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Community of Environmental *Streptomyces* Related to Geosmin Development in Chinese Liquors

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ABSTRACT: Diverse *Streptomyces* species act as geosmin producers in the Chinese liquor-making process. In this paper, the ecology of these *Streptomyces* species was analyzed using denaturing gradient gel electrophoresis (DGGE) of amplified *Actinobacteria*-specified rDNA. The result showed that *Streptomyces* were widely distributed during *Daqu* incubation, and multiple processing, geographic, and climate factors can affect their distribution and diversity. The genes associated with geosmin production were characterized in four geosmin-producing *Streptomyces* strains, all of which were isolated from geosmin-contaminated Daqu. On the basis of this information, a real-time PCR method was developed, enabling the detection of traces of *Streptomyces* in complex solid-state matrices. The primer was targeted at the gene coding for geosmin synthase (*geoA*). The real-time PCR method was found to be specific for geosmin-producing *Streptomyces* and did not show any cross-reactivity with geosmin-negative isolates, which are frequently present in the Chinese liquor-brewing process. Quantification of *geoA* in the Chinese liquor-making process could permit the monitoring of the level of geosmin producers prior to the occurrence of geosmin production. Comparison of the qPCR results based on the gene encoding geosmin synthase and *Actinobacteria*-specified rDNA showed that about 1−10% of the *Actinobacteria* carry the geosmin synthesis gene.

KEYWORDS: *earthy odor, geosmin, real-time PCR, Streptomyces, geosmin synthase, Chinese liquor*

INTRODUCTION

Geosmin, a volatile microbial metabolite that is responsible for an earthy odor, frequently and seriously deteriorates the aroma of foods and aquatic products. In former studies, we have identified geosmin as the source of the earthy odor in Chinese liquor.[1] Solid-state fermentation based on starters (*Daqu* in Chinese), with complex and stable microbial ecosystems, is a major characteristic of Chinese liquor. The functional mold, bacteria, and yeast inoculated by nature provide numerous flavor compounds to affect liquor quality. However, diverse *Streptomyces* species act as geosmin producers in the Chinese liquor-making process.[2] *Streptomyces* is the largest genus of *Actinobacteria*, but the ecology of these microbes in liquormaking is not well understood. Therefore, it is necessary for us to explore the community structure of geosmin-producing *Streptomyces*.

Molecular fingerprinting methods, such as denaturing gradient gel electrophoresis (DGGE) and temporal temperature gradient gel electrophoresis (TTGE), have been widely applied for profiling environmental or food-associated microbial ecosystems, including soils,[3] wood,[4,5] pasture,[6] fermented products,[1,2] or grapes[7] and wine.[8] To our knowledge, common molecular fingerprinting methods have not been introduced in Chinese liquormaking as discriminatory methods to evaluate the risk of off-flavor development.

Additionally, geosmin detection and relevant information are most valuable for Chinese liquor producers to manage the development of off-odor events. Usually, the detection of geosmin is performed by gas chromatography−mass spectrometry (GC-MS). In former studies, we found that geosmin would be introduced to the fermentation process and then concentrated in liquor after distillation.[2] Therefore, when this compound is detected in liquor, it usually means that the off-flavor producers are well established in the Chinese liquor-making process. Early detection of off-flavor-metabolite producing *Streptomyces* species can be very useful in preventing the occurrence of such molecules by appropriate operating actions.

Up to now, the biological synthesis of geosmin is well understood. Geosmin is produced by the conversion of farnesyl diphosphate (FPP) into germacradienol and then geosmin, by a sesquiterpene synthase. The geosmin synthase consists of N- and C-terminal domains. Each domain catalyzes independently the consecutive biochemical reaction. The homologous genes encoding this synthase were also found in *Streptomyces coelicolor* A3 (2) (cyc2), *Streptomyces avermitilis* (*geoA*), and *Streptomyces peucetius* (Spterp13).[9,10] Moreover, species-specific primer systems of these microorganisms have been developed.[11,12] These gene sequences could be applied for discriminating interesting species from the complex microbial ecosystems. The detection and quantification of genes involved in the synthesis of geosmin and related microorganism can be a relevant indicator of the beginning of off-flavor events during the Chinese liquor-making process. Also, comparing the qPCR results based on geosmin synthase-specific primer system and the previously described *Actinobacteria*-specific primer system will help to provide a better understanding of the diversity and

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structure of geosmin-producing microbes in a complex microecology.

