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.
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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

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

numbers SAMN04159251, SAMN04159252 and SAMN04159253.

2.4. Reversion of spo0A in P. polymyxa SC2-M2

The suicide plasmid pRN5101 (Institute of Plant Protection, Chinese Academy of Agriculture Sciences) is a temperature-sensitive *Bacillus thuringiensis−Escherichia coli* shuttle vector carrying the erythromycin resistance cassette. A chloramphenicol resistance cassette was PCR-amplified from plasmid pDG1661 (Institute for Biophysical Chemistry, Pierre et Marie Curie, Paris, France). *E. coli* DH5α cells (TransGen Biotech, Beijing, China) acted as hosts for the recombinant plasmids and were cultivated at 37 °C on LB agar. For antibiotic selection, the media were supplemented with a final concentration of 5 μg/ml chloramphenicol, 10 μg/ml erythromycin and 100 μg/ml ampicillin.

2.5. Vector construction

The strategy for replacement of His 211 by Arg and two other amino acids (Asp and Ala) is illustrated in Fig. 1. Briefly, a 782-bp fragment, which included the latter part of the open reading frame of the *spo0A* gene and part of its downstream intergenic region, was constructed by PCR amplification using primers ArgF/ArgR, AspF/AspR, or AlaF/AlaR (Table 3). The fragment was then ligated into pRN5101 via EagI and HindIII cleavage sites to obtain plasmid pRN5101-Arg/Asp/Ala. The *cat* cassette encoding chloramphenicol resistance, constructed by PCR amplification using primers CatF and CatR (Table 3) from pDG1661, was ligated into pRN5101-Arg/Asp/Ala via ApaI cleavage sites. The previously constructed 782-bp fragment was then inserted by the *cat* cassette, generating 336-bp and a 446-bp homologous flanking regions, resulting in plasmid pRN5101-Arg/Asp/Ala (cm+).

2.6. Electrotransformation

Plasmid pRN5101-Arg/Asp/Ala (cm+) was then introduced into *P. polymyxa* SC2-M2 by electrotransformation as follows. To prepare competent cells, a single *P. polymyxa* SC2-M2 colony was picked from solid LB medium and shaking-cultured in LB broth supplemented with 0.5 M sorbitol at 37 °C. When the cells reached an OD_{600} of 0.75−0.85, the culture was placed on ice for 10 min and then centrifuged at $5000 \times g$ for 10 min at 4 °C. After washing the cells twice with SM buffer (0.5 M sorbitol, 0.5 M mannitol and 0.5 M trehalose dihydrate), the competent cells were resuspended in SM buffer. DNA (100 ng) was then mixed with 70 μl of competent cells, transferred to 1-mm cuvettes and placed on ice for at least 5 min. Electroporation was performed using a Gene Pulser (Bio-Rad, Hercules, CA) with a voltage of 2.3 kV and a time of 4.5−5 ms. One milliliter of LB broth supplemented with 0.5 M sorbitol and 0.38 M mannitol was added to the cell suspension and then incubated at 28 °C for 2.5 h. Transformants were selected on LB agar plates supplemented with 10 μg/ml erythromycin and cassette insertion was confirmed with PCR.

2.7. Genome recombination

Transformants were cultured in LB at 39 °C. Every 12 h, the cultures were inoculated into fresh LB. After four to five inoculations, the cultures were diluted and selected on LB agar plates supplemented with 10 μg/ml erythromycin or 5 μg/ml chloramphenicol. The cultures that grew only on chloramphenicol were chosen and PCR was performed with primers (CatF/R; Table 3) for confirmation.

2.8. Sporulation efficiency

Sporulation efficiency was determined on an LB plate cultured for 48 h, as cfu per ml (spores) after heat treatment by incubation at 80 °C for 10 min, compared with cfu per ml (viable count) of the preheat treatment sample.

2.9. Quantitative real-time PCR (qRT-PCR)

Six sporulation-related genes (*spo0A*, *spoIIAA*, *spoIIAB*, *spoIIE*, *sigE* and *sigF*) were selected to validate the transcriptome sequencing data of SC2, SC2-M1 and SC2-M2 by qRT-PCR. The primer pairs used for the qRT-PCR are listed in S4 Table.

