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EXPERIMENTAL ARTICLES

Phylogeny of 16S rRNA and *nifH* Genes and Regulation of Nitrogenase Activity by Oxygen and Ammonium in the Genus *Paenibacillus*[1]

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Abstract—All *Paenibacillus* 16S rDNA sequences, except for that of *Paenibacillus massiliensis* T7, formed a coherent cluster, distinct from gram-positive nitrogen-fixing *Clostridium pasteurianum* and *Heliobacterium chlorum*. All *Paenibacillus NifH* sequences formed two main clusters. Cluster I encompassing the *NifH* sequences from most of members of *Paenibacillus* spp., such as *Paenibacillus azotofixans* NifH1 and NifH2, *Paenibacillus polymyxa* and *Paenibacillus macerans*. Cluster II including only *P. azotofixans* NifH3. Curiously, three copies of *nifH* genes of *Paenibacillus sabine* T27 clustered within *P. azotofixans* cluster I (NifH1 and NifH2). The effect of O_2 and ammonium on nitrogenase activity was studied with 14 different nitrogen-fixing *Paenibacillus* strains. The optimal oxygen concentration level for all *Paenibacillus* strains is in the 0 to 0.05% range, similar to that for *Klebsiella pneumoniae*. In all *Paenibacillus* strains, the highest nitrogenase activity is obtained in the condition of 0–0.1 mM NH_4Cl and the increase of NH_4Cl from 0.1 to 5 mM caused a rapid inhibition of nitrogenase activity. However, the inhibition was reversible in the presence of 200 mM NH_4Cl in some *Paenibacillus* strains. It is the first time to use almost all of the recognized nitrogen-fixing *Paenibacilus* spp. to investigate the phylogeny of 16S rRNA and *nifH* genes. The data that the inhibition of O_2 and ammonium on nitrogenase acitivity will provide a base for studying the molecular regulatory mechanism of nitrogen fixation in the genus *Paenibacillus*.

Keywords: Paenibacillus, nitrogenase, *nifH*, 16S rDNA, nitrogen-fixing

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Nitrogen fixation has been studied extensively in a variety of gram-negative diazotrophs such as *Klebsiella pneumoniae* [1], *Azotobacter vinelandii* [2], *Azospirillum brasilense* [3, 4], *Rhodospirillum rubrum* [5], *Herbaspirillum seropedicaet* [6]. These bacteria are unable to fix nitrogen in the presence of high concentration of ammonia and O_2. They can fix nitrogen only under conditions of nitrogen limitation and anaerobiosis or at a very low dissolved O_2 tensions.

Paenibacillus is a genus of gram-positive, facultative anaerobic, endospore-forming bacteria, originally included within the genus *Bacillus* and then reclassified as a separate genus in 1993 [7]. At that time, the genus *Paenibacillus* encompassed 11 species, including the three nitrogen-fixing species *Paenibacillus polymyxa*, *P. macerans* and *P. azotofixans* [7]. Since then, continuous transfers of *Bacillus* spp. to the genus and descriptions of novel *Paenibacillus* spp. have increased the number of recognized *Paenibacillus* spp. considerably [8]. At this time of writing, this genus encompasses at least 19 nitrogen-fixing species, including the following 6 novel species described by our lab: *P. sabinae* [9], *P. zanthoxyli* [10], *P. forsythiae* [11], *P. sonchi* [12], *P. sophorae* [13] and *P. jilunlii* [14]. In addition, *nifH* gene has been found in *P. massiliensis* T7 [15] and *P. stellifer* [15]. Although the members of nitrogen-fixing *Paenibacillus* have great potential uses as a bacterial fertilizer in agriculture, the effects of ammonium and oxygen on nitrogenase activity have not been studied carefully.

In this study, five nitrogen-fixing *Paenibacillus* strains are isolated and their full-length 16S rDNA and partial *nifH* gene are cloned and sequenced. Further we investigate the comparative phylogeny of 16S rRNA and *nifH* genes of the five *Paenibacillus* strains, together with those from all of the 19 recognized nitrogen-fixing *Paenibacillus* species. The effects of oxygen and ammonium on nitrogenase activity of the five *Paenibacillus* strains and the selected 9 recognized nitrogen-fixing *Paenibacillus* species are studied.

