World J Microbiol Biotechnol (2014) 30:3129–3136
DOI 10.1007/s11274-014-1740-y

ORIGINAL PAPER

Investigation of antibacterial activity of *Bacillus* spp. isolated from the feces of Giant Panda and characterization of their antimicrobial gene distributions

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Received: 22 January 2014 / Accepted: 10 September 2014 / Published online: 17 September 2014

Abstract *Bacillus* group is a prevalent community of Giant Panda's intestinal flora, and plays a significant role in the field of biological control of pathogens. To understand the diversity of *Bacillus* group from the Giant Panda intestine and their functions in maintaining the balance of the intestinal microflora of Giant Panda, this study isolated a significant number of strains of *Bacillus* spp. from the feces of Giant Panda, compared the inhibitory effects of these strains on three common enteric pathogens, investigated the distributions of six universal antimicrobial genes (*ituA*, *hag*, *tasA*, *sfp*, *spaS* and *mrsA*) found within the *Bacillus* group by PCR, and analyzed the characterization of antimicrobial gene distributions in these strains using statistical methods. The results suggest that 34 strains of *Bacillus* spp. were isolated which has not previously been detected at such a scale, these *Bacillus* strains could be classified into five categories as well as an external strain by 16S rRNA; Most of *Bacillus* strains are able to inhibit enteric pathogens, and the antimicrobial abilities may be correlated to their categories of 16S rRNA; The detection rates of six common antimicrobial genes are between 20.58 %(7/34) and 79.41 %(27/34), and genes distribute in three clusters in these strains. We found that the antimicrobial abilities of *Bacillus* strains can be one of the mechanisms by which Giant Panda maintains its intestinal microflora balance, and may be correlated to their phylogeny.

Keywords *Bacillus* · Antimicrobial activities · Antimicrobial genes · Giant Panda

Introduction

The Giant Panda (*Ailuropoda melanoleuca*), a bamboo specialist, is one of the most endangered animals in the world. With only 2,500–3,000 individuals left in western China (Zhan et al. 2006), these mammals consume about 12.5 kg bamboo each day (Schaller et al. 1985). With such a huge appetite, a lot of appurtenance, including soil and pathogens on the bamboo, is likely into the panda's digestive tract with the bamboo, that may cause panda intestinal diseases. Researches have shown that intestinal disease is the major one of the factors which caused pandas' death (Qiu and Mainka 1993), thus, it is important to control pathogens of panda's gut to keep the intestinal microflora balance.

In animal applications, *Bacillus* group is widely used as probiotics and food additives, these species have the remarkable ability of secreting a variety of products, including enzymes, antibiotics, amino acids, and insecticides (Lutz et al. 2006). For the biological control of pathogens, *Bacillus* group has also shown a significant ability of inhibition, which is mainly contributed by

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antimicrobial peptides (Abriouel et al. 2011). According to the literature (Zhao et al. 2013), antimicrobial peptides produced by *Bacillus* strains have the following two kinds: Ribosome synthesis antimicrobial proteins contain *lantibiotics*, membrane degrading enzymes as well as some unidentified inhibitory proteins; And non-ribosomal synthesis antibiotics primarily include lipopeptide antibiotics, such as *Surfactin*, *Iturin* and *Fengyein*. These substances act against competitive microorganisms, thereby generating a selective advantage for their producers.

Most of antimicrobial peptides display a high degree of target specificity against related bacteria, although many have a wider spectrum of activity, these substances generally coding by a mainly structural gene and a range of other regulatory genes (Hancock and Chapple 1999). *Subtilin* is one of the earliest discovered antimicrobial peptides in *Bacillus* strains (Salle and Jann 1945), as a lantibiotic, *subtilin* causes the dissipation of the transmembrane proton motive force as a result of pore formation, its precursor peptide chain is encoded by *SpaS* gene (Klein and Entian 1994). *Mersacidin* is a spherical lantibiotic, can bind with lipid II, an important synthesis of bacterial peptidoglycan precursors, to prevent the bacterial cell wall polymerization from monomer (Brötz et al. 1998), its biosynthesis gene cluster include a structural gene *mrsA*, and a range of other regulatory genes (Guder et al. 2002). *Iturin* is a large class of lipopeptides which can strongly inhibit pathogen growth, the family includes seven isomers connected by peptides and β-aminoadipic acid (Stein 2005); Huang et al. (1993) found lpa-14 gene encoding lipopeptide antibiotics *iturin A*. *Surfactin* family is a group of β-hydroxyhexanoic acid assembled by heptapeptides, it can penetrate the cell membrane, and has a strong inhibition to bacteria and fungi, *sfp* gene is required for *surfactin* (Nakano et al. 1992). Asano et al. (2001) cloned the *hag* gene, the antimicrobial activity of its expressed product *flagellin* explained the inhibitory appearance of many *Bacillus* strains. A 31-kDa sporulation protein coding by *TasA* gene, has a broad-spectrum antibacterial activity in the environment, conferring a competitive advantage to the spore (Stöver and Driks 1999).

