MEDS Public Health and Preventive Medicine (2021) 1: 18-22
Clausius Scientific Press, Canada

DOI: 10.23977/phpm.2021.010103
ISSN 2616-1915

Isolation and Identification of Aquamicrobium Strains from a Fecal Contaminated Sludge Sample

Chengru Wu[1], Chunyan le[1], Tianwen Gao[1], Yingsong Zheng[2], Jinjun Ji[3*]

1.College of Life Science, Zhejiang Chinese Medical University, Hangzhou, Zhejiang, China

2.the First Clinical Medical College, Zhejiang Chinese Medical University, Hangzhou, Zhejiang, China

3. College of Basic Medicine, Zhejiang Chinese Medical University, Hangzhou, Zhejiang, China

**CorrespondingAuther*

Keywords: Cholesterol degradation, Bacterial isolation, Biochemical classification, Physiological classification, 16s rdna taxonomic study

Abstract: The metabolism of cholesterol by bacteria may impact human health directly and indirectly. To elucidating the degradation process of cholesterol is of great benefit to understand the relationship between environmental microbes and human health. Isolation and identification cholesterol-degrading microorganisms is a basic work to obtaining superior experimental materials. Serial dilution agar plating and agar plate streaking were used to obtain single and pure bacterial colony. Sanger sequencing, Gram staining, and some physiological and biochemical reaction assays were used to characterize isolated bacteria. Two strains, Aqu1 and Aqu2, were isolated and purified from a fecal contaminated sludge sample. The colonies of Aqu1 and Aqu2 were variety of milky white-color. Both strains were gram negative, spherical shaped Aquamicrobium genus bacteria. The 16s rDNA of Aqu1 and Aqu2 was highly identity to that of A. aestuarii and A. lusatiense, respectively.

1. Introduction

Cholesterol is a cyclopentane polyhydrophenanthrene compound, consisting of a steroidal nucleus and an alkane chain. This structure is very stable and difficult to degrade in the environment[1]. Cholesterol is a functional compound in cell membrane and blood plasma of vertebrates[2]. Otherwise, it is the major precursor of sterol/steroidal compounds in eukaryotes and can be metabolized to sterol hormones, bile salts, and vitamin D[3,4]. However, the eukaryotes do not have the ability to degrade cholesterol. The alkene chain of cholesterol can be used as carbon source by microorganisms. And the remaining steroidal nucleus is transformed into various sterol hormones[5]. These sterol hormones, even at extremely low concentrations, can interfere the growth and development of creatures in animalia[6-8]. Therefore, cholesterol has been used as a biomarker for environmental assessment[9]. Hence, studies of cholesterol degrading bacteria has received much attention in recent years. In current research, a fetal contaminant sludge sample was selectively incubated with 10% (g/v) cholesterol for years. And the bacteria were isolated and identified.

2. Materials and Methods

2.1 Materials

Peptone, yeast powder, sodium chloride, agar powder, and other chemical/biochemical material for medium preparation were purchased from Sinopharm Chemical Reagent Co., Ltd. Bacterial DNA extraction kit and PCR amplification kit were purchased from Takara Co., Ltd. Gram staining reagents were purchased from Zhejiang Tuzhi Pharmaceutical Technology Co., Ltd. SHZ-82 constant temperature shaker was obtained from Changzhou Guohua Electric Co., Ltd. ABI7500 PCR instrument was purchased from Thermo Fisher Scientific (China) Co., Ltd. Agarose gel electrophoresis instrument was purchased from Beijing Liuyi Instrument Factory. YS100 optical microscope was purchased from Nikon Corporation, Japan.

2.2 The Source of Cholesterol Enrichment

The sludge sample were collected from streamlet in Hupao spring park in Hangzhou, China. The fetal is from a 3 years old, healthy boy. This 1 g fetal and 10 g cholesterol were mixed with 100 mL sludge sample in a sterile rubber stopper bottle, and incubated at room temperature for 3 years.

2.3 Isolation of Microorganisms

The cholesterol-enriched sample was serial diluted with saline to obtain 1×10^{-1}-10^{-6} dilutions. 100 μL of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} dilutions were separately plated onto the LB agar, and grown in a 30°C incubator until a single colony appears. The representative colonies were picked up and cultured in the LB medium in a 30°C shaker at 160-180 rpm overnight. The broth was serial diluted and plated onto LB agar, as described. The purified colonies were obtained by repeated serial plating and streaking.