RNA was extracted from SC2, SC2-M1 and SC2-M2 as described above and then reverse-transcribed into cDNA using a TransScript All-in-One First-Strand cDNA Synthesis SuperMix for PCR (TransGen Biotech) according to the manufacturer's protocol. qRT-PCR was carried out in a 20-μl volume using TransStart Tip Green q-PCR SuperMix (TransGen Biotech) as recommended by the manufacturer. All measurements were independently conducted in triplicate on independent plates.

3. Results

3.1. Morphological differentiation

Wild-type SC2 (Fig. 2a) formed a thick white colony on Luria−Bertani (LB) agar. Spontaneous mutant SC2-M1 (Fig. 2b) formed a thin partially transparent colony, and
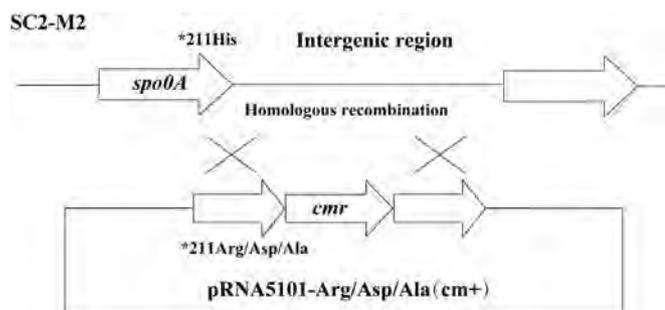

Fig. 1. Schematic representation of the strategy used for restoration and substitutions of His 211 in SC2-M2.
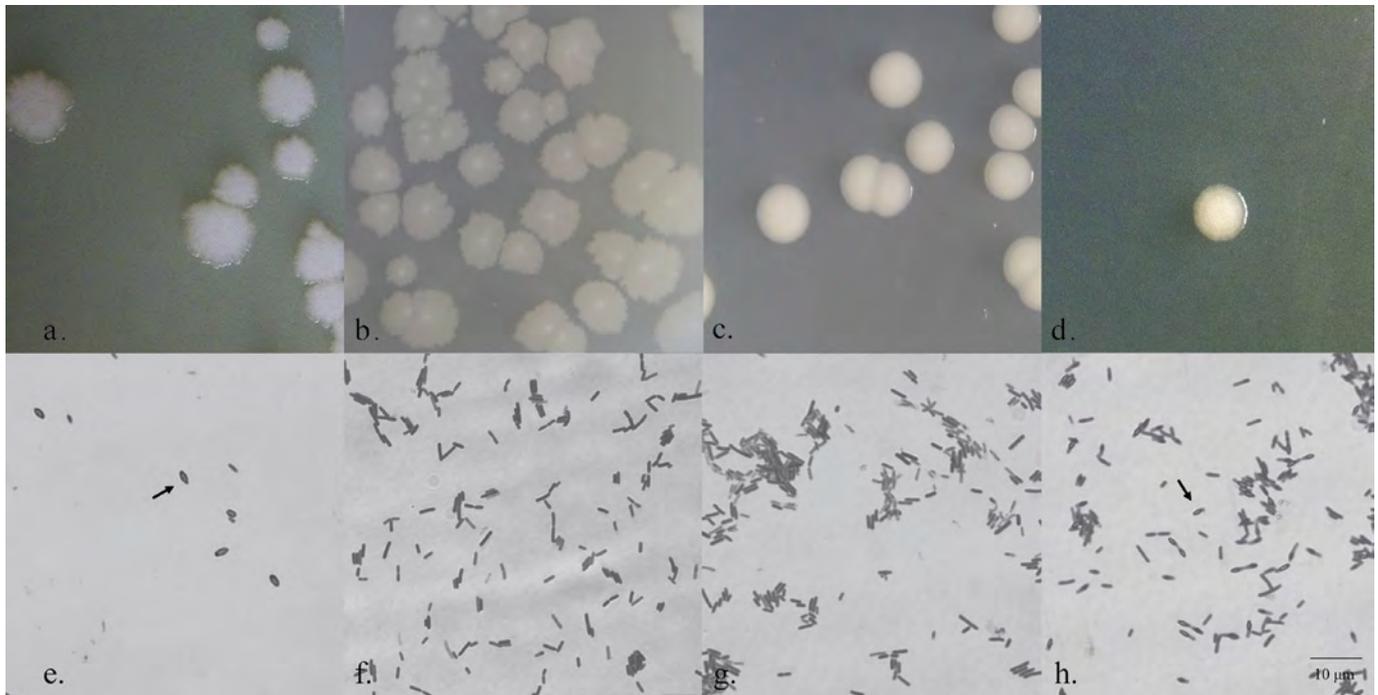
Fig. 2. Colony morphology and light micrographs of *P. polymyxa* SC2, SC2-M1, SC2-M2 and SC2-M2 (Arg 211). Letters a, b, c and d show colony morphology of SC2, SC2-M1, SC2-M2 and SC2-M2 (Arg 211), respectively. All colonies were grown on LB agar for 48 h at 37 °C. Letters e, f, g and h show light micrographs of SC2, SC2-M1, SC2-M2 and SC2-M2 (Arg 211), respectively. Bar = 10 μm for all four micrographs. Arrowheads (e, h) indicate endospores.