Our previous research (Zhou et al. 2013) showed that some of seven *Bacillus* strains from Giant Panda's gut have an improving decomposition of cellulose, which could help their host digesting bamboos. Here, we illustrate the diversity of *Bacillus* strains found in Giant Panda in a much larger scale and demonstrate that most of them are able to inhibit enteric pathogens, which might be one of the mechanisms in maintaining panda's intestinal microflora balance. This study also investigate the distribution of six common antimicrobial genes in these *Bacillus* strains, and discuss their correlation of gene phylogeny.

Materials and methods

Isolation and purification of *Bacillus*

Permission to conduct this research was granted by the Director at the Giant Panda Base. Fresh stool samples were collected in sterile sampling bags from healthy pandas from Ya'an Bifengxia Base of China Conservation and Research Center for the Giant Panda (CCRCGP). The samples were preserved in an icebox, and sent to the laboratory for detection. Samples of 5.0 g were weighed in a sterile environment, mixed in 50 mL PBS, and placed in a water bath at 80 °C for 20 min. *Bacillus* spores are resistant to this temperature, whereas, the spores of other bacteria are killed (Travers et al. 1987). After gradient dilution, the solutions are spread on LB agar medium and left at 37 °C for 24 h. After that time, different strains of *Bacillus* were identified and separated repeatedly to obtain pure strains. After that, the *Bacillus* strains were inoculated into LB medium for cultural expansion.

16S rRNA sequence analysis and phylogenetic tree construction

Total DNA from each strain was extracted using the TIANGEN bacterial DNA extraction kit (Beijing TIANGEN Science and Technology Co., Ltd.) according to the recommended protocol. DNA was eluted in a final concentration of ~50 ng/μL using elution buffer and then stored at −20 °C. 16S rRNA PCR amplification was amplified using universal primers (Maiwald et al. 1994) 8F: 5′-AGAGTTTGATCATGGCTCAG-3′ (10 μmol/L), 1492R: 5′-ACGGTTACCTTGTTACGACTT-3′ (10 μmol/L). Each PCR amplification system was 25 μL, contained 2 μL of purified DNA template, 12.5 μL of 2 × Taq PCR Master Mix (Beijing TIANGEN Science and Technology Co., Ltd.), 1 μL of forward primer, 1 μL of reverse primer, and 8.5 μL of ddH_2O. Cycling conditions were as follows: 95 °C for 5 min, followed by 35 cycles of 95 °C for 40 s, 55 °C for 45 s, and 72 °C for 2 min, with a final extension period of 10 min at 72 °C and 4 °C termination reaction. Elution buffer of the DNA extraction kit was included throughout the PCR steps to serve as a negative control. The PCR product was detected by electrophoresis in 1 % agarose gel, and photographed using the BIO-RAD GelDoc XR System. The PCR product was purified by TIANgel Midi Purification Kit (Beijing TIANGEN Science and Technology Co., Ltd.) and sent for sequencing (Shanghai Invitrogen Biotechnology Co., Ltd.).

Sequencing data were applied to retrieve homologous sequences, using the BLAST algorithm in GenBank of NCBI, after that these sequencing data were updated to GenBank. The phylogenetic tree was constructed using the

neighbor-joining method (Saitou and Nei 1987) and maximum-parsimony method (Fitch 1971), using MEGA5 software. The confidence values for branches of the phylogenetic tree were determined using bootstrap analyses, based on 1,000 re-samplings. The tree graphics were edited using Dendroscope 3 (Huson and Scornavacca 2012).

