JOURNAL OF PURE AND APPLIED MICROBIOLOGY, APRIL 2014. Vol. **8**(2), p. 1321-1326

Study of Fungus Community Compositions in Dunhuang Frescoes and Dominant Bacteria Reduction Mechanism

Sun Qiong

Tourism Institute of Beijing Union University, Beijing, China.

(Received: 18 February 2014; accepted: 21 April 2014)

This paper uses modern molecular biological techniques to examine and analyze the fungus species leading to Dunhuang frescoes corrosion. After the extraction of total DNA in sample and target fragment amplification, clone library construction together with sequence determination and comparison, it can be seen that the series of fungus in Dunhuang frescoes mainly attach to aspergillus, Phialosimplex and Engyodontium. And then, it obtains a reduction Cr (VI) of dominant bacteria Brevibacillus through study, especially taking it as object and using ultrasonic method to get its extracellular polymeric substances (EPS), to analyze the restoring absorption contributions of EPS to Cr (VI). The experimental results show that the removal rate of bacteria EPS to Cr (VI) and total Cr absorptions is 18.58% and 21.12% respectively and absorption contribution is 48.49% and 64.75%, respectively.

Key words: Dunhuang frescoes; Fungus species; Dominant bacteria.

The Dunhuang frescoes are important components of our cultural heritage, but after a long time of ecological and environmental evolution, many microorganism has emerged which has caused a serious threat to preserve frescoes. The study of microbial ecology and its environmental protection in the surface of frescoes and historical heritages can be traced to the late 1950s. Due to the different materials and crafts in different frescoes with diverse remaining environments, the biological communities associated with them also have a big difference. Current researches indicate that microorganisms in frescoes are fungus mainly containing penicillium, aspergillus, acremonium and Engyodontium, bacteria mainly of bacillus, arthrobacter, micrococcus and pseudomonas, algae with dominant species mainly of nostoc, lyngbya and chlorococcum, and actinomyces and crustose lichens of streptomyces and nocardia[1]. These funguses' growth and metabolism not only can affect the aesthetic values of frescoes, but also will cause structural damage to the frescoes. Studies have found that the haunt of tourists has a great impact on the composition and structure of air microbial communities inside the Mogao grottoes cave. These findings suggest that tourists' activities will generate a great disturbance to the original environment which can lead to a serious strike on the fragile ecosystems. In recent years, the method of Molecular Biological Detection is widely favored by researchers due to its sensitivity and being independent for cultivation of microorganisms in samples. Also, by this method, we can get the important information on the composition and structure of microbial communities in the short term[2]. Currently, the domestic study on microbial groups causing frescoes corrosion is rarely found, let alone microbial investigation of tomb.

Our study uses molecular detection technology to examine the funguses of frescoes and analyze the composition of fungal communities. It provides new theoretical basis and technical support for domestic microbiological corrosion monitoring of historical relics, which is conductive

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

to in-depth studies on microbiological corrosion mechanism and long-term preservation under the tomb conditions. In addition, this paper based on the dominant bacteria (*Brevibacillus* sp) acquired by preliminary screening, analyzes the removal contribution of its EPS to Cr (VI) reduction, to further explore the mechanism of dominant bacteria to reduce and remove Cr (VI).

MATERIALS AND METHODS

Sample Collection and Total Extraction of DNA

In corrosion regions, it uses sterile scalpel to scrap frescoes samples with a small amount of plaque, and put into sterile Eppendorf tube, weighing 0.1g by analytical balance; using Power Soil™ (MOBIO Laboratories, Solanabeach, CA, USA) DNA extraction kit, according to operating instructions to complete the total DNA extraction, and then keeping portions and saving at -20°C.

Target fragment amplification

With the total DNA as amplified template, the internal transcribed spacer (ITS) in fungal rRNA gene is amplified by universal primers ITS1 and ITS4. The reaction system (25μl) contains 1U Taq polymerase, NTP with final concentration of 0.2mM, 2.5μl10×reaction buffer, 0.2mM of single primer concentration and 2.0μl template (about with 10ng DNA). The amplification procedure includes: pre-degeneration for 5 min, 94°C, and then the denaturation for 1 min, 94°C, annealing at 55°C for 1 min, and extending at 72 °C for 1 min, to complete 30 cycles for amplification with final extension for 10 min at 72°C. In addition, it uses 1% agarose gel electrophoresis to examine the size and specificity of fragment.

