J. Microbiol. Biotechnol. (2006), **16**(10), 1650–1655

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Genome Snapshot of *Paenibacillus polymyxa* ATCC 842^T

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Received: May 11, 2006
Accepted: June 22, 2006

Abstract Bacteria belonging to the genus *Paenibacillus* are facultatively anaerobic endospore formers and are attracting growing ecological and agricultural interest, yet their genome information is very limited. The present study surveyed the genomic features of *P. polymyxa* ATCC 842^T using pulse-field gel electrophoresis of restriction fragments and sample genome sequencing of 1,747 reads (approximately 17.5% coverage of the genome). Putative functions were assigned to more than 60% of the sequences. Functional classification of the sequences showed a similar pattern to that of *B. subtilis*. Sequence analysis suggests nitrogen fixation and antibiotic production by *P. polymyxa* ATCC 842^T, which may explain its plant growth-promoting effects.

Key words: Pulse-field gel electrophoresis (PFGE), survey sequencing, plant growth-promoting rhizobacteria (PGPR)

The genus *Paenibacillus* has been proposed as a branched-off group from the genus *Bacillus* based on comparative analyses of 16S rRNA sequences [2]. The genus can also be readily distinguished from other bacilli based on such characteristics as facultative anaerobic growth, the production of spores in definitely swollen sporangia, a 45 to 54% G+C content, the secretion of various polysaccharide-hydrolyzing enzymes, and nitrogen fixation [1, 23]. Recently, more and more species are either being identified as *Paenibacillus* or those formerly designated as *Bacillus* spp. are being reclassified as *Paenibacillus* [21, 23], implying that this group may constitute a larger proportion of *Bacillales* than previously thought. Various *Paenibacillus* species have also been isolated in Korea, from natural habitats such as rhizosphere and soil, and their useful traits have been analyzed [3, 6–8, 10, 15, 22, 26]. At present, the genus *Paenibacillus* consists of 84 species (NCBI Taxonomy Homepage at http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html, February 2006).

Nonetheless, despite the growing interest in *Paenibacillus*, its genomic information is very scarce. Most of the completely sequenced organisms currently belong to the *Bacillaceae* family, in particular to the *Bacillus* genus, whereas data on *Paenibacillaceae* sequences is limited even at the draft level. *P. polymyxa*, the type species of the genus *Paenibacillus*, is also of great ecological and agricultural importance, owing to its abundance in soil and potent plant growth-promoting activity. Accordingly, the present study conducted a "genome snapshot" assay on *P. polymyxa* ATCC 842^T as a model organism using pulse-field gel electrophoresis (PFGE) and genome survey sequencing. *P. polymyxa* E681, a strain isolated from the rhizosphere of winter barley cultivated in the southern part of Korea with potent plant growth-promoting activity, was then chosen for a comparison of the PFGE patterns [22].

To assess the relatedness of *P. polymyxa* ATCC 842^T and E681, where the genome sequencing for the latter is near completion, and estimate the genome sizes of both strains, the genomic DNA was digested with rare-cutting enzymes and the DNA fragments separated with pulse-field gel electrophoresis. Both setups yielded approximately 40 NotI fragments ranging from 15 to 430 kb (Fig. 1). The combined fragment size was 5.7 Mb (ATCC 842^T) and 5.5 Mb (E681), and since the latter was well consistent with the genome size obtained from the genome sequencing (data not shown), the PFGE results appeared to give good estimates of the chromosome sizes. However, even though the two strains had similar-sized chromosomes, their digestion patterns were unique. Previous studies have reported genetic and phenotypic diversity in the *Paenibacillus* genus [6, 17]. Additional intraspecies genetic heterogeneity also