Inhibitory spectrum of *Bacillus*

Bacillus strains were grown in 100 mL LB medium at 37 °C in a shaker at 150 rpm for 24 h. After cultivation, the cells were removed by centrifugation at 12,000 rpm for 10 min. The bacterial-free supernatants were extracted by rotary evaporator at 40 °C (Telke et al. 2011) and diluted to ~1 mg/mL as crude extracts. The pathogens were purchased from China Center for Type Culture Collection (CCTCC), included *Escherichia coli* (CCTCC AB 212358), *Staphylococcus aureus* (CCTCC AB 91053), and *Salmonella enteritidis* (CCTCC M 95026), which are common microbial gut isolates, were diluted to 1×10^6 previously. The antimicrobial abilities of *Bacillus* strains were assessed using the Oxford Cup method (Wheat 2001): 50 μL of each pathogen was coated on LB solid medium with the Oxford Cup in. 10 μL crude extract of each strain was then added at the midpoint of the Oxford Cup, cultivated at 37 °C for 24 h, and the inhibition zone was measured. LB liquid medium without any bacteria was also detected as a control. Inhibition zones by each strain were measured three times to reduce experimental error.

The characterization of between pathogen inhibitions and phylogeny of strains was also detected, by using Factors Loading Analysis by the Principal Component Extraction Method, through software STATISTICA, ver. 10, which is used to describe relatively few unobserved latent variables connected between variables as factors to reflect most of the information, to explain the original correlation (Comrey and Lee 2013).

Table 1 Specific primers for the functional genes of the peptides

Genes	Primers
ituA (D21876.1)	F: 5′-atgaaaatttacggagtatatatg-3′
	R: 5′-ttataacagctcttcatacgtt-3′
hag (AB033501.1)	F: 5′-atgagaatcaaccacaatatcgc-3′
	R: 5′-ttaacctttaagcaattgaagaac-3′
tasA (JF791687.1)	F: 5′-atgggtatgaaaaagaaattaag-3′
	R: 5′-ttagtttttatcctcactgtga-3′
sfp (EU882341.1)	F: 5′-atgaagatttacggaatttatatg-3′
	R: 5′-ttataaaagctcttcgtacgag-3′
spaS (DQ452514.1)	F: 5′-atgtcaaagttcgatgatttcga-3′
	R: 5′-ttatttagagattttgcagttaca-3′
mrsA (Z47559.1)	F: 5′-atgagtcaagaagctatcattcg-3′
	R: 5′-ttaacaaatacattcagaagttag-3′

Investigation of antimicrobial genes in *Bacillus*

The distribution of the six common antimicrobial genes found within the *Bacillus* group was detected in these strains from Giant Panda by PCR. Specific primers for the functional genes of antimicrobial peptides (Zhao et al. 2013; Abriouel et al. 2011), *ituA* (*Iturin A*), *hag* (*Flagellin*), *tasA* (*TasA*), *sfp* (*Surfactin*), *spaS* (*Subtilin*), and *mrsA*

Table 2 Names of strains and their GenBank accession numbers of 16S rDNA

Group	Name	Accession number	Reference strain	Identify (%)
A	HH1	KF860134	KC172005.1	99
A	GG3	KF860131	KC456632.1	100
A	GG1	JX489620	HM854728.1	99
A	GG2	KF860130	KF410851.1	100
A	TT1	KF860141	JX126865.1	99
A	YS1	JX489621	JQ308561.1	99
A	SY1	JX489618	GQ861467.1	100
A	YY1	JX482116	CP006881.1	100
A	HH2	KF860135	CP006881.1	99
A	JX2	KF860138	KC172005.1	99
A	JX1	KF860137	EU256502.1	99
B	FF2	JX489622	KC915229.1	99
B	FF1	JX489619	JX843447.1	99
C	ZX1	JX444509	KF601957.1	100
C	ZX2	KF860151	KF601957.1	100
C	AA1	KF860125	CP006863.1	99
C	QY1	KF860139	CP006863.1	99
C	CF1	KF860129	KF475814.1	100
C	XM1	KF860144	EU661712.1	99
D	WJ1	KF860142	KF208484.1	100
D	HC3	KF860133	JQ936679.1	99
D	YH5	KF860146	KF254668.1	100
D	YH4	KF860145	KF254668.1	99
D	YH7	KF860148	KF254668.1	99
D	AA5	KF860127	AB680477.1	99
D	AA6	KF860128	AB680477.1	99
	XL4	KF860143	AB680892.1	99
E	QY2	KF860140	AB680399.1	99
E	HC2	KF860132	NR_041490.1	99
E	YH6	KF860147	KF054925.1	99
E	YH8	KF860149	KF054925.1	99
E	YY2	KF860150	KF054925.1	99
E	HH3	KF860136	HQ202814.1	99
E	AA2	KF860126	AB115960.1	99

