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Isolation and Identification of Exiguobacterium Profundum Strain from Cholesterol-Enriched Fetal Sample

Heqiang Yuan, Hangjie Fu, Chengru Wu, Yuxiao Li, Guorong Yang, Bin DING*

College of Life Sciences, Zhejiang Chinese Medical University, Hangzhou, Zhejiang 310053, China
**Corresponding Author*

ABSTRACT. *Background: Cholesterol is metabolized into various sterol hormones in living organisms. Excessive use of cholesterol results in high amounts of sterol hormones with adverse effects on environment. Objective: The aim of this study was to identify and isolate a bacteria strain for cholesterol degradation. Methods: Bacterial enrichment, cultivation, serial dilution and agar plating were used for bacterium isolation. Isolated bacteria were identified and characterized through morphological, molecular, physiological and biochemical methods. Further, multi substrate metabolism and drug sensitivity tests were carried out on isolated bacteria. Results: Sanger sequencing and BLAST analysis showed high homology (99.78%) of the isolated bacterial strain with Exiguobacterium profundum strain 10C. The isolated bacterial strain was, therefore named EP01. Further analysis showed the strain was rod or spherical shaped, gram negative and peritrichous. In addition, the strain occurred in single or in pairs assembling bacterium, and produced orange or yellow clone on LB agar plate. The strain required a temperature range of 27-45 ℃ (optimum temperature was 37 ℃), pH range of 5-11 (optimum pH was 9), and NaCl range of 0% - 5% (optimal concentration of sodium chloride was 1%) for growth. The doubling time of the EP01 under the optimal conditions was 1.22h. EP01 showed positive catalase, amylase and alkaline protease activities. Further, EP01 tested positive for kinetics, hydrogen sulfide, gelatin hydrolysis and mannitol tests. On the contrary, tested negative for oxidase, urease, citrate, Tween-20, nitrate reductase and phenylalanine decarboxylase activities. EP01 metabolized glucose, xylose, raffinose, fructose and lactose. However, EP01 showed no growth on cholesterol, estradiol, testosterone, ergosterol, carbazole, phenanthrene, tetrachlorobiphenol A, 4-hydroxyphenylacetic acid, n-octanoic acid, n-nonanoic acid, decanoic acid, lauric acid, myristic acid, palmitic acid and arabinose as the only carbon source. Furthermore, EP01 was sensitive to 1μg/μL penicillin potassium, chloramphenicol and erythromycin, but not to metronidazole. Conclusion: EP01 is a Exiguobacterium profundum strain with strong alkaline resistance. EP01 can be used for environmental remediation to degrade contaminants.*

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KEYWORDS: *Exiguobacterium profundum, Isolation, Identification, Multi substrate experiment, Drug sensitivity test*

1. Introduction

Cholesterol is a cyclopentane polyhydrophenanthrene compound. Cholesterol consists of a stero nucleus, three six-carbon rings, one five-carbon ring, and an alkaloid side chain. Cholesterol structure is very stable and difficult to degrade in the environment due to the small functional groups and high hydrophobicity [1]. Cholesterol plays important roles in animals. It is a component of cell membrane and a precursor for biosynthesis of steroid hormones (such as estradiol, testosterone, glucocorticoid and halocorticoid), bile salts and vitamins [2]. However, eukaryotes cannot degrade cholesterol, therefore, it is discharged into the environment, and deposited in soil or water [3]. A previous study reports presence of cholesterol in a 558-million-year-old fossil [4]. Increase in livestock production and sterol hormone drug production, increases the negative impact of cholesterol and sterol hormones on the environment. China practices large-scale agriculture, animal husbandry and has high population, therefore, the country is at high risk of adverse effects from compounds such as cholesterol carried in feces [5]. Cholesterol and its derivatives are difficult to fully degrade, therefore, they are used as reference biomarkers for analysis of environmental pollutants [6-7].

