Characterization and Disruption of Exonuclease Genes from *Streptomyces aureofaciens* B96 and *S. coelicolor* A3(2)

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**ABSTRACT.** *Streptomyces aureofaciens* B96 produces several intra- and extracellular enzymes with deoxy-ribonuclease activity. According to the sequence of the previously published gene *exoSc* from *S. coelicolor* A3(2), the DNA sequence from *S. aureofaciens* B96 was amplified, cloned and expressed in *E. coli*. The protein product of *exoSa* gene, recExoSa, was also an exonuclease with DNAase and 5´-phosphomonoesterase activities at optimum temperature 37 °C and pH 8.0. It degraded only linear DNA (chromosomal, double-stranded and single-stranded) and linear plasmid DNA from both ends, with a preference to blunt ends in comparison with overhang ends. The purified enzyme exhibited no RNAase activity. Both *exoSc* and *exoSa* genes were interrupted by the apramycin resistance gene; constructed fragments were transformed into particular streptomyces protoplasts. Mutation caused by *exoSa* disruption in *S. aureofaciens* chromosome and mutation by interrupted *exoSc* in *S. coelicolor* were lethal.

**Abbreviations**

ccc covalently closed circular MM minimal medium ss single-stranded

ds double-stranded mMM modified MM

Streptomyces are the most widely studied and well known genus of the actinomycete family. They usually inhabit soil and are important decomposers. These microorganisms are used to produce the majority of antibiotics applied in human and veterinary medicine and agriculture, as well as anti-parasitic agents, herbicides, pharmacologically active metabolites (e.g., immuno-suppressants) and several enzymes important in the food and other industries (Kitani et al. 2008).

The putative occurrence of an autolytic phenomenon in the nonsporulating aerial mycelium of *S. coelicolor* was reported by Wildermuth (1970). The author described such lysis as a dynamic process and reported the presence of membrane segments outside the cell wall. This interesting observation was confirmed by several authors (Migüélez et al. 1999, 2000; Nicieza et al. 1999; Fernández and Sanchez 2002; Manteca et al. 2006) who revealed the existence of two death rounds in streptomyces. The first round takes place very soon after spore germination, affecting young substrate mycelium. The second round in the case of *S. antibioticus* starts at ≈30 h and affects the aerial mycelium. The accompanying event in the processes of such hyphal death was the nucleoid degradation, together with the alteration of cytoplasmic membrane and massive destruction of the cell wall. It was also demonstrated that nucleic acid degradation was indeed an early event in the cell dismantling and accompanied both death rounds in *S. antibioticus*. Furthermore, numerous nucleases of different molecular mass (18–44 kDa) and lacking sequence specificity were shown to be involved in these processes (Cal et al. 1995; Nicieza et al. 1999; Fernández and Sanchez 2002; Manteca et al. 2006).

Similarly to other streptomyces, *S. aureofaciens* B96 produces under submerged conditions several intra- and extracellular nucleases (Brnáková et al. 2005). According to the fact that nucleases could be one of the cell-death-related proteins and are involved in the recycling of nucleic acids from substrate mycelium, the first step of programmed cell-death studies should be characterization of their genes and protein products. Moreover, it is very labor- and time-consuming to characterize and separate nucleases directly from streptomyces. In vitro cloning, expression and biochemical characterization in heterologous systems is easier and, at present, it precedes molecular-biological characterization of individual genes in their natural hosts. There have been a few reports about the presence of nucleases in *S. antibioticus*, *S. glaucescens* (Nicieza et al. 1999; Manteca et al. 2006) and *S. thermonitrificans* (Patil et al. 2005) and their biochemical characterization. However, scarce information is available on the function of nucleases in the biology of these microorganisms, their
roles having been only predicted. Recently, the cloning and partial characterization of *S. coelicolor* A3(2) exonuclease recExoSc (Brnáková et al. 2007) was published; here, its homologue from *S. aureofaciens* B96, exonuclease exoSa, was amplified. The main aim of this work was cloning of the exoSa gene, its expression in *E. coli*, characterization of its protein product and inactivation of exoSa and exoSc genes in their natural hosts.