2.4 Identification of Bacteria

The purified colonies were fixed and stained with crystal violet firstly, then washed with iodine solution following by decolorization with 95% ethanol, counterstaining with saffron. After water washing, the colored colonies were air dried and observed under microscopy. The identification of physiological and biochemical reactions refers to the “Manual for Identification of Common Bacterial Systems”[10]. And mineral salt medium with vitamin and mineral metals were used for substrate utility tests. For 16s rDNA sequencing, the fresh grown bacterial strain was centrifugally collected, and the DNA was extracted with phenol/chloroform method. The PCR reagent assay was provided by Takara to amplify the bacterial 16s rDNA, with the universal primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The sequence of amplified sequence was identified with Sanger sequencing method by Zhejiang Tianke High-tech Development Co., Ltd. And the homologous sequences were derived by BLAST (https://blast.ncbi.nlm.nih.gov). And MEGA7.0 was used to construct a phylogenetic tree.

3. Results

3.1 Morphological Study of Purified Bacteria

Two strains were obtained, named Aqu1 and Aqu2. In LB agar, Aqu1 and Aqu2 showed round shape, smooth and moist, variety of milky white color colonies (Figure 1 A, B). Both of them were spherical and gram-negative bacteria (Figure 1 C, D).

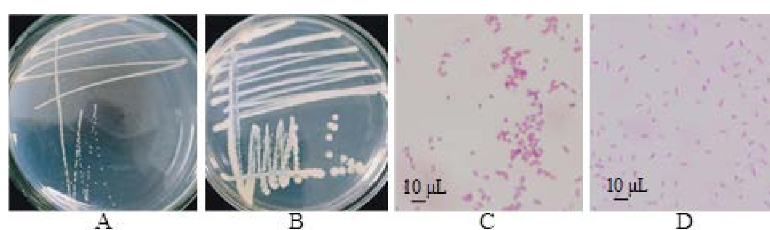

Fig.1 : Morphological Study of Aqu1 and Aqu2. a and C Are Colony and Bacterial Cell of Aqu1; B and d Are Colony and Bacterial Cell of Aqu2, Respectively.

3.2 Blast Result

BLAST analysis indicated that Aqu1 and Aqu2 belong to *Aquamicrobium* genus. The 16s rDNA sequence of Aqu1 is 99.4% identity to that of *A. aestuarii*, and Aqu2 is 100% identity to *A. lusatiense*. A phylogenetic tree was generated based on a binomial matrix, containing the data of 16 fragments (Figure 2).

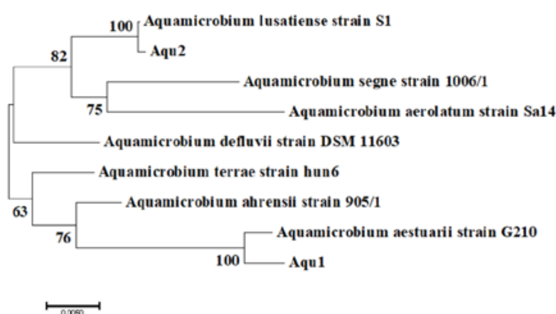

Fig.2 : Phylogenetic Tree

3.3 Physiological and Biochemical Characters

The two strains present identity characters in physiological and biochemical study. Only glucose can be used as carbon source by Aqu1 and Aqu2. Aqu1 and Aqu2 showed positive in oxidase and catalase test assays. The two strain can reduce thiosulfate to H_2S. And they have dynamic capability. The results of physiological and biochemical study were listed in Table 1.

