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Original article

A single amino acid mutation in Spo0A results in sporulation deficiency of *Paenibacillus polymyxa* SC2

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Abstract

Sporulating bacteria such as *Bacillus subtilis* and *Paenibacillus polymyxa* exhibit sporulation deficiencies during their lifetime in a laboratory environment. In this study, spontaneous mutants SC2-M1 and SC2-M2, of *P. polymyxa* SC2 lost the ability to form endospores. A global genetic and transcriptomic analysis of wild-type SC2 and spontaneous mutants was carried out. Genome resequencing analysis revealed 14 variants in the genome of SC2-M1, including three insertions and deletions (indels), 10 single nucleotide variations (SNVs) and one intrachromosomal translocation (ITX). There were nine variants in the genome of SC2-M2, including two indels and seven SNVs. Transcriptomic analysis revealed that 266 and 272 genes showed significant differences in expression in SC2-M1 and SC2-M2, respectively, compared with the wild-type SC2. Besides sporulation-related genes, genes related to exopolysaccharide biosynthesis (*eps*), antibiotic (fusaricidin) synthesis, motility (*flgB*) and other functions were also affected in these mutants. In SC2-M2, reversion of *spo0A* resulted in the complete recovery of sporulation. This is the first global analysis of mutations related to sporulation deficiency in *P. polymyxa*. Our results demonstrate that a SNV within *spo0A* caused the sporulation deficiency of SC2-M2 and provide strong evidence that an arginine residue at position 211 is essential for the function of Spo0A.

Keywords: *Paenibacillus polymyxa*; Genome resequencing; Transcriptome sequencing; Sporulation

1. Introduction

Sporulation is one of the most complicated bioprocesses performed by bacteria of the classes Bacilli and Clostridia. The process of sporulation takes 6−8 h and has a high energy requirement, including hundreds of genes. Vegetative cells go through a series of morphological changes and differentiate into metabolically dormant cells, called endospores. Endospores are composed of a partially dehydrated central core (containing the genome) surrounded by several concentrically arranged protective layers. This structure allows bacteria to survive harsh environmental conditions such as heat, drought, UV radiation, chemical exposure and nutrient limitation [1]. Sporulation is a last-resort adaptive response adopted by bacteria.

Paenibacillus polymyxa, formerly called *Bacillus polymyxa* [2], is a plant-growth-promoting rhizobacterium. By producing various antibiotics such as fusaricidin and polymyxin, which are synthesized by a non-ribosomal peptide synthetase, *P. polymyxa* targets a variety of plant pathogens [3−7]. *P. polymyxa* also secretes cytokinin, auxin and indolic and

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phenolic compounds which promote plant growth [8–15]. These features make *P. polymyxa* a reliable biocontrol agent for agricultural application. Furthermore, *P. polymyxa* forms endospores, which are considerably better for storage and transport compared with vegetative cells when used for agricultural purposes.

P. polymyxa SC2 was first isolated from the rhizosphere of pepper plants in Guizhou, China [16]. This bacterium significantly inhibits various pathogenic microorganisms because its genome contains many antibiotic-related genes, including those that encode proteins for the synthesis of fusaricidin, polymyxin, iturin, lantibiotic, bacillorin, polyketides and bacitracin. However, in a laboratory environment, *P. polymyxa* SC2 shows highly unstable colony morphology during successive cultivation on Luria–Bertani (LB) agar. Two spontaneous mutants, named SC2-M1 and SC2-M2, formed different colonies compared with the wild-type SC2. More importantly, both SC2-M1 and SC2-M2 lost their ability to form endospores.

Sporulation deficiency was first recorded in *Bacillus subtilis* [17]. Following propagation for 6000 generations without selection for sporulation, the ability of *B. subtilis* to sporulate was either severely reduced or completely lost (sporulation frequency $<2 \times 10^{-6}$). Although genome sequencing analysis revealed mutations in some sporulation-related genes (such as *cotX* and *spsI*, encoding a spore coat protein and a polysaccharide, respectively), the exact cause of the sporulation deficiency remains unclear [18]. A recent study of differences in colony morphology in laboratory-evolved strains of *B. subtilis* suggested that adaptive specialization of biofilm-forming species could occur through mutations that modulated biofilm formation [19]. Up to now, however, few studies have systematically investigated spontaneous sporulation mutations that occur in *P. polymyxa*. Additionally, because of its poor transformation efficiency, research into the development of sporulation and functions of many other *P. polymyxa* genes has been slow.