**MATERIALS AND METHODS**

**Strains, plasmids and culture conditions.** Strains used were *Streptomyces aureofaciens* B96 and *S. coelicolor* A3(2) (Collection of Microorganisms, Institute of Molecular Biology, Slovak Academy of Sciences), *Escherichia coli* NovaBlue [E. coli K-12 endA1 hsdR17(rK12− mK12+) supE44 thi-1 recA1 gyrA96 relA1 lac F’[proA+ B+ lacB+ Z+ mlbK15::Tn10 (TetR)] (Novagen) and *E. coli* BL21(DE3) [E. coli B834 F− ompT hsdSB(rB− mB+) gal dcm (DE3)] (Novagen) (Studier and Moffat 1986). *E. coli* ET12567 (dam− dcem− hsdM−) (MacNeil et al. 1992) was used for preparation of nonmethylated plasmid DNA. Conditions for growth, transformation of competent *E. coli* (Novagen) cells and expression of target exoSa gene were set according to Novagen’s pET System Manual. Plasmids pETBlue-2 and pET-21a(+) (Novagen) were used for *E. coli* cloning and expression experiments. Plasmid pJ6021 (Kieser et al. 2000) was used for expression in streptomycyes and promoter-probe shuttle vector pKJ2 (Nazarov et al. 1990) was used as a control for transformation experiments.

Manipulations with streptomycetes strains were done according to Kieser et al. (2000). *S. aureofaciens* B96 was cultivated (after inoculation with ½ of total volume of overnight culture at 200 rpm for 2 d at 30 °C) in liquid TSBG (TSB (Oxoid) + 0.7 % glycine) medium for the DNA isolation and GPY (Brnáková et al. 2005) medium for isolation of extracellular proteins with nucleolytic activity. Growth of *S. aureofaciens*, protoplast preparations and their transformation were done according to Kieser et al. (2000) with a few modifications: 0.64 % glycine in YEME, 2 mg/mL of lysozyme in buffer, and 3-h incubation with lysozyme. Growth of *S. coelicolor* A3(2), protoplast preparation and their transformation were done according to Kieser et al. (2000).

Solid media, sporulation agar, MM, medium 16 (in g/L: agar 25, dextrine 15, peptone 5, sucrose 3, beef extract 1, yeast extract 1; in mg/L: NaCl 50, K2HPO4 50, MgSO4, pH 7.2, urea 10), and mM + sucrose (mM + 103 g/L sucrose), mMMS (mM + 103 g/L sucrose), R2YE and R5 were used for regeneration of protoplasts. Plates were overlaid with soft nutrient agar with particular antibiotics.

**DNA manipulations in *E. coli*** were done according to Sambrook et al. (1989). Isolation of chromosomal DNA (by salting-out procedure) and further DNA manipulations in *Streptomyces* were done according to Kieser et al. (2000).

**Cloning and overexpression of recombinant recExoSa.** An approximately 0.7 kb DNA fragment was amplified by PCR using *S. aureofaciens* B96 genomic DNA as template and oligonucleotide primers NucF and NucR containing Ndel and XhoI restriction sites at the 5’- and 3’-ends, respectively, for subsequent cloning. Primers were annealed to the chromosomal DNA for 1 min at 55 °C and extended for 2 min at 72 °C. Thirty cycles of PCR were performed with DyNAzyme™ DNA polymerase (Finzymes). The amplified fragment was purified following agarose gel electrophoresis and cloned directly into pETBlue-2, digested with EcoRV, with Novagen’s Perfectly Blunt® cloning kit following manufacturer’s instructions. The pETBlue_exoSa plasmid carrying the particular gene was used for the sequence examination. The 0.7 kb DNA fragment (exoSa, without stop codon), encoding *S. aureofaciens* recExoSa recombinant protein, was amplified by PCR from *S. aureofaciens* genomic DNA under the afore-mentioned conditions with primers NucF and newly designed NucSaR with XhoI restriction site. Consequently, the amplified fragment was inserted into pET-21a(+) replacing its Ndel–XhoI sequence. The pET21a_exoSa plasmid was introduced into *E. coli* BL21(DE3). These cells were grown in liquid Luria–Bertani medium containing 100 µg/mL of ampicillin (LBA) at 200 rpm and 37 °C until *A*600 reached 0.5. Then they were induced with 1 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG) and the cell culture was further incubated (200 rpm, 3 h, 30 °C).

Primers used:

NucF 3’-GGA ATT CCA TAT CCT CAC CGT GAC CTC CGT CAA CGT C-3’

NucR 3’-CCG CTC GAG GCC GTC GTA GAC CAC CGT GAC CGG-3’

NucSaR 3’-CCG CTC GAG CCG TCG TAG ACC ACC GTC ACC GGG-3’

**Construction of vectors for the exoSa and exoSc inactivation.** The gene disruption constructs were prepared by fusion PCR (Kuwayama et al. 2000). Two DNA fragments of each of the nuclease genes, up-