spontaneous mutant SC2-M2 (Fig. 2c) formed a thick, white smooth colony. More importantly, SC2-M1 and SC2-M2 both lost their ability to form endospores compared with the wild-type (Fig. 2e–g).

3.2. Genome resequencing analysis

Genome resequencing generated a total of 5,929,781 reads and 1,494,206,802 bp for SC2-M1 and a total of 5,566,642 reads and 1,402,719,005 bp for SC2-M2 after filtration using an Illumina HiSeq 2500 system (Illumina, San Diego, CA). All reads were then aligned with the reference *P. polymyxa* strain SC2 genome and variants found in the genomes of SC2-M1 and SC2-M2 are listed in Table 1.

Of all the variants, only one was located in sporulation-related gene *spo0A*, which is the master regulator of the sporulation bioprocess. The variation was a G > A substitution at nucleotide position 3,383,926, which causes an amino acid polymorphism at position 211 (Arg to His) in the helix-turn-helix (HTH) domain of Spo0A. Another single nucleotide variation (SNV) occurred in the important two-component system regulator gene *degU*, which plays a part in many cellular responses, such as genetic competence, motility, biofilm formation, exoprotease production and complex colony architecture [22–26]. Variants were also found in genes with functions that included chemotaxis (PPSC2_21240), transport (sugar transporter *lacF* and peptide transporter *pstB*), purine metabolism (*purR*), stress response (*yvqE* and *sigB*) [27,28] and cell separation (*ywbG*) [29].

3.3. Comparative transcriptomic analysis

To obtain more information on gene expression in strains SC2, SC2-M1 and SC2-M2, we compared gene transcript abundances. A total of 266 of the 6117 genes analyzed (4.5%) from SC2-M1 were transcribed at levels significantly different ($P < 0.05$) from those of the wild-type, with 10 genes upregulated and 256 genes downregulated (S1 Table and Fig. 3a). A total of 272 of the 6117 genes analyzed (4.4%) from SC2-M2 were transcribed at levels significantly different ($P < 0.05$) from those of the wild-type, with 10 genes upregulated and 262 genes downregulated (S2 Table and Fig. 3b). Many of the downregulated genes were clustered together in the genomes, indicating they may be transcribed as an operon. Some examples include the sporulation-related genes, exopolysaccharide biosynthesis-related genes and fusaricidin biosynthesis-related genes.

Consistent with the sporulation deficiency observed in strains SC2-M1 and SC2-M2, a large number of the downregulated genes (122/256 in SC2-M1 and 68/262 in SC2-M2, Fig. 3a, b) were related to sporulation and could be characterized by the timing of expression, from sporulation stage 0/I to stage VI. From heat map analysis, we determined that expression of most of these genes was very low. Two sigma factor genes, *sigF* and *sigE*, which encode sporulation-specific RNA polymerase sigma factors that regulate early-stage gene expression in the forespore and mother cell showed a 29.86- and a 149.1-fold change in SC2-M1 and a 37.27- and a 187.4-fold change in SC2-M2, respectively. The expressions of six genes (*spo0A*, *spoIIE*, *spoIIAA*, *spoIIAB*, *sigE* and *sigF*) associated with sporulation were confirmed by

