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Note

Comparing methods of genetic manipulation in *Bacillus subtilis* for expression of recombinant enzyme: Replicative or integrative (CRISPR-Cas9) plasmid?

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ABSTRACT

The present study evaluated the stability of *Bacillus subtilis* strains transformed with a replicative or integrative plasmid (via CRISPR-Cas9) to express a recombinant phytase. Both transformation methods did not affect the growth of *B. subtilis*, but the stability of the construct and the enzymatic activity was reduced in the strain transformed with the replicative plasmid.

Although there are a variety of microorganisms with potential for the production of recombinant proteins, *Bacillus subtilis* has been noted not only for its probiotic characteristics but also for its superior ability to secrete proteins (Illing, 2002; Zweers et al., 2008). *B. subtilis* can be easily manipulated with replicative plasmids, and there is a common sense that the higher the number of plasmid copies in the cell, the greater the amount of recombinant protein produced. However, episomal plasmids can cause problems since they can be transferred by conjugation to other bacteria, including to different species. This process may also result in loss of the plasmid by the original strain along with its ability to produce the recombinant protein. It is already established that, depending on the type of bacteria, a large number of plasmid molecules can lead to a high energy cost to the cell and compromise the production of the heterologous protein (Gill et al., 2009; Kroll et al., 2010). Otherwise, the homologous recombination can provide greater stability of the genetic construct that integrates the genome of the host cell. However, this process generally results in the integration of only one copy of the genetic construct, which in theory reduces the ability of the cell to synthesize the recombinant protein. Recently, the innovative CRISPR-Cas9 genomic editing technology has been successfully applied in *B. subtilis* (Altenbuchner, 2016).

In the present study, *B. subtilis* was manipulated to express and secrete a recombinant phytase of fungal origin. Phytases are enzymes that degrade phytic acid (phytate), which is the main form of phosphorus storage in plants, and monogastric animals do not assimilate this form of phosphorus. Thus, this enzyme has importance as an additive in feed used in animal production, since it allows the inclusion of vegetable ingredients in higher amounts in the diets.

Our objective was to evaluate if phytase production would be altered in *B. subtilis* transformed with replicative plasmid or with integrative plasmid based on CRISPR-Cas9 technology. For this purpose, the KM0 strain was first manipulated with the replicative plasmid pJJ5 (Fig. 1A), which contains two origins of replication: one for Gram-negative bacteria derived from the pUC18 plasmid, and the other for Gram-positive bacteria derived from the pUB110 plasmid, a spectinomycin resistance gene and a genetic unit consisting of a promoter that is activated during the stationary phase of the *B. subtilis* (Pylb; Yu et al., 2015) growth, which controls the expression of phytase from the fungus *Aspergillus fumigatus* (GenBank accession number AHZ62778).

The phytase gene was optimized for expression in *B. subtilis* through OPTIMIZER software (http://gnemos.urv.es/OPTIMIZER). In addition, the secretion signal of a levansucrase (SacB; GenBank accession number CAA26513) from *B. subtilis* was added to the 5′-region of the phytase gene to produce a fusion protein to facilitate secretion. The *B. subtilis* strain transformed with the replicative pJJ5 plasmid was named BsJJ5. Spectinomycin resistance was used to evaluate the stability of the pJJ5 plasmid.

For the genomic edition of *B. subtilis*, the plasmid pJOE9620 (kindly provided by Dr. Josef Altenbuchner, University of Stuttgart, Germany) was used as a basis for cloning. This plasmid contains the GFPmut1 gene driven by the xylose-inducible promoter (Pxyl) in order to label the transformed *B. subtilis* strain. It also contains the Cas9 gene under the control of mannose-inducible promoter PmanP and sgRNAs for integration between the *yjaZ* and *trpS* genes. This genome region is rich in oligopeptide transporters genes, which are not essential for *B. subtilis* growth. The sgRNA was directed to the *appD* gene, coding for an

