JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Nov. 2013. Vol. **7**(Spl. Edn.), p. 735-740

Screening and Cultivation Conditions of a *Bacteroides* Strain from Tideland Soil

Quanquan Liu[1], Lei Yang[2] and Jingtang Zheng[1]*

[1]State Key Laboratory of Heavy Oil Processing, China University of Petroleum, Qingdao 266580, Shandong, P.R. China.
[2]Institute of Apiculture, Chinese Academy of Agricultural Sciences, Beijing 100093, P.R. China.

(Received: 27 September 2013; accepted: 04 November 2013)

A novel strictly anaerobic, heterotrophic bacterium, strain D-2, was isolated from the tideland soil of East China sea. This organism was identified as a member of the genus *Bacteroides* on the basis of its 16S rRNA gene sequence. Cells stained Gram-negative, were gram-negative, no-spore, appeared as irregular rod. Cells were 0.5 μm wide and 1–3 μm long, and occurred in clusters of two to four cells. Its optimum temperature, pH and NaCl concentration for growth were 37 ºC, 7.0 and 20 g·L^{-1}, respectively. Under the optimum growth conditions, the doubling time was approximately 91.8 min. Strain D-2 fermented a variety of simple and complex substrates such as glucose, cellobiose, ribose, raffinose(2 mM), lactose, xylan, arabinose and so on. The end products identified during growth on glucose were acetate, propionate, butyrate, CO_2, and H_2. The DNA G+C content of this organism was 43.9 mol%. Based on the phenotypic and phylogenetic characteristics, it is proposed that this organism represents a novel species in the genus *Bacteroides*. The Genbank Number is GU384203.

Key words: *Bacteroides*, Tideland Soil, Cellulose, Phylogenetic, Identification.

Cellulose as the most widely distributed on earth, the most abundant carbohydrate, its degradation is central to a natural carbon cycle. Therefore, it is significant to solve the world energy crisis, food shortages, environmental pollution and other issues. Cellulose have played an important role in many industries since it is used by human[1].

All the time, the cellulose degradation bacteria isolated place have mainly confined to land soil, making the source of cellulose degradation bacteria has been greatly restricted. Although only a few scholars have studied marine cellulose degradation microorganisms, it was shown that marine microbial has an attractive prospect[2]. This paper mainly study on the cellulose degradation bacteria which separated from tideland soil and the focus on cultivation conditions and characteristics of physiological and biochemical, provide a more broad space for the study of cellulose degradation bacteria.

Bacteroides are a gram-negative, no-spore, obligate anaerobic bacterium. Cells are ball-shaped, most no flagella, nitrate reduction negative, catalase negative, peptone or glucose decomposition and produce succinic acid, acetic acid, formic acid, lactic acid and propionic acid, etc. A few species produce dark pigment. The G+C content of the genomic DNA is 40-50%. According to the biochemical reaction and pigment, *Bacteroides* include 91 species and 5 subspecies. In the present paper, author describe a novel bacterium, strain D-2, isolated from tideland soil of

* To whom all correspondence should be addressed.
E-mail: jtzheng03@163.com

the East China Sea and study the physiological, biochemical properties and metabolism of strain D-2.

MATERIALS AND METHODS

Samples collection

Samples down to a 5-15cm depth were collected from tideland soil of the East China Sea (30°32' E, 120°81'N) and directly transported to the laboratory and stored at 4 ºC before use as a source of inoculum.

Cultivation Condition

Enrichments under anaerobic conditions were prepared using a modified Hungate technique[3]. For enrichment, the following medium was used (L^{-1} distilled water): $(NH_4)_2SO_4$ 2.0 g, KH_2PO_4 1.0 g, $MgSO_4$ 0.5 g, ascorbate 1.0g, NaCl 30 g, quantitative filter paper 2.0 g, peptone 2.0 g, yeast extract 1.0 g, Vitamin solution 10 mL, trace elements 10mL. Cellobiose and glucose were carbon source in identification medium and culture medium separately. The pH was adjusted to 7.8 by using 5 M KOH before autoclaving and the medium was dispensed into serum bottles with N_2/CO_2(4 : 1, 150 kPa) in the headspace. $NaHCO_3$ were injected from sterile stock solutions to final concentrations of 0.5% before inoculation. After incubation without shaking at 25 ºC until filter paper begins to be disintegrated. Fresh culture (5mL)was then transferred anaerobically into a new bottle of sterile medium. Single colonies were picked and purified by repeated use of the Hungate roll-tube method [4]. The purity of each culture was checked by microscopic examination.