Table 1
Variants identified in SC2-M1 and SC2-M2.[a]

CDS region variants

Gene ID	Gene name	Product	Variant type	Codon	Protein change	Mutant
PPSC2_15030	spo0A	Sporulation transcription factor	g 3383926 a	CGC → CAC	R211H	SC2-M1 and SC2-M2
PPSC2_23095	degU	Two-component regulator	a 5193781 g	CAA → CGA	Q218R	SC2-M1 and SC2-M2
PPSC2_01770	lacF	Sugar ABC transporter permease	g 412041 a	AGC → AAC	S36N	SC2-M1
PPSC2_05440	purR	HTH-type transcriptional repressor	ins G after 1263263	Frameshift		SC2-M1 and SC2-M2
PPSC2_05785	yvqE	Histidine kinase	c 1340409 t	CAA → TAA	Q476(stop)	SC2-M1
PPSC2_10405		Hypothetical protein	c 2282552 t	TCC → TTC	S128F	SC2-M1 and SC2-M2
PPSC2_13030	pstB	Peptide ABC transporter ATPase	c 2950888 t	ACG → ATG	T178M	SC2-M1 and SC2-M2
PPSC2_24035	ywbG	Putative autolysin	Δ6 bp (5401047)	Frameshift		SC2-M1 and SC2-M2
PPSC2_21215	sigB	RNA polymerase sigma 70	ITX (4732584)	Frameshift		SC2-M1
PPSC2_21240		Histidine kinase	c 4739953t	CAG → TAG	Q56 (stop)	SC2-M2

Intergenic regions

POS	Variant	Intergenic region	Comment[b]			Mutant
113087	C to A	PPSC2_00420 (fusTE)−PPSC2_00425	No feature found			SC2-M1 and SC2-M2
5360859	G to A	PPSC2_23840−PPSC2_23845	No feature found			SC2-M1 and SC2-M2
5660508	G to A	PPSC2_25195−PPSC2_25200	No feature found			SC2-M1
2041429	GT to G	PPSC2_09290 (napB)−PPSC2_09295	Putative DegU − binding site			SC2-M1
26720 (plasmid)	A to T	PPSC2_25920−PPSC2_25925	No feature found			SC2-M1

[a] Single nucleotide variations (SNV), indels, and intra-chromosomal translocations (ITX) were identified by aligning the whole genome of SC2-M1 and SC2-M2 with the reference genome of *Paenibacillus polymyxa* SC2.

