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DegQ regulates the production of fengycins and biofilm formation of the biocontrol agent *Bacillus subtilis* NCD-2

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ABSTRACT

Bacillus subtilis NCD-2 is an excellent biocontrol agent for tomato gray mold and cotton soil-borne diseases. The fengycin lipopeptides serve as a major role in its biocontrol ability. A previous study revealed that insertion of *degQ* with the mini-Tn*10* transposon decreased the antifungal activity of strain NCD-2 against the growth of *Botrytis cinerea.* To clarify the regulation of *degQ* on the production of fengycin, we deleted *degQ* by in-frame mutagenesis. Compared with the wild-type strain NCD-2, the *degQ*-null mutant had decreased extracellular protease and cellulase activities as well as antifungal ability against the growth of *B. cinerea in vitro*. The lipopeptides from the *degQ*-null mutant also had significantly decreased antifungal activity against *B. cinerea in vitro* and *in vivo*. This result was confirmed by the decreased fengycin production in the *degQ*-null mutant that was detected by fast protein liquid chromatography analysis. Quantitative reverse transcription PCR further demonstrated that *degQ* positively regulated the expression of the fengycin synthetase gene. In addition, the *degQ*-null mutant also had a flatter colony phenotype and significantly decreased biofilm formation ability relative to the wild-type strain. All of those characteristics from *degQ*-null mutant could be restored to the strain NCD-2 wild-type level by complementation of intact *degQ* in the mutant. Therefore, DegQ may be an important regulator of fengycin production and biofilm formation in *B. subtilis* NCD-2.

Introduction

Botrytis cinerea is an airborne plant pathogenic fungus causing the gray mold over 200 crop species, which is the most common and most serious disease in vegetables and fruits (e.g., tomato, cucumber, cabbage, beans, strawberry, grape and blackberry) and resulted in considerable economic losses (Ten et al., 1998; Williamson et al., 2007). For the increasing concern of the consumers' health and environmental pollution, chemical fungicides in disease management are limited (Chen et al., 2008) . *Bacillus subtilis* has shown a strong antagonistic effect on hyphal growth and spore germination and a reduction of tomato gray mould caused by *B. cinerea* (Walker et al., 1998; Toure et al., 2004; Chen et al., 2008; Cawoy et al., 2015). In addition, beneficial *B. subtilis* is also a potential biocontrol agent for suppressing plant soil-borne diseases. The main mechanisms include the direct inhibition of plant pathogen growth by producing a variety of bioactive metabolites (Yánez-Mendizábal et al., 2011), competition for nutrients and ecological niches with pathogens (Kumar et al., 2011), and induction of plant systemic resistance (Lahlali et al., 2013). The production of active antifungal compounds is shared by most *B. subtilis* with potential biological ability. The iturin, surfactin, and fengycin families of lipopeptides are the predominant active antifungal compounds produced by *B. subtilis* (Stein, 2005). Iturins exhibit strong antifungal activities against many pathogenic fungi and restrict antibacterial activities (Maget-Dana et al., 1994). Surfactin is a highly powerful biosurfactant, and it has antibacterial and antiviral abilities; in addition, surfactin shows strong synergistic actions when applied in combination with iturin A or fengycin (Maget-Dana et al., 1992; Romero et al., 2007). The fengycin family shows strong antifungal activity, specifically against filamentous fungi (Vanittanakom et al., 1986; Stein, 2005). Besides their direct antimicrobial activities, surfactin and fengycin have been identified as bacterial elicitors of induced systemic resistance in the host plant (Ongena et al., 2007).

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To suppress plant soil-borne diseases, rapid and effective colonization in the rhizosphere is considered a prerequisite for direct inhibition of the growth of phytopathogenic fungi and competition for nutrients and niches with pathogens (Kumar and Johri, 2012). Low root colonization efficiency usually leads to lower biocontrol activity (Bull et al., 1991; Bais et al., 2004). The root colonization ability of *B. subtilis* is associated with its ability to form biofilms (Yaryura et al., 2008). The colonization and biocontrol efficiency of *Bacillus* could be significantly improved by improving its ability to form biofilms (Weng et al., 2012). Biofilms are dynamic biological systems and complex structured communities that are encased in self-produced extracellular matrix (Hall-Stoodley et al., 2004). Biofilm formation could increase *B. subtilis* resistance to environmental stresses (e.g., antimicrobial agents, ultraviolet exposure, and pH changes) and allow it to colonize in the plant rhizosphere more steadily (Hall-Stoodley et al., 2004; Stewart and Franklin, 2008; López et al., 2010; Vlamakis et al., 2013).

B. subtilis possesses complex regulatory pathways and multilayered regulatory mechanisms that control biofilm formation (Kunst and Rapoport, 1995). The phosphorylated global regulator Spo0A activates the transcription of the *eps* and *tapA-sipW-tasA* operons that encode the biofilm matrix by repressing the transcription of *abrB* (Hamon and Lazazzera, 2001) or activating the transcription of SinI. SlrR/SlrA is homologous to SinR/SinI and also is positively regulated by Spo0A to activate the transcription of the *eps* and *tapA-sipW-tasA* operons and other important genes for biofilm formation in *B. subtilis* (Kobayashi, 2008). In *B. subtilis*, biofilm formation also is regulated by the DegU/DegS two-component system. DegQ stimulates phosphotransfer from DegS-P to DegU, and the phosphor-DegU level induces the transition from a motile cell state to a biofilm-forming state.

