Abstract  Previously, it was shown that inactivation of the tricarboxylic acid cycle aconitase gene acnA impairs the morphological and physiological differentiation of Streptomyces viridochromogenes Tü494, which produces the herbicide phosphinothricin tripeptide (PTT). In order to further characterize the role of the aconitase in the Streptomyces life cycle, aconitase activity was analyzed during growth of S. viridochromogenes in liquid culture. Two prominent maxima were measured in cell-free crude extracts. The first maximum was found at an early stage of growth, which is correlated with a decrease in pH when rapid glucose consumption is initiated. The second, lower maximum was detected at the beginning of the expression of the PTT-specific biosynthetic gene phsA, implying the onset of secondary metabolism. These results were confirmed by examining transcription of the acnA promoter in time-course experiments. The highest transcription rate was found during the early growth phases. In order to identify putative regulatory mechanisms, the transcriptional start site of the acnA transcript and subsequently the promoter were identified. Several putative, regulatory protein binding sites (e.g. regulators of oxygen stress or iron metabolism) were detected in the promoter region of acnA, which suggested complex regulation of acnA.

Keywords  Aconitase regulation · Streptomyces viridochromogenes · Phosphinothricin tripeptide

Introduction

Members of the genus Streptomyces are Gram-positive soil bacteria that are characterized by a complex life cycle. Beginning with an arthrospore, they first form an interconnected dense mycelium within the substrate. Following changes in the environmental conditions, e.g. nutrient depletion, aerial mycelium and subsequently arthrospore formation begin. In addition to the morphological differentiation, physiological differentiation also takes place. During this secondary metabolism, several interesting metabolites are produced, e.g. alkaloids, glycosides and other bioactive compounds. The most interesting group of secondary metabolites is the antibiotics. In streptomycetes, antibiotic production generally occurs in a growth-phase-dependent and developmentally coordinated manner, and is subjected to pathway-specific and pleiotropic control. Several regulatory proteins, e.g. AbsA (Aceti et al. 1998; Anderson et al. 2001) and AfsR (Floriano et al. 1996) in Streptomyces coelicolor, as well as ppGpp (Hesketh et al. 2001), phosphate (Rebello et al. 1989), cAMP (Süssstrunk et al. 1998; Kang et al. 1999) and glucose concentrations (Kim et al. 2001) are involved in these pleiotropic effects. Investigations in Streptomyces viridochromogenes (Schwartz et al. 1999), S. coelicolor (Viollier et al. 2001a, b) and Streptomyces noursei (Jonsbu et al. 2001) have shown that the enzymes of the tricarboxylic acid (TCA) cycle, especially aconitase, also pleiotropically affect the production of antibiotics during secondary metabolism.

In addition to the major TCA cycle aconitase (AcnA) (Schwartz et al. 1999), an aconitase-like protein (Pmi) that is involved in the synthesis of the antibiotic phosphinothricin tripeptide (PTT) has been identified in S. viridochromogenes (Heinzellmann et al. 2001). Together with other PTT biosynthetic gene products, such as the peptide synthetase PhsA, which participates in non-ribosomal synthesis of the antibiotic (Schwartz et al. 1996; Grammel et al. 1998), Pmi represents a secondary-metabolite-specific protein. Biochemical and genetic analysis re-
revealed that Pm1 is unable to catalyze the TCA cycle reaction (Heinzelmann et al. 2001). Furthermore, a minor aconitase activity has been identified in an acnA/pmi double mutant (Schwartz et al. 1999). It is not known whether the activity is due to a second TCA cycle aconitase or to an unrelated hydratase-dehydratase having a broad substrate specificity that includes citrate (Schwartz et al. 1999). A TCA cycle aconitase (AcnA) mutant is unable to develop aerial mycelium and to sporulate, resulting in a bald phenotype. An aconitase mutant of S. coelicolor is characterized by similar defects in differentiation (Viollier et al. 2001b). The authors speculated that this is partially due to medium acidification and citrate accumulation. Furthermore, the S. viridochromogenes mutant does not produce PTT, demonstrating that AcnA also affects physiological differentiation (Schwartz et al. 1999).

For a better understanding of the role of aconitase in the Streptomyces life cycle, the activity of the TCA cycle aconitase and transcription of the corresponding gene during the life cycle of the PTT producer Streptomyces viridochromogenes were examined.