[b] Intergenic regions were imported into DBTBS (http://dbtbs.hgc.jp/) and searched for putative promoters or binding sites, for accessory transcription factors, and for transcriptional terminators.
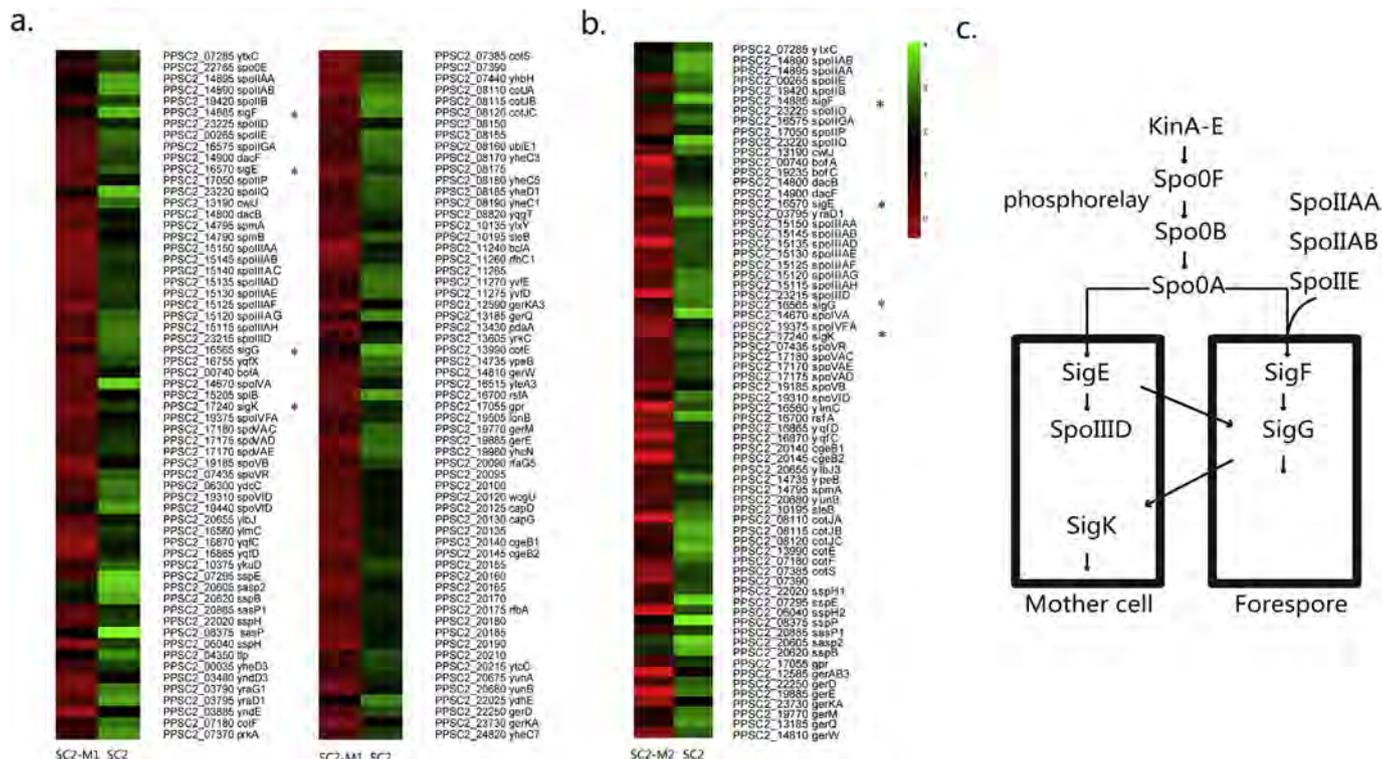

Fig. 3. Heat maps comparing expression levels of genes associated with sporulation from three *P. polymyxa* strains. (a) Heat maps comparing gene expression levels for SC2 and SC2-M1. (b) Heat map comparing gene expression levels for SC2 and SC2-M2. The heat maps are based on $\log_{10}$ FPKM (fragments per kb of coding sequence per million mapped reads) values. Corresponding locus tags and gene names are listed at the right of the maps. The red and green bands indicate low and high gene expression levels, respectively. All differently transcribed genes had a significance of $P < 0.05$. Asterisks indicate expression of *sigF*, *sigE*, *sigG* and *sigK*. (c) Schematic representation of phosphorelay and four main sporulation-specific sigma factors closely related to Spo0A that are involved in sporulation of *B. subtilis* (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).
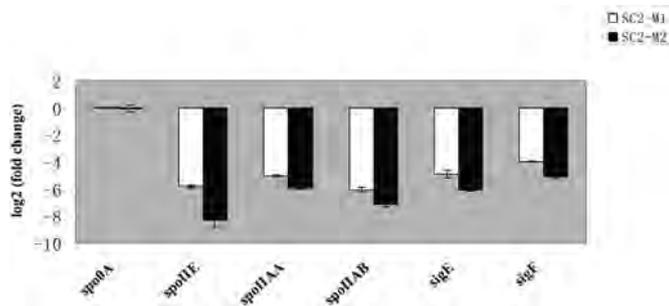
Fig. 4. Relative expression levels of six sporulation-related genes in SC2-M1 and SC2-M2 compared with SC2.

quantitative real-time polymerase chain reaction (qRT-PCR) analysis (Fig. 4). Consistent with transcriptome sequencing results, sharp decreases in expressions of *spoIIE*, *spoIIAA*, *spoIIAB*, *sigE* and *sigF* were observed in SC2-M1 and SC2-M2, while expression of *spo0A* was similar in SC2-M1, SC2-M2 and SC2.

In addition to sporulation-related genes, decreases in expression were also noted in many other genes. A large gene cluster, from PPSC2_05890 to PPSC2_05945, consisting of 12 genes and spanning 13,265 bp of the genome, is presumed to be the exopolysaccharide biosynthesis cluster (*eps* operon). Genes within this operon showed at least a 19.7-fold reduction in expression in SC2-M1 and a 31.34-fold reduction in SC2-M2. Significant decreases in expression were also observed for genes in the fusaricidin synthesis cluster, membrane/transport-related genes, metabolism-related genes and genes encoding other proteins and enzymes (S1 and S2 Tables).

