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Identification of antibiotic-producing *Bacillus sensu lato* isolated from national parks of Hoang Lien and Phu Quoc in Vietnam

Phân loại các loài vi khuẩn Bacillus sensu lato sinh kháng sinh phân lập tại vườn Quốc Gia Hoàng Liên và Phú Quốc

Research article

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Many lipopeptide antibiotics produced from *Bacillus sensu lato* (*Bacillus s. l.*) against drug-resistant bacteria have been recently reported. To explore the potential production of the antibiotics from this group of bacteria in Vietnam, we collected 38 soil samples from two national parks of Hoang Lien and Phu Quoc and isolated 411*Bacillus s. l.* strains. Of those, 22 strains had antagonistic activity against both susceptible *S. aureus* and *E. coli*. The strains were further tested on drug-resistant bacteria collected at Bach Mai hospital and 20 strains demonstrated antagonistic activity against at least 2 of 18 drug-resistant bacteria *K. pneumoniae*, *E. coli*, *A. baumannii* and *S. aureus*. Analysis of 16S rDNA sequence showed that most of the broad spectrum antibiotic producers were *Paenibacillus* species whereas narrow spectrum antibiotic producers were *Bacillus* species. Strains PQH 0103 and PQH 0410 were probably produced novel antibiotic agents as they suspected to be novel taxa in *Paenibacillus* genus. Moreover, strains PQH 1509 and PQH 1702 produced broad spectrum antibiotics were identified as *P. chitinolyticus*. To our best knowledge, there is no report on antibiotic production from this species. Further elucidation of chemical structure of antibiotics produced from *Bacillus s. l.* isolated in soils of Vietnam is needed.

Gần đây, nhiều kháng sinh lipopeptide từ vi khuẩn *Bacillus sensu lato* (*Bacillus s. l.*) có khả năng sinh kháng sinh kháng vi khuẩn kháng thuốc đã được phát hiện và công bố. Để tìm hiểu khả năng sinh kháng sinh của nhóm vi khuẩn này ở Việt Nam, chúng tôi tiến hành thu thập 38 mẫu đất tại vườn Quốc gia Hoàng Liên và vườn Quốc gia Phú Quốc và phân lập được 411 chủng vi khuẩn *Bacillus s. l.*. Trong số đó, 22 chủng có khả năng sinh kháng sinh kháng 2 chủng vi khuẩn *S. aureus* và *E. coli* nhậy cảm thuốc. 20 trong số 22 chủng này có hoạt tính kháng ít nhất từ 2 đến 18 chủng kiểm định kháng thuốc thuộc các loài *K. pneumoniae*, *E. coli*, *A. baumannii* và *S. aureus*. Dựa trên kết quả phân loại bằng trình tự gene 16S rRNA, các chủng *Bacillus s. l.* sinh kháng sinh phổ rộng là các loài *Paenibacillus* trong khi đó các loài sinh kháng sinh phổ hẹp là *Bacillus*. Trên cây phát sinh chủng loại, chủng PQH 0103 và PQH 0410 có thể là những loài mới và có tiềm năng sinh các chất kháng sinh mới. Hơn nữa, chủng PQH 1509 và PQH 1702 sinh kháng sinh phổ rộng được phân loại vào loài *P. chitinolyticus*. Đây là loài chưa có công bố nào về khả năng sinh chất kháng sinh và có thể là loài sinh chất kháng sinh mới. Nghiên cứu giải mã cấu trúc chất kháng sinh từ các loài *Bacillus s. l.* phân lập tại Việt Nam cần được làm rõ trong tương lai.

Keywords: antibiotic, antimicrobial agent, *Bacillus sensu lato*, biodiversity, drug-resistant bacteria and identification.