Table 1 : Physiological And Biochemical Study of Aqu1 and Aqu2

Tested assay	Glucos	Lactose	Raffino	Arabin	Xylose	Oxidas	Catalas	Amylas	H_2S	Methyl	Citrate	Mannit	Tween	Dynami
Aqu1	+	-	-	-	-	+	+	-	+	-	-	-	-	+
Aqu2	+	-	-	-	-	+	+	-	+	-	-	-	-	+

Note: "+" means positive in reaction; "-" means negative in reaction

4. Discussion

In this study, Aqu1 and Aqu2, two *Aquamicrobium* strains were isolated and purified from a fetal contaminated sludge sample, which was cholesterol specifically enriched. Both Aqu1 and Aqu2 are spherical, gram-negative. The colony of Aqu1 is slightly smaller and lighter than that of Aqu2. The result of 16s rDNA sequencing and BLAST analysis indicated that Aqu1 and Aqu2 may be strains belong to *Aquamicrobium aestuarii* and *Aquamicrobium lusatiense* respectively. In five different carbohydrate substrates, only glucose can be used as carbon source to grow. Which means these

trains have a relatively narrow substrate spectrum. This character limits the distribution of these strain in environment. The oxidase and catalase positive means that Aqu1 and Aqu2 can grow aerobically. Hydrogen sulfide positive indicated various respiration model of the strains. Dynamics test positive illustrated that they may have flagella. These physiological and biochemical characters of Aqu1 and Aqu2 are complete identity.

The genus *Aquamicrobium* was first proposed by Bambauer et al. in 1998[11]. In comparison with *Phyllobactrium*, in Phyllobacteriaceae, it is still a relatively new bacterial species. Currently, 7 species in the genus were isolated and taxonomically identified, which are *A. defluvii*[11], *A. aerolatum*[12], *A. aestuarii*[13], *A. ahrensii*[14], *A. segne*[14], *A. lusatiense*[15], *A. soli*[16] and *A. terrae*[17]. *Aquamicrobium* is a gram-negative bacteria, aerobic bacteria, this genus is found in the activated sludge[11], air[12], chemical industry center[17] and Bohai Sea[18], can degrade thiophene-2-carboxylate[11], petroleum[18], polychlorinated biphenyls (PCB)[19], biphenyl[20], cyhalofop-butyl[21]. The species of the *Aquamicrobium* genus have been isolated from polluted environments such as sewage treated factory, active sewage sludge and biological filters. Some of degradation capability on the specific pollutants has been studied[11], which indicates that members of this genus may have potential ability for environmental reparation. But none previous studies elucidate the cholesterol modifying and catabolizing capability of the species of the *Aquamicrobium*. What is the function of *Aquamicrobium* in the environment and How *Aquamicrobium* influences human health are still unclear. The cholesterol degradation of Aqu1 and Aqu2 remains for further studied.

5. Acknowledgments

This study was financially supported by Hangzhou Zhimo Biotechnology Co., Lid.

References

[1] Bhatti H.N., Khera R.A. "Biological transformations of steroidal compounds: a review". Steroids, vol.77, no.12, pp.1267-90, 2012.

[2] Slaytor M., Bloch K. "Metabolic transformation of cholestenediols". Journal of Biological Chemistry. vol. 240, no. 12, pp.4598-602, 1965.

[3] Miller W.L. "Molecular biology of steroid hormone synthesis". Endocrine Reviews. vol. 9, no. 3, pp. 295-318, 1988

[4] Björkhem I., Eggertsen G. "Genes involved in initial steps of bile acid synthesis". Current Opinion in Lipidology. vol. 12, no. 2, pp.97-103, 2001.

[5] Wei S.T., Wu Y.W., Lee T.H., et al. "Microbial Functional Responses to Cholesterol Catabolism in Denitrifying Sludge". mSystems. vol. 3, no. 5, pp.e00113-18, 2018.

[6] Hudson S.B., Kluever B.M., Webb A.C., et al. "Steroid hormones, energetic state, and immunocompetence vary across reproductive contexts in a parthenogenetic lizard". General and Comparative Endocrinology. vol. 288, pp.113372, 2020. [online] Available: https://doi.org/10.1016/j.ygcen.2019.113372.

[7] Hoffman M., Mann L.K., Won J.H., et al. "Steroid Hormone Levels in Recipient Amniotic Fluid in Twin-Twin Transfusion Syndrome and Their Association with Preterm Delivery". American Journal of Perinatology. vol. 37, no. 6, pp.562-569, 2020.

