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Sporulation and spore stability of *Bacillus megaterium* enhance *Ketogulonigenium vulgare* propagation and 2-keto-L-gulonic acid biosynthesis

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ABSTRACT

Bacillus spp. is widely used as the companion bacterium in the two-step biosynthesis of 2-keto-L-gulonic acid (2-KLG), which is the direct precursor in the production of vitamin C by *Ketogulonigenium vulgare*. To understand the effects of sporulation and spore stability on 2-KLG production, the *spo0A* and *spoVFA* deletion mutants of *Bacillus* megaterium were constructed. The sorbose conversion rates of *spo0A* and *spoVFA* mutant co-culture systems were 33% and 70% lower, respectively, than that of the wild-type co-culture system. In addition, *K. vulgare* cell numbers in the two mutant systems declined by 15% and 49%, respectively, compared to the value in the wild-type system. Correlation analysis indicated that the 2-KLG concentration is positively related to sorbose dehydrogenase activity and the *K. vulgare* cell number. This study demonstrated that sporulation and spore stability of the wild-type companion play key roles in the enhancement of *K. vulgare* propagation and 2-KLG biosynthesis.

1. Introduction

Vitamin C is an essential water-soluble vitamin for humans and some mammals (Bremus et al., 2006). The synthesis of vitamin C from D-glucose via a two-step fermentation process, a method originally developed in China, has been adopted by almost all of the main vitamin C producers. In the first step of the fermentation, *Gluconobacter suboxydans* converts D-sorbitol to L-sorbose (Macauley-Patrick et al., 2005). In the second step, a mixed culture of *Bacillus megaterium* and *Ketogulonigenium vulgare* converts L-sorbose to 2-keto-L-gulonic acid (2-KLG). *B. megaterium* is generally considered to be a companion strain that generates and releases metabolites to the fermentation broth that, in turn, stimulate *K. vulgare* propagation and 2-KLG accumulation (Zhang et al., 2010). In addition to *B. megaterium,* many other *Bacillus* spp. (e.g., *B. subtilis, B. cereus,* and *B. thuringiensis)* also significantly enhance 2-KLG accumulation via *K. vulgare* (Yin et al., 1997). These companion bacteria mainly remain in the form of spores during the later phase of co-culture. However, the effects of sporulation and the spore stabilities of these Gram-positive companions on *K. vulgare* propagation and 2-KLG accumulation have not been reported thus far.

The main regulator, Spo0A (encoded by *spo0A*), is a conserved regulator of sporulation (Olmedo et al., 1990). Spo0A is activated initially by phosphorylation of its regulatory domain through a multicomponent phosphorelay chain when nutrients are limited (Cervin and Spiegelman, 1999). The phosphorylated Spo0A (Spo0A-P) can then recognize and bind to a specific DNA sequence, named '0A-box', with the result that gene transcription is activated or repressed (Castilla-Llorente et al., 2006; Trach et al., 1991). When Spo0A-P has accumulated to a certain level, sporulation is initiated by the synthesis or activation of a cascade of σ factors at the appropriate time and location (Kroos et al., 1999). Many studies have indicated that the *spo0A* mutant of *B. subtilis* blocks entry into sporulation (Asayama and Kobayashi, 1993; Chibazakura et al., 1991).

In *Bacillus* spp. spores, abundant pyridine-2,6-dicarboxylic acid (DPA) helps to reduce core water content and makes the spores resistant to various environmental stresses. DPA is synthesized by DPA synthase, which is the product of the *spoVF* operon, composed of *spoVFA* and *spoVFB*. Inactivation of either *spoVFA* or *spoVFB* results in deficient DPA synthesis (Errington, 1993). The core region of DPA-less spores appears to be more hydrated than that of normal spores (Paidhungat et al., 2000). Further studies indicated that *spoVF* mutation leads to spore instability and sensitivity to wet heat and DNA damage because the cortex-lytic enzyme SleB is activated by the hydrated core (Magge et al., 2008; Paidhungat et al., 2000; Paredes-Sabja et al., 2011).

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As it provides powerful host cells for heterologous protein production (Gamer et al., 2009; Jahn et al., 2010), *B. megaterium* has been studied intensively and the complete genome sequences of two important strains were recently reported (Ravel et al., 2011). *B. megaterium* possesses *spo0A* and *spoVF* operons encoding proteins with high identity to those proteins in *B. subtilis*. In the current study, the effects of *B. megaterium* sporulation and spore stability on promoting *K. vulgare* propagation and 2-KLG accumulation were investigated by constructing *spo0A* and *spoVFA* mutant co-culture systems, respectively. Distinct performances were found for these mutants compared to those of the wild-type co-culture system. The results indicate that sporulation and spore stability play important roles in stimulating *K. vulgare* propagation and 2-KLG accumulation.

2. Methods

2.1. Bacterial strains and plasmids

The strains and plasmids used in this study are listed in Table 1. The *spo0A* and *spoVFA* mutants were derived from *B. megaterium* WSH-002 by double-crossover homologous recombination. All bacterial strains were stored at −80 °C.