Morphology observation

An Olympus BH-2 phase-contrast microscope with Olympus OM-2 camera were used routinely to observe cells. An Amray-1000B scanning electron microscope was used to observe the microstructure of strain D-2[5, 6]. Gram reaction was determined and susceptibility tests were in PBS buffer, modified as described by Boone & Whitman [7].

Phylogenetic analysis

Genomic DNA was isolated and purified by using the method of Marmur[8] as modified by Jarrellet[9]. The 16S rRNA gene was selectively amplified with a TaKaRa Thermocycler Dice TP600, using a TaKaRa 16S rDNA Bacterial Identification PCR kit (TaKaRa). The universal primer 27f/1492r [10] was used for 16S rRNA gene amplification. PCR products were purified with a TIANgel Midi Purification Kit and sequenced with an ABI 3770 sequencer. The partial 16S rRNA gene sequences (1411 bp) were compared with reference published sequences in the GenBank database by using the BLAST program[11], and then aligned with closely related sequences in the order *Bacteroides* by using CLUSTAL_X software [12]. The phylogenetic tree constructed via the neighbour-joining method in MEGA 5.2[13]. Bootstrap values were calculated after 1000 replications[14].

The G+C content of the genomic DNA

The G+C content was determined by the thermal-denaturation method[15], using a Beckman DU 800 spectrophotometer. Escherichia coli K12 DNA was used as a reference.

Physiological and biochemical characteristics

Growth was monitored by measuring the increase of the OD_{600} with a UV/Visible Beckman DU800 spectrophotometer. Effects of pH and temperature on strain D-2 were determined in triplicate in basal medium with glucose(120 mM). The pH range for growth was determined in the culture medium with various buffers at a concentration of 10 mM [16]. For studies of NaCl requirements, NaCl was weighed directly in the tubes before the medium was dispensed. To investigate the sensitivity of strain D-2 to antibiotics, rifampicin, acheomycin, kanamycin, streptomycin, ampicillin, chloramphenicol and erythromycin (all at 100 $\mu g \cdot mL^{-1}$) were added to sterile basal medium with glucose (120 mM) at 25 ºC. Growth was observed on complex substrates such as beef extract, peptone and trypticase. The utilization of single substrates was studied using basal medium supplemented with 0.01 % yeast extract and the test carbon source. Yeast extract was required for growth with carbohydrates. The basic medium was supplemented with vitamin and trace mineral solutions (5 $mL \cdot L^{-1}$ each) [17]. To confirm growth on any of the substrates, the novel isolate was cultured third in the same medium.

RESULTS

Strain D-2 was a strictly anaerobic, fermentative organism. Cells of isolate D-2 appeared irregular globular and rods(Fig. 1) and

stained gram-negative. Strain D-2 occurred in clusters of two to four cells. Spherome formed during the early exponential phase. They were non-motile and no spore formation was observed, which is characteristic for members of the order *Bacteroides*. Cells were 0.5 µm wide and 1–3 µm long. Typically, Strain D-2 appeared irregular shaped in all phases of growth. Strain D-2 was cultured in cellobiose solid medium for 7 days to form a white, edge blur colonies, and produced yellow pigment in decline phase. When cultured in a liquid culture medium, Strain D-2 produced flocculent precipitate and sank to the bottom of the medium in decline phase.

