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Isolation and characterization of deodorizing bacteria for organic sulfide malodor

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Abstract: Strain JII screened out from different odor origins can efficiently degrade methyl mercaptan and ethanethiol whereas has no ability to remove dimethyl sulfide. The results indicated that the strain JII breaks only the C-SH bond. The optimum temperature and pH of JII are 20—30℃ and 6.0—8.3 respectively. A systematic identification method—16S rDNA gene sequence comparison, for deodorizing bacteria was carried out. The 16S rDNA gene sequence analysis of strain JII showed the highest level of 97% homology to *Rape rhizosphere*.

Keywords: 16S rDNA gene; methyl mercaptan; Ethanethiol; *Rape rhizosphere*

Introduction

Volatile organic sulfides emitted from wastewater treatment plants, wood-pulping industry, oil refineries and sewer systems are not only nuisance, but also cause health problems for workers and residents nearby (Joanna, 2001). There are three methods for odor control, biological, chemical, and physical method. Now much attention is paid to biological method of odor treatment due to its high efficiency, low-cost, and environmental acceptability. Deodorizing bacteria play key roles in biological deodorization techniques. Since the 1980s, many highly efficient deodorizing sulfide bacteria have been insolated and domesticated, for example, *Thiobacillus thioparus* DW44 (Kyeoungpsuk, 1992), *Thiobacillus thioparus* strain HA43 (Cho, 1991) and *Thiobacillus thioparus* strain CH11 (Chung, 2001; 1996) isolated from peat deodorizing biofilter, *Thiobacillus thioparus* TK-m (Gould, 1992; Kanagawa, 1989) and *Thiobacillus thiooxidans* (Kyung-suk, 2000) from activated sludge, *Thiobacillus thioparus* Strain E6 (Smith, 1988) from limicola forma in lake, *Thiobacillus thioparus* Strain T5 (Cho, 1991; Chung, 2001) from sludge in sea and etc. MM (methyl mercaptan), DMS (dimethyl sulphide), H_2S can be efficiently removed from contaminated air by these autotrophic bacteria. However, most of them are usually difficult to proliferate mainly because their growth rates are significantly lower than those of heterotrophic bacteria.

Some heterotrophic bacteria are isolated to degrade organic sulfide malodor such as *Pseudomonas acidovorance* (Zhang, 1991; Kanagawa, 1982), *Hyphoicrobium* sp. *Hyphoicrobium* sp. S (De Bont, 1981), *Hyphoicrobium* sp. EG (Suylen, 1987), *Hyphoicrobium* sp. I55 (Pedersen, 1995)) and *Xanthomonas* sp. (Kyeoungpsuk, 1992) etc. *Hyphoicrobium* sp. (De Bont, 1981) can degrade DMS into formaldehyde and MM, and the product MM is further oxidized to formaldehyde and S^{2-}. *Xanthomonas* spp. has special metabolic pathway and can oxidize MM to polysulfide, which is similar to elemental sulfur. *Pseudomonas acidovorance* can only degrade DMDS (dimethyl disulfide) to DMS but cannot decompose DMS further. For their short domestication period, all these deodorizing bacteria have been inoculated into biofilter to improve the removal for degrading malodor.

As most isolated deodorizing bacteria have very narrow selectivity range of organic sulfide malodor, lost of screening work are needed in order to obtain highly efficient, good quality deodorizing sulfate bacteria. The isolated bacteria are to be further identified and classified. The microseq 16S rDNA gene kit allows identification of bacteria based on the sequence of their 16S rDNA gene. Genomic DNA extracted from bacteria is first amplified by PCR method and then sequenced. In this paper, molecular classification using 16S rDNA gene technology is more accurate and more rapid than physiological and morphological identification.

1 Materials and methods

1.1 Deodorizing bacteria sources

The following materials are exploited to initiate culture media for the isolation of organic-sulphide-degrade bacteria: peat in deodorizing biofilter, mud of Songhuajiang River, black clay-soil, activated sludge, and cattle manure.

