewed [25].

Some proteobacteria were shown to convert CA. For example, the strains of *Xanthomonas*, *Pseudomonas* and *Acinetobacter* genera transformed CA to a mixture of 3α,12α-dihydroxy-7-oxo-cholanic, 3α-hydroxy-7,12-dioxo-cholanic and 3,7,12-triketo-cholanic acids [26]. Under anoxic conditions *Pseudomonas* sp. NCIB 10590 modified CA mainly to 12β-hydroxy-androsta-4,6-diene-3,17-dione while 12α-hydroxy-3-oxochola-4,6-dien-24-oic acid was detected in minor amounts [27].

Certain proteobacteria, mainly, pseudomonads, and anaerobic bacteria of *Clostridium*, *Eubacterium* and *Bifidobacterium* genera were reported to oxidize hydroxyl group at C-3 [16]. Few *Pseudomonas* sp. mutant strains were selected capable of accumulating 12α- and 12β-hydroxyandrosta-1,4-diene-3,17-dione stereoisomers, and 3α,12β-dihydroxy-9(10)-secoandrosta-1,3,5(10)-triene-9,17-dione as major products from DCA [28]. *Rhodococcus ruber* transformed CA and DCA to 3α,7α,12α-trihydroxy-9-oxo-9,10-seco-23,24-bisnorchola-1,3,5(10)-trien-22-oic acid and 3,12α-dihydroxy-9-oxo-9,10-seco-23,24-bisnorchola-1,3,5(10)-trien-22-oic acid, correspondingly [29].

In spite of the progress in the field of steroid microbial conversion which was observed at the past decade, the discovery of the suitable strains capable of performing desired reactions is still a challenge, and along with bioinformatics approach, traditional experimental screenings are necessary.

In our previous work, the strains of actinobacteria capable of regio- and stereospecific hydroxylation of LCA at positions 7α, 7β and 12α and those with 3α-hydroxysteroid dehydrogenase (3-HSDH) activity were revealed on the base of a broad screening [24].

In this study, we investigated the ability of selected actinobacteria of different taxonomy belongings to transform DCA and CA, identified key metabolites formed and revealed the most active biocatalysts that can be exploited further for the production of potentially physiologically active cholanic acids.

2. Experimental

2.1. Materials

Deoxycholic acid (3α,12α-dihydroxy-5β-cholanic acid, DCA), lithocholic acid (3α-hydroxy-5β-cholanic acid, LCA) and ursodeoxycholic (3α,7β-dihydroxy-5β-cholanic acid, UDCA) acids were obtained from ACROS Organics (USA). Cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid, CA); ursocholic acid (3α,7β,12α-trihydroxy-5β-cholan-24-oic acid, UCA); chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid, CDCA); 3-keto-deoxycholic acid (12α-hydroxy-3-oxo-5β-cholan-24-oic acid, 3-keto-DCA), 7-keto-deoxycholic acid (12α-hydroxy-7-oxo-5β-cholan-24-oic acid, 7-keto-DCA) and 12-keto-chenodeoxycholic acid (3α,7α-dihydroxy-12-oxo-5β-cholan-24-oic acid, 12-keto-CDCA) were kind gifts from Prodotti Chimici e Alimentari S.p.A. (Basaluzzo, Italy). Randomly methylated β-cyclodextrin (RAMEB) was purchased from Wacker Chemie (Germany); yeast extract - from Difco (USA), cornsteep solids - from Sigma-Aldrich (USA). Methoxyamine hydrochloride, *N,O-bis*(trimethylsilyl)trifluoroacetamide, *N*-trimethylsilylimidazole, trimethylchlorosilane and pyridine were purchased from SERVA (Germany). All other reagents were of the best purity grade from commercial suppliers (Russia).

2.2. Microorganisms

The strains were obtained from All-Russian Collection of Microorganisms (VKM IBPM RAS) and working collection of Laboratory of Microbiological Transformation of Organic Compounds (MTOC) at G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (IBPM RAS).

2.3. Growth of strains on DCA, or CA as sole carbon sources

1 mL of cell suspension containing 1×10^6 CFU were sprayed onto agar plates with mineral medium composed of (g/L): KH_2PO_4 – 1.0; $(NH_4)_2SO_4$ –3.0; $MgSO_4$ – 0.2; $FeSO_4$ – 0.01; $ZnSO_4$ – 0.002; pH 7.0 supplemented with either DCA (1 g/L), or CA (1 g/L). The plates were incubated for 5–7 days at 30 °C. For each strain, the controls without BAs were used to quantify any background growth.

2.4. Bioconversion

The strains were grown in a nutrient medium (50 mL) containing (g/L): glucose – 7, yeast extract – 4.5, corn steep solids – 5, $CaCO_3$ – 0.05, dissolved in distilled water (pH 7.0). For DCA, or CA bioconversion, 5% (v/v) of the seed culture was inoculated in the transformation medium (medium A, or B) to a final volume of 100 mL. Medium A was composed of (g/L): glycerol – 5; KH_2PO_4 – 1, K_2HPO_4 – 4, $(NH_4)_2SO_4$ – 3, carbamide – 0.25, $MgSO_4$ – 0.2, $FeSO_4$ – 0.01, $ZnSO_4$ – 0.002; pH 7.0. Medium B of the same composition as medium A additionally contained RAMEB (6.7 g/L).

DCA, or CA (100 mg) was added as a hot methanol solution to a final concentration of 1 g/L. Final solvent concentration did not exceed 2% (v/v). Bioconversions were carried out aerobically in Erlenmeyer flasks (750 mL) on a rotary shaker (200 rpm) at 29 °C for 48–120 h.

2.5. Isolation of metabolites

Steroids were isolated from the culture medium by extraction with ethyl acetate (EtOAc). After the rotary evaporation

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