Side chain and other functional groups of cholesterol can be degraded or transformed by microorganisms in the environment to form various steroid hormones [8-9]. Sterol hormones are a class of endocrine disruptors. Sterol hormones mainly affect their own balance, metabolism, immunity, reproductive development and other physiological processed by blocking, interfering or inhibiting synthesis and transport of hormones in eukaryotic organisms. Steroid hormones significantly affect health even at a very low concentration (ng/L) [10-13]. Therefore, it is very important to explore approaches for degradation of cholesterol in the environment. Bacteria are known to degrade cholesterol by oxidizing it to 4-cholesten-3-one through cholesterol enzyme. The aim of this study was to isolate cholesterol degrading bacterium from cholesterol enriched-sludge sample and explore efficiency and mechanism of cholesterol degradation.

2. Materials and Methods

2.1 Materials

2.1.1 Reagents and Medium

K_2HPO_4, NaH_2PO_4, $CaCl_2$, $MgCl_2$ and NH_4Cl were obtained from Shanghai Silian Chemical Plant. Cholesterol, testosterone, estradiol, ergosterol and phenanthrene were purchased from Shanghai Boao Biotechnology Co., Ltd. Glucose, xylose, raffinose, fructose, lactose, arabinose, tetrachlorobiphenol A and carbazole

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were sourced from Beijing Kangpu Huiwei Technology Co., Ltd. Further, n-octanoic acid, nonanoic acid, decanoic acid, lauric acid, myristic acid and palmitic acid were purchased from Wuhan Qiongge Company. 16S rDNA bacterial identification PCR kit was sourced from Bao Bioengineering Co., Ltd.

Inorganic salt medium (MS) consisted of 40 mmol/L Na_2HPO_4/KH_2PO_4, 0.1 mmol/L $CaCl_2$, 10 mmol/L NH_4Cl, 0.8 mmol/L $MgCl_2$, 7 vitamins and 8 trace elements.

LB medium was prepared using 2g peptone, 1g yeast powder, 2g sodium chloride and 200ml distilled water. Solid LB plate was obtained by adding 1% - 2% agar. MS and LB media were sterilized by autoclaving at 1×10^5 Pa for 20 min.

2.1.2 Main Instruments

Instruments used in this study included constant-temperature shaker, agarose gel electrophoresis instrument (Beijing Liuyi Company); autoclave, ultracentrifuge (Zhejiang Nader Scientific Instrument Co., Ltd.); super-clean workbench (Beijing Ataron Instrument Technology Co., Ltd.); full-wavelength spectrophotometer (British Biochrom Co., Ltd.); optical microscope (Carl Zeiss Stock Company); scanning electron microscope (Hitachi SU-8010 scanning electron microscope); ABI7500 PCR thermal cycler (Thermo Fisher Technology Co., Ltd.).

2.2 Methods

2.2.1 Isolation of Bacterium

Soil sample was enriched in MS medium with cholesterol as the single carbon source for 3 years. 1 ml of the enrichment media was diluted with normal saline to obtain 10^{-1}-10^{-6} gradient solution. The gradient solution was then coated on LB medium at pH 7 and cultured at 30 ℃. After growing single bacterial colony, 3-4 pieces were zoned on LB solid medium to obtain pure monoclonal bacterium. The colony was then picked and stored in a - 80 ℃ refrigerator.

2.2.2 Sanger Sequencing and Phylogenetic Tree Construction

After culturing the bacteria for 12-16 hours, the colony was collected. After DNA extraction, 16S rDNA was amplified following the PCR reaction kit provided by Takara company. Universal sequencing primers used in this study were 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). After denaturing at 94℃, annealing at 55℃, extending at 72℃ and cycling for 35 times, 16S rDNA sequence of the colony was amplified in vitro. Amplified products were sequenced by Zhejiang Tianke Biological Co., Ltd using the Sanger sequencing method. Homologous sequences of the 16S rDNA obtained after sequencing were retrieved from NCBI database. MEGA7.0 was used to construct a phylogenetic tree of these sequences using Neighbor-Joining method.