1.2 Enrichment cultures

The enrichment culture is prepared as follows: 500 ml bottles each with 200 ml LB culture medium is the first sealed with rubber stopper and then 10 ml ET is injected into the bottle through the seal using a syringe.

1.3 Experimental apparatus

The experimental equipment used in this work is shown in Fig. 1. Each suspended-growth reactor consists of a 40-cm tall glass column with a 5 cm inner diameter. At the bottom of the reactor a sparge stone is attached to disperse the contaminated air entering the reactor. The reactors are fed up and 0.5 L nutrient solution was added into through the nozzle on the top everyday, which contains all necessary macronutrients and micronutrients to microorganisms. ET (Ethanethiol) or DMS in a small bottle is put in a wide mouthed bottle and air is introduced. The inlet ET or DMS

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concentration is controlled by air dilution. MM (99%) is withdrawn by a peristaltic pump and mixed with air to create appropriate mixture concentration.

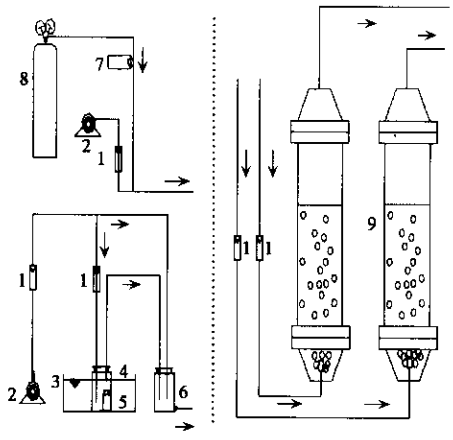

Fig.1 Schematic diagram of the experimental devices used in this study
1. flow meter; 2. air compressors; 3. thermostat; 4. glass bottle; 5. DMS or ET liquid; 6. mixed air bottle; 7. peristaltic pump; 8. MM gas cylinder; 9. suspended-growth reactors. Left: odors origin; Right: bioreactor

1.4 Analytical methods

Concentrations of ET and MM are measured using a gas chromatograph (Chromatography N2000, Zhejiang University) equipped with a stainless steel column and a flame ionization detector. The operating conditions are set at: injector 200℃, column 80℃. The pH is determined using PHS-3C types pH meter. The sulphate content is measured by barium chromate photometer.

1.5 Sequence analysis of 16S rDNA gene

Genomic DNA is extracted from selected deodorizing bacteria using the method according to the literature (Tang, 1998) and 16S rDNA is amplified using the following primers: Forward primer, BSF8/20: 5′-AGAGTTTGATCCTGGCTCAG-3′, 20nt; Reverse primer, BSR534/18: 5′-ATTACCGCGGCTG CTGGC-3′, 18nt.

Amplifications are carried out in a volume of 20 μl (Takara PCR kit), which contains 1 μl of template DNA, 2 μl 10 × buffer, 0.6 μl forward primer, 0.6 μl reverse primer, 1.6 μl dNTP, 0.1 μl Taq enzyme, 15.1 μl ddH_2O. The reactions are performed on a programmable thermal cycler (PTC-100, MJ Research). The amplification cycle is: 94℃ for 1 min, 58℃ for 30 s and 72℃, 30 s repeated 30 times followed by a final cycle with an extension phase of 10 min at 72℃. The PCR products (8 μl) are loaded on horizontal, 0.8% agarose gels, and subjected to electrophoresis for 40 min at 3 V/cm in tris-borate buffer. Gels are stained with ethidium bromide, and photographed under UV-light transillumination.

PCR products are purified and reclaimed by reagent kit of MOBIO Co. in U.S.. The PCR products of isolated bacteria are sequenced in BIOAISA Co. in Shanghai. With the microbial identification and BLAST analysis software, sequence sample files are assembled and the final sequence was compared with 16S rDNA gene sequence in the GenBank. The similar sequence is analyzed and identified by Clustalw or Multalin software (Tang, 1998).