In the present study, a PCR-DGGE method was applied to the study of the microbial composition of Daqu samples related to geosmin production. The development of a qPCR protocol was reported for the detection and quantification of the genes related to geosmin biosynthesis during liquormaking.

MATERIALS AND METHODS

Strains and Culture Conditions. The strains used in the present study are listed in Table 2. Five *Streptomyces* strains (LBG-FXJ, HX, QC-1, QC-2, and QC-3) were identified as geosmin producers. Among them, LBG-FXJ was isolated from *Qingcha Daqu* of Laobaigan Distillery. HX was isolated from *Hongxin Daqu* of Fenjiu Distillery. QC-1, QC-2, and QC-3 were isolated from Qingcha Daqu of Fenjiu Distillery.

For some species, different strains were analyzed to evaluate the specificity of the designed primer set. All above strains were cultivated on potato dextrose agar (PDA) at 30 °C for 3 days before genomic DNA extraction.

Genomic DNA Extraction and PCR Amplification. Colonies were picked directly from agar plates, and nucleic acids were extracted using a modified method of Hoffman et al.[13] The final DNA of each sample was dissolved in 30 μL of H_2O. The DNA sample of each isolate was diluted 10 times to reduce PCR inhibitors. Purified DNA was quantified at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). In addition, the absence of extensive DNA degradation was verified by 1.5% agarose gel electrophoresis and ethidium bromide staining. PCR was performed with a C1000 Thermal Cycler provided by Bio-Rad Laboratories (Hercules, CA, USA). All reagents for PCR amplification and DNA purification, including the Taq DNA polymerase, were from Takara Biotechnology (Dalian, China).

Sample and Geosmin Quantification. Different stage samples of Daqu Qingcha (QC), Hongxin (HX), and Houhuo (HH) were obtained from Fenjiu Distillery Co. Ltd. (Fenyang, China). Qinke Daqu (QK) was obtained from Qinghai Mutual Aid Distillery Co. Ltd. (Xining, China). Concentrations of geosmin in these Daqu samples were quantified by headspace−solid phase microextraction−gas chromatography (HS-SPME-GC-MS) according to what we have reported.[2]

DGGE Analysis. PCR amplification for DGGE was performed with the *Actinobacteria*-specified primers of 16S rRNA, Com2xf (5′-AAACTCAAAGGAATTGACGG-3′) and Ac1186r modified with a GC-clamp (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG-CTTCCTCCGAGTTGACCC-3′) was employed after modification with a GC-rich clamp for DGGE. The PCR products were analyzed by DGGE using a Bio-Rad DCode Universal Mutation Detection System (Bio-Rad). Samples were applied to 8% (w/v) polyacrylamide gels in 1× Tris/acetate/EDTA (TAE) buffer [20 mmol L^{-1} Tris, 10 mmol^{-1} acetate, 0.5 mmol L^{-1} EDTA (pH 8.0)]. Optimal separation was achieved with a 30−50% urea−formamide denaturing gradient [100% correspondent to 7 mol L^{-1} urea and 40% (v/v) formamide]. The gels were electrophoresed for 200 min at 100 V and then stained with SYBR green I (Amresco, Solon, OH, USA) for 45 min. The gel was photographed with the GelDos 2000 system (Bio-Rad).

Cloning and Sequencing of *GeoA* from Geosmin-Producing *Streptomyces*. Geosmin synthase genes of different strains were obtained from NCBI. Sequences were downloaded from GenBank and aligned with both primers using the software package Vector NTI Advance 10. On the basis of the theoretically amplified fragment, sequences were verified again using BLAST search. Designed primers, 248F (5′-TCTTCTTCGACGACCACTTCCT-3′) and 1832R (5′-CCCTCGTACTCGATCTCCTTCT-3′), were employed for PCR isolating geosmin synthase from *Streptomyces* isolated from Daqu. The PCR amplification for each isolate was performed in a 25 μL volume containing 12.5 μL of GC buffer I (Takara Biotechnology, Dalian, China), 0.5 μL of either forward or reverse primer (25 μmol L^{-1} each), 1 μL of template DNA (50 ng), and 10.5 μL of H_2O. The following conditions were used in PCR: an initial preheat for 5 min at 95 °C, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 0.5 min, extension at 72 °C for 1.5 min, and termination with a final extension at 72 °C for 10 min. The amplicon of each isolate was purified and sequenced by the sequencing facility of Sangon Biotech (Shanghai, China).