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1. Introduction

Since the discovery of penicillin, hundreds of antibiotics originated from microorganisms have been discovered, developed and used in medical clinics for treating infectious diseases (Walsh, 2003; Mandell et al., 2005). However, pathogenic bacteria exposed to antibiotic-containing environments can mutate their DNA content or acquire resistant genes by the mean of horizontal gene transfer in order to develop their own resistant mechanisms which result in the formation of drug-resistant bacteria (Toenover, 2006; Alekshun and Levy, 2007).

Emergence of drug-resistant bacteria is major global health threat associated with high mortality rates and medical costs. In a recent joint technical report, the European Medicines Agency (EMA) in collaboration with Action on Antibiotic Resistance (ReAct) estimated that at least 25,000 patients die each year in the EU from an infection due to drug-resistant bacteria (Freire-Moran et al., 2011). In USA, the death occurs in one of five patients infected with methicillin-resistant *Staphylococcus aureus* (MRSA) and there are more death arising from invasive MRSA infection than from AIDS patients (Dougherty and Pucci, 2012).

Although antibiotic resistance occurs in many bacteria species, *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumanni*, *Pseudomonas aeruginosa* and *Escherichia coli* (or the ESKAPE bacteria) are the most considerable group of drug-resistant bacteria (Shlaes, 2010). This group possesses a high risk of hospital acquired infection and can relate to outbreaks of diseases (D'Souza et al., 2010; Taneja et al., 2010). Thus, discovery of new antibiotics exhibiting the activity against this bacterial group is crucial needed.

Recently, many lipopeptide antibiotics produced from *Bacillus sensu lato* (*Bacillus s. l.*) have been reported (He et al., 2007; Wu et al., 2010; Guo et al., 2012). This group of antibiotics shows highly effectiveness against drug-resistant bacteria in *vitro* and *invivo* (Ding et al., 2011; Huang and Yousef, 2014). In order to explore the antibiotics from microbial resource in Vietnam, we isolated and identified antibiotic-producing *Bacillus s. l.* in two national parks of Hoang Lien and Phu Quoc.

2. Materials and methods

2.1 Bacterial indicators

Two common susceptible bacteria of *Escherichia coli* VTCC-B-482 and *Staphylococcus aureus* VTCC-B-658 available at Vietnam Type Culture Collection were involved into primary screening test. Additionally, 18 drug-resistant bacteria of *S. aureus* (3 strains), *E. coli* (6 strains), *K. pneumoniae* (6 strains) and *A. baumannii* (3 strains) were kindly received from Department of Medical Microbiology, Bach Mai hospital. Antibiotic susceptibility of the bacteria was tested using disk diffusion method. The resistance profiles were interpreted using documents guided by Clinical Laboratory and Standard Institute (CLSI). All of viable drug-resistant bacteria were performed under biological safety cabinet class II in a restricted area of the Institute.

2.2 Soil sampling and bacterial isolation

Thirty-eight soil samples were collected at a depth of 10 cm at two national parks of Hoang Lien (18 samples) and Phu Quoc (20 samples) in November, 2010 and 2011, respectively. For each sample, 10 grams of soil were dispersed into a 250 ml Erlenmeyer flask containing 90 ml of NaCl 0.85%. After shaking at 220 rpm for 30 min, the bacterial supernatants was collected and heated at 80^{o}C for 10 min in order to eliminate vegetable cells. The treated supernatant was then diluted and plated into Nutrient agar (Becton Dickinson and Company, France). After incubation at 25^{o}C for 5 days, colonies demonstrated distinct characteristics of size, colour, surface texture and margin were collected and stored at -70^{o}C in LB broth containing 20% glycerol.

2.3 Antagonistic activity test

From fresh cultures grown on nutrient agar, *Bacillus s. l.* strains were streaked on TSB agar (Becton Dickinson and Company, France) and incubated at 28^{o}C for 28 h. Using sterile steel roll with a diameter of 6 mm, agar plugs were prepared and transferred onto nutrient agar inoculated with certain bacterial indicators. After overnight incubation at 35^{o}C, a clear inhibition zone surrounding agar plugs was reported as positive result.

