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Original Article

Regulator DegU is required for multicellular behavior in *Lysinibacillus sphaericus*

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ABSTRACT

DegS and DegU make up a two component system belonging to a class of signal transduction systems that play important roles in a broad range of bacterial responses to the environment. However, little study has been done to explore the physiological functions of DegS-DegU in mosquitocidal *Lysinibacillus sphaericus*. In this study, it was found that deletion of *degU* or *degS-degU* inhibited the swarming motility, biofilm formation, sporulation and binary toxin production through regulating the related genes, and phosphorylation was necessary for the functions of DegU. Based on the findings, a regulation network mediated by DegU was delineated. Both DegU-pi and Spo0A-pi positively regulates genes which are linked with the transition from stage II to the end of the sporulation process and also influences the production of binary toxins via regulation on *sigE*. Both DegU-pi and Spo0A-pi negatively regulate *abrB*/*sinR* and influence the biofilm formation. DegU-pi can positively regulate the motility via the regulation on *sigD*. Whether the regulations are directly or indirectly need to be explored. Moreover, Spo0A-pi may indirectly regulate the swarming motility through negatively regulating DegU. It was concluded that DegU is a global transcriptional regulator on cell swarming motility, biofilm formation, sporulation and virulence in *L. sphaericus*.

1. Introduction

Sporulation is an important multicellular process playing extensive physiological function in spore-forming bacteria. By forming spores, bacteria enter a dormant state surviving from adverse environmental conditions and turn to the active life cycle through germination when favorable conditions are available [16]. The sporulation process starts with the formation of an asymmetric septum, which separates mother cell and forespore in cell [26]. The forespore then becomes a double membrane-bound protoplast in the mother cell cytosol during the engulfment process. A cortex is then formed between the double membranes and a series of coat layers around the forespore. Upon the completion of forespore maturation, the mother cell undergoes programmed lysis releasing the mature spore into the environment [25].

The sporulation process has been vastly studied in *Bacillus subtilis*, a model strain of *Bacillus* spp., which identified rather complicated regulation network with abundance regulatory proteins involved. The most important regulatory protein in this signal transduction network is Spo0A, which is activated by phosphorylation and required for bacterial development. The signal transduction pathway in *B. subtilis* sporulation involves several steps: the kinase proteins KinA and KinB phosphorylate the response regulator protein Spo0F and the phosphoryl group from Spo0F is passed to Spo0B then to the response regulator Spo0A. As a master transcriptional regulator, the activated Spo0A leads to the sequential activation of the downstream regulators (SpoIIE and SpoIIAA) initiating the polar septum formation [13,15]. The SpoIIAA then antagonizes the binding of anti-sigma factor SpoIIAB to the SigF and the free SigF activates the expression of *sigG* and *spoIIR* [12,24]. The SpoIIR is then secreted into the intermembrane space activating the SpoIIGA protease to remove an inhibitory propeptide from SigE in the mother cell [31]. This drives the transcription of genes encoding a hydrolase complex SpoIIM/SpoIID/SpoIIP that mediates engulfment, resulting to sporulation [34].

In addition to sporulation, swarming motility and biofilm formation are the other two important multicellular behaviors to adapt to various survival pressures during the bacteria's life cycle.

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Swarming motility represents one kind of flagellum-driven motility that the bacteria move rapidly and colonize on nutrient-rich solid substrates. Biofilm formation is a social behavior of the bacteria by producing extracellular polymeric matrix commonly comprised of polysaccharides, proteins and DNA and aggregating on a surface [27]. It was found that in *B. subtilis*, the swarming requires the secretion of a surfactant and an increase in flagellar density on the surface of the cell. Most genes within a 27 kb large *fla/che* operon, including sigma factors (e.g. sigD), flagellar biosynthesis and chemotaxis related genes (e.g. *fliM, flgB, motA, motB, cheA, cheB* and *swrD*), are involved in swarming motility [21]. Besides, SwrA and DegS-DegU are two important regulators for swarming [33]. DegS and DegU, a histidine kinase (HK) and an intracellular response regulator (RR), respectively, make up a two-component system (TCS) belonging to a class of signal transduction systems which are widely presented in eukaryotic cells and play important roles in a broad range of bacterial responses [22,45]. After receiving extracellular signal, DegS can be autophosphorylated and transfers phosphoryl group to DegU resulting to a structural change of DegU. DegU acts as a molecular switch, whose transcriptional regulator activity is dependent on the degree of phosphorylation [9,36]. It was found that a low level of phosphorylated DegU (DegU-Pi) is required for the swarming but a high level of DegU-Pi inhibits the swarming mobility [36]; DegU-Pi activates the transcription of *fla/che* operon with the presence of SwrA whereas represses the transcription of *fla/che* operon with the absence of SwrA [32,33]. During biofilm development, many flagellar biosynthesis and chemotaxis related genes within *fla/che* operon were repressed and the *eps* operons encoding enzymes involved in exopolysaccharide synthesis and *tapA* encoding a protein fiber were hyperexpressed in *B. subtilis* [6,18]. The SinR is one of the major regulators of the genes required for biofilm formation. The regulation of SinR requires the binding with SlrR, a protein homologous to SinR [29]. Being a multiple regulator that controls a myriad of processes, DegU-Pi also regulates biofilm formation [29,43], extracellular protease production [3,9] and exopolymer poly-γ-DL-glutamic acid (γ-PGA) biosynthesis [42] in *B. subtilis*. *Lysinibacillus sphaericus* is a Gram-positive, aerobic and spore-forming bacterium, which is ubiquitous in nature. Some strains are toxic against mosquito larvae and play an important role in combating the vector of etiological agent of deadly and debilitating human diseases such as dengue, chikungunya, filariasis, malaria and west nile fever [39]. These strains produce several kinds of mosquitocidal toxins, of which the binary toxins (BinA and BinB) produced in sporulation stage are the major toxicity contributor [7,38]. The BinA and BinB are highly toxic to the larvae of *Culex* and *Anopheles* mosquitoes and both are required to achieve maximal toxicity [41]. Besides, *L. sphaericus* has a long persistence in mosquito larvae breeding sites and not easily affected by nutritional deficiency or pollution [26]. This character becomes an advantage for *L. sphaericus* as a biopesticide in field application for mosquito biocontrol. The spores and the exosporium, the outside layer of the spore which packed the spores and the crystals formed by BinA and BinB, are believed to be the main reason for the long persistence as they help *L. sphaericus* to resist the complicated and continual environment challenges [14]. Furthermore, the spores of *L. sphaericus* were also found to be toxic to mosquito larvae [1]. Previous research have shown that the production of toxin proteins BinA and BinB is associated with sporulation in *L. sphaericus* [7,38]. Therefore, the sporulation process and the regulatory mechanisms that govern spores and toxin proteins formation in this bacterium are very important and thus require further exploration.