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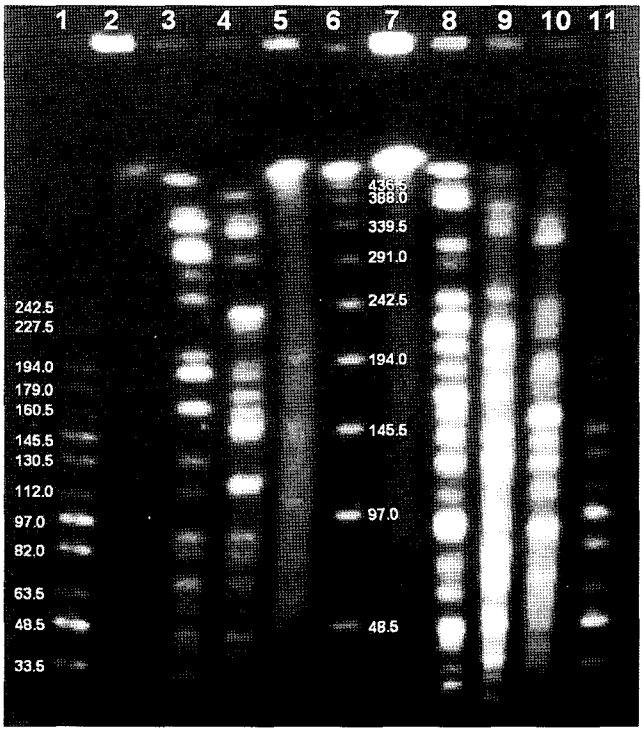

Fig. 1. Pulse-field gel electrophoresis of restriction fragments of *Paenibacillus polymyxa* ATCC 842^T (lanes 2–5) and E681 (lanes 7–10).

High-molecular-weight DNA was prepared in 1% pulse-field certified agarose blocks (Bio-Rad) from cells grown until $OD_{600\,nm}$ 0.3–0.4 in 50 ml of LB containing 2% glucose. The blocks were processed (including enzyme digestion) according to the manufacturer's instructions. The electrophoresis was performed using a CHEF-DR® II (Bio-Rad) in 0.25×TBE buffer at 14°C and a pulse time of 2 to 20 s for 20 h at 6 Vcm^{-1}. Lanes: 1 and 11, midrange ladder; 6, lambda concatemer; 2, 5, and 7, uncut genomic DNA; 3 and 8, NotI; 4, PmeI; 9, SfiI; and 10, Sse8387I. The numbers on lanes 1 and 6 refer to the DNA fragment size in kb.

appears in *Paenibacillus* (*e.g.*, *P. polymyxa*) [9], which would explain the difference in the restriction patterns between the two strains.

The genomic DNA was partially digested with Sau3AI and fragments of 1.2–2 kb were cloned into a pBC KS+ vector. The sequencing was performed with an ABI 3700 DNA analyzer. Out of 2,016 reads, 1,747 (86.7%) high quality sequences with an average length of 571 bp were selected for further analyses after low-quality or vector regions had been trimmed (Table 1). Close to one million (996,603) base pairs were determined in total and deposited in the genome survey sequence division of the GenBank (dbGSS), under accession numbers DU532978 thru DU534724. Assuming a genome size of 5.7 Mb for ATCC 842^T, this represents 17.5% of the entire genome. Meanwhile, according to the Lander-Waterman model for random sequencing, the fraction of the sequenced genome was 16.1% with a sequencing coverage of 17.5% [13]. The average G+C content of the sequences was 46.1%, which was similar to that of other *Paenibacillus* species. Assembly using PHRAP (http://www.phrap.org/) produced 365 contigs, consisting of 907 reads (48.1% incorporated). The total length of the contigs and unincorporated sequences collectively amounted to 866 kb (15.2% of the estimated genome size), which is in accordance with the Lander-Waterman model.

A BLASTN search against the NCBI nucleotide database revealed that only 297 (17.0%) of the 1,747 sequences had significant database matches (E-value $<10^{-5}$), demonstrating the current limitation of nucleotide sequence data on *Paenibacillus* species. The taxonomic distribution of the best matching sequences included *P. polymyxa*, *P. popilliae*, *P. jamilae*, various *Bacillus* species, *Oceanobacillus iheyensis*, *Geobacillus kaustophilus*, *Staphylococcus epidermidis*, several *Listeria* species, and a few *Lactobacillales* species. Fifty-seven sequences were homologous to rRNA and tRNA genes. The nucleotide divergence between the ATCC 842^T sequence tags and the E681 contig sequences, as identified by the BLASTN search, was 9.02% (E-value $<10^{-5}$, data not shown), further supporting genetic heterogeneity within the *P. polymyxa* species.