2 Results and discussion

2.1 Selection and biodegradation traits of strain Jll

The results of selection showed that 32 strains bacteria could live on high concentration ET enrichment culture, of which eight strains can degrade ET. One stable, high efficiency-deodorizing bacterium is gained by several repeated transfers. MM gas (50 mg/m^3) was supplied at a rate of 0.4 m^3/h to the aeration tube into which Jll was inoculated (Fig. 1). Gas bubbles reach the surface within 4 s. The initially low removal is often attributed to the adaptation period for the bacteria to accustom to new conditions. However, within 5 h after initiation MM removal efficiency of the reactor exceeds 70%, after which the equilibrium is reached and MM removal efficiency stays steady and constant in the rage of 80%—84%. Compared with about 10% adsorption removal of MM by liquid nutrition without inoculation, Jll proves to be very useful for malodorous MM removing. Just the same as MM, Jll can also degrade ET efficiently. The removal pattern of ET is similar to MM with only strain Jll in the basal medium. After 7 h operation, the ET removal efficiency reaches a maximum of 72% with operational conditions being: inlet gas flow rate 0.5 m^3/h and inlet gas concentration 35 mg/m^3. But the strain has no ability to remove DMS, as is shown in Fig. 2. Therefore, this study demonstrated that strain Jll seems to break only the C-SH bond. Most anions containing sulfur in the reactor effluent are SO_4^{2-} in the process of biodegrading MM and ET. pH value of the reactor is 6.8—7.4 before initiation and during operation pH value ranged from 6.4 to 7.1.

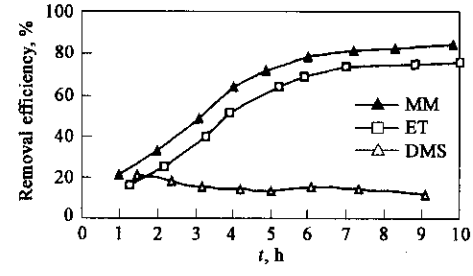

Fig.2 Relationship between operating time and removal efficiency of MM, ET and DMS by strain Jll

2.2 Physiological and morphological characteristics of strain Jll

Scanning electron photomicrograph and photomicrograph of the bacteria are shown in Fig. 3 and Fig. 4 respectively. Their morphological characteristics are as follows: colony morphology on nutrient-agar plate is less than 1 mm, lemon yellow, semi-translucent, round, regular, entire, shiny and high convex; old colony turns brown; cell morphology, rod shaped 0.5 by 0.8—1.0 μm; immobile, no flagellum rods; gram negative; non-spore forming; aerobic. Effects of temperature and pH on the growth of the isolate are also studied. The optimum growth of Jll occurs at 20—37℃ and at pH 6.0—8.3. Table 1 shows that the strain grows very well on selective media with thiosulfate or S^0. Its growth is

not inhibited by the presence of organic substrates. Judging from phenotypic characterization, the isolate is suspected to be *Flavobacterium* sp. It is difficult to determine which species strain Jll belongs to using conventional methods.