2.4 16S rDNA sequencing analysis

Bacterial DNA was extracted by soft lysis method. Briefly, fresh bacterial cells were treated in 0.2 ml of lysis buffer (100 mM Tris HCl, 100mM Na2EDTA, 1.5 M NaCl, 1% cetyltrimethyl ammonium bromide (CTAB), pH 8.0) added with 50 µl of SDS 20%. After mixing vigorously for 20 sec, the cells were incubated at 65^{o}C for 2 h. An equal volume of chloroform : isoamyl alcohol (49 : 1) was added to precipitate protein by a centrifuge step at 16,000 g for 5 min. The upper layer of DNA solution was transferred into a new tube, precipitated with cooled isopropanol, and washed with cooled ethanol 70%. The DNA pellet was suspended into TE buffer and stored at -20^{o}C for further analysis.

For 16S rRNA gene amplification, approximately 50 ng of template DNA was incorporated into 25-µl reactions containing 1x PCR buffer, 1U Taq polymerase (KaTaRa), 125 nM of each dNTP, and 400 nM of each universal primers 27F and 1525R. The PCR conditions were 95^{o}C for 5 min, following by 35 cycles of 30 sec at 95^{o}C, 30 sec at 55^{o}C and 1min 45 sec at 72^{o}C. The PCR products were confirmed by gel electrophoresis and then purified using a commercial DNA extraction kit (Qiagen). Nucleotide sequences were analyzed with universal primers 1492R, 800R and 518F on 3100 Avant Genetic Analyzer (Applied Biosystem). Quality of nucleotide chromatogram was checked and edited with Chromas lite 2.1. Nearly complete 16S rRNA gene sequences (~1.5 kb) were assembled using Clone Manager 8.0.

2.5 Phylogenetic analysis

All of the 16S rDNA nucleotide sequences were retrieved from GenBank. The sequences were aligned using Clustal W. Phylogenetic tree was constructed by neighbour-joining method using MEGA 5.

3. Results and discussion

3.1 Isolation of *Bacillus sensu lato*

Bacillus s. l. is a large and diverse group of endospore-forming bacilli that occupies in various natural habitats. This group consists of at least 37 genera belonging to three families Bacillaceae, Paenibacillaceae and Alicyclobacillaceae (Goldman and Green, 2009). Based on characteristic of heat-resistant endospores, vegetative bacterial cells of heterotrophic soil bacteria were eliminated and the isolation of endspores was performed. Totally, 411 *Bacillus s. l.* strains were isolated from 38 soil samples collected at Hoang Lien and Phu Quoc national parks.

3.2 Screening for antibiotic-producing *Bacillus sensu lato*

3.2.1 Primary screening

Primary screening for antibiotic-producing *Bacillus s. l.* was carried out using two susceptible *S. aureus* and *E. coli* available at VTCC. Of 411 *Bacillus s. l.* strains, 66 (16%) strains demonstrated antagonistic activity against one or two bacterial indicators. Of those, 17 strains had activity against only Gram-positive *S. aureus*; 27 strains against only Gram-negative *E. coli*; and 22 strains against both Gram-positive *S. aureus* and Gram-negative *E. coli* (Figure 1).

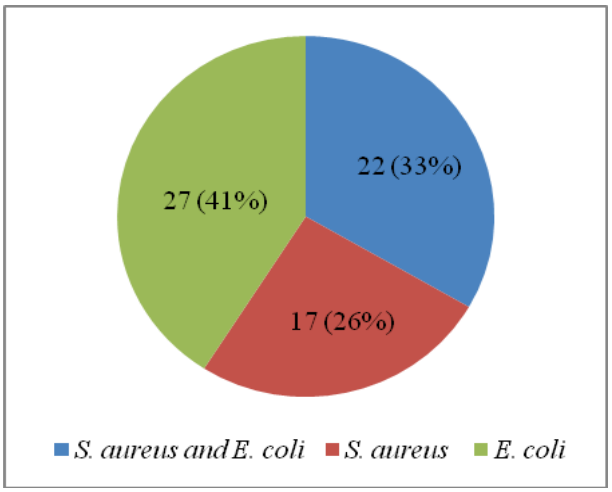

Figure 1. Number of *Bacillus sensu lato* strains produce antibiotics against susceptible *S. aureus* and *E. coli*.