Table 1. Features of the ATCC 842^T genomic library.

Library insert size (range in bp)	1,200–1,500	
Total number of sequencing reactions (M13 reverse primer only)	2,016	
Low-quality reads	206	
Reads generated from clones with no or short insert	66	
High-quality reads	1,747	
Average length of high-quality reads (bp)[a]	570.5	
Homology search results		
Sequence matching:		
RNA[b]	57	(3.3%)
Protein-coding genes (known function)	1,063	(60.8%)
Protein-coding genes (unknown function)	296	(16.9%)
No database match	331	(18.9%)
Total	1,747	(100.0%)

[a]Short stretches of upstream vector sequences were excluded.

[b]If a given sequence included both an RNA gene and a protein-coding gene, they were regarded as sequence matching protein-coding genes.

Table 2. Phylogenetic classification of sequences with putative protein-coding genes.

Classification	No. of sequences
Firmicutes	**1,092 (80.4%)**
Bacillales	947
Family *Bacillaceae*	845
Genus *Bacillus*	670
Other *Bacillaceae*	175
Family *Listeriaceae*	36
Family *Staphylococcus*	36
Family *Paenibacillacea*	28
Other *Bacillales*	2
Clostridia	103
Other Firmicutes	42
Proteobacteria	**102 (7.5%)**
Actinobacteria	**63 (4.6%)**
Cyanobacteria	**25 (1.8%)**
Bacteroidetes	**19 (1.4%)**
Other	**58 (4.3%)**
Total	**1,359 (100.0%)**

Putative protein-coding regions were identified with BLASTX searches against the UniProt KnowledgeBase database. Of the queries 1,359 (77.7%) had significant database matches, and functions could be assigned to 60.8% of the input sequences. As expected, homology searches based on the translated sequence tags yielded much higher coverage than the nucleotide comparison alone. In brief, 1,416 sequences were assigned putative functions by comparison to nucleotide and amino acid databases (Table 1). The phylogenetic distribution of 1,359 putative protein-coding sequences is shown in Table 2. Most of the best hits were classified as belonging to the *Bacillus* genus (49.3%). Only 28 sequence tags fell into the *Paenibacillaceae* family, again reflecting the scarcity of *Paenibacillus* sequence information. The putative functions of the predicted protein-coding genes in each sequence tag are summarized in Table 3, according to a classification adapted from Moszer *et al.* [18] for *Bacillus subtilis*. As the sequence assembly did not significantly reduce the total sequence number (69.4% reduction including singlets), it was deemed appropriate to use the entire sequence set rather than contigs alone for the functional analyses, regardless of redundancy.

The generated *P. polymyxa* ATCC 842^T sequence tags were limited, yet represented a genome snapshot that could approximately describe the lifestyle of this strain. As shown in Table 3, the gene distribution was similar in *P. polymyxa* ATCC 842^T and *B. subtilis*. The most common classes include transporters (18.4%), carbohydrate metabolism (19.3%), and RNA synthesis (9.6%). The number of genes putatively involved in antibiotic production was somewhat over-represented in the ATCC 842^T genome (10.4%), which may have been the result of the gigantic size of the gene cluster encoding enzymes for polyketide or nonribosomal peptide antibiotic biosynthesis. As expected for a soil-dwelling bacterium, *P. polymyxa* ATCC 842^T was rich in genes for carbohydrate metabolism (including transporters) and nitrogenous compound metabolism. Extracellular hydrolytic enzymes, such as xylanases, inulinase, glucanases, and polyphosphatases, may be helpful in the rhizobial lifestyle benefiting from plant-derived compounds.