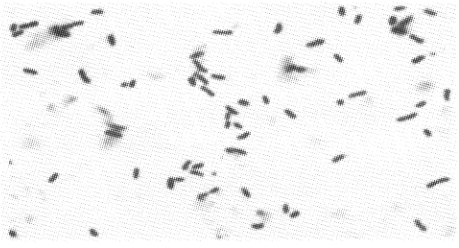

Fig. 3 Microscopic photo of strain Jll at 1500 magnification

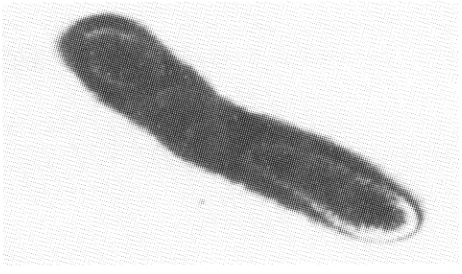

Fig.4 Transmission electron micrograph of strain Jll at 20000 magnification

2.3 Amplification of strain Jll's 16S rDNA

After analysis of 16S rDNA gene sequence, it is found that sequence of about 1500 bp of the 16S rDNA gene contain nine high divergent regions (V1—V9, Fig. 5) and eight conservative regions (U1—U8) (Gray, 1984; Wuyts, 2002). Conservative regions are good targets of primer in PCR amplification. Conservative sequence in 8th to 27th 16 (+), 683rd to 702nd N3R and 1512th to 1492nd 16(−) are often selected as sequencing primer to define the part sequence of 16S rDNA including high divergent regions of V1, V2 and V6. V2 has enough information to identify bacteria species. According to reference, this study gains small subunit rDNA from Internet as primer: forward primer, BSF8/20; reverse primer, BSR534/18. Sequence of 500 bp containing high divergent regions of V1, V2, and V6 will be amplified from bacterial genome.

The products of PCR are analyzed by electrophoresis in a 1.0%(w/v) low-melt agarose gel to confirm correct product size. Single strip of approx. 500 bp of Jll and *E. coli* are obtained in anticipation(Fig.6). The PCR products of Jll are used as the sequencing template directly. PCR products are purified to remove impurity of primer and template using DNA mini-columns. The sequences are assembled using automated sequencer. A 483 bp 16S rDNA sequence is obtained as shown in Table 2.

Table 1 Physiological characteristics of strain Jll

Physiological characteristics	Strains Jll
Starch hydrolysis	−
Glutin hydrolysis	+
Indoxylacetate hydrolysis	−
M.R	−
V.P	−
4% NaCl	+
Oxidase	+
Catalase	+
Urease	−
Nitrate reduction	−
Lactose	+
Glucose	+
Mannitol	−
Sucrose	+
Sodium citrate	−
Sodium thiosulfate	+
H_2S	+
S^0	+

Notes: +. positive growth or reaction; −. negative growth or reaction

Table 2 Partial list of Jll 16S rDNA gene sequences

1	TTATTCGTAT	AGTACCTTCA	GCTACTCTCA	30
31	CGAGAGTAGG	TTTATCCCTA	TACAAAAGAA	60
61	GTTTACAACC	CATAGGGCCG	TCGTCCTTCA	90
91	CGCGGGATGG	CTGGATCAGG	CTCTCACCCA	120
121	TTGTCCAATA	TTCCTCACTG	CTGCCTCCCG	150
151	TAGGAGTCTG	GTCCGTGTCT	CAGTACCAGT	180
181	GTGGGGGATC	ACCCTCTCAG	GCCCCCTAAA	210
211	GATCGTAGAC	TTGGTGAGCC	GTTACCTCAC	240
241	CAACTATCTA	ATCTTGCGCG	TGCCCATCTC	270
271	TATCCACCGG	AGTTTTCAAT	ATCGAATGAT	300
301	GCCATTCAAT	ATATTATGGG	GTATTAATCT	330
331	TCCTTTCGAA	AGGCTATCCC	CCAGATAAAG	360
361	GCAGGTTGCA	CACGTGTTCC	GCACCCGTAC	390
391	GCCGCTCTCT	CATTTCCGAA	GAAACAATAC	420
421	CGCTCGGCTT	GCATGTGTTA	GGCCTCCCGC	450
441	TAGCGTTCAT	TCTGAGCCAG	GATCAACTCT	480
481	AT			510

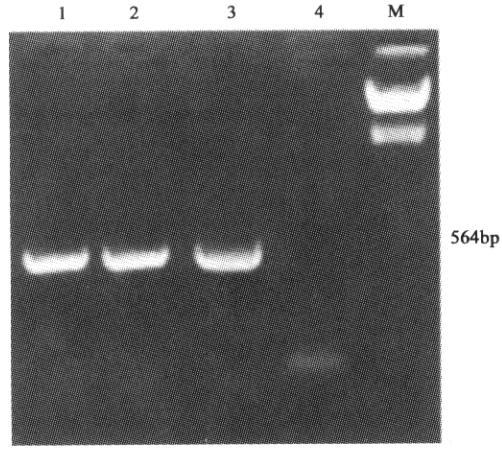

Fig.6 An ethidium bromide-agarose gel (0.8%) of 16S rDNA gene PCR amplification
lanes 1, 2: two Jll samples; lane 3: *E. coli*; lane 4: negative control; M: λ/hind Ⅲ