Little study has been done about sporulation regulation of DegU in *Bacillus* spp. or physiological functions of DegS-DegU in mosquitocidal *L. sphaericus*. In a previous research in our laboratory, a random mariner-based transposon insertion mutant library of *L. sphaericus* was constructed, which revealed accidentally that *degU* was probably related to sporulation in *L. sphaericus* [44]. But the physiological roles and regulatory mechanisms of *degU* in mosquitocidal *L. sphaericus* was not further studied.

In this study, the mutants with the deletion of genes *degS* (Bsph_1142), *degU* (Bsph_1143), *spo0A* (Bsph_3503), *sigE* (Bsph_1443), *sigF* (Bsph_1714) and the operon *degS-degU*, respectively, were constructed by homologous recombination. The comparison on the phenotypes and transcriptional levels of the wild-type and the mutants were performed to explore the regulatory roles of the DegS-DegU on motility, biofilm formation, sporulation and toxicity in *L. sphaericus*. The data helps to understand the physiological functions and mechanism of DegS-DegU in *L. sphaericus*.

2. Materials and methods

2.1. Bacterial strains, plasmids, culture condition and primers

Escherichia coli and *L. sphaericus* strains were routinely cultured in LB medium at 37 °C and 30 °C, respectively. Spores of L. *sphaericus* strains were cultured in MBS medium (pH 7.2–7.4, 0.68% KH_2PO_4, 0.03% $MgSO_4 \cdot 7H_2O$, 0.002% $MnSO_4$, 0.002% $Fe_2(SO_4)_3$, 0.002% $ZnSO_4 \cdot 7HO_2$, 0.002% $CaCl_2$, 1% Tryptase, 0.2% yeast extract) at 30 °C. Antibiotics were added at the following concentrations (μg/mL): 100 μg/mL ampicillin, 10 μg/mL kanamycin, 10 μg/mL erythromycin for *L. sphaericus* and 50 μg/mL kanamycin for *E. coli*. The primers used in this study are listed in Table S1. Genomic DNA from *L. sphaericus* strain C3-41 (GenBank accession number CP000817.1) was used as the template in all PCRs.

2.2. Mutant construction

A 750 bp upstream and a 750 bp downstream fragments of *degS* and a kanamycin-resistance gene (*kan*) were amplified using primers F*degS*-up/R*degS*-up, F*degS*-down/R*degS*-down and F*kan*/R*kan*, respectively. The three obtained PCR products were mixed together and used as template for the second round PCR with primer pair F*degS*-up/R*degS*-down. The resulting PCR product was digested with *Nhe*I/*BamH*I and ligated into the same digested temperature-sensitive suicide vector pRN5101, giving the recombinant plasmid pRN5101-*degS*. The pRN5101-*degS* was transformed into *L. sphaericus* C3-41 by electroporation and a mutant with the deletion of *degS*, named Δ*degS*, was screened from the transformant by homologous recombination and heat treating based on the method described previously [40]. Similar methods were used to construct the mutants with the deletion of *degU* (Δ*degU*), *spo0A* (Δ*spo0A*), *sigE* (Δ*sigE*), *sigF* (Δ*sigF*) and with the deletion of *degS-degU* operon (Δ*degSU*) using the primers listed in Table S1.

For construction of *degU* and *degSU* complementation plasmid, the fragments of *degU* and *degS-degU* were amplified from C3-41 genome using primers F*degU*-com/R*degU*-com and F*degSU*-com/R*degSU*-com. The obtained PCR products were digested with *Bam*HI/*Xho*I and ligated between the equivalent sites of plasmid pHT315-8E21b generating plasmid pHT315-*degU* and pHT315-*degSU* respectively. PHT315-*degU* and pHT315-*degSU* were introduced into Δ*degU* and Δ*degSU* by electroporation, resulting in the complementation strains of *degU* (named *degU*-com) and *degS-degU* (named *degSU*-com).

In addition, a *degU* mutant was constructed using point mutation, in which a glycine acid had taken place of the original aspartic acid predicted as a phosphorylation site at NCBI (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Firstly, two partial *degU* sequences were amplified using primers F*degU*Asp1/R*degU*Asp1 and F*degU*Asp2/R*degU*Asp2, respectively. The two obtained PCR products were then

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