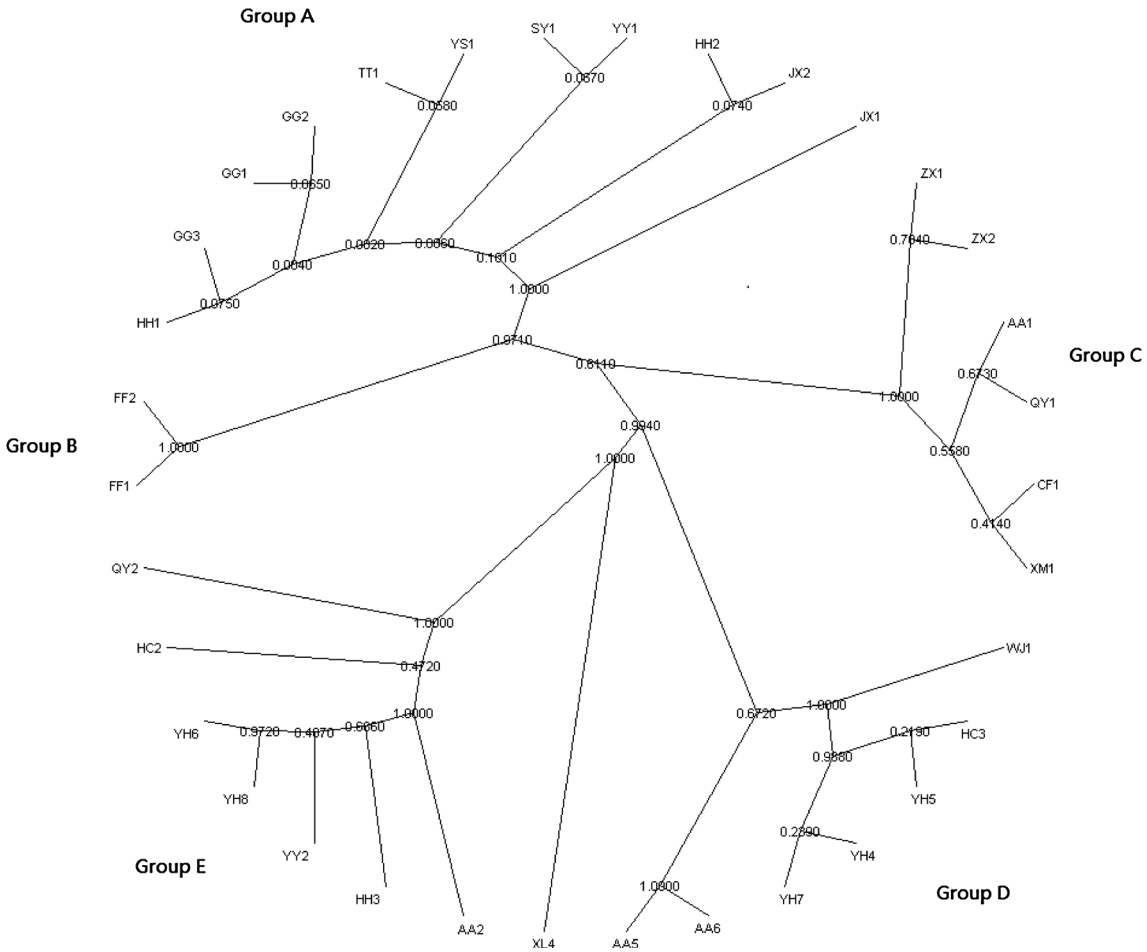

Fig. 1 The homologous tree of *Bacillus* strains. The homologous tree was constructed by MEGA5 and edited by Dendroscope 3, based on 16S rRNA genes of 34 *Bacillus* strains isolated from feces of Giant Pandas. Groups were classified by apparent branches of the homologous tree, since the XL4 was not situated in any branch, it was classified to an external strain. The number of members in these groups was fairly evenly distributed, with the exception of Group B, which had fewer numbers than others