B. subtilis strain NCD-2 showed strong inhibition against the growth of phytopathogenic fungi *in vitro* and significant control efficiency against cotton seedling damping-off and verticillium wilt in field trials over 10 consecutive years (Li et al., 2005). Previous studies showed that both the production of fengycin lipopeptides and colonization in the cotton rhizosphere played important roles in the strain NCD-2 control of cotton seedling damping-off (Guo et al., 2010). Our previous study revealed that insertion of *degQ* by the transposon mini-Tn*10* decreased the antifungal activity of strain NCD-2, but the regulation of *degQ* on the production of fengycin was not confirmed (data not shown). DegQ is a small pleiotropic regulatory protein. It consists of 46 amino acids that control the expression of degradative enzymes, intracellular proteases, and several other secreted enzymes (Koumoutsi et al., 2007). Increased expression of the pleiotropic regulator DegQ in *B. subtilis* 168 results in a 7–10-fold increase in antibiotic production (Tsuge et al., 1999, 2005), and a *degQ* mutation led to decreased pellicle formation (Kobayashi, 2007b). Therefore, in this study, we investigated the role of DegQ in fengycin production and biofilm formation in strain NCD-2. This study will contribute to a better understanding of the biocontrol mechanisms and will improve biocontrol efficiency in future practical applications.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *B. subtilis* strains were stored at −80 °C in Luria–Bertani broth (LB) with 30% glycerol (v/v). Routinely, fresh bacterial cultures were retrieved from frozen stocks before each experiment and grown at 37 °C on LB agar for maintenance or in Landy medium (Landy et al., 1948) for lipopeptide production at 30 °C for 48 h with 180 rpm rotary shaking. *Escherichia coli* DH5α was used for plasmid replication and was cultured at 37 °C in LB medium. When necessary, antibiotics were added at the following concentrations: for *B. subtilis*, 10 μg/mL tetracycline and 1 μg/mL erythromycin; for *E. coli*, 100 μg/mL ampicillin and 10 μg/mL of tetracycline. *Botrytis cinerea* (deposited as CGMCC No. 3.15253 in the China General Microbiological Culture Collection Center, CGMCC) was isolated from the diseased tomato leaf and maintained on potato dextrose agar (PDA) and incubated at 25–28 °C.

Table 1
Microorganisms and plasmids used in this study.

Strains or plasmids	Characteristics	Reference or Source
B. subtilis strain		
NCD-2	Wild-type strain, producer of fengycin	Laboratory stock
MQ	*degQ* deletion mutant, derivative of strain NCD-2. Tet^r	Current study
CQ	Complementary strain, containing the intact *degQ* gene; derivative of strain MQ. Tet^r	Current study
NCDΔfen	Fengycin deficient	Laboratory stock (Guo et al., 2014)
E. coli DH5α	*recA1 endA1 hsdR17 deoR* thi*21 supE44 gyrA96 relA1*	Tiangen Biotech
B. cinerea	Pathogen of tomato gray mold	CGMCC No. 3.15253
Plasmids		
pHY300PLK	*E. coli* and *B. subtilis* shuttle vector, Origin of replication: pAMα1, *Streptococcus faecalis*. Tet^r	Takara Biotech
pMAD	*E. coli* and *B. subtilis* shuttle, temperature-sensitive vector. Ap^r Em^r	(Arnaud et al., 2004)
pMADΔdegQ	pMAD with *degQ* deletion box. Ap^r Em^r	Current study
pHBdegQ	A 1,649 bp *Eco*RI–*Bam*HI fragment containing intact *degQ* cloned into pHY300PLK. Tet^r	Current study

Tet^r, Ap^r, Em^r indicate resistance to tetracycline, ampicillin and erythromycin, respectively.

Strain construction

To generate an internal deletion in the *degQ* gene of strain NCD-2, the temperature-sensitive vector pMAD was used (Arnaud et al., 2004). The upstream region of *degQ* was amplified with the primer pair *degQ*-P1: 5′-CGCGGATCCCCTCACGAAGGAACCCAA-3′ (*Bam*HI restriction site underlined) and *degQ*-P2: 5′-CGGGGTACCCGACAGATTCATTACGAAACAT-3′ (*Kpn*I restriction site underlined). The downstream region of *degQ* was amplified with the primer pair *degQ*-P3: 5′-CGGGGTACCTTTTCCATCGTTTCCACA-3′ (*Kpn*I restriction site underlined) and *degQ*-P4: 5′-CCGGAATTC GCAAAGAGCAGCCTAACA-3′ (*Eco*RI restriction site underlined). The two PCR products were introduced into the *Kpn*I site, and a 2,747-bp fragment was obtained by PCR amplification using the primer pair *degQ*-P1 and *degQ*-P4. This PCR fragment was digested with *Bam*HI and *Eco*RI and then inserted into the *Bam*HI and *Eco*RI sites of the shuttle vector pMAD to generate pMADΔdegQ. The recombined plasmid pMADΔdegQ was transformed into the wild-type strain NCD-2 by the protoplast fusion method (Guo et al., 2010). An in-frame deletion of the *degQ* gene in *B. subtilis* NCD-2 was carried out following the previously described procedure with a modification (Arnaud et al., 2004). Colonies with no erythromycin resistance were selected, and the *degQ*-deleted mutants were confirmed by PCR amplification and sequencing using the primer pair *degQ*-P1 and *degQ*-P4.