Clone Library Construction

The PCR products are purified by applying agarose DNA kit[3], and then extracts 7ul connecting with the pGM-T carrier, cloning to the competent cell prepared by recipient bacterium of *E.coli* DH-5α (Tiangen Co., Beijing, China). According to blue-white selection flat (AMP concentration of 100μg ml^{-1} in LB, X-Gal of 60μg ml and IPTG of 20μg ml), it picks 150 to 250 white patches from every transformation plate, using T7-SP6 primer amplification directly to verify the appropriate size of inserted fragment; Placing the positive clones spots correctly determined in the LB medium (containing 12μg ml of Amp) at 37°C overnight, drawing the 1ml solution to the sequencing company completed the sequencing.

Sequencing and Sequence Alignment

It employs CONTIGEXPRESS software (Informax, MD, USA) to compile the sequence to be measured, completing the Chimera detection online by using chimera detecting system. The obtained sequence should achieve the comparison and similar sequence screening, to compare with the given ITS genetic sequence of fungi groups with the BLAST procedure.

Dominant Bacteria Reduction Mechanism

Dominant bacteria: *Bacillus brevis* (*Brevibacillus* sp.);

Mode wastewater: preparing with potassium dichromate, the concentration of Cr (VI) is about 100 mg/L.

Basic fluid medium: 0. 5 g/L $KHPO_4$, 2. 0 g/L (NH)SO, 0.1 g/L NHCl, 0.5 g/L NaSO, 0. 1 g/L CaCl, 0.5 g/L NaHCO, 0.1g/L $MgSO_4$, 2.0 g/L yeast extract, 3. 0 g/L sodium lactate, 0.02 g/L VB, 0. 02 g/L VC, 6mL trace metals supplement liquid and PH value as 6.8 to 7.2, to prepare the bacterial suspension of dominant bacteria.

Basic solid medium: adding 15g/L agar to the basic fluid medium for colony counts.

Methods

To test the concentration of sugar and protein in EPS solution and make qualitative analysis of DNA; based on the above analysis results; it filters the conditions of EPS extracted by ultrasound to get the optimum. Taking six aliquots of equal bacteria suspensions, four of which are ultrasonic processed to acquire two aliquots of EPS solutions and substrate solutions with the removal EPS respectively, to measure the blank bacterial suspension without ultrasonication, EPS solution after ultrasonication and the removal rate of substrate solution with removal EPS to chromic, so as to analyze the absorption removal contribution of EPS to chromic.

RESULTS

Clone Library Sequence Alignment

Through the construction of clone library, frescoes can get No.138 and No.224 item of fungal ITS sequence respectively. The results of each sequence alignment in NCBI database is presented in table 1.

Table 1. Typical ITS sequences alignment in NCBI database on frescoes

Clone numbers	Total percentage	Similar species	Sequence No.	Coverage rate	Maximum Likelihood	Sequence source
35	25.35%	Phialosimplex	GQ169326	99%	93%	Animal pathogenic bacteria
		Fungi strain	GU566292	99%	93%	Rhizosphere Soil
93	67.38%	*Aspergillus*	GU566286	99%	91%	Rhizosphere Soil
		Aspergillus	HQ631021	99%	87%	Plant endophytes
10	7.26%	*Aspergillus*	GU566217	99%	88%	Rhizosphere Soil
		Fungi strain	GU566292	99%	93%	Rhizosphere Soil
63	28.12%	Aspergillus	GU797139	99%	100%	Plant endophytes
		Uncultured fungi	FJ213546	100%	99%	Urban air
15	6.71%	Uncultured fungi	FN667943	83%	95%	Plant endophytes
		Unidentified fungi	GU566292	100%	93%	Rhizosphere Soil
8	3.58%	Eupenicillium	HQ607978	97%	93%	Ant symbiotic bacteria
		Penicillium	GU566244	100%	92%	Rhizosphere Soil
17	7.58%	*Aspergillus*	AY373861	94%	99%	Genome DNA
		Uncultured fungi	FJ524295	100%	90%	Rice endomycorrhizae
51	22.76%	*Eupenicillium*	AF033464	90%	96%	Genome DNA
		Engyodontium	HQ827787	100%	94%	the fungi to break down polycarbonate
5	2.22%	Uncultured fungi	GU174374	100%	99%	Forest floor
		Capnodiales	HQ212300	100%	99%	Arctic tundra
7	3.13%	*Cladosporium*	HQ380770	93%	100%	Mogao grottoes air
		Uncultured fungi	HQ212300	100%	97%	Arctic tundra
3	1.35%	Uncultured fungi	HM537059	100%	99%	Degradation of deciduous endophytes
		Uncultured fungi	GU721670	100%	99%	Filter dust
6	2.69%	Uncultured fungi	HQ832973	97%	99%	Speleothem
		Uncultured fungi	GU721238	100%	99%	Filter dust
6	2.69%	Uncultured fungi	FJ820737	97%	95%	Air suspended particles
		Uncultured fungi	FN397250	100%	90%	Fungi- contaminated soil
5	2.22%	*Beauveria*	AM176689	86%	99%	sedimentary rocks in deep sea
		Cordyceps	AB027368	100%	82%	Plant endophytes
9	4.01%	Uncultured fungi	HQ607928	97%	99%	Ant symbiotic bacteria
		Uncultured fungi	GU055997	100%	87%	Ant symbiotic bacteria
19	8.49%	Uncultured fungi	FJ235861	93%	99%	Copper contaminated soil
		Uncultured fungi	GU055997	100%	87%	Ant symbiotic bacteria
10	4.45%	Uncultured fungi	FJ235979	86%	93%	Ruins of wood painting fragments
		Ochrocladosporium	EU040233	100%	88%	Wood fiber