Strain D-2 was not able to grow in oxygenated media. The strain grew between pH 6.0 and 9, optimum growth occurring at pH 7.0; however, no growth was observed at pH 5.5 or 9.5. The temperature range for growth was between 13 and 40 ºC, optimum growth being at 37 ºC. Strain D-2 required 0-40 g NaCl L^{-1} for growth; optimum growth occurred at 20 g NaCl L^{-1}. Under optimal growth conditions (temperature, pH and NaCl), the doubling time of strain D-2 was approximately 91.8 min.

The compounds that strain D-2 was able to use for growth were glucose (5 mM), cellobiose(5 mM), ribose(5 mM), raffinose(2 mM), synanthrin(5 mM), fructose (5 mM), xylose (5 mM), maltose (2 mM), lactose (2 mM), xylan (2 mM), arabinose(2 mM), and sorbose (2 mM). No growth occurred on mannose (5 mM), rhamnose(5 mM), tartrate(10 mM), glutamate (10 mM), malate (10 mM), citric acid (10 mM), fumarate (10 mM), amygdalin (2 mM), melezitose (2 mM), salicin (2 mM) and mannitol (10 mM) (Table 1).

The glucose fermentation products of isolate D-2 were analyzed after incubation of a cell suspension at 37 ºC. The fermentation end products from glucose (50 mM) were determined by an 850 Professional ion chromatograph (Metrohm) and SHIMADZU GC2010 chromatography. Acetate (10.7 mM), propionate (24.6 mM), butyrate (1.5 mM), CO_2, and traces of H_2 were produced. The end products increased in response to growth and glucose consumption. However, propionate significantly inhibited the growth and metabolism. When the concentration is greater than 2%, the strain stop growing.

Table 1. Discriminating characteristics between strain D-2 and closely related *Bacteroides* type strains

Characteristic Isolation source	1 Tideland Soil	2 Human intestinal	3 Human intestinal	4 Human faeces	5 Human faeces	6 Termit e gut
Cell size (µm)	05/1-3	0.8–1.5/ 0.5–5.0	0.8–1.5/ 0.5–5.0	0.8/ 1.5–4. 5	1.0/ 1.5-2.0	0.8 /1.7–2.5
Optimal temperature(ºC)	37	37	37	37	37	37
Acid is produced from:						
Rhamnose	-	-	-	+	+	+
Arabinose	+	-	+	+	+	+
Cellobiose	+	+	+	+	+	+
Glucose	+	+	+	+	+	-
Mannose	-	+	+	+	+	-
Raffinose	+	+	+	+	+	+
Sucrose	-	+	+	+	+	-
Xylose	+	+	+	+	+	+
Salicin	-	-	-	+	-	+
Trehalose	-	-	-	-	-	-
Lactose	+	-	-	+	+	-
Maltose	-	-	-	+	+	-
G+C mol%	43.9	41.4	42.0	43.0	42.7	44.9

Taxa:1,Strain D-2; 2, *Bacteroide. nordii* WAL11050^T[19]; 3, *Bacteroide. salyersiae* WAL10018^T[19]; 4, *Bacteroides finegoldii* DSM17565^T [20]; 5, *Bacteroides faecis* JCM16478^T[21]; 6, *Bacteroides reticulotermitis* JCM10512^T[22]; +, Positive; - , negative