MATERIALS AND METHODS

Bacterial strains. Five nitrogen-fixing *Paenibacillus* strains used in this study were isolated from the

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Table 1. Strains used for determining regulation of oxygen and ammonium on nitrogenase activity

Bacterial species	Strains	Sequence accession no.	
		16S rDNA	*nifH*
			AJ515294
Paenibacillus azotofixans	ATCC35681^T	AJ251192	AJ299453
			AJ299454
Paenibacillus graminis	RSA19^T	NR_028886	AJ223994
Paenibacillus jilunlii	Be17	GQ985392	GQ985393
Paenibacillus sophorae	S27	GQ985394	GQ985395
Paenibacillus sonchi	X19-5	EU867444	DQ358736
			DQ349125
Paenibacillus sabine	T27	NR_043729	HM583799
			HM583800
Paenibacillus massiliensis	T7	AY373370	AY373364
Paenibacillus zanthoxyli	JH29	DQ364788	DQ471303
Paenibacillus forsythia	T98	DQ349124	DQ338443
***Paenibacillus* sp.**	**WLY 1-18**	**JN873139**	**JN873136**
***Paenibacillus* sp.**	**WLY TD94**	**JN873142**	**JQ003558**
***Paenibacillus* sp.**	**WLY 1-43**	**JN873140**	**JN873137**
***Paenibacillus* sp.**	**WLY 1-49**	**JN873141**	**JN873138**
***Paenibacillus* sp.**	**WLY 78**	**JN873143**	**JQ003557**
Klebsiella pneumoniae	M5al	JQ003559	JQ003560

Five *Paenibacillus* strains isolated in this study is in bold.

rhizospheres of wheat, maize, scutellaria and bamboo, planted in different areas of China, based on their growth on nitrogen-free medium after heating at 100°C for 10 min [15]. Strains used for studying effect of oxygen and ammonium on nitrogenase activity are listed in Table 1 and they include the 5 *Paenibacillus* strains and the 9 selected recognized nitrogen-fixing *Paenibacillus* spp. Other reference type strains used for studying phylogeny of *rrs* and *nifH* genes are shown in Figs. 1 and 2 in the part of Results.

Acetylene reduction assay (ARA) of nitrogenase activity. For nitrogenase assays, all *Paenibacillus* strains and reference *K. pneumoniae* are grown at 30°C in the following media described by [17]. Nitrogen-free medium contains (per liter) 10.4 g Na_2HPO_4, 3.4 g KH_2PO_4, 26 mg $CaCl_2 \cdot 2H_2O$, 30 mg $MgSO_4$, 0.3 mg $MnSO_4$, 36 mg Ferric citrate, 7.6 mg $Na_2MoO_4 \cdot 2H_2O$, 10 μg *p*-aminobenzoic acid, 5 μg biotin and 4 g glucose as carbon source. Nitrogen-deficient medium contains 2 mM glutamate as nitrogen source supplemented in nitrogen-free medium. Nitrogen-excess medium contains 20 mmol/L NH_4Cl supplemented in nitrogen-free medium. For measuring the effect of ammonium on nitrogenase activity, 0~200 mmol/L NH_4Cl is used as nitrogen source and 0.5% O_2 is used. For measuring the effect of oxygen on nitrogenase activity, nitrogen-deficient medium containing 2 mM glutamate as nitrogen source was used. All *Paenibacillus* strains and *K. pneumoniae* were grown overnight in nitrogen-deficient medium (containing 2 mM glutamate as nitrogen source). The cultures were collected by centrifugation, washed three times with 0.9% saline water and then resuspended in a 10-mL flask with nitrogen-free medium to OD_{600} 0.1, supplemented with 0–200 mM NH_4Cl. The flasks were capped and filled with argon, and the oxygen concentration was adjusted, and at the same time 10% (v/v) acetylene was added. Cultures were incubated at 30°C. Ethylene production was analyzed by gas chromatography after incubation at 3, 4, 5, 6, 7 and 8 hours. All treatments were in three replicates and all the experiments were repeated three or more times. Nitrogenase activity was expressed as nmol ethylene/h/mg protein. Nitrogenase activity shown in Tables 2 and 3 is average of those obtained at at 3, 4, 5, 6, 7 and 8 hours.