Prokaryotic nitrogen fixation into ammonia is of great agricultural importance, and nitrogen fixation is considered to be one of the main rhizobacterial plant growth-promoting activities in addition to phytohormone production, antibiotic activity, and solubilization of organic phosphates. Only one subset of *Paenibacillus* species is known to fix nitrogen among aerobic endospore-forming *Firmicutes* [1]. Nitrogen fixation is hence a key feature separating *Paenibacillus* spp. from *Bacillus* spp. In the present study, several genes relevant to nitrogen fixation were identified among the ATCC 842^T sequence tags. Three sequence tags encoding NifU, dinitrogenase reductase-activating glycohydrolase (DraG), and NifS, respectively, were identified by BLASTX. However, no genes for dinitrogenase (NifD/NifK) were found. Traditionally, identification of sequences homologous to the nitrogenase reductase gene (*nifH*) by a PCR has been widely used as an indicator of nitrogen fixation [25]. However, in the current study, no sequence tags homologous to *nifH* were identified by sample sequencing, although a partial *nifH* sequence (GenBank Accession No. AJ223997) has been previously reported in this strain [1]. The protein DraG is known to be involved in the posttranslational regulation of nitrogenase activity. Although well characterized in several proteobacterial species, it has never been found in *Firmicutes*. Thus, the present finding of DraG in the ATCC 842^T genome may give additional insights into the mechanism of nitrogenase regulation in *Paenibacillus* species. Eight species including *P. polymyxa* are reportedly nitrogen-fixing in *Paenibacillus*, yet the distribution of genes encoding a nitrogen-fixing function within *P. polymyxa* species seems to vary depending on the particular strain. Previous work suggests that the *P. polymyxa* type strain may be nitrogen-fixing, but methodological limitations still require optimization [20].

The plant growth-promoting activity of soil bacteria has been partly attributed to the production of phytohormones, such as auxin-related compounds, cytokinins, and gibberellins. Auxin production has already been reported in environmental *P. polymyxa* strains [14] and E681, plus a putative biosynthetic pathway has been postulated in strain E681 (Ghim S. Y., personal communication). However, the presence of genes responsible for the key steps in auxin production (*e.g.*, indole-3-pyruvate decarboxylase) could not be confirmed in any of the 1,747 sequence tags in this study. In addition, no sequences homologous to bacterial isopentenyltransfersase were identified, which has been identified as the key enzyme for cytokinin biosynthesis.

Table 3. Functional classification of sequences with protein-coding genes in *P. polymyxa* ATCC 842^T and comparison with *Bacillus subtilis*.

Functional categories[a]	ATCC 842	*B. subtilis*[a]
Cell envelope and cellular processes		
Cell wall	28 (2.7)	89 (3.5)
Transport/binding proteins and lipoproteins	190 (18.4)	400 (15.6)
Sensors (signal transduction)	14 (1.4)	39 (1.5)
Membrane bioenergetics (electron transport and ATP synthase)	29 (2.8)	82 (3.2)
Motility and chemotaxis	22 (2.1)	55 (2.1)
Protein secretion	7 (0.7)	26 (1.0)
Cell division	2 (0.2)	22 (0.9)
Sporulation	12 (1.2)	164 (6.4)
Germination	13 (1.3)	26 (1.0)
Transformation/competence	1 (0.1)	25 (1.0)
Intermediary metabolism		
Metabolism of carbohydrates and related molecules	200 (19.3)	271 (10.6)
Metabolism of amino acids and related molecules	55 (5.3)	201 (7.8)
Metabolism of nucleic acids and related molecules	24 (2.3)	92 (3.6)
Metabolism of lipids	29 (2.8)	89 (3.5)
Metabolism of coenzymes and prosthetic groups	35 (3.4)	103 (4.0)
Metabolism of phosphate	5 (0.5)	10 (0.4)
Metabolism of sulfur	5 (0.5)	8 (0.3)
Information pathways		
DNA replication	14 (1.4)	26 (1.0)
DNA restriction/modification and repair	14 (1.4)	42 (1.6)
DNA recombination	17 (1.6)	19 (0.7)
DNA packaging and segregation	6 (0.6)	11 (0.4)
RNA synthesis	99 (9.6)	254 (9.9)
RNA modification	6 (0.6)	28 (1.1)
Protein synthesis	21 (2.0)	101 (3.9)
Protein modification	7 (0.7)	35 (1.4)
Protein folding	6 (0.6)	12 (0.5)
Other functions		
Adaptation to atypical conditions	6 (0.6)	81 (3.2)
Detoxification	20 (1.9)	89 (1.4)
Antibiotic production	108 (10.4)	35 (1.4)
Phage-related functions	20 (1.9)	87 (3.4)
Transposon and IS	4 (0.4)	10 (0.4)
Miscellaneous	15 (1.5)	30 (1.2)
Total	1,034 (100)	2,562 (100)