2.2. Medium and culture conditions

Medium compositions for co-culture seed culture and fermentation were described previously (Zhang et al., 2011). *B. megaterium* and its mutants were cultured in Schaeffer's sporulation medium (SSM) (Schaeffer et al., 1965) or Luria–Bertani (LB) medium to determine the sporulation efficiency or growth curve. *Escherichia coli* DH5α, *B. megaterium*, and recombinant strains were grown in LB medium or on LB agar. When required, the medium was supplemented with chloramphenicol at 15 μg/ml for *E. coli* or 2.5 μg/ml for *B. megaterium* mutants and ampicillin at 100 μg/ml for *E. coli*.

2.2.1. Seed preparation and fermentation of the co-culture system

The seeds for *K. vulgare* WSH-001 and *B. megaterium* WSH-002 or the mutant co-culture systems were prepared as follows. (1) *K. vulgare* WSH-001 and *B. megaterium* WSH-002 (or its mutants) were individually streaked on seed culture medium containing 2% agar and incubated at 30 °C until a single colony formed; (2) about 30 *K. vulgare* WSH-001 single colonies were dispersed in 3 ml of sterilized physiological solution, 0.9% NaCl in H_2O (labeled as solution A); (3) a sterilized toothpick was dipped in a *B. megaterium* WSH-002 (or mutant) colony and stirred in 100 ml of sterilized physiological solution (labeled as solution B); (4) 200 μl of solution A was spread on 50 ml of solidified seed medium slant, then a spreading rod dipped with solution B was streaked over the surface of this seed medium slant; (5) the streaked seed medium was cultured at 30 °C for 36 h; (6) the mixed culture on the seed medium was rinsed and suspended in 10 ml of sterilized physiological solution. Subsequently, 200 μl of the mixed culture were inoculated into a 750-ml flask containing 75 ml of seed medium and cultivated at 30 °C for 18 h on a 200 r/min orbital incubator.

Fermentations were performed in 750-ml flasks containing 75 ml of fermentation medium, buffered by 5 g/l $CaCO_3$, at 30 °C for 72 h on a 200 r/min orbital incubator. The inoculum amount was 10% (v/v). All fermentation experiments were performed with three replicates.

2.2.2. Culture conditions for growth measurements and DPA content analysis

For the growth curve determinations, single colonies of *B. megaterium* and its mutants were separately transferred to a 500-ml flask containing 50 ml of LB medium and cultivated at 30 °C and 200 r/min. Every 2 h, 100 μl of culture were diluted to 3 ml with LB medium and the OD600 was measured. For DPA content analysis, strains were individually preincubated overnight in LB medium (30 °C, 200 r/min), and then cultured in SSM medium (37 °C, 200 r/min) with 1% (v/v) inoculum. About every 6 h, 1 ml of culture was collected and centrifuged (10,000×*g*, 5 min); the pellets were then washed with distilled water twice. DPA quantitative analysis was performed as previously described (Warth, 1983).

2.2.3. Determination of sporulation efficiency

The sporulation efficiency of *B. megaterium* strain was determined on LB agar plates. The number of viable cells at the onset of the stationary phase in SSM was counted as total colony-forming units (CFUs) on LB plates (30 °C for 16 h). The spore number after 48 h of culture in SSM was determined as heat-resistant (incubated at 80 °C in water for 15 min) CFUs on LB plates (30 °C for 16 h). The sporulation efficiency was defined as the percentage of heat-resistant CFUs to total CFUs on the LB plates.

2.3. Plasmid construction and gene deletion

All plasmid constructions were amplified with *E. coli* DH5α using standard methods. For deletion of *spo0A* and *spoVFA* in *B. megaterium* WSH-002 by homologous recombination (Fig. 1), the recombination flanks of *spo0A* and *spoVFA* CDS were constructed and individually cloned into pMD18-T simple vector (Takara), resulting in vectors pMD-RF0A and pMD-RFVFA. Recombination flanks of *spo0A* (RF0A) and *spoVFA* (RFVFA) were individually amplified from the *B. megaterium* WSH-002 chromosome by PCR as follows: flank A (862 bp) of *spo0A* and flank A (859 bp) of *spoVFA* were generated with the primer pairs spo0AP1/spo0AP2 and spo-

Table 1
Bacterial strains and plasmids used in this study.

Strains/plasmids	Relevant characteristics	Reference
K. vulgare WSH-001	Wild type	Zhang et al. (2010)
B. megaterium WSH-002	Wild type	Zhang et al. (2010)
B. megaterium Δ*spo0A*	Δ*spo0A*::*cat*	This study
B. megaterium Δ*spoVFA*	Δ*spoVFA*::*cat*	This study
E. coli DH5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Takara
pMD18-T simple	Amp^r [a]	Takara
pMD-RF0A	Amp^r *lacZ*::RF0A	This study
pMD-*cat*	Amp^r *lacZ*::*cat*	This study
pMD-RF0A::*cat*	Amp^r *lacZ*::RF0A::*cat*	This study
pMD-RFVFA	Amp^r *lacZ*::RFVFA	This study
pMD-RFVFA::*cat*	Amp^r *lacZ*::RFVFA::*cat*	This study
pT7-RNAP	Cm^r [a]	Gamer et al. (2009)

[a] Amp^r and Cm^r are ampicillin and chloramphenicol resistance, respectively.