Amplification, cloning and sequencing of 16S rDNA. Full-length 16S rDNAs (ca.1500 bp) were amplified as described by Yanagi and Yamasato [18]. The PCR products were ligated with vector pUC18 and then sequenced [15].

Amplification and sequencing of *nifH* gene. A 324 bp of *nifH* fragment from the five strains was amplified as described by [15] using the following primers: for-

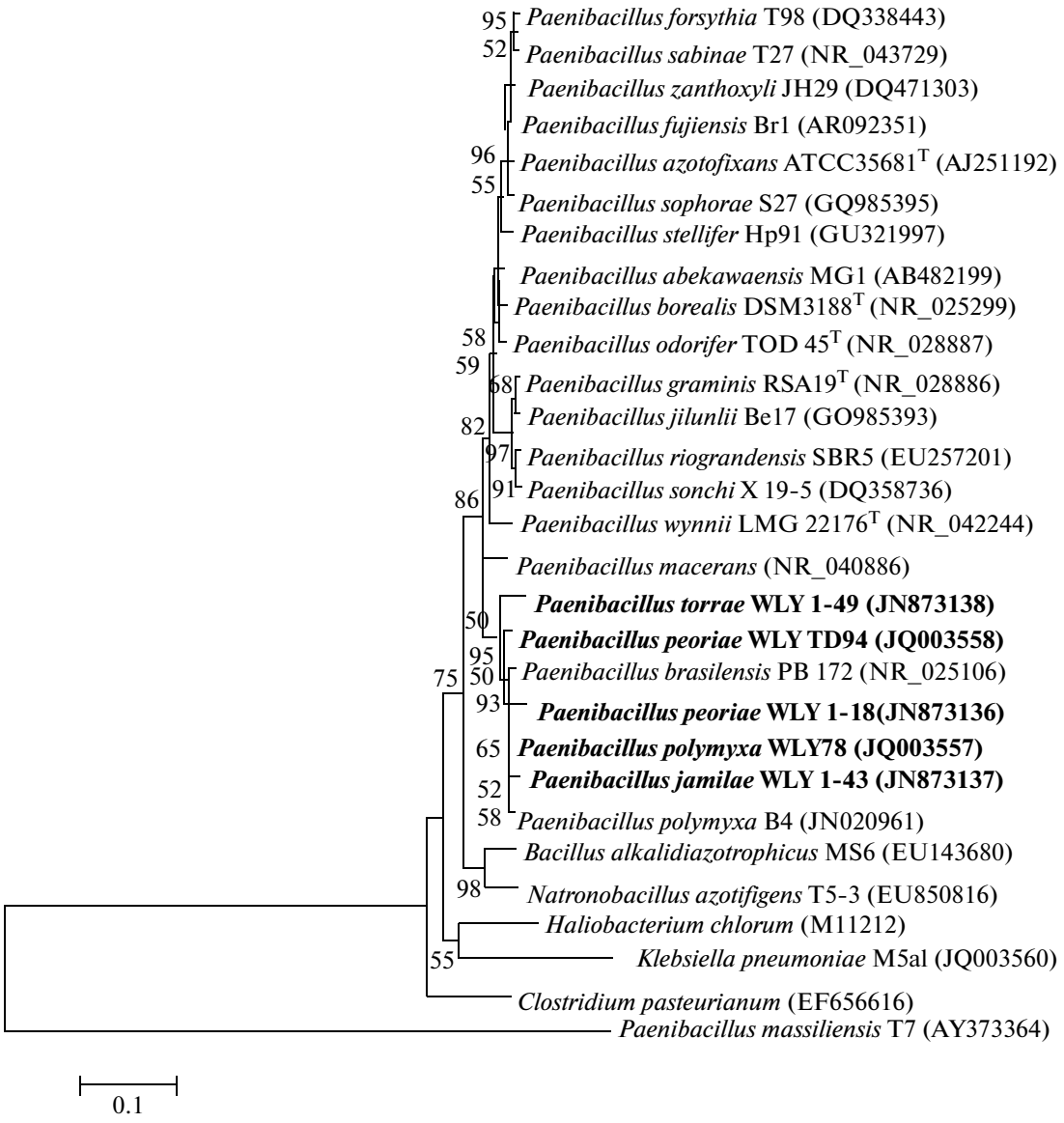

Fig. 1. Phylogenetic tree based on 16S rDNA sequences, including the sequences of the five isolated strains and 19 recognized *Paenibacillus* spp. and other sequences from the database. Trees were constructed by the neighbour-joining method and bootstrap values above 50% from 1000 bootstrap replicates are shown for each node. Graphic representation of the tree was made using MEGA 4.0 software.