**Material and methods**

**Bacterial strains and growth conditions**

S. viridochromogenes Tü494 was cultivated in yeast malt medium (YM) (Schwartz et al. 1996) in a 10-l Biostat S bioreactor (Braun, Melsungen, Germany) at 37 °C, 300 rpm and an aeration rate of 0.5 vvm. The reactor was inoculated with 500 ml preculture (two 1-l Erlenmeyer flasks with 250 ml YM medium inoculated with 3×10⁸ spores and incubated for 48 h at 30 °C). The pH and partial pressure of oxygen (pO₂) values were measured online. At several time points (6.5–94.5 h), samples were taken. Cells and supernatant were separated by centrifugation (10 min, 5000 × g, 4 °C) and frozen at –20 °C until testing of the samples.

**Determination of the glucose concentration**

The glucose concentration in the supernatant was determined by HPLC using a Capcell SG80 amino column (particle size 5 µm, 125x4.6 mm; precolumn 20x4.6 mm) (GROM, Herrenberg, Germany). The eluent was a 3:1 mixture of acetonitrile/water and the flow rate was 1 ml/min. The calibration was between 0.1 and 10 mg D-glucose /ml. For this experiment, an isocratic high pressure pump (HP 1050), a refractometer detector (HP 1037A) (tempered at 40 °C) and a 3396A integrator (all instruments supplied by Hewlett Packard, Waldbronn, Germany) were used.

**Determination of dry weight**

The sample volume was determined by weighing. The supernatant was removed by centrifugation and the dry weight of the cell pellet was determined after 2 days at 100 °C.

**Determination of the PTT concentration in the supernatant**

PTT concentration in the fermentation broth was determined in a biological assay. Supernatant (10 µl) was spotted onto paper disks (6 mm, Macherey and Nagel, Düren, Germany). The disks were placed on plates with Bacillus subtilis as indicator organism. After overnight incubation at 37 °C, the sizes of the inhibition zones were determined. Calibration was carried out using PTT solutions with known concentrations (0.01 – 1.00 mg/l).

**SDS PAGE and Western blotting**

SDS PAGE and Western blotting experiments using anti-PhsA antibodies were carried out as described by Schwartz et al. (1996).

**Determination of aconitase activity**

Cell pellets of the bioreactor samples were washed once with 10 ml washing buffer (20 mM Tris/HCl, pH 8.0) and then resuspended in 4 ml ice-cold lysis buffer (20 mM Tris/HCl, pH 8.0, 5 mM dithiothreitol). Mycelium was broken down by ultrasonic treatment in an ice/ethanol bath as described previously (Bruntner et al. 1998). Cell-free crude extracts were obtained by centrifugation for 30 min at 20,000×g and 4 °C. Aliquots were removed for protein estimation by the Bradford method and for immunoblotting experiments. Aconitase activity was assayed spectrophotometrically at 240 nm after the conversion of citrate to isocitrate, and the specific activity was calculated using an absorption coefficient of 3.6 mM⁻¹cm⁻¹ (Kennedy et al. 1983). One unit (U) of enzyme activity converted 1 nmol of substrate per minute; cellular activities are expressed as U·mg⁻¹.

**RNA preparation**

To investigate transcription of the acnA region, S. viridochromogenes was grown in sucrose-containing yeast extract/malt extract (YEME) medium (Kieser et al. 2000) inoculated with 1 ml precul- ture (500-ml Erlenmeyer flasks with 200 ml YM medium inoculated with 9×10⁸ spores and incubated at 30 °C). At several times (24 h, 48 h, 72 h, 96 h), 20-ml samples were taken. Cells were harvested by centrifugation (5 min, 5,000×g) and washed with 5 ml L1 buffer (25 mM Tris/HCl, 25 mM EDTA, 300 mM sucrose, pH 8.0). Total RNA was isolated using the peqGold RNAPure kit (Peqlab, Erlangen, Germany) as recommended by the manufacturer.

**Primer extension**

The transcriptional start point(s) of the acnA transcript was mapped by primer-extension as described by Tesch et al. (1996) and using the synthetic oligonucleotide Pex2 (5′-GCTGCGGCG-GTCAAGCTTGGCAGC-3′), which covers positions 1–30 (relative to the translational start site) in the complementary strand of the acnA gene. Reverse transcripts were separated on 5% polyacrylamide-urea gels in parallel with dideoxy sequencing reactions.

**DNA nucleotide sequencing**

The nucleotide sequence was determined by the dideoxy chain-termination method using the T7 sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden), deaza-dGTP reaction mixture and pSK27 (pUC18 carrying a 2.7-kb SacI DNA fragment containing a part of acnA including the promoter region) as a template with the 32P-labeled acnA-specific primer Pex2.

**Regulatory binding sites**

The consensus sequences of the regulatory binding sites identified in the promoter region of acnA have been published by Robison et al. (1998), Hahn et al. (2000) and Günter et al. (1993). The program DNASSIS (Amersham Pharmacia Biotech) was used for sequence alignments and identification of inverted/direct repeats.