(*Mersacidin*) are listed in Table 1. The PCR procedure was the same for 16S rRNA, except the primer, with cycling conditions as follows(Zhao et al. 2013): 95 °C, 3 min; 95 °C, 30 s, 51 °C, 60 s, 72 °C, 90 s, 30 cycles; 72 °C, 5 min; 4 °C termination reaction. The PCR product was detected by electrophoresis in 1 % agarose gel, and photographed using the BIO-RAD GelDoc XR System. Ten positive PCR products were selected randomly and sent for sequencing and validation (Shanghai Invitrogen Biotechnology Co., Ltd.). Sequencing data were applied to retrieve homologous sequences using the BLAST algorithm in GenBank. The regularities of distribution of six antimicrobial genes were detected using Principal Component Analysis (PCA) of STATISTICA, ver. 10. PCA is to investigate the correlation between multiple variables and find a few main components to reveal the internal structure (Abdi and Williams 2010).

Results

Isolation of *Bacillus* and 16S rRNA sequence analysis

To survey *Bacillus* species in the Giant Panda gut, *Bacillus* strains were isolated from Giant Panda stools, where *Bacillus* spores can survive due to their high temperature tolerance properties. After purification, 34 strains of bacteria were isolated and named by their hosts. Table 2 lists the names of these strains and the GenBank accession numbers for the 16S rRNA sequences. Using an analysis of the 16S rRNA sequence, we classified these strains into five categories. Since the XL4 was not similar with any group, it was classified as an external strain. The homologous tree is described in Fig. 1. The results demonstrate an abundant diversity of *Bacillus* in Giant Panda, since no one group was found to have significant dominance.