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2.2.3 Gram Staining and Electron Microscopic Observation

Target bacterium, Staphylococcus aureus and Escherichia coli were cultured overnight. After culturing, bacteria were stained with Gram's reagent, observed and photographed under oil microscope. Target bacteria were cleaned, fixed and sprayed gold, and observed and photographed under scanning electron microscope.

2.2.4 Optimum Conditions for the Experiment

Optimal temperature of the experiment was determined by inoculating the bacterium into LB medium and culturing at different temperatures to measure change in OD_{600} before and after culturing. Optimum pH of the experiment was determined by inoculating bacterium into LB medium with different pH to measure change in OD_{600} before and after culturing. Optimal salt tolerance test was determined by inoculating bacterium into LB medium containing different concentrations of NaCl to measure change in OD_{600} before and after culturing bacterium. Bacterium was cultured at the optimal temperature, pH and NaCl concentration conditions, and OD_{600} was determined.

2.2.5 Identification of Physiological and Biochemical Tests

Morphological, physiological and biochemical characteristics of the strain were analyzed following guidelines by the second edition of Laboratory Experiments Microbiology [14].

2.2.6 Substrate Metabolic Capacity Test

Bacterium was inoculated in MS medium with different substrates as the sole carbon source. Ability of the bacterium to metabolize the substrate was determined based on OD_{600} before and after culture and the turbidity change of the culture medium. Steroids, polycyclic and monocyclic compounds, medium and long chain saturated fatty acids, and sugars were used as substrates. All tests were carried out in triplicates for each substrate. Blank control groups were set without bacterium.

2.2.7 Drug Resistance Test

Round filter papers (6 mm in diameter) containing 1μg/μL penicillin potassium, erythromycin, chloramphenicol and metronidazole were pasted on the plate containing bacterium. After 18-24 h of culture, the size of inhibition zone was measured by cross-over method. Test for each antibiotic was carried out in triplicates.

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3. Results and Analysis

3.1 Colony and Morphological Observation

A bacterial strain named EP01 was isolated from cholesterol-enriched fetal sample. EP01 is a round-shaped, aerobic, gram negative bacteria (Fig.1 A) with orange or yellow color. The strain had no spores, was short rod or spherical shaped, occurred in pairs or single and showed a periflagellum (Fig.1 B).

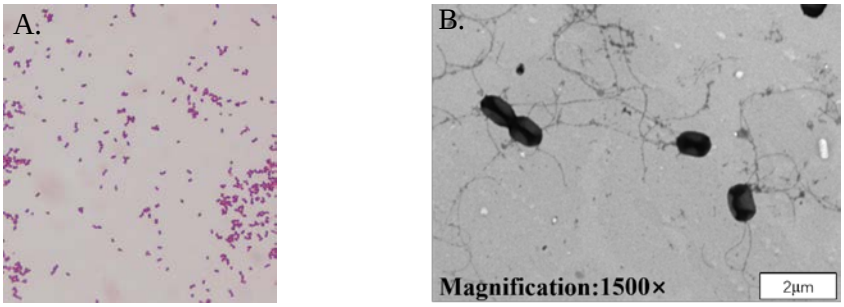

Fig.1 Gram Staining of Strain EP01(A), Morphology of Strain EP01 under SEM (B)

3.2 Phylogenetic Tree

Sanger sequencing and BLAST analysis showed that strain EP01 is highly homologous with E. profundum strain 10C (99.78%). A phylogenetic tree of EP01 and homologous sequences is shown in Fig. 2.