Real-Time PCR Analysis. Two real-time PCR methods were developed for the screening of Daqu DNA samples. Primers used for qPCR for *geoA* were 245F (5′-TCTTCTTCGACGACCACTTCCT-3′) and 551R (5′-CGGCGCATCTCGATGTACTC-3′), generating an amplicon of 270 bp. The nucleotide sequence of primer Com2xf/Ac1186r, specific for *Actinobacteria* 16S rRNA gene sequences, was obtained from the paper reported by Schäfer et al.[12] Primers used for qPCR for 16S were Com2xf (5′-AAACTCAAAGGAATTGACGG-3′) and Ac1186r (5′-CTTCCTCCGAGTTGACCC-3′), generating an amplicon of 300 bp.

Each qPCR reaction was performed in 20 μL with SsoFast EvaGreen Supermix (Bio-Rad), 300 nM of each primer, and the template DNA. Ten nanograms of total DNA extracted from Daqu sample was used. The amplification conditions were as follows: preheating at 98 °C for 2 min and then 40 cycles of 98 °C for 5 s, 56 °C (for *geoA*) or 60 °C (for 16S) for 5 s, and 68 °C for 15 s. Melting curves were performed to confirm the purity of the amplified product.

RESULTS AND DISCUSSION

Communities of *Actinobacteria* in Different Types of Daqu. The ecology of *Actinobacteria* spp., which may act as geosmin producers in the Chinese liquor-making process, was analyzed using DGGE of amplified rDNA. The DNA exacted from Daqu samples was used as a template for PCR with the *Actinobacteria*-specific primers of 16S rRNA. The amplicons were analyzed using DGGE. Four strains isolated from Daqu, which were well identified as geosmin producers by GC-MS analyses, were treated as markers in the DGGE analysis. Positive PCR amplification could be obtained for most Daqu samples, except for the samples ready for incubation. As shown in Figure 1A, DGGE profiles revealed the presence of a fairly simple *Actinobacteria* community with no more than five different bands in a single sample. At the early stage of QC Daqu incubation, the bands of samples were simple (Figure 1A, 8), less than three bands. As the incubation period persisted, fingerprint richness got higher in QC Daqu samples (Figure 1A, 10−17). This indicated that the *Actinobacteria* community became more complex during the incubation period of QC Daqu. HX (Figure 1A, 21 and 22) and HH (Figure 1A, 23) Daqu showed similar levels of fingerprint richness. On the bais of the DGGE analysis, a similarity in community structure was found between QC and HX Daqu samples. In contrast to marker, the bands representing QC-1 and FXJ, respectively, were the most frequently present among the Daqu samples made by Fenjiu Distillery, including QC, HX, and HH Daqu. However, the DGGE profile of QK Daqu (Figure 1A, 18), which was made by different distilleries, was significantly different from those of all the other Daqu samples obtained from Fenjiu Distillery. A definitely different community structure was observed between Daqu samples made by Qinghai Mutual Aid Distillery and Fenjiu Distillery. The results indicated that multiple processing, geographic, and climate factors could affect the distribution and diversity of geosmin-producing *Streptomyces* bacteria.

Genes Encoding Geosmin Synthase in Five Isolated Geosmin-Producing *Streptomyces* Strains. Six genes of geosmin synthase in different *Streptomyces* species have been reported (listed in Table 1). On the basis of the conserved regions of these gene sequences, a primer pair, 248F (5′-