3.2.2 Secondary screening

Antibiotic production was further tested on 18 bacteria *S. aureus*, *K. pneumoniae*, *E. coli* and *A. baumannii*. Based on the antibiotic susceptibility profiles, 3 strains of *S. aureus* were sensitive to phenicol, glycopeptide and oxazolidinone but resistant to penicillins, second generation of cephalosporin, amiloglycoside, fluoroquinolone, fosfomycin and macrolide; 6 strains of *E. coli* and 6 strains of *K. pneumoniae* were completely resistant to penicillins, all generations of cephalosporin, fluoroquinolone and fosfomycin; 3 strains of *A. baumannii* were resistant to penicillins, all generations of cephalosporin, amiloglycoside, fluoroquinolone, fosfomycin and polymyxin. As the pathogenic bacteria resistant to antibiotics in more than three antimicrobial categories, they were considerable to be multidrug-resistant bacteria (Magiorakos et al., 2011).

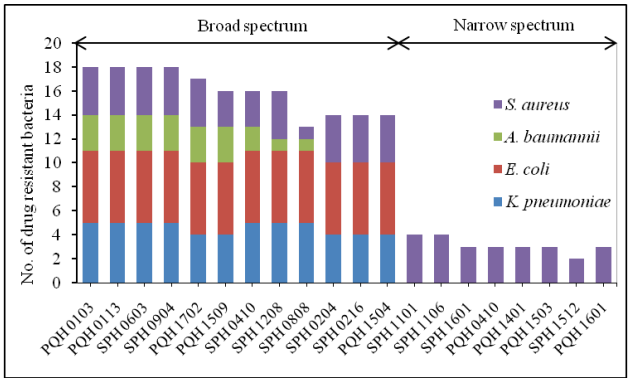

Figure 2. Antagonistic pattern of 20 *Bacillus sensu lato* strains against drug-resistant bacteria

Of 22 *Bacillus s. l.* strains selected for further test, 9 strains produced antibiotics against 4 species of drug-resistant bacteria *K. pneumoniae*, *E. coli*, *A. baumannii* and *S. aureus*; 3 strains had antagonistic activity against 3 species of drug-resistant bacteria *K. pneumoniae*, *E. coli* and *S. aureus*; 8 had antagonistic activity against only drug-resistant *S. aureus*; and the other 2 strains did not show antibiotic activity (Figure 2). Based on antagonistic pattern, the first 12 strains were classed into broad spectrum antibiotic producer and the latter 8 strains were classed into narrow spectrum antibiotic producers (Figure 2). Of broad spectrum antibiotic producers, 4 strains PQH 0103, PQH 0113, SPH 0904 and SPH 0603 had antibiotics against all of the tested drug-resistant bacteria.

3.3 Identification of antibiotic-producing *Bacillus sensu lato*

16S rDNA nucleotide sequences of 22 *Bacillus s. l.* strains were analyzed. Based on the closest match with type strains in the Eztaxon-e database, the bacterial strains were classified into 4 genera *Bacillus* (7 strains), *Paenibacillus* (13 strains), *Tumebacillus* (1 strain), and *Viridibacillus* (1 strain). In *Bacillus*, the bacteria were identified as *B. aryabhattai* (1 strain), *B. cereus* (3 strains), *B. pumilus* (1 strain), *B. safensis* (1 strain) and *B. vireti* (1 strain). *Paenibacillus* had *P. elgii* (2 strains), *P. jamilae* (3 strains), *P. chytinolyticus* (2 strains), *P. peoriae* (1 strain), *P. terrae* (4 strains) and *P. chibensis* (1 strain). Only one species obtained in each of the two last genera was *T. permanentifrigoris* (1 strain) and *V. arenosi* (1 strain).