ward 5'-GGCTGCGATCC(CGA)AAGGCCGA (CT)TC(CGA)ACCCG-3' and reverse 5'-CTG(GCA) GCCTTGTT(CT)TCGCGGAT(CG)GGCATGGC-3'. The *nifH* PCR product was purified and ligated to vector pMD18-T and then sequenced.

Data analysis. Sequences were aligned using the CLUSTALX software [19]. The evolutionary distances were calculated using DNADIST program in software package TREECONW. The phylogenetic tree was generated by the neighbour-joining method using the software package TREECONW [20].

RESULTS

Sequencing and phylogeny of 16S rDNA. In this study, five nitrogen-fixing strains, designated WLY 1-18, WLY 1-43, WLY 1-49, WLY TD94, WLY A78, are isolated from the rizhospheres of wheat (WLY 1-18), maize (WLY 1-43, WLY TD94), scutellaria (WLY TD94) and bamboo (WLY A78). The nearly complete 16S rDNA sequences of the five strains were amplified and sequenced (accession numbers shown in Table 1). Comparative 16S rDNA sequence analysis has demonstrated that the five strains belong to the genus *Paenibacillus*. The five strains showed high levels of

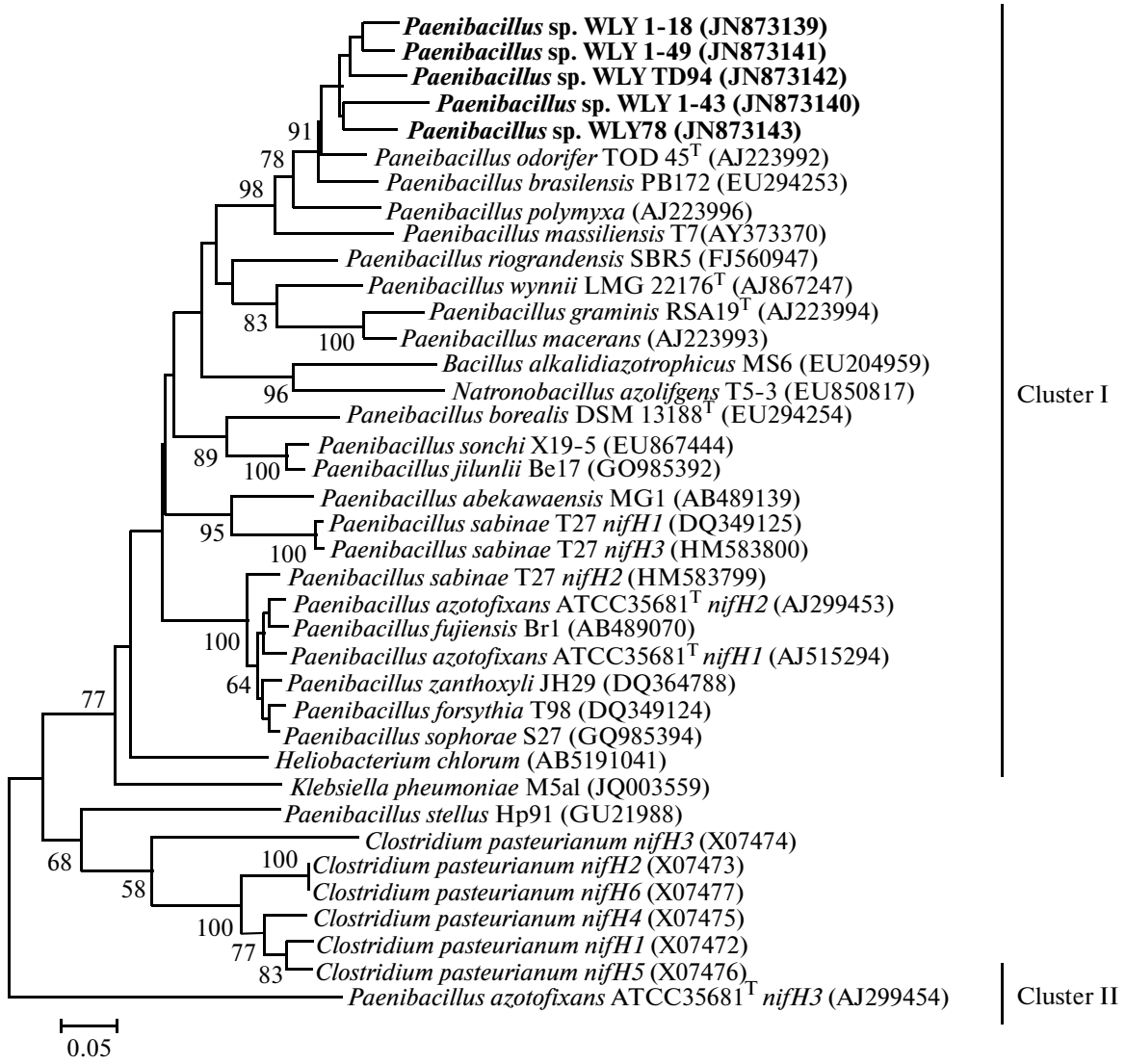

Fig. 2. Phylogenetic trees for NifH sequences analysed by the neighbour-joining method. Bootstrap values above 50% are shown for each node.

similarity with *P. peoriae* (97.5–99.3%), *P. jamilae* (97.7–99.7%), *P. brasilensis* (98.5–98.7%), *P. polymyxa* (97.7–98.7%), *P. terrae* (97.8–99.1%) and *P. kribbensis* (97.5–98.4%). Levels of 16S rDNA sequence similarity among the five novel strains were 95–96%. Fig. 1 shows the phylogenetic tree based on the 16S rDNA sequence of the five strains and the 19 recognized