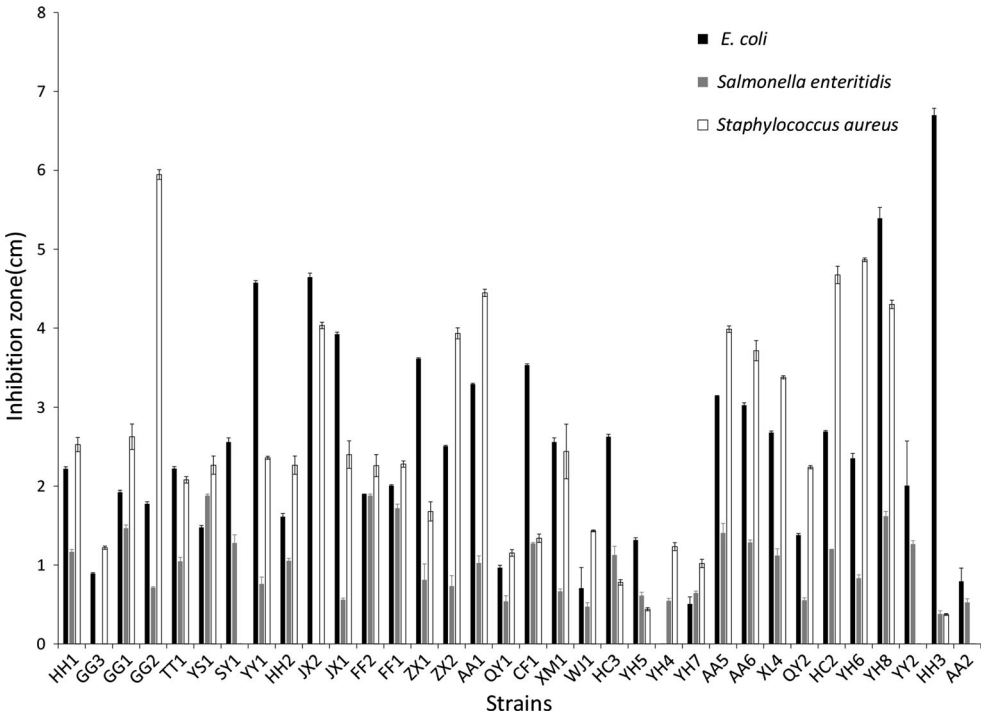

Fig. 2 The inhibition zones of *Bacillus* strains. The spectrum of antimicrobial activity of these *Bacillus* strains was detected by pathogen growth inhibition of crude extract of *Bacillus* strains, using Oxford Cup method. The inhibition zone were based on average and standard deviation of three-time-measured sizes of 34 *Bacillus* strains on three pathogens. These strains had highly inhibitory effect to *E. coli* (2.45 ± 0.05 cm in average) and *Staphylococcus aureus* (2.34 ± 0.07 cm in average), but not to *Salmonella enteritidis* (0.95 ± 0.05 cm in average)

Inhibitory spectrum of *Bacillus*

The spectrum of antimicrobial activity of these *Bacillus* strains was detected by pathogen growth inhibition, as observed using agar plates and the Oxford Cup method. We found that most of *Bacillus* strains were highly sensitive to *E. coli* and *Staphylococcus aureus*, however, they had no obvious inhibitory effect on *Salmonella enteritidis*. HH3 was the most active inhibitor against *E. coli* (6.70 ± 0.08 cm); YS1 showed the most inhibition toward *Salmonella enteritidis* (1.88 ± 0.02 cm); GG2 had the most inhibition to *Staphylococcus aureus* (5.95 ± 0.06 cm). Comparing this with the homologous tree of 16S rRNA, we found that, in the groups, Group E has the best average inhibition against three pathogens compared other groups, however Group D has the least. Details of the inhibition zones of *Bacillus* strains are described in Fig. 2.

We also analyzed the determinants of these strains' antibacterial abilities by Factor Analysis. In Fig. 3, the members' distributions of Groups A and B are mainly affected by Factor 2, but little affected by Factor 1; on the contrary, Group C–E are mainly affected by Factor 1 and little by Factor 2. Comparing the result with the categories of 16S rRNA, we found that the inhibitions of near-distance groups are affected by the same factors, so we assume that the two factors are related to their genetic homology. However, statistics could only predict but not define specific hidden variables or reasons, in future, we will undertake verification experiments to further explore the specific reasons or genes, affecting these two factors, to identify the correlation between their phylogeny and function.

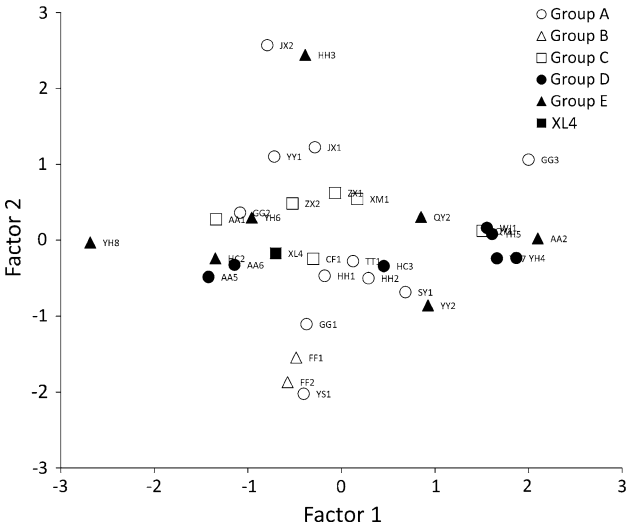

Fig. 3 Factors loading for antimicrobial activities of *Bacillus* strains. Factor analysis of antimicrobial activities of *Bacillus* strains was by Factors Loading Analysis by the Principal Component Extraction Method, through software STATISTICA, ver. 10., based on the inhibition zone data of *Bacillus* strains, the result without shaft. Factor 1 and Factor 2 were the assumed two major elements affected their antimicrobial activities, predicted by the software. Members' distributions of Groups *A* and *B* are mainly affected by Factor 2, but little affected by Factor 1; on the contrary, Group *C–E* are mainly affected by Factor 1 and little by Factor 2. Comparing with the homology of 16S rRNA, the inhibitions of near-distance groups are affected by the same factors