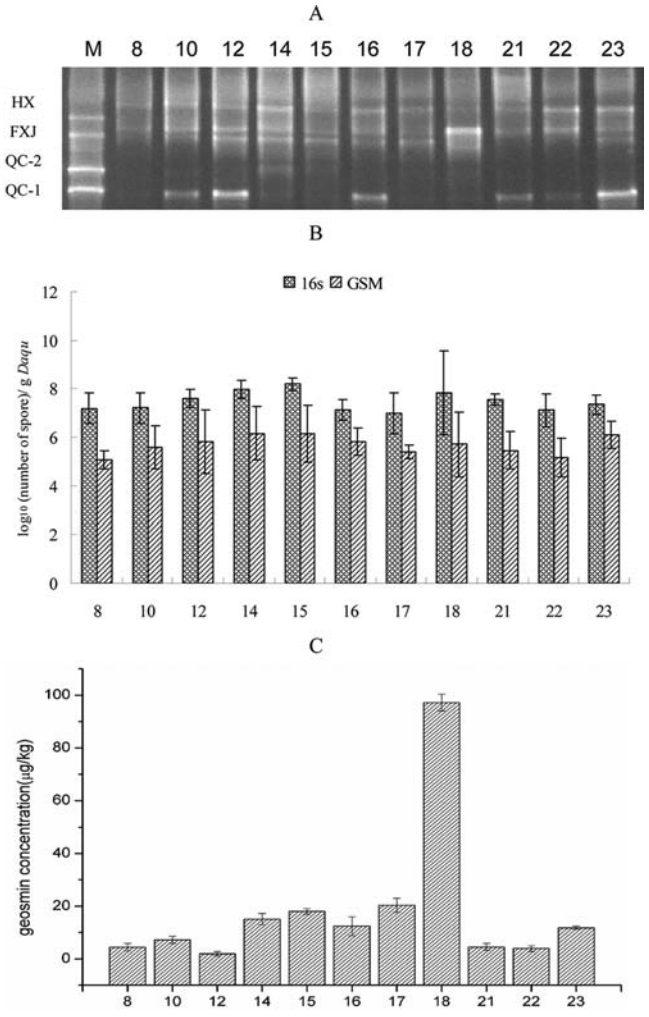

Figure 1. DGGE profile employed by Com2xf/Ac1186r (A), spore densities determined by the real-time PCR with the corresponding primers (GSM, 245F/551R; 16s, Com2xf/Ac1186r) (B), and geosmin concentrations (C) of Daqu belonging to different incubation periods and different areas (8−17, QC Daqu different incubation periods; 18, QK Daqu; 21−22, HH Daqu; 23, HX Daqu; M, HX, *Streptomyces fradiae* HX; FXJ, *Streptomyces albus* LBG-FXJ; QC-2, *Streptomyce sampsonii* QC-2; QC-1, *Streptomyces radiopugnans* QC-1).

Table 1. Strains with Published Geosmin Synthase Gene (*geoA*) Sequences from GenBank and Primer Pairs Used in This Study as well as Expected Length of the Amplicons

	expected length of the amplicon (bp)		
strain	248F/ 1832R	245F/ 551R	Com2xf/ Ac1186r
Streptomyces griseus subsp. *griseus* NBRC 13350	~1500 bp	~300 bp	~270 bp
Streptomyces peucetius ATCC 27952			
Kitasatospora setae KM-6054			
Streptomyces avermitilis MA-4680			
Streptomyces scabiei 87.22			
Streptomyces coelicolor A3 (2) SCO6073			

TCTTCTTCGACGACCACTTCCT-3′) and 1832R (5′-CCCTCGTACTCGATCTCCTTCT-3′), was designed so that the forward primer sequence would be anchored on the conserved aspartate-rich motif of the N-terminal domain, whereas the reverse primer flanked the C-terminal DDYYP or NSE triad regions.[14] Using the *geoA* cloning PCR procedure, about 1500 bp DNA fragments were amplified from all geosmin-producing *Streptomyces* strains isolated from Daqu, which were isolated and identified in a former paper.[2]

Design, Validation, and Specificity of the Geosmin Synthase-Specific Primer Set for qPCR. The geosmin synthase-specific primers were designed on the basis of the alignment of geosmin synthase gene. As shown in Figure 2, the forward 245F primer sequence would be anchored on the conserved aspartate-rich motif of the N-terminal domain, whereas the reverse 551R primer flanked the NSE triad region normally with 140 amino acids downstream of the aspartate-rich motif. The primer set amplified about 300 bp of the fragment of a partial sequence of N-terminal domain geosmin synthase. The primer set was tested in silico for sequences (gene sequences available from the GenBank data library). The theoretical fragment using the primer set was correctly amplified after BLAST search (http://www.ncbi.nlm. nih.gov/). Tests using isolates of selected geosmin-producing species with both conventional PCR and real-time PCR demonstrated that the primer pair 245F/551R can amplify all of the tested *Streptomyces* spp.