nitrogen-fixing *Paenibacillus* spp., compared with those of gram-positive *C. pasteurianum* and *H. chlorum*, and gram-negative *K. pneumoniae*. Except for *P. massiliensis* T7, all nitrogen-fixing *Paenibacillus* spp., such as *P. azotofixans, P. graminis, P. jilunlii, P. sophorae* and the new isolated five strains, formed a monophyletic cluster distinct from those of gram-positive *C. pasteurianum* and *H. chlorum*, and gram-negative *K. pneumoniae.* However, the 16S rDNA sequence of *P. massiliensis* T7 is not clustered within the *Paenibacillus* cluster.

Sequencing and phylogeny of *nifH* gene. Our previous work has identified three *nifH* genes from *P. sabine* T27 with gene cloning method (data not published) and their GenBank accession numbers are listed in Table 1. Here the partial *nifH* gene was amplified and sequenced from the five *Paenibacillus* strains and their accession numbers are shown in Table 1. Fig. 2 shows the phylogenetic tree based on the amino acid sequences deduced from the *nifH* fragments amplified from the five strains and *NifH* sequences of the 19 recognized nitrogen-fixing *Paenibacillus* spp. compared with those of gram-positive *C. pasteurianum* and *H. chlorum*, and gram-negative *K. pneumoniae.* The *NifH* tree constructed in this study (Fig. 2) has a topology similar to that of previously published trees of *NifH* phylogeny [21–24]. All *Paenibacillus NifH* sequences formed two main clusters, which are different from those of gram-positive *C. pasteurianum* and

Table 2. Effect of oxygen on the nitrogenase activity (nmol C_2H_4/mg protein hr)