Totally, 13 species were identified from of 22 *Bacillus s. l.* strains (Table 1).

Most of the *Bacillus s. l.* strains had the 16S rDNA nucleotide similarity greater than 99.3%. However, 4 strains designated as PQH 0103, PQH 0410, PQH 1601 and PQH 1005 showed a low 16S rDNA similarity value which was in a range from 97.8% to 98.6% (Table 1). All of the strains were isolated from soils of Phu Quoc national parks and probably presented novel taxa in *Bacillus*, *Paenibacillus* and *Tumebacillus* genera. Interested in the broad spectrum antibiotic producers, the phylogenetic tree of *Paenibacillus* species was constructed. Strain PQH 0103 and *P. tianmuensis* formed a separate clade with other closely related species *P. elgii*, *P. koreensis* and *P. ehimensis*; strain PQH 0410 also located distantly from *P. chibensis* and others (Figure 3). The result confirmed two potential novel species of *Paenibacillus* genus. Further assignment of those strains to species level is needed.

Table 1. Identification of antibiotic-producing *Bacillus sensu lato* based on Eztaxon-e database

	No.	Strains	Species	16S rDNA similarity (%)	Type strain (accession no.)
Broad spectrum antibiotic producer	1	PQH 0103	*Paenibacillus elgii*	98.66	SD 17 (AY090110)
	2	PQH 0113	*Paenibacillus elgii*	99.86	SD 17 (AY090110)
	3	SPH 0603	*Paenibacillus jamilae*	99.77	CECT 5266 (AJ271157)
	4	SPH 0904	*Paenibacillus jamilae*	99.59	CECT 5266 (AJ271157)
	5	PQH 1504	*Paenibacillus jamilae*	99.52	CECT 5266 (AJ271157)
	6	PQH 1702	*Paenibacillus chytinolyticus*	99.93	IFO 15660 (AB021183)
	7	PQH 1509	*Paenibacillus chytinolyticus*	99.93	IFO 15660 (AB021183)
	8	SPH 1208	*Paenibacillus peoriae*	99.36	DSM 8320 (AJ320494)
	9	SPH 0410	*Paenibacillus terrae*	99.49	AM141 (AF391124)
	10	SPH 0808	*Paenibacillus terrae*	99.49	AM141 (AF391124)
	11	SPH 0204	*Paenibacillus terrae*	99.48	AM141 (AF391124)
	12	SPH 0216	*Paenibacillus terrae*	99.49	AM141 (AF391124)
Narrow spectrum antibiotic producer	13	PQH 0410	*Paenibacillus chibensis*	97.84	JCM ^{9*905} (AB073194)
	14	PQH 1401	*Bacillus safensis*	100	FO-036b (AF234854)
	15	PQH 1601	*Bacillus vireti*	98.32	LMG 21834 (AJ542509)
	16	PQH 1503	*Bacillus aryabhattai*	99.93	B8W22 (EF114313)
	17	SPH 1101	*Bacillus pumilus*	99.79	ATCC7061(ABRX01000007)
	18	SPH 1106	*Bacillus cereus*	99.86	ATCC 15479 (AE016877)
	19	SPH 1512	*Bacillus cereus*	100	ATCC 15479 (PRJNA 57957)
	20	SPH 1601	*Bacillus cereus*	99.93	ATCC 15479 (PRJNA 57957)
Non*	21	PQH 1005	*Tumebacillus permanentifrigoris*	98.29	Eur 19.5 (DQ444975)
	22	SPH 0408	*Viridibacillus arenosi*	100	LMG 22166 (AJ627212)

* The strains did not show antagonistic activity against drug-resistant bacteria

Except for strain PQH 0410, all of the strains identified as *Paenibacillus* were grouped into broad spectrum antibiotic producers. Four strains PQH 0103, PQH 0113, SPH 0904 and SPH 0603 identified as *P. elgii* and *P. jamilae* produced antimicrobial agents against all drug-resistant bacteria. *P. chibensis* PQH 0410 and *Bacillus* species produced antibiotics against only Gram-positive *S. aureus*. Although *T. permanentifrigoris* PQH 1005 and *V. arenosi* SPH 0408 demonstrated antibiotic activity against two susceptible *S. aureus* and *E. coli*, none of those could inhibit the growth of drug-resistant bacteria.

