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Crystal protein synthesis is dependent on early sporulation gene expression in *Bacillus sphaericus*

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Abstract

Insertional mutations in the *spo0A* and *spoIIAC* genes of *Bacillus sphaericus* 2362 were prepared by conjugation with *Escherichia coli* using a suicide plasmid containing cloned portions of the target genes. The mutants resembled their *Bacillus subtilis* counterparts phenotypically and were devoid of crystal proteins as determined by electron microscopy, SDS–PAGE and Western blots. The mutants had greatly reduced toxicity to anopheline mosquito larvae compared to the parental strain. We conclude that crystal protein synthesis in this bacterium is dependent on expression of early sporulation genes.

Keywords: *Bacillus sphaericus*; Insecticidal toxin; Asporogenic mutants

1. Introduction

Some strains of *Bacillus sphaericus* produce a protein parasporal body, which is highly toxic if eaten by susceptible mosquito larvae such as those of certain *Anopheles* and *Culex* species. The crystal comprises equimolar amounts of two proteins of 41.9 and 51.3 kDa (BinA and BinB, respectively) that share no homology with the more extensively studied insecticidal crystals (Cry proteins) of *Bacillus thuringiensis* [1,2]. The two *bin* genes are located on the chromosome and transcribed as a bicistronic mRNA although the promoter has not been defined precisely [3].

Synthesis of the binary toxin in *B. sphaericus* is an early event during sporulation. Bin proteins, crystals and toxicity to insect larvae are first observed immediately following septum formation around the start of stage III [4,5] and the crystal is fully formed by stage V. Fusion of the *bin* promoter region to a β-galactosidase reporter gene revealed expression starting at the end of exponential growth (t_0) and continuing into stationary phase for about 10 h (t_{10}) [6]. Asporogenic mutants of *B. sphaericus* 2297 do not possess crystalline inclusions, lack the Bin proteins and are poorly toxic to *Culex pipiens* larvae [7]. Moreover, asporogenic mutants of strain 2362 derived from chemostat culture were found to be 100-fold less toxic to larvae than wild type strains [8].

In the most extensively studied crystalliferous bacterium, *B. thuringiensis*, two different transcriptional mechanisms are responsible for *cry* gene expression in

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stationary phase cells. Transcription of *cry1Aa* and most other *cry* genes is dependent on RNA polymerase associated with the sporulation-specific sigma factors σ^{35} and σ^{28} that are homologous to σ^{E} and σ^{K} of *B. subtilis*, and does not occur in early-blocked sporulation mutants [9–11]. Expression of the *cry3A* gene, on the other hand, is independent of sporulation. This gene is weakly active during the vegetative stage, activated at the end of exponential growth and transcribed from t_0 until t_{10} from a vegetative (σ^{A}) promoter [10,12]. Indeed, *cry3A* expression is increased in an asporogenous *spo0A* host [13].

In this study, we have prepared for the first time directed *spo* mutations in *B. sphaericus* and show that control of *bin* expression is similar to that observed for *cry1Aa* in *B. thuringiensis*, in that it is dependent on early sporulation genes, in particular *spo0A* and *spoIIAC*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

B. sphaericus ASB 13269, a restriction-deficient derivative of the highly toxic strain 2362 [14] was used for construction of the mutants. The wild type strain 2362 and the low toxicity strain *B. sphaericus* SSII-1 were from our collection [15]. *Escherichia coli* TOP10F′ (Invitrogen) was used as a host for general cloning procedures and strain CA448 was used as a donor in conjugation experiments. The latter is a derivative of HB101 containing R702, the helper plasmid for conjugation. *B. sphaericus* strains were grown in NYSM broth and agar [16], which comprises nutrient broth supplemented with yeast extract (0.05%) and salts (50 μM $MnCl_2$, 0.7 mM $CaCl_2$ and 1 mM $MgCl_2$), and *E. coli* strains in Luria–Bertani (LB) broth and agar. Strains were grown at 37 °C unless stated otherwise.