Strains	Various concentrations of O_2						
	0%	0.05%	0.5%	1%	5%	10%	21%
K. pneumonia M5al	4371.3 ± 79.5	3681.5 ± 13.1	2650.5 ± 61.7	2422 ± 89.5	372.9 ± 29.1	0	0
P. azotofixans ATCC35681^T	2013.1 ± 24	2511.5 ± 20.1	1082.9 ± 15.2	120.6 ± 8.4	0	0	0
P. graminis RSA19^T	4316.9 ± 25.4	5228.4 ± 72	3348.7 ± 20	2413.8 ± 28.8	0	0	0
P. forsythia T98	114.6 ± 3.7	76.9 ± 8.7	37.7 ± 0.7	20.3 ± 0.8	0	0	0
P. massiliensis T7	748 ± 35.3	408.2 ± 22.8	54 ± 5.4	34.1 ± 4.4	0	0	0
P. jilunlii Be17	1171 ± 18.6	845.2 ± 8.0	639.2 ± 59.3	541.3 ± 17.5	0	0	0
P. sabine T27	2407.1 ± 26.3	2659.7 ± 26.3	1864.2 ± 5.0	1753.8 ± 13.8	1442.2 ± 13.2	0	0
P. sophorae S27	840.6 ± 13.6	770.7 ± 22.3	457.4 ± 13.5	169.7 ± 7.6	0	0	0
P. sonchi X19-5	1024.4 ± 39.4	882.7 ± 21	377.2 ± 11.4	302.6 ± 22.9	0	0	0
P. zanthoxyli JH29	3397 ± 13.2	3165.1 ± 48.4	478.9 ± 5.4	398.1 ± 13.1	0	0	0
Paenibacillus sp. WLY 1-18	152.1 ± 1.7	102.4 ± 3.9	52.8 ± 1.3	41.6 ± 0.6	0	0	0
Paenibacillus sp. WLY TD94	754.5 ± 24.6	574.4 ± 17.8	376.0 ± 1.0	249.9 ± 4.8	0	0	0
Paenibacillus sp. WLY 1-43	2706.2 ± 378.7	2600.0 ± 29.4	785.0 ± 31.4	428.2 ± 30.4	0	0	0
Paenibacillus sp. WLY 1-49	400.2 ± 16.3	378.4 ± 14.0	238.3 ± 16.5	176.5 ± 5.9	176.3 ± 4.1	0	0
Paenibacillus sp. WLY78	874.3 ± 22.1	1541.2 ± 49.2	626.1 ± 9.5	412.0 ± 14.1	247.3 ± 19.5	0	0

H. chlorum, and gram-negative *K. pneumoniae*. Cluster I encompasses the *NifH* sequences from most of members of *Paenibacillus* spp., such as *P. azotofixans NifH1* and *NifH*2, *P. polymyxa, P. macerans* and the five *Paenibacillus* strains. Cluster II only includes *P. azotofixans NifH3*. Curiously, three copies of NifH from *P. sabine* T27 clustered with *P. azotofixans* cluster I (*NifH1* and *NifH2*), different from that the three *NifH*s of *P. azotofixans* formed two clusters.

Effect of oxygen on the nitrogenase activity. To determine the effect of oxygen on the nitrogenase activity of *Paenibacillus* spp., we monitored their acetylene reduction rates in a vigorously stirred chamber at different O_2 levels. Table 1 presents the nitrogenase activity of all *Paenibacillus* spp. in comparison with that of *K. pneumonia* under various O_2 concentrations. In *K. pneumonia*, the highest nitrogenase activity was found in the absence of O_2, and the increase of O_2 concentration from 0.05 to 5% (5% O_2 approximately 1.0 kPa O_2) caused a rapid inhibition of nitrogenase activity. The optimal oxygen concentration level for all *Paenibacillus* strains is in the 0 to 0.05% range, similar to that of *K. pneumonia* (as shown in Table 2). The addition of 5% O_2 cause a rapid inhibition of nitrogenase activity in most *Paenibacillus* spp., while nitrogenase activity remained in a few *Paenibacillus* spp. The addition of 10% O_2 completely inhibited nitrogenase activity in all *Panebacillus* spp. and *K. pneumonia*.

Effect of NH_4Cl on the nitrogenase activity. The effect of the NH_4Cl concentration on the nitrogenase activity of *Paenibacillus* spp. was studied. Table 3 shows the inhibition pattern of NH_4Cl on the nitrogenase activity of *Paenibacillus* spp. in comparison with that of *K. pneumonia*. In *K. pneumonia*, the highest nitrogenase activity is obtained in the absence of NH_4Cl and the increase of NH_4Cl from 0.1 to 5 mM caused a rapid inhibition of nitrogenase activity. In some *Paenibacillus* strains, the inhibition pattern by NH_4Cl was similar with that of *K. pneumonia*. However, in other *Paenibacillus* strains, the nitrogenase activity remained even in the presence of 200 mM NH_4Cl, indicating the inhibition was reversible in the presence of high concentration of NH_4Cl.