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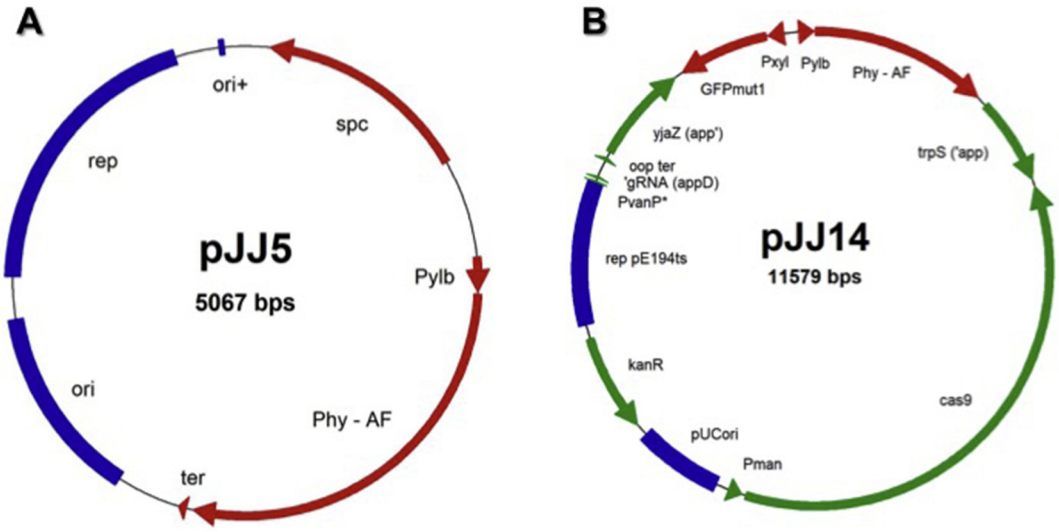

Fig. 1. Plasmids used for *B. subtilis* transformation. (A) Replicative plasmid pJJ5. (B) Integrative plasmid pJJ14.

oligopeptide transporter. The same genetic construct used to express phytase in pJJ5 plasmid was cloned into the *Sfi*I site of plasmid pJOE9620. The resulting plasmid was named pJJ14 (Fig. 1B), pJJ14 is composed of the promoter Pxyl what is activated by xylose; GFPmut1: gene coding for green fluorescent protein; Pman: promoter activated by mannose; gRNA: RNA guide; YjaZ: gene used for homologous recombination. The *B. subtilis* strain transformed with the integrative pJJ14 plasmid was named BsJJ14, which also carries a chloramphenicol resistance gene integrated by CRISPR-Cas9 technology as well (data not shown). Chloramphenicol resistance was used to evaluate the stability of the integrated genetic construct.

The strain *B. subtilis* KM0 was transformed with pJJ5 or pJJ14 plasmid according to the standard two-step procedure with minimal Spizizen medium (Anagnostopoulos and Spizizen, 1961). Transformed colonies were selected in solid LB (Luria Bertani) medium containing spectinomycin (100 µg/mL) or chloramphenicol (10 µg/mL) after overnight incubation at 37 °C. Bacterial growth was evaluated by optical density (OD600) spectrophotometer (BioMate3, ThermoScientific, USA). A single colony of each strain was individually inoculated into 10 mL LB medium and grown overnight, shaking at 250 rpm at 37 °C. Thereafter, the growth was diluted to an optical density OD600 = 0.05 nm, and grown under the conditions cited above. Periodically, a 1 mL aliquot was used to measure optical density. The stability of the genetic constructs present in the BsJJ5 and BsJJ14 strains was determined according to De Gelder et al. (2007), with modifications. A pre-inoculum was incubated in LB medium for 16 h, shaken at 250 rpm at 37 °C, with the selection antibiotic (spectinomycin or chloramphenicol). After reaching the stationary growth stage, the growth was centrifuged at 8000 ×*g* for 10 min at 4 °C. For removal of the antibiotic, the plate was washed with sterile saline (0.85% NaCl). Then, an aliquot was diluted to an optical density (OD600) = 0.05 nm and growth in non-selective LB medium until the growth reached a (OD600) = 0.1 nm. This procedure was repeated 13 times, and in each stage aliquots of the growth were plated in medium with or without antibiotic. Stability was determined by the ratio between the colony forming units (CFU) of the selective and non-selective plaques.

After about 65 generations, a single colony of each strain was inoculated into 5 ml of selective LB medium at 37 °C, and grown overnight. Subsequently, growths were centrifuged at 8000 ×*g* for 10 min at 4 °C for removal of the antibiotic and the plates were washed with sterile saline (0.85% NaCl). Optical density (OD600) was measured, the growth was diluted in 10 mL of non-selective LB medium and new growth was established with initial OD600 = 0.05 nm. The growth was continued for 16 h for the growth to reach the stationary phase and activate the Pylb promoter for phytase expression. After, the growth was centrifuged at 8000 ×*g* for 30 min at 4 °C. The supernatant was collected for the determination of phytase activity using the method of Heinonen and Lahti (1981), with slight adaptations for assays in 96-well plates. The activity was determined in the supernatant having 1 M sodium phytate (C6H6O24P6Na12; Sigma-Aldrich) as a substrate buffered with 2 M sodium acetate (pH 5.0). The reaction occurred for 30 min at 40 °C and determined by the addition of the colour reaction (AAM) prepared with acetone, 5 M sulfuric acid and 10 mM ammonium molybdate (2:1:1, *v*/v). After 30 s, the adding of 1 M citric acid quenched the reaction. The released inorganic orthophosphate was measured. The assay was performed in five-fold. Absorbance was read at 405 nm. One unit of phytase activity was defined as the amount of enzyme needed to release 1 µmol of phosphate per minute under the assay conditions.