[a]Functional classification was adapted from the SubtiList database [18]. Numbers in parentheses indicate the percentage of putative protein-coding genes in each group.

The production of antibiotic substances in microorganisms is borne out of competition with other organisms in the natural environment. In the rhizosphere, such bacterially generated antibiotics can inhibit the growth of pathogenic microorganisms. Thus, in the present study, a large proportion of the sequence tags (10.4%) were found to harbor genes dedicated to antibiotic production, whereas a sequence homology comparison revealed a wide spectrum of antibiotic substances produced by *P. polymyxa* ATCC 842^T, covering various forms of lipopeptides, lantibiotics, peptide antibiotics, and polyketide antibiotics. As many as 10.4% of the sequence tags were homologous to biosynthetic genes for bacillomycin D/L (24 hits), mycosubtilin (15 hits), iturin A (11 hits), gramicidin S (7 hits), lichenysin (5 hits), tyrocidine (5 hits), and some other antibiotics, where most of these are produced by *B. subtilis* [19, 24], *B. licheniformis* [11], and *B. amyloliquefaciens* [12].

Although the existence of peptide antibiotics in *P. polymyxa* has long been established, the genes responsible for their biosynthesis have not yet been described. For example, polymyxins are a family of nonribosomally generated peptide antibiotics that have been known and used for more

than fifty years [5]. Among them, polymyxin B produced by *P. polymyxa* has been most extensively studied and widely used. More recently, polymyxin M (also known as "mattacin") from *P. kobensis* M was characterized [16]. Independent groups have also reported on the molecular structure of the fusaricidin-type antifungal antibiotics produced by *P. polymyxa* PKB1 [4]. Taking into account that the genes for nonribosomal peptide synthetases and polyketide synthetases are generally very large (often encoding proteins with thousands of amino acids) and complex, the apparent wide spectrum of the putative final product may result from multiple sequence reads generated from a single gigantic gene. Consequently, the current snapshot sequence analysis is useful as regards providing a glance of the antibiotic-producing capability of ATCC 842^T, yet careful interpretation is required in dealing with sequences generated through low-coverage survey sequencing that may have statistically little representational value for the full biosynthetic capacity of the sequenced genome.

In conclusion, a genome snapshot of *P. polymyxa* ATCC 842^T was produced by low-coverage DNA sequencing and pulse-field gel electrophoresis of DNA fragments cut with highly selective restriction endonucleases to predict the size and generic features of the *Paenibacillus polymyxa* genome, while exploring its similarities to the naturally occurring E681 strain with potent plant growth-promoting activity. Various genes were identified as possibly involved in nitrogen fixation and antibiotic production, both of which are common in plant growth-promoting rhizobacteria. With the genome sequencing and annotation of *P. polymyxa* E681 near completion by the authors' team, a further detailed comparison with ATCC 842^T will soon be possible, allowing new insights into the functions and evolution of the *Paenibacillus* genomes.

Acknowledgments

We thank Mi-Young Chu, Nam-Soon Kim, and other members of the Functional Genomics Research Center for technical assistance. We are also indebted to Cheol-Goo Hur, Doil Choi, and Yong Sung Kim for their dedicated support to this project. This work was supported by the BioGreen 21 Program of Rural Development Administration, the 21C Frontier Microbial Genomics and Applications Center Program of the Ministry of Science and Technology, and the KRIBB Research Initiative Program, Republic of Korea. J. F. K. and H. J. contributed equally to this work.

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