DISCUSSION

In this study, five nitrogen-fixing *Paenibacillus* strains are selectively obtained and their 16S rDNA sequence and *nifH* gene are PCR amplified and sequenced. Comparative phylogeny analysis shows that all *Paenibacillus* 16S rRNA sequences except that of *P. massiliensis* T7 formed a coherent cluster. The reason that *P. massiliensis* T7 is phylogenetically distinct from other *Paenibacillus* spp. is duo to that *P. massiliensis* sp. nov. (type strain 2301065T), isolated from blood of a patient and without nitrogen-fixning ability, has lower (87.6–94.4%) similarity with the existing *Paenibacillu*s spp. [25]. *P. massiliensis* T7, isolated from the rizhosphere of willow, is a nitrogen fixer and it has 98% similarity with its type strain 2301065^T at the 16S rDNA sequence level.

As shown in Fig. 2, all *Paenibacillus NifH* sequences formed two main clusters. Cluster encompasses *P. azotofixans NifH1* and *NifH2* and the *NifH* sequences from most of members of *Paenibacillus* spp., such as

Table 3. The effect of NH_4Cl on the nitrogenase activity (nmol C_2H_4/mg protein hr)

Strains	Concentration of NH_4Cl (mM)									
	0	0.1	0.5	1	5	10	20	50	100	200
K. pneumonia M5al	1593.4 ± 14.2	1346.1 ± 21.6	462.8 ± 11.3	279.4 ± 14.1	52.7 ± 8.4	0	0	0	0	0
P. azotofixans ATCC35681^T	1573.1 ± 16.4	1400.4 ± 12.4	1143.3 ± 12.8	955.8 ± 14.1	424.1 ± 14.6	0	0	15.4 ± 1.7	40.6 ± 3.3	53.9 ± 2.2
P. graminis RSA19^T	2751 ± 15.6	2747.3 ± 44.7	3128.2 ± 7.6	2406.2 ± 26.9	139.4 ± 26.3	183.7 ± 28.7	123.4 ± 2.1	617.8 ± 65.2	1848.5 ± 29.5	808.7 ± 16.2
P. forsythia T98	411.3 ± 18.5	370.3 ± 45.4	162.5 ± 9.4	47.3 ± 3.3	5.7 ± 0.5	0	0	0	0	0
P. jilunlii Be17	531.8 ± 76	305.1 ± 20	143.7 ± 16.7	66.8 ± 4.9	4.5 ± 1.9	6.3 ± 1.5	9.9 ± 5.2	46.2 ± 9.3	2.5 ± 0.9	0
P. massiliensis T7	1135.6 ± 46.6	1879.8 ± 64.8	1356.3 ± 43.7	883.9 ± 20.6	457 ± 37.4	0	0	0	0	0
P. sabine T27	2353.6 ± 38.6	2656.8 ± 88.3	2156.5 ± 71.3	1475.7 ± 4.1	0	19.8 ± 9.6	29.3 ± 15.9	605.2 ± 14.2	1676.5 ± 1.6	1693.4 ± 33
P. sophorae S27	911.6 ± 16.9	532.3 ± 18.3	300.6 ± 2.7	142 ± 2.5	0	0	0	0	0	0
P. sonchi X19-5	582.1 ± 28.3	743.9 ± 23.9	265.5 ± 45.8	166.9 ± 2.9	85.4 ± 3.4	0	0	0	273.3 ± 15.5	6.0 ± 1.5
P. zanthoxyli JH29	4942.5 ± 26	5455.1 ± 28.5	3974.2 ± 12.4	3522.3 ± 17.4	39.5 ± 4.1	128.8 ± 21.5	202.2 ± 7.8	692.8 ± 74.5	1480.3 ± 16.7	1053.3 ± 116.4
Paenibacillus sp. WLY 1-18	326.1 ± 5.1	702.5 ± 20.2	160.9 ± 2.5	48.6 ± 1.1	26.3 ± 1.4	0	0	0	0	0
Paenibacillus sp. WLY TD94	2855.8 ± 10.0	1976.2 ± 65.1	1331.8 ± 10.0	1029.3 ± 29.4	0	0	0	0	0	0
Paenibacillus sp. WLY 1-43	1308.2 ± 27.6	1071.6 ± 32.3	1070.3 ± 11.7	685.4 ± 14.1	243.9 ± 2.6	0	0	0	0	0
Paenibacillus sp. WLY 1-49	560.5 ± 15	605.3 ± 19.0	336.7 ± 10.1	194.7 ± 2.4	34.6 ± 8.2	0	0	0	0	0
Paenibacillus sp. WLY78	8379.9 ± 17.7	7625.7 ± 16.3	3836.4 ± 12.1	2546.8 ± 11.1	208.7 ± 6.4	0	0	0	0	0