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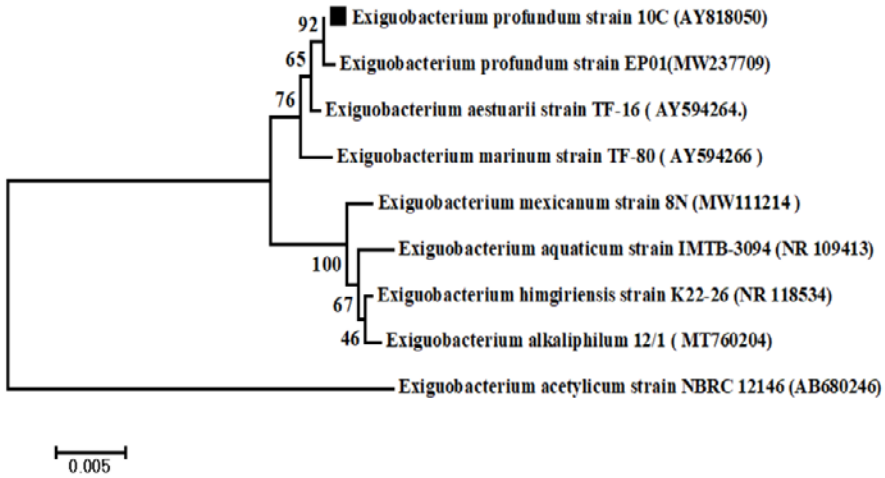

Fig.2 Phylogenetic Tree of 16S rDNA Gene Sequence of Strain EP01 and homologous sequences

3.3 3 Optimum Culture Conditions and Growth Curve

Change in OD_{600} (▲ OD_{600}) within the same time was used to determine rate of bacterial proliferation under optimal conditions. Proliferation temperature of EP01 ranged from 25℃ to 45℃, and the optimum growth temperature was 37℃ (Fig.3 A). Proliferation pH of EP01 ranged between pH 5 to 12, and the optimal culture pH was 8-9 (Fig.3 B). Optimal salt concentration for EP01 growth was 1% (Fig.3 C). The doubling time of EP01 was 1.22 h and the growth curve under optimum culture conditions is shown in Fig.3 D.

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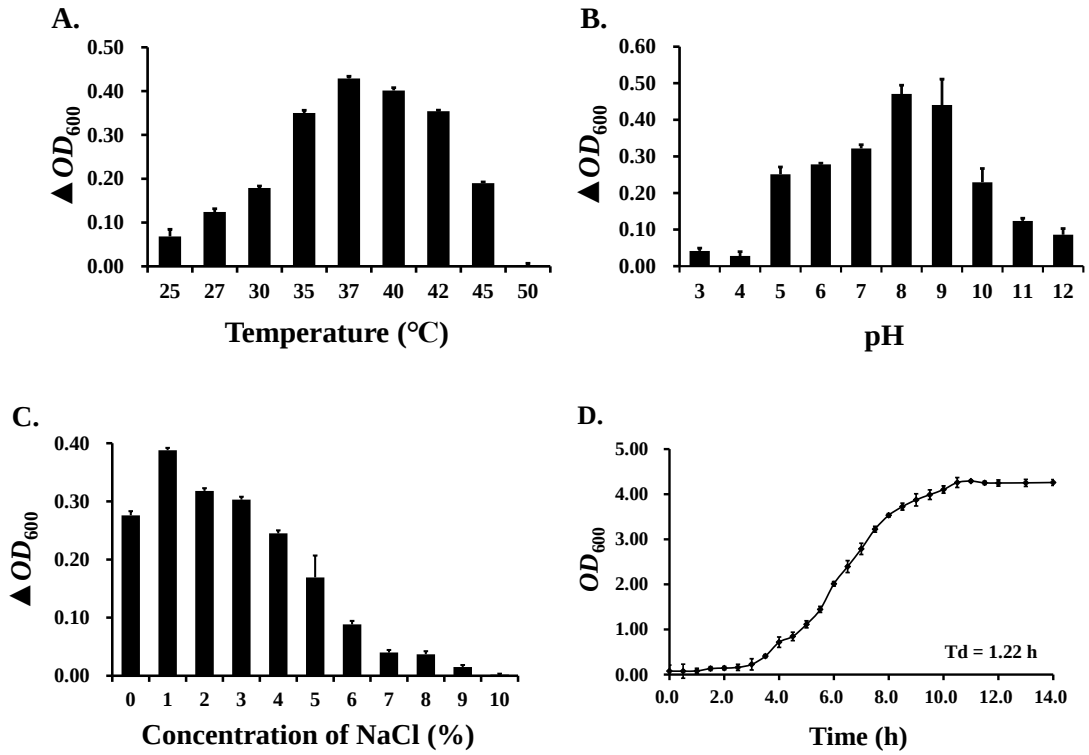