3.4. Reversion of spo0A in P. polymyxa SC2-M2

Reversion of *spo0A* was performed in SC2-M2 because SC2-M2 had less variations in the genome compared with SC2-M1.

The His 211 residue was reversed by Arg using a genome recombination strategy. Other amino acids (Asp and Ala) were also selected for substitution of His 211 using the same strategy (Fig. 5). The results indicated that only Arg 211 in Spo0A produced complete recovery of sporulation (Fig. 2h and S1 Fig.). Sporulation efficiency is listed in Table 2.

4. Discussion

Sporulation deficiency exhibited by some laboratory-evolved environmental bacterial strains such as *B. subtilis* WN624B, WN624D and WN624E has recently gained attention. Mutations in the genome of these strains are likely to play a major role [17,18]. In the present study, we isolated two spontaneous mutants from *P. polymyxa* SC2, named SC2-M1 and SC2-M2. Both of them formed partially transparent colonies compared to the wild-type. Additionally, SC2-M2 formed much smoother colonies with regular edges compared to SC2 and SC2-M1 (Fig. 2a−c). More importantly, both SC2-M1 and SC2-M2 lost the ability to form endospores.
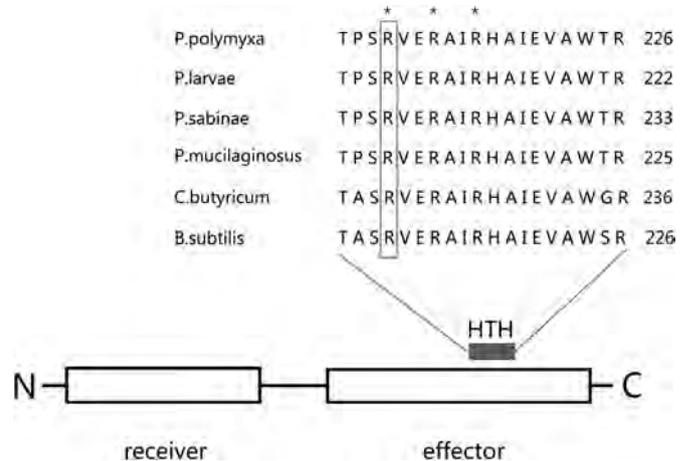

Fig. 5. Domain structure of Spo0A and comparison of amino acid sequences of Spo0A in related species. The alignment shows the amino acid sequences in the helix-turn-helix (HTH) region of Spo0A from *P. polymyxa*, *P. larvae*, *P. sabinae*, *P. mucilaginosus*, *Clostridium butyricum* and *B. subtilis*. Asterisks indicate three key positively-charged arginine residues that form favorable electrostatic contacts with the negatively charged ribose-phosphate backbone of DNA [25]. The box indicates the mutated arginine residue at position 211 (Arg 211) in *P. polymyxa* SC2 that is conserved in related species. Sequences were aligned using CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2).

Table 2
Sporulation efficiency of strains used in this study.

Strain	Sporulation efficiency
SC2	0.939
SC2-M1	0
SC2-M2	0
SC2-M2 (Arg 211)	0.924
SC2 (Asp 211)	0
SC2-M2 (Ala 211)	0

Table 3
Primers used in this study.

Primer	Sequence (5′−3′)[a]
CatF	*CCGGGCCC* TCATGTTTGACAGCTTAT
CatR	*CCGGGCCC* CCACGCCGAAACAAGCGC
ArgF	*CCCGGCCG* CTAACAACCTTCCCTAGT
ArgR	*CCAAGCTT* GCGTAGAACGCGCCATT
AspF	*CCCGGCCG* CTAACAACCTTCCCTAGT
AspR	*CCAAGCTT* CTGTAGAACGCGCCATT
AlaF	*CCCGGCCG* CTAACAACCTTCCCTAGT
AlaR	*CCAAGCTT* CGGTAGAACGCGCCATT

[a] Restriction sites are in italics.