During Daqu incubation, filamentous fungi (*Rhizopus*, *Aspergillus*, *Monascus*, and other genera), yeasts (*Saccharomyces*, *Hansenula*, and other genera) and bacteria (*Bacillus*, *Clostridium*, and other genera), which are considered to be the functional microbiota, reproduce at a high number and act as the main community. These microorganisms are responsible for the formation of a range of enzymes, substrates for alcoholic fermentation, and flavor compounds.[8,15−17] Table 2 demonstrates that the real-time PCR with 245F/551R is able to detect isolates of *Streptomyces* species, all of which were GC-MS positive for geosmin. The real-time protocol did not amplify DNA from any GC-MS geosmin-negative isolates such as *Saccharomyces*, *Bacillus*, and *Mucor* sp., clearly implying that the presence of a geosmin synthase gene is invariably associated with geosmin production by the host, thereby validating the utility of the real-time PCR gene detection as a reliable diagnostic assay for geosmin producers.

In pure culture situation, DNA extracted from spore suspension for *Streptomyces sampsonii* QC-2 was serial diluted. As shown in Table 3, the standard deviations (SD) of triplicate Ct values were from 0.07 to 0.14. Amplification efficiency was 101.8% at the range of diluted 10^6 times. When the extract genome was diluted 10^6 times, genomic DNA was 0.51 pg. It is reported that 0.01 pg of genomic DNA represents approximately one genome equivalent to a single bacterial cell.[18,19] From this point of view, about 51 cells could be detected in the pure culture. In other words, the developed qPCR method is sensitive enough to detect the trace containment of geosmin-producing *Streptomyces*.

Finally, these analyses showed that the primer set 245F/551R was the best consensus between a short amplicon and a good specificity and coverage of genes encoding geosmin synthase from different strains. The 245F/551R primer set seems suitable for combination with real-time qPCR.

Experimental Determination of the Sensitivity and Efficiency of the Real-Time qPCR Approach. Found predominantly in soil and decaying vegetation, most *Streptomyces* produce spores[20] and are noted for their distinct "earthy" odor that results from production of a volatile metabolite, geosmin.[21] Significant correlations between the

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Figure 2. Multiple sequence alignment of geosmin synthase genes from *Streptomyces albus* LBG-FXJ (L1 VECTER), *Streptomyces fradiae* HX (L2 VECTER), *Streptomyces radiopugnans* QC-1 (L3 VECTER), *Streptomyces avermitilis* MA-4680 (MA-4680), *Streptomyces peucetius* ATCC 27952 (S peucetius), *Streptomyces griseus* subsp. *griseus* NBRC 13350 (S griseus subsp.), *Streptomyces coelicolor* A3 (2) SCO6073 (S SCO6073), *Streptomyces scabiei* 87.22 (S scabiei), and *Kitasatospora setae* KM-6054 (K setae). The red-boxed areas indicate the location of geosmin synthase-specific primer, 245F/551R, based on the alignment of geosmin synthase genes.

Table 2. Specificity of Designed Primer 245F/551R for qPCR[a]

strain	geosmin production	qPCR
Streptomyces albus LBG-FXJ	+	+
Streptomyces fradiae HX	+	+
Streptomyces radiopugnans QC-1	+	+
Streptomyce sampsonii QC-2	+	+
Streptomyces albus QC-3	+	+
Hanseniaspora sp. BJY-10	−	−
Saccharomyces cerevisiae C-1	−	−
Penicillium chrysogenum S-13	−	−
Bacillus amyloliquefaciens 644	−	−
Absidia corymbifera S-11	−	−
Mucor circinelloides S-12	−	−
Trichoderma viride S-34	−	−

[a]+, with geosmin production or positive qPCR result ($0 < Ct \leq 30.0$); −, without geosmin prodction or negative qPCR result ($Ct = 0$ or ≥ 40.0).

Table 3. Reliability of the Real-Time PCR Method for *Streptomyces sampsonii* QC-2 Amplified with 245F/551R at 10× Serial Dilutions of DNA Concentrations

dilution times	log DNA concn (ng/μL)	replication	average Ct value	SD[a]
1	2.71	3	13.84	0.09
10	1.71	3	16.63	0.07
10^2	0.71	3	19.46	0.13
10^3	−0.29	3	22.97	0.10
10^4	−1.29	3	26.62	0.14
10^5	−2.29	3	29.65	0.10
10^6	−3.29	3	33.38	0.11

[a]Standard deviation of Ct value.