P. polymyxa NifH and *P. macerans NifH*. Cluster includes the only *P. azotofixans NifH3*. Our data are in agreement with the *NifH* phylogenetic tree reported by [21, 22]. Unlike in *P. azotofixans,* three *nifH* genes in *P. stellifer* T27 are included in cluster. Although both *Paenibacillus* ssp. and *C. pasteurianum* are gram-positive bacteria, they fall in distinct clusters.

Previous studies showed that nitrogenase activity is inhibited by O_2 and ammonium in many gram-negative bacteria, such as *K. pneumoniae*, *A. vinelandii*, *A. brasilense*, *R. rubrum* and *H. seropedicae*. In these bacteria, almost all of the *nif* genes, including *nifHDK* encoding nitrogenase, are transcribed from σ^{54} promoters (–24/–12) [26] and NifA, the transcriptional activator, is required for expression of *nif* genes. Both fixed nitrogen and oxygen control the synthesis of nitrogenase (NifHDK) by inhibiting NifA expression and activity. Besides regulation at the gene expression level, nitrogenase also is regulated at the enzymatic level in many organisms such as *A. brasilense*, *R. rubrum* and *H. seropedicae*.

O_2 and ammonium inhibit nitrogenase activity in gram-positive nitrogen fixing *C. pasteurianum,* just as does in gram-negative nitrogen fixing bacteria. *C. pasteurianum* has six *nifH* (*nifH1*) and *nifH*-like (*nifH2*, *nifH3*, *nifH4*, *nifH5* and *nifH6*) genes and the presumed promoters for the *nif* genes have sequences either identical to or very similar to the *Escherichia coli* σ^{70}-dependent –35 and –10 consensus promoter [27, 28]. This bacterium lacks a *nifA*-like gene. It means that the *nifH* promoters of *C. pasteurianum* are very different from that of *K. pneumoniae*. However, the molecular regulatory mechanism of nitrogen fixation in *C. pasteurianum* has not been well demonstrated.

In all *Paenibacillus* strains, the highest nitrogenase activity is obtained in the condition of 0–0.1 mM NH_4Cl and the increase of NH_4Cl from 0.1 to 5 mM caused a rapid inhibition of nitrogenase activity. However, the inhibition was reversible in the presence of 200 mM NH_4Cl in some *Paenibacillus* strains, such as *P. sonchi* X19-5 and *P. zanthoxyli* JH29 indicating the inhibition was reversible in the presence of high concentration of NH_4Cl. It has been shown that besides regulation at the gene expression level, nitrogenase activity is also reversibly inhibited by low levels of ammonium ions in many organisms, such as *Rhodospirillum rubrum, Rhodobacter capsulatus* and *Azospirillum brasilense*. This effect, called nitrogenase switch-off/switch-on was be due to the ADP-ribosylation of an arginine residue of one of the Fe protein (dinitrogenase reductase) subunits by the enzyme dinitrogenase reductase ADP-ribosyl transferase (DRAT). Removal of the ADP-ribosyl moiety and reactivation of the Fe protein is catalyzed by the dinitrogenase reductase-activating glycohydrolase (DRAG) [29]. However, we do not know there is *draT*/*draG* genes or not. Thus, the molecular regulatory mechanism of nitrogen fixation in *Paenibacillus* needs to be investigated.

However, we do not know the molecular regulatory mechanism of nitrogen fixation in *Paenibacillus*. We also do not know there is transcriptor NifA or not in *Paenibacillus*. Thus, the molecular regulatory mechanism of nitrogen fixation in *Paenibacillus* needs to be investigated.

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