2.2. Cloning of internal regions of the spo0A and spoIIAC genes

Chromosomal DNA from *B. sphaericus* 2362 was prepared by phenol extraction [15] for use as template in PCR. Parts of the *spo0A* and *spoIIAC* genes were amplified using two pairs of primers; Spo0F2 (5′-TATGCCTCATCTAGATGG-3′; bases 174–191 relative to the start codon) and Spo0R2 (5′-AACTTCAATGGCATGACG-3′; bases 637–654) derived from the *spo0A* sequence of *B. sphaericus* ATCC 14577 [17] were used to amplify *spo0A* and Spo2F2 (5′-CAACGAGAGGTGTTGAGC-3′; bases 152–169) and Spo2R3 (5′-CGGTTCAAGCCTTCTAAGCAC-3′; bases 589–609) targeted to the *spoIIAC* gene of strain 2362 [18] were used to amplify *spoIIAC*. PCR amplifications were routinely carried out in 100 μl reaction volumes using 100–500 ng template DNA, 100 pmol of each primer, 200 μM of each dNTP, 2.5 mM $MgCl_2$, *Taq* polymerase buffer, 2 U of *Taq* DNA polymerase (Promega) and sterile Millipore water. The amplification programs were 30 cycles of denaturation at 95 °C, 1 min; annealing at 47 °C, 1 min (for *spo0A*) or 51 °C, 1 min (for *spoIIAC*); and extension at 72 °C, 3 min followed by a final extension at 72 °C, 10 min. PCR products were cloned in pCR2.1 using the original TA cloning kit (Invitrogen) according to the manufacturer's instructions and the identity of the cloned fragments was confirmed by DNA sequencing.

Recombinant pCR2.1 containing the relevant inserts was digested with *Eco*RI for 4 h and electrophoresed in 3% NuSieve GTG agarose (FMC). The gel bands containing inserts were excised and ligated into *Eco*RI-digested, dephosphorylated (calf intestinal alkaline phosphatase) pMTL30, the *E. coli* cloning vector pMTL20 (Ap^R Em^R) containing the *oriT* region of RK2 for mobilization [19]. Recombinants were transformed into *E. coli* CA448 by electroporation using a Gene Pulser (Bio-Rad) set at 2.5 kV, and 25 μF with a pulse controller set at 200 Ω. Plasmids isolated from colonies obtained on LB plates containing 100 μg ml^{-1} ampicillin and 50 μg ml^{-1} kanamycin were purified and characterized by restriction enzyme digestion and electrophoresis. Two plasmids named pMTL30Spo0A and pMTL30SpoIIA were used for strain construction.

2.3. Conjugation between E. coli and B. sphaericus

Transfer of pMTL30Spo0A and pMTL30SpoIIA from *E. coli* CA448 to *B. sphaericus* ASB 13269 was achieved by filter mating at 30 °C [20]. In brief, the *E. coli* donor strain was grown to stationary phase in LB broth supplemented with ampicillin (100 μg ml^{-1}) and kanamycin (50 μg ml^{-1}) to select for the pMTL30 derivative and the R702 helper plasmid, respectively. The recipient *B. sphaericus* culture was grown in NYSM to the stationary phase. Donor and recipient cells were recovered by centrifugation at 4000*g* for 10 min, washed twice with holding buffer (25 mM potassium phosphate buffer, pH 7 and 1 mM $MgSO_4$) and resuspended in the same volume of holding buffer. Donor and recipient cells were mixed in a 10:1 ratio and 5 ml of the suspension passed through a 0.45 μm nitrocellulose membrane. The filters were transferred to the surface of Nutrient Agar plates and incubated for 15 h at 37 °C. Cells were washed from the membrane by vortexing in 1 ml NYSM. T4 phage (10^{10} pfu ml^{-1}) was added to counterselect the donor, and incubated for 2 h at 37 °C. Aliquots were then plated on NYSM plates containing 5 μg ml^{-1} erythromycin.