Bacillus s. l. species have been known to produce a dozen of peptide and lipopeptide antibiotics. Based on biosynthetic mechanisms, the antimicrobial agents are classified into two types of ribosomally synthesized peptides and nonribosomally synthesized peptides. Ribosomally synthesized peptides or bacteriocins can exhibit a relatively narrow range of antimicrobial activity as paenibacillin produced from *P. polymyxa* (He et al., 2007), paenibacillin P and paenibacillin N produced from *P. alvei* (Anandaraj et al., 2009), pumilicin 4 produced from *B. pumilus* (Aunpad and Na-Bangchang, 2007), lichenicidin produced from *B. licheniformis* (Dischinger et al., 2009; Begley et al., 2009) and haloduracin produced from *B. halodurans* (Lawton et al., 2006). Our *Bacillus* strains and *P. chibensis* PQH 0410 showing antagonistic activity

against only Gram-positive *S. aureus* probably produced bacteriocin antibiotics.

Nonribosomally synthesized peptides show broader spectra of activities against bacteria or fungi (Abriouel et al., 2011; Lee and Kim, 2011). After discovery of penicillin, some of lipopeptide antibiotics from *Bacillus s. l* . have been used in medical clinics for treatment of infectious diseases; they are gramicidin (from *Brebacillus brevis*), bacitracin (from *B. subtilis*) and polymyxin (from *P. polymyxa*). Of those, polymyxin is playing an important agent in antibiotic therapy of Gram-negative resistant bacteria such as *A. baumannii*, *P. aeruginosa*, *K. pneumonia* và *E. coli* (Urban et al., 2010). In phylogenetic tree, the *P. jamilae* strains SPH 0603, SPH 0904 and PQH 1504 and *P. peoriae* SPH 1208 were grouped in the same cluster of *P. polymyxa* (data not shown). Therefore, the strains probably produced antibiotics related to polymyxin.

Search for new polymyxin derivatives that are less toxicity to human and higher efficiency against resistant bacteria is being received a considerable attention in antibiotic discovery from *Bacillus s. l.*. Many new lipopetide antibiotics are recently discovered and reported from *Paenibacillus* species such as pelgipeptins from *P. elgii* strain B69, cyclic lipopeptide antibiotics PE1 and PE2 from *P. ehimensis* strain B7 and octapeptin B5 from *P. tianmuensis* strain F6-B70 (Ding et al., 2011; Qian et al., 2012; Huang et al., 2013). In phylogenetic tree, those species were grouped in the same cluster (Figure 3) but the strain PQH 0103 formed a distinct clade which is expected to produce novel lipopeptide antibiotic.