Table 3 Distribution of antimicrobial genes in 34 *Bacillus* strains

Group	Strais	Detection of antimicrobial genes					
		tasA	*spaS*	*ituA*	*hag*	*sfp*	*mrsA*
A	HH1	−	+	−	+	+	−
A	GG3	−	−	+	+	+	−
A	GG1	−	−	−	+	+	−
A	GG2	+	−	−	+	+	−
A	TT1	−	+	−	−	+	−
A	YS1	−	−	−	+	+	−
A	SY1	−	−	−	+	+	−
A	YY1	−	+	+	+	−	+
A	HH2	−	−	−	−	+	−
A	JX2	+	−	−	+	+	−
A	JX1	−	−	+	+	+	−
B	FF2	−	−	+	+	+	−
B	FF1	−	−	+	+	+	−
C	ZX1	−	−	−	+	+	−
C	ZX2	+	+	+	+	−	−
C	AA1	+	+	+	+	−	+
C	QY1	−	+	−	−	−	−
C	CF1	−	−	−	+	−	−
C	XM1	−	+	−	−	−	+
D	WJ1	−	−	+	−	−	−
D	HC3	−	−	−	−	+	+
D	YH5	+	+	−	+	−	−
D	YH4	−	+	−	−	−	−
D	YH7	−	−	−	+	−	−
D	AA5	−	+	−	+	−	+
D	AA6	−	+	−	+	−	+
	XL4	−	+	+	+	−	+
E	QY2	−	−	−	+	−	−
E	HC2	−	+	+	+	+	+
E	YH6	−	+	−	+	−	+
E	YH8	+	+	+	+	+	+
E	YY2	+	+	−	+	+	+
E	HH3	−	+	−	+	−	+
E	AA2	−	−	−	+	+	−

+ detection positive, − detection negative

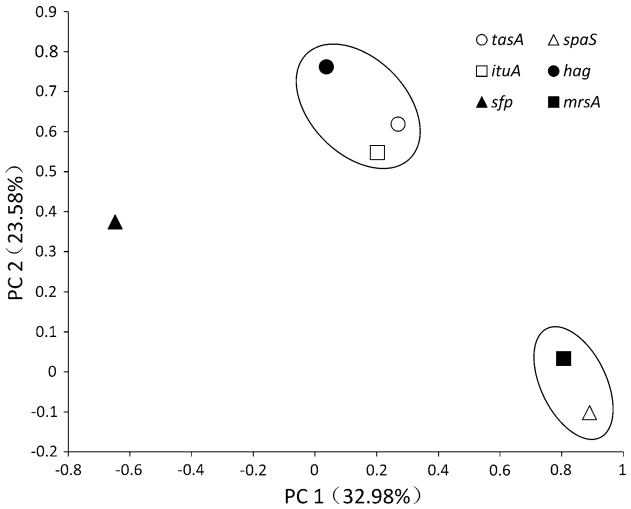

Fig. 4 Principal component analysis of distribution of six antimicrobial genes. The regularities of distribution of antimicrobial genes were detected using Principal Component Analysis of STATISTICA, ver. 10, based on the detection data of six antimicrobial genes by PCR. PC1 and PC2 were the two largest Principal Component sort of Contribution. Six genes distributed in three clusters, *hag*, *tasA*, and *ituA* formed a group, and *mrsA* and *spaS* had a short distance; however, *sfp* was at some distance from the others, which means *hag*, *tasA*, and *ituA* had some relevance to the genome; *mrsA* and *spaS* also appeared together easily; however, *sfp* showed little association with the others in these *Bacillus* strains