To identify the cause of this deficiency, we carried out global genomic and transcriptomic analysis of *P. polymyxa* SC2, SC2-M1 and SC2-M2. Genome resequencing analysis revealed that fewer variations occurred in the genome of SC2-M2 compared with SC2-M1. However, both of these strains shared one SNV located in the sporulation-related gene *spo0A*, which codes for the master regulator of the entire bioprocess of sporulation and is particularly important in the initiation stage. To investigate this, we reversed His 211 by Arg in mutant strain SC2-M2. To confirm the specific role of Arg 211 in Spo0A, we also substituted His 211

by Asp and Ala, but only SC2-M2 (Spo0A) substituted with Arg 211 exhibited complete recovery of sporulation (Fig. 2f). This result confirmed that the sporulation deficiency exhibited by SC2-M2 was caused directly by the G > A point mutation located in *spo0A*.

As an important two-component regulator, Spo0A directly regulates the expressions of at least 122 genes, including activating the genes necessary for sporulation [31], especially those involved in the initial stages. As a switch for entry into sporulation, Spo0A is activated first through a phosphorelay system that consists of a series of histidine kinases (KinA/B/C/D/E) and Spo0F and Spo0B. The activated Spo0A (Spo0A-P) then initiates expression of *sigF* and *sigE*, which subsequently activates *sigG* and *sigK* expression, thereby regulating the expression of hundreds of genes in the forespore and mother cell (Fig. 3c). Spo0A consists of two functional domains, an N-terminal phosphorylation and dimerization domain (receiver) and a C-terminal DNA binding (effector) domain, which are separated by a hinge region [32]. When Spo0A is phosphorylated at the receiver domain, it forms a dimer [33,34], which binds to the "0A box" (consensus sequence 5′-TGTCGAA-3′) located in the promoter region of target genes via three helices, αC, αD and αF and two loops αA-αB and αB-αC. Three positively charged Arg residues (Arg 211, Arg 214 and Arg 217), located on the successive turns of the recognition helix αD, interact with the negatively charged DNA backbone [35]. The amino acid sequences in this HTH region show a high degree of similarity to corresponding regions in other endospore-forming microorganisms (Fig. 5). In our study, the single mutated nucleotide changed Arg to His at position 211 of Spo0A (Fig. 5). A previous study showed that amino acid substitutions (Arg > Gly or Arg > Ala) at position 214 caused a sporulation-deficient phenotype in *B. subtilis*; however, attempts to substitute the Arg residue at position 211 with Gly or Ala failed [36]. The complete recovery of sporulation in the SC2-M2 (Arg 211) strain in the present work provides strong evidence that Arg 211 is also a required amino acid for the efficient function of Spo0A.

Additionally, the morphology of SC2-M2 and SC2-M2 (Arg 211) colonies was much smoother at the edges compared with that of SC2 and SC2-M1 colonies (Fig. 2a−c). This phenomenon is most likely caused by mutation of the chemotaxis-related histidine kinase gene PPSC2_21240, which had a stop codon that terminated the peptide sequence at the 56th amino acid. Chemotaxis is always coupled with the movement of flagella, so whether this gene is important for flagella assembly remains to be investigated.

Sporulation is considered a competitive advantage that is acquired after long periods of evolution. Strains from both *B. subtilis* [17,18] and *P. polymyxa* have shown defects in sporulation during their successive cultivation in laboratory environments. Whether this phenomenon also occurs in other sporulating organisms is yet to be investigated. The causes of sporulation deficiencies may vary, but genome mutations play major roles. The question remains as to why these mutations occur so frequently and whether this is an adaptive response to a special environment possessed by sporulating organisms.

In conclusion, this study provides the first comprehensive analysis of sporulation deficiency in *P. polymyxa* SC2 at the genomic and transcriptomic levels. Most importantly, it demonstrated that a single nucleic acid mutation (G > A) within *spo0A* is responsible for the sporulation deficiency and that the corresponding amino acid (Arg 211) of Spo0A is required for its efficient function.

Conflict of interests

The authors declare that they have no conflict of interests.

Authors' contributions

BD, YD and CW designed the study. XH and XY performed the laboratory work and analyzed the data. XH drafted and wrote the manuscript. KL, SZ and LY provided reagents and materials. All the authors read and approved the final manuscript.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2016.05.002.

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