The genetic manipulation method used did not affect the growth of both BsJJ5 and BsJJ14 strains (Fig. 2A). However, the stability test of the genetic construct over the generations shown in Fig. 2B pointed to a significant loss of the antibiotic resistance characteristic in the strain transformed with the replicative plasmid (BsJJ5). In this case, only about 11% of the cells maintained resistance to spectinomycin at the end of the 65 generations. Differently, the strain BsJJ14 remained without loss of the resistance phenotype throughout the experiment. Finally, Fig. 2C shows that phytase activity was even higher in the BsJJ14 strain than in the BsJJ5 strain at the end of the 65 generations. This about 20% of difference shows that the replicative plasmid, although present in a less number of cells, still manages to produce a reasonable amount of enzyme probably due to the high copy number maintained in each cell. However, loss of the plasmid has important implications that concern not only the short short-life of the strain from a commercial point of view, but also the possibility of horizontal transfer. Sharing characteristics such as resistance to antibiotics with other bacteria can generate multi-resistant strains with unpredictable consequences for organisms and the environment. In conclusion, the use of integrative plasmid via CRISPR-Cas9 technology seems to be a viable alternative for the expression of recombinant enzymes in *B. subtilis*, generating stable strains and with low risk of horizontal transfer of the manipulated characteristics.

Declaration of Competing Interest

The authors declare no conflict of interest.

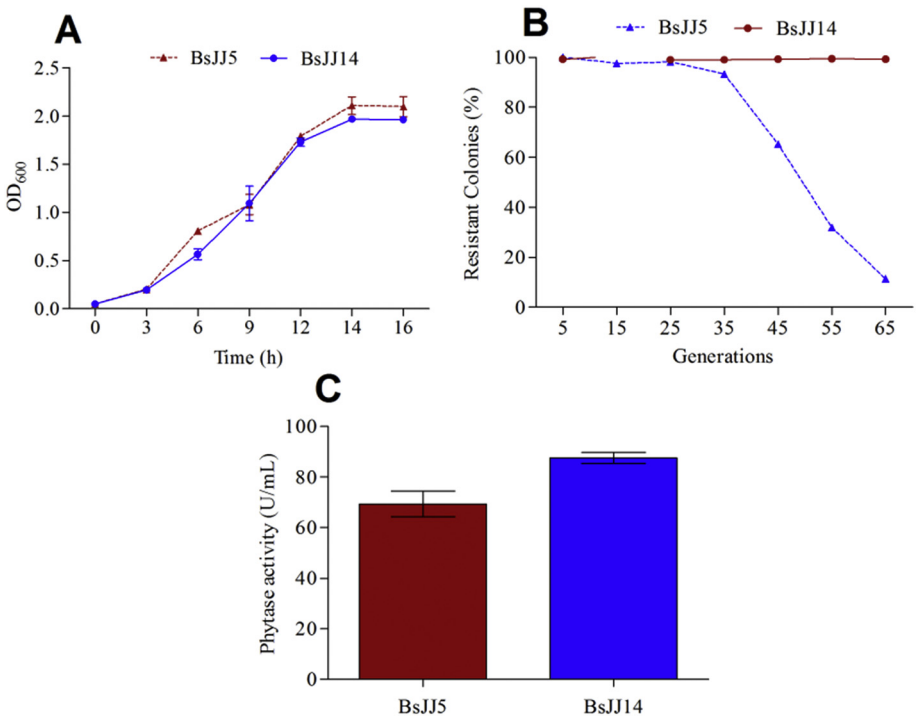

Fig. 2. Transformation of *Bacillus subtilis* with plasmid replicative plasmid (strain BsJJ5) and integrative plasmid (strain BsJJ14). (A) Growth curve of *B. subtilis* (B) Stability analysis of *B. subtilis* strains grown for 65 generations. (C) Analysis of phytase activity at the end of 65 generations in BsJJ5 and BsJJ14 strains. Data in (A) and (C) are presented as average ± standard error from three independent replicates (coming from different growth cultures).

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