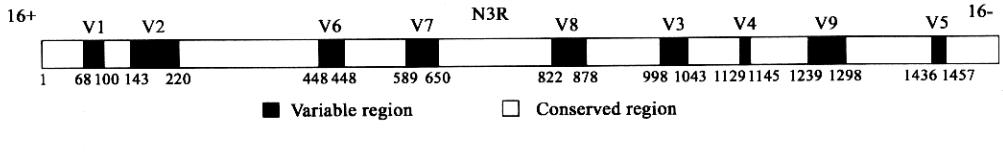

Fig.5 Diagram of 16S rDNA gene structure

2.4 Analysis of Jll PCR products

By comparison of the partial 16S rDNA sequence (483 bp) of Jll with a BLAST search in GenBank database, it is found that the closest similarity to strain Jll are *Rape rhizosphere bacterium* M9024, *Chryseo-bacterium* sp. U3, *Chryseobacterium scophthalmum*, *Flavobacterium indologenes*. These and other related sequences are used to construct phylogenetic tree(*E. coli* as extra-group) based on maximum likelihood method (seed member, 1000 bootstrap samples, Fig.7). The result has shown that *Rape rhizosphere* is more close to strain Jll than any other bacteria, which has 97% identity with strain Jll. Because the difference rate of over 2.5% is generally considered to be a new species within a given genus (Chung, 2001). The 16s rDNA sequence divergence among these strains suggested that it is a new species. Strain Jll is further differentiated from extant *Rape rhizosphere* sp. by a number of phenotypic traits. For example, Jll can make use of S^- and sodium thiosulfate and grow on high concentration NaCl.

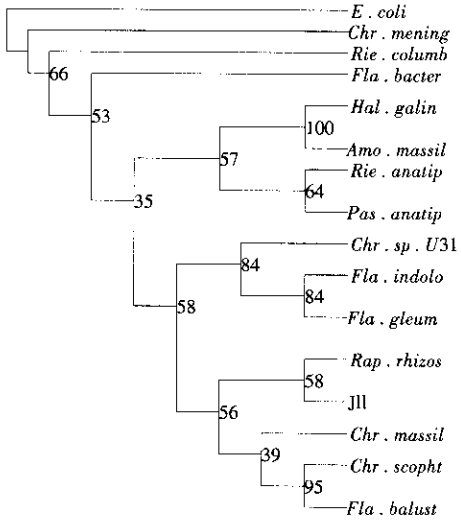

Fig. 7 Phylogenetic tree based on maximum likelihood analysis of 16S rDNA gene sequences of strain Jll and other similar bacteria. Numbers at the nodes represent the proportion of 1000 bootstrap re-samplings that support the topology shown

3 Conclusions

In this study, we successfully use LB culture containing ET to isolate a highly efficient deodorizing bacterium: strain Jll. It can efficiently degrade MM and ET. Their removal efficiencies can remain 84% and 72% respectively, running at activated sludge. But the strain Jll has no ability to remove DMS. The results proved that strain Jll seem to break only the C-SH bond. Jll grew on MM, ET and stoichiometrically oxidized them into SO_4^{2-} in the process of biodegrading. The optimum pH of Jll are 6.0—8.3.

The isolate is suspected to be *Flavobacterium* sp. based on morphology and biochemical characteristics. However, the biochemical identification of strain Jll is difficult and time-consuming. Therefore, analysis of strain Jll's 16S rDNA gene sequences showed that it is a new species close to *Rape rhizosphere*.

References:

Burgess J E, Parsons S A, Richard M S, 2001. Developments in odour control and waste gas treatment biotechnology: a review [J]. Biotechnology Advances, 19: 35—63.

Cho, K S, Zhang L, Hirai M *et al.*, 1991. Removal characteristics of hydrogen sulfide and methanethiol by *Thiobacillus* sp. isolated from peat in biological deodorization[J]. Ferment Bioeng, 71: 44—49.