Investigation of antimicrobial genes in *Bacillus*

The distribution of the six common antimicrobial genes in *Bacillus* from Giant Panda was detected by PCR. Two or three antimicrobial genes were detected in most of the strains, with detection rates between 20.58 % (7/34) and 79.41 % (27/34). Among these, *hag* was the most distributed antimicrobial gene and *tasA* was the least. The sequences of randomly selected positive PCR products showed 96–99 % similarity with purpose genes, through BLAST proved the accuracy of PCR. Complete PCR results for the antimicrobial genes are presented in Table 3. In this result, the distributions of some groups' antimicrobial genes were similar. For example, *hag* and *sfp* could be simultaneously detected in most members of Group A; *spaS*, *hag*, and *mrsA* could be detected in Group F; and the distribution of genes in Group B was exactly same. The result of Principal Component Analysis found that the six antimicrobial genes are distributed in three clusters (Fig. 4), that demonstrate *hag*, *tasA*, and *ituA* had some relevance to the genome; *mrsA* and *spaS* also appeared together easily; however, *sfp* showed little association with the others in *Bacillus*.

Discussion

According to the literature (Zhu et al. 2011), *Bacilli* are the second most prevalent group of the Giant Panda's intestinal flora, the other large proportion of species include *Clostridia* and *Proteobacteria*. However, no research has, so far, specifically studied this important group. In this study, 34 strains of *Bacillus* from Giant Panda and their antimicrobial activities were investigated, which is the first time at such a scale.

Generally, *Bacillus* is not strictly a soil organism, instead, it has a biphasic life cycle, obtaining nutrients from the soil

and the gut. *Bacillus* plays an important role in maintaining a balance within the intestinal environment of its host gut, such as inhibiting the growth of pathogens and improving the digestibility of food matter (Roughead et al. 2012; Leonel Ochoa-Solano and Olmos-Soto 2006; Tam et al. 2006). Moreover, Some *Bacillus* strains have been used as probiotics for food additive and nutriceutical applications, based on functional properties such as improving body weight in farm animals and poultry (Cartman et al. 2008). One of the most important properties of these *Bacillus* strains is their significant effect in maintaining colonic microflora balance, and keeping Giant Panda healthy. Our previous research (Zhou et al. 2013) showed that some of strains obtained in this study could have various advantages, such as improving stress resistance and the decomposition of cellulose, both with potential probiotic applications. Here, we demonstrated that most of 34 strains are able to inhibit enteric pathogens, that may have significant effects to maintain Giant Panda intestinal microflora balance, and keeping Giant Panda healthy.

According to the literature, *Iturin A, Flagellin* and *TasA*, have their specific antimicrobial spectrum, however, *Surfactin, Subtilin*, and *Mersacidin* show antimicrobial activities in the nanomolar range against a broad spectrum of bacteria and fungi. Besides spectrum, their antimicrobial activities were also affected by skills of gene expression of producers. Both of them will affect these *Bacillus* strains to be potential sources of antibiotics. In this study, six antimicrobial genes were all detected from 34 *Bacillus* strains, and they are supposed to produce these peptides theoretically. Next step, we will try to purify and identify these antimicrobial substances, detect their specific activities, and investigate their expressions of strains in different conditions, to filter a potential source of new antibiotics.

Previous research indicates that, the homology of 16S rRNA of microbes could be an index to predict their functions (Langille et al. 2013). By using statistic methods, we found the that these strains' antimicrobial abilities may be correlated to the categories of 16S rRNA. A similar situation was also found in six common antimicrobial gene distributions, that these genes distribute in three clusters, and there may have some correlations between the gene distribution and phylogeny in these strains. However, we do not have an exactly evidence to prove the sufficiently linked between phylogeny and function in these strains, which we need more experiments to explore and identify. Furthermore, we will undertake verification experiments to further explore the specific reasons, or genes, affecting factors in antimicrobial effect.

In summary, this study investigated the diversity of *Bacillus* from the gut of the Giant Panda, analyzed their phylogenetic relationships based on 16S rRNA, compared their inhibitory effects on three common intestinal pathogens, and detected the presence of genes for antimicrobial peptides. We have shown that antimicrobial abilities of *Bacillus* might be one mode of action to benefit the intestinal health of Giant Panda. Further screening could yield a probiotic application or antibiotics supplement to treat Giant Panda in the future.

Acknowledgments This work was supported by the National Natural Science Foundation of China (Number 31272620), Sichuan Provincial Department of Science and Technology Support Program (Number 2011NZ0060) and the Program for Changjiang Scholars and Innovative Research Team in University (Number IRT0848).

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