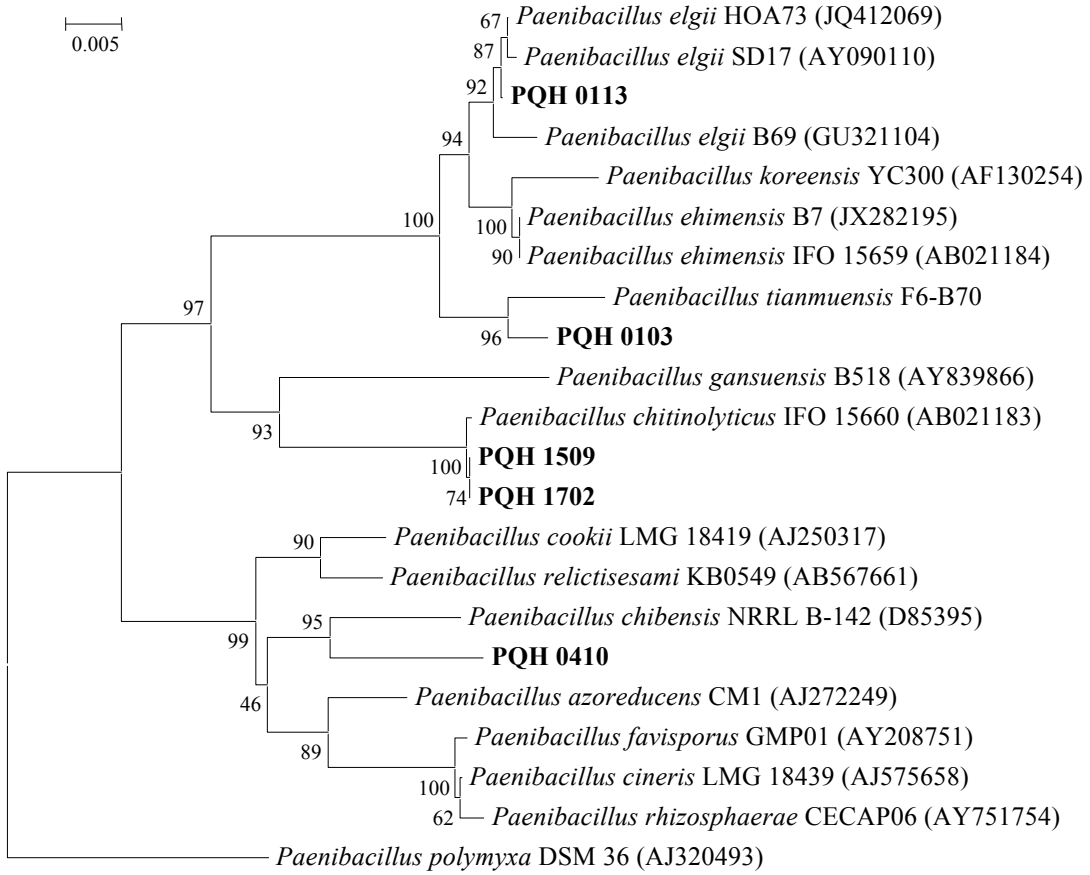

Figure 3. Phylogenetic tree based on 16S rDNA nucleotide sequences of *Paenibacillus* species

Antibiotic produced from *P. chibensis* also published by Lorentz et al., 2006. This study showed the strong ability against fungal pathogens of antibiotic produced from *P. chibensis* contributed to enhance biocontrolling potential. Specially, to our best knowledge, in six researched species from *Paenibacillus* genus, there was no report on antibiotic production from *P. chytinolyticus*. Therefore, we expected that this species produced a potential novel antibiotic.

4. Conclusions

Of 411 *Bacillus sensu lato* strains isolated from national parks of Hoang Lien and Phu Quoc, 22 strains had antagonistic activity against both susceptible *S. aureus* and *E. coli*. Of those, 20 strains demonstrated antagonistic activity against at least 2 of 18 drug-resistant bacteria of *K. pneumoniae*, *E. coli*, *A. baumannii* and *S. aureus*. Analysis of 16S rDNA sequence showed that most of the broad spectrum antibiotic producers were *Paenibacillus* species whereas narrow spectrum antibiotic producers were *Bacillus* species. Strains PQH 0103 and PQH 0410 were probably produced novel antibiotic agents as they suspected to be novel taxa in *Paenibacillus* genus. Moreover, strains PQH 1509 and PQH 1702 identified as *P. chitinolyticus* produced broad spectrum antibiotics. To our best knowledge, there is no report on antibiotic production from this species. Further elucidation of chemical

structure of antibiotics produced from the *Bacillus s. l.* isolated from soils in Vietnam is needed.

5. Acknowledgements

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