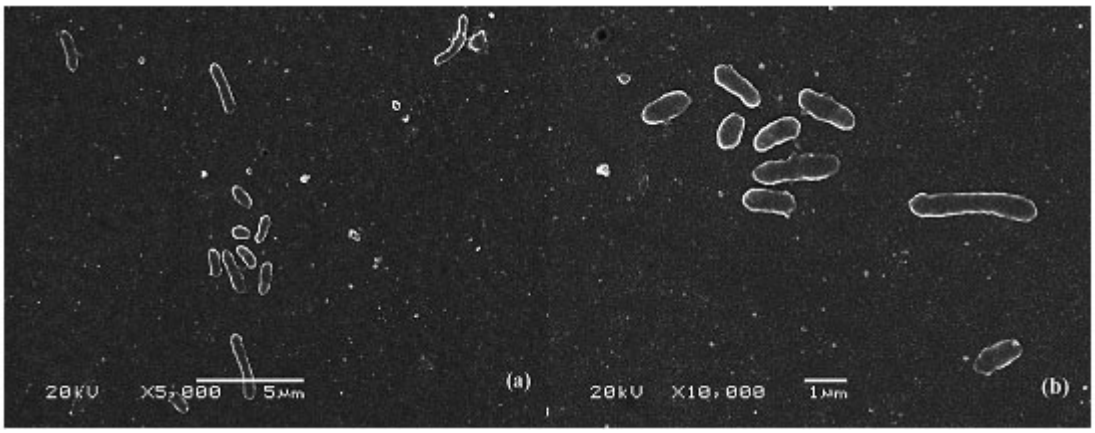

Fig. 1. Electron micrograph of D-2(a.5000×, b.10000×)

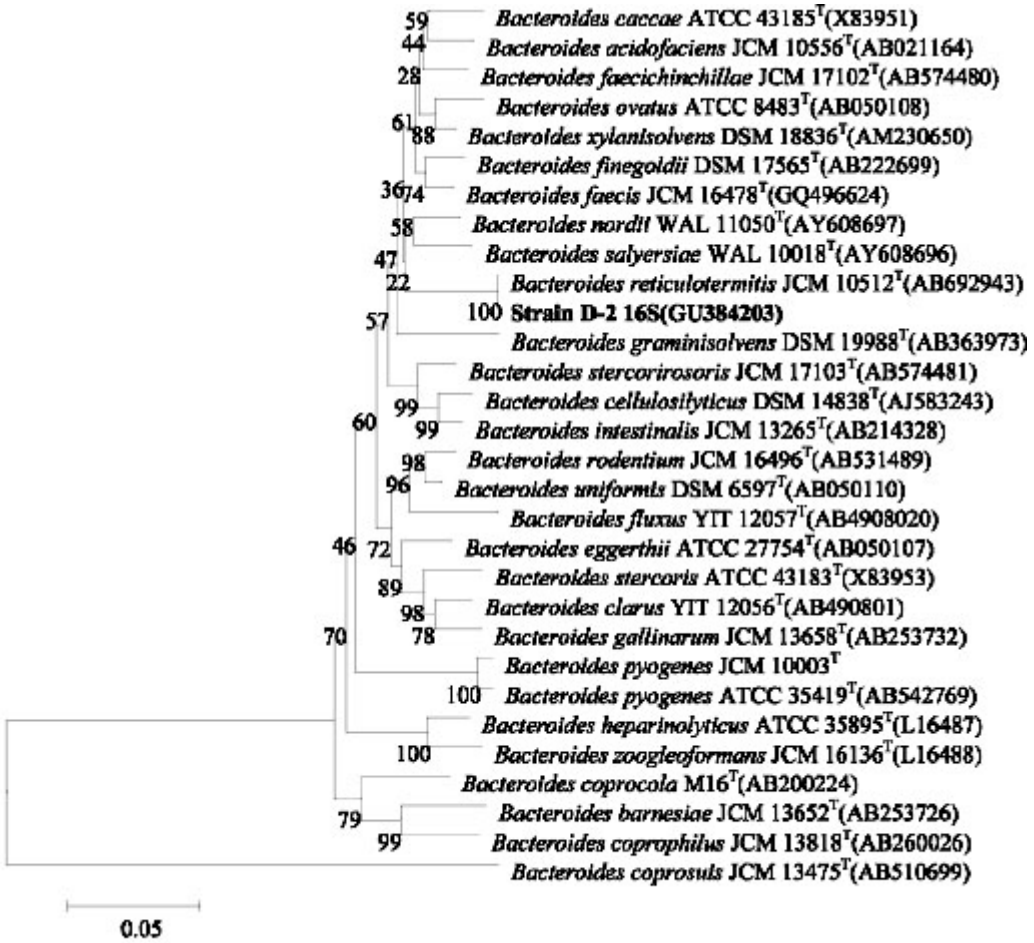

Fig. 2. A phylogenetic dendrogram based on 16S rRNA gene sequences showing the position of strain D-2 (bold type) among members of the order *Bacteroides*. The phylogenetic tree was constructed by using the neighbour-joining method in MEGA 5.2 software, based on 1411 unambiguous bases and 1000 bootstrap replications. Bar, 5 nucleotide substitutions per 100 nucleotides