Figure.3 Optimum Growth Condition of Strain EP01 and Growth Curve
A.Optimum temperature B. Optimum pH C. Optimum Salt concentation D. Growth Curve under Optimum Conditions

3.4 Physiological and Biochemical Tests

Physiological and biochemical characteristics of EP01 as identified using Berger's bacteria identification manual are shown in Table 1.

Table 1 Biochemical and Physiological Characteristics of Strain Ep01

Experiment content	Result	Experiment content	Result
Oxidase	-	gelatinase	+
Catalase	+	citrate	-
urease	-	Mannitol	+
amylase	+	Tween-20	-
alkaline protease	+	nitrate reductase	-
Motility	+	Phenylalanine decarboxylase	-

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hydrogen sulfide	+	methyl red	+

"+" means positive result and "-" means negative result

3.5 Substrate Metabolism Test

Diversity in bacterial substrate determines distribution of bacteria in the environment. EP01 was inoculated into MS medium containing cholesterol, testosterone, estradiol, ergosterol, carbazole, tetrachlobisphenol A, phenanthrene 4-hydoxyphenyl acetic acid, n-caprylic acid, nonanoic acid, decanoic acid lauric acid, myristic acid, palmitic acid, glucose, xylose, raffinose, fructose, lactose and arabinose as sole carbon source to determine the effect of each substrate on metabolic capacity of the bacterium. The results were as shown in Table 2.

Table 2 Multi-Substrate Test Results of Strain Ep01

Substrate	Result	Substrate	Result	Substrate	Result
Cholesterol	-	4-hydroxypheny lacetic acid	-	Glucose	+
Testosterone	-	n-Caprylic acid	-	Xylose	+
Estradiol	-	Nonanoic Acid	-	Raffinose	+
Ergosterol	-	Decanoic Acid	-	Fructose	+
Carbazole	-	Lauric acid	-	Lactose	+
Tetrachlorobisphenol A	-	Myristic acid	-	Arabinose	-
Phenanthrene	-	Palmitic acid	-		

"+" means that the culture is significantly turbid and "-" means that the culture is not turbid.

3.6 Drug-Sensitivity Test

Results of drug sensitivity test showed that inhibition circles of penicillin potassium, erythromycin, chloramphenicol and metronidazole were 29.00±1.00 mm, 8.70±0.58 mm, 8.30±1.15 mm and 6.00±0.48 mm, respectively. The diameter of the filter paper was 6.00 mm, therefore, it showed that EP01 is sensitive to penicillin potassium, erythromycin and chloramphenicol, but not to metronidazole. Sensitivity of EP01 on the antibiotics decreased from penicillium to chloramphenicol in the order penicillin potassium> erythromycin> chloramphenicol.3.Discussion

A bacterial strain was isolated from the cholesterol-enriched fetal sample through gradient dilution and agar plating. Sanger sequencing and BLAST analysis showed that E. profundum strain EP01 was highly homologous with E. profundum strain 10C (99.78%). Strain EP01 is a short rod or spherical shaped, single or paired bacterium with periflagellum. The strain is an aerobic, gram negative bacterium with orange or yellow color. The strain grew at temperatures between 27-45℃, pH between 5-11 and 0% - 5% NaCl. Optimum growth conditions were a temperature of 37 ℃, pH of 9 and 1% salt concentration. The doubling time of the strain was