The Optimum Condition for EPS Extraction

It designs an orthogonal test with two factors (Ultrasonic Power and Ultrasound Time) and four levels, the results are shown in table 2.

It can be seen from table 2 that sugar concentration of EPS solution is the highest as 0.0187 mg/mL when ultrasonic power is 120 W and ultrasonic time is 3 min (considered as 120W/3min, below); the second is that concentration is 0.0183 mg/mL at 100 W /3min. While 100 W /3min, protein concentration is the highest as 0.1930 mg/mL. Regardless of which group is testing, ultrasonic power is greater than the ultrasonic time, which indicates that ultrasonic power has more effects on processing than ultrasonic time, and then the final determination of the optimum condition is 100W/3 min.

Table 2. Orthogonal test results

Test No.	Ultrasonic Power /W	Ultrasound Time /min	Sugar/ $mg \bullet mL^{-1}$	Protein $mg \bullet mL^{-1}$
1	60	1	0.0146	0.0631
2	60	2	0.0152	0.0486
3	60	3	0.0157	0.0871
4	60	4	0.0154	0.0667
5	80	1	0.0153	0.0578
6	80	2	0.0135	0.0521
7	80	3	0.0122	0.0415
8	80	4	0.0146	0.0342
9	100	1	0.0138	0.0625
10	100	2	0.0162	0.173
11	100	3	0.0182	0.192
12	100	4	0.0146	0.176
13	120	1	0.0147	0.168
14	120	2	0.0172	0.112
15	120	3	0.0188	0.144
16	120	4	0.0177	0.172
K_1	00609 (02655)	00587 (03521)		
K_2	00559 (01854)	00621 (03855)		
K_3	00628 (06024)	00651 (04656)		
K_4	00686 (05970)	00623 (04471)		

Removal Efficiency of EPS to Cr (VI)

The structures of EPS are destroyed by the ultrasonic waves, which cannot separate the EPS solutions after chromium absorption. Thus, it uses indirect analytical method to calculate the absorption removal rate of EPS to chromium, which respectively determines the absorption removal rate of equivalent complete bacterial suspension and substrate solution after removing EPS to chromium, and then subtraction to obtain the removal rate of EPS on chromium.

The test results show that the removal rate of dominant bacteria to Cr (VI) and total Cr is 38.32% and 32.62% respectively, and for substrate solution after removing EPS is 19.74% and 11.5% respectively. It is obvious that the removal rate for Cr (VI) is higher than the total Cr, indicating that there exists Cr reduction reaction in absorption removing, and the reduzate Cr (VI) is still in aqueous phase. It can indirectly reckon that the absorption removal rate of EPS to Cr (VI) and total Cr is respectively 18.58% and 21.12%, and the contribution rate for removing Cr (VI) and total Cr is 48.49% and 64.75% respectively.

DISCUSSIONS

Studies show that gas carbon dioxide, moisture that tourists exhale and volatile gases produced by body itself are the main causes of brick frescos color change, and moisture is the

biggest media medium. Meanwhile, tourist activities will change the microbial community structures, and proteobacteria microorganisms inside the Lascaux Caves have increased by 45-50% before tourism opening to 98% [5]. There are many cases about caves and tomb paintings infected by microorganisms caused by visitors. Castanar de Ibor caves of Spain natural monuments opening since 2003, owing to the fragments brought by visitors, it results in widespread outbreak of Fusarium and Mucor circinelloides. Therefore, closing the tourism for underground tomb plays an important role in maintaining the diversity of original biocenosis in the surface of frescoes and ecosystem stability, but long-term close may easily lead to the poor air exchange, bringing a potential threat to preserve tomb paintings. Thus, both open and close year round are not conductive to the long-term preservation of brick frescoes. Based on indoor temperature and humidity monitoring data, it suggests formulating open programs to promote exchange and circulation of the air inside and outside the cave, in order to ensure reasonable protection of brick frescoes.