$\log_{10}$ number of the spore number in Daqu samples and those quantified with the real-time PCR were obtained for geosmin synthase gene (*geoA*) and *Actinobacteria*-specific 16S rRNA gene (16S), respectively (Figure 3). The results indicate that the method provided accurate estimates of the geosmin-producing *Actinobacteria* in Daqu samples. The Ct of the no-templates assay was at least 3.3 cycles higher than that of the most diluted standard. In both cases, the limits of detection (LOD) were found to be 505 (for *geoA*) and 212 (for 16S) spores/g Daqu. The PCR assay was thus sensitive enough to allow the detection of traces of geosmin-producing *Actinobacteria* in a complex matrix, such as Daqu.

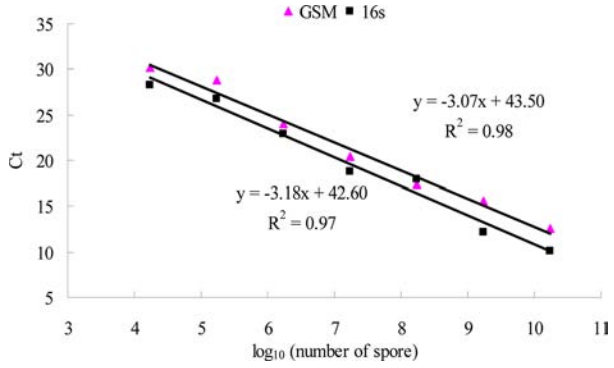

Figure 3. Relationship between the threshold cycle (C(t)) and $\log_{10}$ number of spores for *Streptomyces sampsonii* strain QC-2 amplified with the primer pair 245F/551R (GSM) and Com2xf/Ac1186r (16s). Template DNA was extracted from Daqu samples in which 10× serial dilutions of conidial suspensions of each strain were used to infest each corresponding Daqu sample prior to DNA extraction.

The efficiency of the real-time qPCR method for Daqu DNA extracts was tested by serial dilution of DNA templates derived from Daqu samples. The relationship between the Ct value and the logarithm of the amount of DNA template in the PCR was linear and highly significant (Figure 3). Both standard curves showed high linearity in a spike range of 10^4−10^{10} spores/g Daqu. The slopes of the calibration curves were −3.07 and −3.18, indicating amplification efficiencies of 111.7 and 106.3%, respectively. Initially, Com2xf/Ac1186r primer is designed to be specific for *Actinobacteria*.[12] Through our study, this primer system was also suited for real-time PCR to quantify the biomass of *Actinobacteria* in complex Daqu samples.

Application of qPCR to Daqu Samples and Validation of the Method. The real-time PCR method was applied for the detection of geosmin-producing *Actinobacteria* in various Daqu samples. The biomasses ($\log_{10}$ number of spores/g Daqu) determined by qPCR amplified by 245F/551R were compared to the values determined by Com2xf/Ac1186r. The numbers using geosmin synthesis gene were always less than those using the 16S rRNA gene; this is mostly 1−2 logs on the scale (Figure 1B). This implies that about 1−10% of the *Actinobacteria* carry the geosmin synthesis gene. On the other hand, we inferred that there were some *Actinomyces* species

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which did not produce geosmin in the Daqu samples, so the qPCR method based on genes encoding geosmin synthase provided more accurate estimates of the geosmin-producing microbes in Daqu samples.

Moreover, the spore numbers in most Daqu samples, determined by the developed qPCR method, were correlated with the results of geosmin concentration determined by GC-MS (Figure 1C). In Figure 1C, the concentrations of geosmin ranged from 1.93 to 97.20 μg/kg Daqu. The content of geosmin in QK Daqu was highest. However, the fluorescence quantitative result showed that the microbial biomass in QK Daqu was not much higher than that in the other analyzed samples. The possible reason is that there was a different geosmin-producing *Streptomyces* structure as found in Figure 1A. In QK Daqu, there may be some strong geosmin-producing *Streptomyces* species. Among the three types of Daqu, FJ-QC, FJ-HX, and FJ-HH, made in the same distillery, the biomass in FJ-QC Daqu sample (Figure 1B, 17) determined by the qPCR method was highest. We also have found the highest geosmin content in FJ-QC Daqu sample.[2] The quantification based on the gene encoding geosmin synthase could be determined as the geosmin marker in Daqu sample contaminated by geosmin-producing *Streptomyces* bacteria.

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Notes

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