Growth of strain D-2 was inhibited completely by chloramphenicol (200 μg·mL^{-1}), ampicillin (200 μg·mL^{-1}), rifampicin (200 μg·mL^{-1}), acheomycin (200 μg·mL^{-1}) and kanamycin (200 μg·mL^{-1}); streptomycin (200 μg·mL^{-1}) and erythromycin (125 μg·mL^{-1}) caused partial inhibition of growth. The other antibiotics had no effects on the growth of strain D-2.

The G+C content of the genomic DNA of strain D-2 was 43.9 mol%, which fell within the limits of the range reported for the genus *Bacteroides* that have a G+C content of 40–48 mol%[18].

Analysis of the 16S rRNA gene sequences revealed that strain D-2 was members of the genus *Bacteroides* (Fig.2). Strain D-2 was phylogenetically affiliated to the strain *Bacteroides reticulotermitis* [19]. The phylogenetic position of the strains D-2 among *Bacteroides species* and representative members of the genera *Bacteroides* are shown in Fig.2. Close phylogenetic relatives of strain D-2 was *Bacteroides reticulotermitis* JCM 10512^T. Sequence similarity values were calculated using the program Blast. 16S rRNA gene sequence similarities of strain D-2 with *Bacteroides reticulotermitis* JCM 10512^T was 99%. Strain D-2 showed 93% sequence similarity with *Bacteroides salyersae* WAL 10018^T. However, strain D-2 could be clearly distinguished from *Bacteroides reticulotermitis* by a 1 mol% lower G+C content of its DNA(43.9 mol%) (Table 1). The main fermentation products of strain *Bacteroides reticulotermitis* JCM 10512^T were succinic and acetic acids. For strain D-2 the main products from glucose fermentation were acetate, propionate, butyrate. In comparison with *Bacteroides reticulotermitis* JCM 10512^T, strain D-2 had a different kind of substrate utilization.

DISCUSSION

Strain D-2, the similarity values with the type strain *Bacteroides reticulotermitis* JCM 10512^T is highest reaching 99% by comparison with the older species of the genus *Bacteroides*. But only from similarity comparison cannot distinguish the taxonomic status of Strain D-2 in genus *Bacteroides*. However through the analysis of physiological and biochemical experiments, the substrate utilization and the main fermentation products from glucose between strain D-2 and *Bacteroides reticulotermitis* JCM 10512^T are different (Table 1). There are some other differences on physiological characteristics. So far, the taxonomic status of the strain D-2 needs DNA hybridization method to be further identified.

Cellulose macromolecule is one of the important raw materials of production biomass on reuse waste recycling in China. However, the cellulose is a polymeric compound, insoluble in water and common organic solvents. It is difficult to biodegrade. The main problems of biotransformation of cellulose is unable to penetrate the structure. Therefore the rate-limiting step of the biological fermentation process is the hydrolysis of cellulose macromolecules. The strain D-2 can degrade cellulose material on the optimum conditions, which is important in helping reasonable utilization of cellulosic materials. And the metabolic product of strain D-2 is propionic acid, the speed of propionic acid transformed to methane is very slow during the fermentation process, which often leading to accumulation of system imbalance. So to study the physiological and biochemical characteristics of strain D-2 and identify the metabolism relationship between it and other strains can be a crucial role in the controllability of red tide pollution at East China Sea offshore.

East China Sea offshore is the area of eutrophication ,which the residue of red tide seriously affect the ocean environment. So it is the key area of environmental governance in recent years. Strain D-2 is isolated from tideland soil of the East China Sea, can grow at low temperature and high salt tolerance and degrade cellulose to produce fatty acid and gas, and its decomposition products are an important intermediate metabolite of bacterial fermentation process. The study of strain D-2 has a great significance not only for cellulose degradation but also for the isolation and identification of microbial resources in the East China Sea area.