To determine the cause of sporulation deficiency in *P. polymyxa* mutants SC2-M1 and SC2-M2, a global analysis of the wild-type strain SC2, SC2-M1 and SC2-M2 was conducted. We first compared differences between strains using a genome resequencing strategy, and then quantitatively compared the abundance of transcription of each gene in each of the strains under the same conditions. Using an optimized transformation method, we also conducted a reversion experiment in SC2-M2 to confirm our results.

2. Materials and methods

2.1. Bacteria and culture condition

P. polymyxa SC2 was isolated from the rhizosphere of a pepper plant in Guizhou, China. This was authorized by the owner, Guiyang Nanming Lao Ganma Flavor Food Co., Ltd. [16]. SC2-M1 and SC2-M2 were isolated from the successive cultivation of SC2 spread on LB plates. SC2, SC2-M1 and SC2-M2 were deposited at −80 °C.

2.2. Genome resequencing

Genomic DNA was extracted using a commercial DNA isolation kit (TianGen, Beijing, China) from 50-ml overnight cultures grown in LB medium inoculated with a single SC2-M1 and SC2-M2 colony. A genomic DNA library was generated using an Illumina genomic DNA library generation kit by the commercial sequencing company Biomarker Technologies (Beijing, China) following the manufacturer's protocol. In brief, genomic DNA was first fragmented by nebulization. Then, adapters were ligated to the ends of the DNA fragments. The adapter-modified DNA fragments were enriched by PCR. Finally, Solexa was used to remove any sequences that contained an "adapter" [20]. Then the filtered reads were aligned to the *P. polymyxa* SC2 reference sequence (GenBank: NC_014622.2 (Chr) and NC_014628.2 (Plsm)) for detection rates of SNVs, deletions and insertions. SNVs and indels initially located in the coding regions of functional genes were validated by PCR using FastPfu DNA polymerase (TransStart, Beijing, China) (primers are listed in S3 Table). The sequences obtained in this study have been deposited in the Sequence Read Archive (SRA) database (www.ncbi.nlm.nih.gov/sra) under accession numbers SAMN04121040 and SAMN04121041.

2.3. Total mRNA extraction and sequencing

Strains SC2, SC2-M1 and SC2-M2 were cultured on LB agar for 24 h at 37 °C, when most of SC2 step into the progress of sporulation, and then washed with sterile H_2O. Total RNA was extracted using an EasyPure RNA kit (TransStart) according to the manufacturer's protocol. Pure RNA samples were sent to the commercial sequencing company GENEWIZ (Beijing, China) for mRNA enrichment and transcriptome sequencing on an Illumina sequencing platform.

Raw mRNA sequence data were filtered to discard any reads that did not meet overall quality values using the Next-Generation Sequencing Quality Control Toolkit, version 2.3 (http://59.163.192.90:8080/ngsqctoolkit/). The output data were stored in the standard FASTQ format and mapped against the *P. polymyxa* SC2 reference sequence (GenBank: NC_014622.2 (Chr) and NC_014628.2 (Plsm)) using Bowtie version 2.1.0 (http://sourceforge.net/projects/bowtie-bio/files/). Mapped read count normalization was applied to the data based on the number of fragments per kb of coding sequence per million mapped reads (FPKM) [21]. Differences in gene expression profiles were analyzed using EdgeR software version 2.13 (http://bioconductor.org/news/bioc_2_13_release/) with the EdgeR algorithm. More than one difference in gene expression profiles with a false discovery rate of ≤ 0.05 was considered significant. The reads obtained in this study have been deposited in the Sequence Read Archive (SRA) database (www.ncbi.nlm.nih.gov/sra) under accession

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