Chung Y C, Huang C, Tseng C P, 1996. Operation optimization of *Thiobacillus thioparus* CH11 biofilter for hydrogen sulfide removal [J]. Journal of Biotechnology, 10(1): 1—6.

Chung Y C, Huang C P, Tseng C P *et al.*, 2001. Biotreatment of H_2S and NH_3 containing waste gases by co-immobilized cells biofilter[J]. Chemosphere, 43: 1043—1050.

Chung W K, King G M, 2001. Isolation, characterization, and polyaromatic hydrocarbon degradation potential of aerobic bacteria from marine macrofaunal burrow sediments and description of *Lutibacterium anuloederans gen. nov.*, sp.[J]. Applied and Environment Microbiology, 67(12): 5585—5592.

De Bont A M, Van Dijken J P, Harder W, 1981. Dimethyl sulphoxide and dimethyl sulphide as a carbon, sulphur and energy source for growth of *Hyphomitcrobium*[J]. Microbiol, (127): 315—323.

Gould W D, Kanagawa T, 1992. Purification and properties of methyl mercaptan oxidase from *Thiobacillus Thioparus* TK-m [J]. Journal of General Microbiology, 138: 217—221.

Gray M W, Sankoff D, Ceclergren R J, 1984. On the evolutionary descent of organisms and organelles: a global phylogeny based on a highly conserved structural core in small subunit ribosomal RNA[J]. Nucleic Acids Research, 12: 5837—5852.

Kanagawa T, Dazai M, Fukuoka S, 1982. Degradation of O, O-dimethyl phosphorodithioate by *Thiobacillus thioparus* TK-1 and *Pseudomonas* AK-2 [J]. Agricultural and Biological Chemistry, 46: 2571—2578.

Kanagawa T, Eiichi M, 1989. Removal of methanethiol, dimethyl sulfide, dimethyl disulfede, and hydrogen sulfide from contaminated air by *Thiobacillus thioparus* TK-m[J]. Journal of General Microbiology Applied and Enviromental Microbiology, 55(3): 555—558.

Kyeoungpsuk Cho, Mitsuyo Hirai, Makoto Shoda, 1992. Degradation of hydrogen sulfide by *Xanthomonas* sp. strain DY44 isolated from peat[J]. Applied and Environmental Microbiology, 58(4): 1183—1189

Kyung-suk Cho, Hee Wook Ryu, Nae Yoon Lee, 2000. Biological deodorization of hydrogen sulfide using porous lava as carrier of *Thiobacillus thiooxidans* [J]. Journal of Bioscience and Bioengineering, 90(1): 25—31.

Pedersen A R, Arvin E, 1995. Removal of toluene in waste gases using a biological trickling filter[J]. Biodegradation, (6): 109—118.

Smith N A, Kelly D P, 1988. Mechanism of oxidation of dimethyl disulpide by *Thiobacillus thioparus* strain E6[J]. J Gen Microbiol, (134): 3031—3039.

Suylen G M H, Large P J, Van Dijken J P *et al.*, 1987. Methyl mercaptan oxidase, a key enzyme in the metabolism of methylated sulphur compounds by *Hyphomicrobim* EG[J]. Journal of General Microbiology, 133: 2989—2997.

Tang Y W, Nicole M Ellis, Marlene K Hopkins, 1998. Comparison of phenotypic and genotypic techniques for identification of unusual aerobic pathogenic gram-negative bacilli[J]. Journal of Clinical Microbiology, 3674—3679.

Wuyts J, Peer Y V D, Winkdmans T *et al.*, 2002. The European database on small subunit ribosomal RNA[J]. Nucleic Acids Research, 30(1): 183—185.

Zhang L, Kuniyoshi I, Hirai M *et al.*, 1991. Oxidation of dimethyl sulfide by *pseudomonas acidoborans* DMR-11 isolated from peat biofilter[J]. Biotechnol Lett, 13: 223—228.

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