ACKNOWLEDGMENTS

This research was supported by the Fundamental Research Funds for the Central Universities.

REFERENCES

1. O'SULLIVAN, A.C. Cellulose: the structure slowly unravels. *Cellulose*, 1997; **4**: 173-207.
2. Schwarz, W. The cellulosome and cellulose degradation by anaerobic bacteria. *Appl Microbiol Biotechnol*, 2001; **56**: 634-649.
3. Ollivier, B., Cayol, J.L., Patel, B., Magot, M., Fardeau, M.L.,Garcia, J.L. *Methanoplanus petrolearius sp*. nov., a novel methanogenic bacterium from an oil producing well. *FEMS Microbiol Lett*,1997; **147**: 51-56.
4. Humane, R. A roll tube method for cultivation of strict anaerobes. *Methods In Microbiology*, 1969; **3**: 117.
5. Cheng, L., Qiu, T.L., Li, X., Wang, W.D., Deng, Y., Yin, X.B.,Zhang, H. Isolation and characterization of *Methanoculleus receptaculi sp*. nov. from Shengli oil field, China. *FEMS Microbiol Lett*, 2008; **285**: 65-71.
6. Mikucki, J.A., Liu, Y., Delwiche, M., Colwell, F.S.,Boone, D.R. Isolation of a methanogen from deep marine sediments that contain methane hydrates, and description of *Methanoculleus submarinus sp*. nov. *Appl Environ Microbiol*, 2003, **69**: 3311-3316.
7. Boone, D.R., Kamagata, Y. Rejection of the species *Methanothrix soehngenii*^{VP} and the genus *Methanothrix*^{VP} as *nomina confusa*, and transfer of *Methanothrix thermophila*^{VP} to the genus *Methanosaeta*^{VP} as *Methanosaeta thermophila comb*. nov. Request for an Opinion. *Int J Syst Bacteriol*, 1998; **48**: 1079-1080.
8. Marmur, J. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J Mol Biol*, 1961; **3**: 201-208.
9. Jarrell, K.F., Faguy, D., Hebert, A.M.,Kalmokoff, M.L. A general method of isolating high molecular weight DNA from methanogenic archaea (archaebacteria). Can *J Microbiol*, 1992; **38**: 65-68.
10. Baker, G., Smith, J.,Cowan, D.A. Review and re-analysis of domain-specific 16S primers. *J Microbiol Methods*, 2003; **55**: 541-555.
11. Altschul, S.F., Gish, W., Miller, W., Myers, E.W.,Lipman, D.J. Basic local alignment search tool. *J Mol Biol*,1990; **215**: 403-410.
12. Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F.,Higgins, D.G. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*,1997; **25**: 4876-4882.
13. Kumar, S., Tamura, K.,Nei, M. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform*, 2004, **5**: 150-163.
14. Felsenstein, J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*,1985; 783-791.
15. Marmur, J.,Doty, P. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol*, 1962; **5**: 109-118.
16. Takai, K., Inoue, A.,Horikoshi, K. Methanothermococcus okinawensis sp. nov., a thermophilic, methane-producing archaeon isolated from a Western Pacific deep-sea hydrothermal vent system. *Int J Syst Evol Micr*,2002; **52**: 1089-1095.
17. Balch, W., Fox, G., Magrum, L., Woese, C.,Wolfe, R. Methanogens: reevaluation of a unique biological group. *Microbiol Rev*,1979; **43**: 260.
18. Shah, H. The genus Bacteroides and related taxa. *The prokaryotes*,1992; **2**: 3593-3607.
19. Sakamoto, M.,Ohkuma, M. Bacteroides reticulotermitis sp. nov., isolated from the gut of a subterranean termite (Reticulitermes speratus). *Int J Syst Evol Micr*, 2013, **63**: 691-695.