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1.22 h. The strain tested positive for catalase, amylase, alkaline protease, kinetics, hydrogen sulfide, gelatin hydrolysis and mannitol activities. On the contrary, EP01 tested negative for oxidase, urease, citrate, Tween-20, nitrate reductase and phenylalanine decarboxylase activities. In addition, EP01 grows in inorganic salt medium with glucose, xylose, raffinose, fructose and lactose as the sole carbon source. However, use of cholesterol, estradiol, testosterone, ergosterol, carbazole, phenanthrene, tetrachlorobisphenol A, 4-hydroxyphenylacetic acid, n-octanoic acid, n-nonanoic acid, decanoic acid, lauric acid, myristic acid, palmitic acid and arabinose as the sole carbon sources showed no growth of EP01 strain. The strain was sensitive to 1μg/μL penicillin potassium, chloramphenicol and erythromycin, but not to metronidazole and the sensitivity to antibiotics was in the order penicillin potassium> erythromycin> chloramphenicol.

The genus Exiguobacterium was first reported approximately a century ago. Members of Exiguobacterium are isolated from various environments such as the Yellow Sea tidal flat in South Korea [15], the sea mud in South China Sea [16], Greenland ice core [17], Siberian permafrost [18], plant rhizosphere soil [19] and atmospheric aerosol [20]. Currently, Exiguobacterium genus comprises of 18 species. Members of Exiguobacterium are characterized by heat resistance, cold resistance, alkali resistance and salt resistance. In addition, members of this genus are gram positive, acid resistant, active, facultative anaerobic, oxidase negative, catalase positive, have no spores, generally rod-shaped, and some strains have spherical shape in logarithmic or stable growth period [21-22]. Most of the colonies formed on nutrient medium have a regular round shape, and they are light yellow or orange in color under aerobic conditions, and the pigment does not diffuse. Some of colonies are milky white under anaerobic conditions [23-24].

E. profundum10C which showed the highest homology with EP01, was first discovered and named in 2007. Its optimum temperature for growth is 45℃, optimum pH is 7, and optimum sodium chloride concentration is 0% - 2% [25]. In contrast, EP01 is not as heat-resistant as E. profundum10C, however it showed more resistance to saline environment, therefore EP01 is an alkali-resistant bacterium. The two strains produces orange or yellow pigment. Furthermore, both strains are short rod-shaped and exist in pairs or singly. These characteristics are consistent with that of Exiguobacterium genus. However, E. profundum 10C and other bacteria of this genus are gram-positive [25], whereas EP01 is gram-negative bacterium. Gram staining shows bacterial cell wall components. Therefore, it is necessary to further explore cell wall components of EP01. EP01 tested positive for catalase and negative for oxidase, which is consistent with facultative anaerobe E. profundum 10C [25], suggesting that EP01 may be facultative anaerobic bacteria. EP01 produced amylase and alkaline protease, a characteristics of E. aurantiacum BK-P23 strain [26], therefore, EP01 can be used in fermentation industry to produce enzymes. E. profundum10C metabolizes various sugars under anaerobic and aerobic conditions. EP01 metabolized glucose, xylose, raffinose, fructose and lactose under aerobic conditions. Studies have shown that Exiguobacterium can degrade azo dyes, organic pesticides, petroleum and transform a variety of heavy metals, and promote growth of plants by acting as plant probiotic [27]. Results of multiple-substrates

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tests showed that EP01 does not metabolize sterols, monocyclic and polycyclic compounds, medium-chain and long-chain saturated fatty acids, so the role of EP01 in environmental remediation should be explored further. In conclusion, EP01 is an Exiguobacterium profundum strain with strong alkali resistance. It has a wide range of growth temperature and pH, therefore, it can adapt to extreme conditions. However, the role of EP01 in environmental remediation should be explored further.

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