[8] Houshdaran S., Oke A.B., Fung J.C., et al. "Steroid hormones regulate genome-wide epigenetic programming and gene transcription in human endometrial cells with marked aberrancies in endometriosis". PLoS Genetics. vol. 16, no. 6, pp.1-40, 2020. [online] Available: https://doi.org/10.1371/journal.pgen.1008601

[9] Veiga P., Juste C., Lepercq P., et al. "Correlation between faecal microbial community structure and cholesterol-to-coprostanol conversion in the human gut". FEMS Microbiology Letters. vol. 242, no. 1, pp.81-86. 2005.

[10] Oren A., Garrity G.M. "Then and now: a systematic review of the systematics of prokaryotes in the last 80 years". Antonie Van Leeuwenhoek. vol. 106, no. 1, pp.43-56, 2014.

[11] Bambauer A., Rainey F.A., Stackebrandt E., et al. "Characterization of Aquamicrobium defluvii gen. nov. sp. nov., a thiophene-2-carboxylate-metabolizing bacterium from activated sludge". Archive of Microbiology. vol. 169, no. 4, pp. 293-302. 1998.

[12] Kämpfer P., Martin E., Lodders N., et al. "Transfer of Defluvibacter lusatiensis to the genus Aquamicrobium as Aquamicrobium lusatiense comb. nov. and description of Aquamicrobium aerolatum sp. nov.". International Journal of Systematic and Evolutionary Microbiology. vol. 59, Pt10, pp.2468-2470. 2009.

[13] Jin H.M., Kim J.M., Jeon C.O. "Aquamicrobium aestuarii sp. nov., a marine bacterium isolated from a tidal flat". International Journal of Systematic and Evolutionary Microbiology. vol. 63, pp.4012-4017, 2013.

[14] Lipski A., Kampfer P. "Aquamicrobium ahrensii sp. nov. and Aquamicrobium segne sp. nov., isolated from experimental biofilters". International Journal of Systematic and Evolutionary Microbiology. vol. 62, pp.2511-2516, 2012.

[15] Fritsche K., Auling G., Andreesen J.R., et al. "Defluvibacter lusatiae gen. nov., sp. nov., a new chlorohenol-degrading member of the alpha-2 subgroup of proteobacteria". Systematic and Applied Microbiology. vol. 22, pp.197-204, 1999.

[16] Xu C.F., Zhang L., Huang J.W., et al. "Aquamicrobium soli sp. nov., a bacterium isolated from a chlorobenzoate-contaminated soil". Antonie Van Leeuwenhoek. vol. 110, pp.305-312, 2017.

[17] Wu Z.G., Wang F., Gu C.G., et al. "Aquamicrobium terrae sp. nov., isolated from the polluted soil near a chemical factory". Antonie Van Leeuwenhoek. vol. 105, no. 6, pp.1131-1137.2014.

[18] Wang X., Jin D., Zhou L., et al. "Draft Genome Sequence of Aquamicrobium defluvii Strain W13Z1, a Psychrotolerant Halotolerant Hydrocarbon-Degrading Bacterium". Genome Announcements. vol. 3, no. 4, pp.e00984-15. 2015. [online] Available: https://doi: 10.1128/genomeA.00984-15.

[19] Chang Y.C., Sawada K., Kim E.S., et al. "Whole-Genome Sequence of Aquamicrobium sp. Strain SK-2, a Polychlorinated Biphenyl-Utilizing Bacterium Isolated from Sewage Sludge". Genome Announcements. vol. 3, no. 3, pp.e00439-15. 2015. [online] Available: https://doi: 10.1128/genomeA.00439-15.

[20] Sugawara H., Koyama D., Sawada K., et al. "Purification and degradation characteristics of biphenyl degrading enzyme bphc from aquamicrobium sp. sk-2". Journal of Japan Society of Civil Engineers, Ser G (Environmental Research). vol. 71, no. 7, pp.III_413- III_419, 2015.

[21] Nie Z.J., Hang B.J., Cai S., et al. "Degradation of cyhalofop-butyl (CyB) by Pseudomonas azotoformans strain QDZ-1 and cloning of a novel gene encoding CyB-hydrolyzing esterase". Journal of Agricultural and Food Chemistry. vol. 59, no. 11, pp.6040-6046. 2011