The funguses in this study are mainly Aspergillus, Phialosimplex and Engyodontium; in Spain La Rabida monastery, the funguses isolated from the frescoes are mainly *Cladosporium, Engyodontium* and *Aspergillus*[6]; In Han Tomb murals of Mi County in Henan, the funguses isolated by applying cultivation method are mainly *Penicillium, Paecilomyces* and *Aspergillus*; in Xi'an Qujiang Bamboo Garden, the funguses isolated from the tomb paintings of West-Han Dynasty are mainly *Penicillium, Paecilomyces* and *Aspergillus*[7]. NCBI sequence alignment has found that gene library sequence which is similar to sequence in fungus ITS clone library is mostly likely to symbiotic bacteria isolated from ants, plant endophytes and animal pathogentic bacterium. A large number of studies indicate that funguses of ancient frescoes are associated with arthropod. It should be still further confirmed that arthropod and atmospheric particulate are main sources of microbial propagation in frescoes.

Regardless of traditional means of isolation and identification, or modern molecular biological identification technology, Aspergillus and Engyodontium play a key role in microorganism groups in corrosive frescoes. Molecular detection results in this study have a certain difference with traditional means of enrichment and separation culture, which is closely related to detection technology itself. Firstly, traditional means of enrichment and isolation culture cab only get about 1% of microorganism, while other 99% of microorganisms related to frescoes discoloration and aging are hard to recover, unable to truly realize the microbial diversity[8]. Secondly, microbial groups in frescoes have dynamics and successions in time and space; micro-environmental and nutritional changes will have new variations in microbial community structures. Modern molecular biological technique has an irreplaceable advantage of traditional isolation culture in fast and accurate detection of corrosive microbial communities, and also has a good prospect in the in-depth of biological corrosion mechanism.

However, after removing EPS by calculating complete bacteria and equivalent bacteria, it can achieve the removal rate difference of substrate solution to Cr (VI) and total Cr, and the removal rate of dominant bacteria EPS indirectly obtained to Cr (VI) and total Cr is 18.58% and 21.12% respectively, the removal contribution rate of 48.49% and 64.75%, which is easy to see that the contribution of EPS in Cr (VI) is significantly lower than to total Cr.

CONCLUSIONS

We can draw a conclusion from the study that: to complete bacteria, EPS is the major site of Cr (VI) absorption, which makes a good reduction for Cr (VI). When the concentration of Cr (VI) in wastewater exceeds a certain range, it will be likely to act on cell wall through concentration gradient, to realize the reduction and absorption of Cr (VI) with the cell wall and spheroplast together. Therefore, continuous investigation on reduction and absorption capacity of spheroplast to Cr (VI) is of great significance for further exploration of the reduction absorption removal mechanism of dominant bacteria to Cr (VI).

REFERENCES

1. FlemmingH C,W ingender J. Relevance ofmicrobial ex-tracellular polymeric substances

(EPSs) Part: structural and ecological aspects. *Water Sci Technol*., 2001; **43**(6): 1-8.

2. Chojnacka K, Chojnacki A, GZrecka H. Biosorption of Cr^{3+}, $Cd^{2+}$and Cuions by blue-green algae Spirulina sp. kinetics, equilibrium and theme chanism of the process. *Chemosphere*, 2005; **59**(1): 75-84.
3. Pepe O., Sannino L., Palomba S., Anastasio M., BlaiottaG., VillaniF., MoschettiG. Heterotrophic microorganisms in deteriorated medieval wall paintings in southern Italian churches. *Microbiol Res*, 2005; **165** (1): 21-32.
4. Dupont J., Jacquet C., Dennetière B., Lacoste S.,Bousta F., Orial G., Cruaud C., Couloux A., Roquebert M.F. Invasion of the French Paleolithic painted cave of Lascaux by members of the *Fusarium solani* species complex. *Mycologia*, 2007; **99**(4): 526-533.
5. Zhang Y,Fang X L,Ye Z L,etal. B iosorption of Cu(II) on extracellular polymers from *Bacillus* sp. F19. *J Environ Sc,i* 2008; **20**(11): 1288-1293.
6. Mohammadi P., Krumbein W.E. Biodeterioration of ancient stone materials from the Persepolis monuments (Iran).. *Aerobiologia*, 2007; **24**(1):27-33.
7. Long LK, Yang SZ, Yao Q, Zhu HH, DNA extraction from arbuscular mycorrhizal fungi and analysis by PCR-denaturing gradient gel electrophoresis. *Mycosystema*, 2005; **24**(4): 564-569 (in Chinese)
8. Yu YG, Zhao B, The interaction and effect of two species of arbuscular mycorrhizal fungi on the growth of *Astragalus sinicus* L. at different pH level. *My cosystema*, 2008; **27**(2): 209-216 (in Chinese).