Univerzita Karlova

1. lékařská fakulta

Autoreferát disertační práce

UNIVERZITA KARLOVA
1. lékařská fakulta

Analýza a mapování vazebných míst regulátorů genové exprese u streptomycet

Analysis and mapping of binding sites of gene expression regulators in the genus of *Streptomyces*

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2020

Doktorské studijní programy v biomedicíně

Univerzita Karlova a Akademie věd České republiky

Obor: Mikrobiologie

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Školicí pracoviště: Ústav imunologie a mikrobiologie, 1. LF UK a VFN v Praze ; Mikrobiologický ústav akademie věd ČR, Praha

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

Abstrakt (CZ)

Streptomycety jsou lékařsky důležité bakterie žijící v půdě, které podléhají morfologickým změnám od spór po vzdušné hyfy a jsou důležitými producenti bioaktivních látek včetně antibiotik. Jejich genová exprese je přísně regulována v časných úrovních transkripce a translace. Během řízení transkripce hrají sigma faktory ústřední roli; modelový organismus *Streptomyces coelicolor* má ohromujících 65 sigma faktorů. Exprese sigma faktorů samotných je řízena na post-transkripční úrovni působením malých asRNA molekul, které modifikují hladinu jejich messengerových RNA. Avšak pouze několik sigma faktorů ve streptomycetách má známé regulony a také jejich regulace prostřednictvím malých RNA molekul nebyla dosud studována.

V závislosti na předtím naměřených datech o genové expresi jsme vybrali několik vysoce exprimovaných sigma faktorů. Pomocí mutantních kmenů nesoucích sigma faktory značené HA tagem byly analyzovány regulony dvou důležitých sigma faktorů, SigQ a HrdB, pomocí techniky ChIP-seq. Další sigma faktory byly dále studovány pomocí metody 5 'a 3' RACE a Northern blottingu, aby se zjistilo, zda mají asRNA.

Naše data potvrzují nezbytnost sigma faktoru HrdB během vegetativní fáze růstu. Ukázalo se, že druhý sigma faktor, SigQ, je důležitým regulátorem metabolismu dusíku a regulátorem reakce na osmotický stres, probíhající během klíčení spór.

Objevili jsme také tři nové *cis*-asRNA sigma faktorů SigH, SigB a SigR; navíc se předpokládá, že poslední dva vytváří complex s RNázou III. Na základě těchto údajů lze navrhnout dráhu pro transkripční řízení asRNA-sigma faktor-regulon.

Klíčová slova: streptomycety, sigma faktory, *cis*-asRNA, mRNA, RACE, Northern blot, RNáza III

Abstract (EN)

Streptomyces are medically important soil-living bacteria that undergo morphological changes from spores to aerial hyphae and are important producers of bioactive compounds including antibiotics. Their gene expression is tightly regulated at the early level of transcription and translation. In the transcriptional control, sigma factors play a central role; the model organism *Streptomyces coelicolor* possesses astonishing 65 sigma factors. The expression of sigma factors themselves is controlled on the post-transcriptional level through the action of asRNAs that modify their mRNA level. However, only several sigma factors in *Streptomyces* have known regulons and also their sRNAs-mediated regulation has not been studied so far.

According to gene expression data, we selected several highly expressed sigma factors. Using HA-tagged mutant strains, regulons of two important sigma factors, SigQ and HrdB, were analyzed by ChIP-seq procedure. Other sigma factors were further studied to see if they possess asRNAs, using 5' and 3' RACE method and northern blotting.

Our data confirm the essentiality of HrdB sigma factor during the vegetative phase of growth. The other, SigQ, has been revealed to be an important regulator of nitrogen metabolism and osmotic stress response coinciding germination.

We also uncovered three novel *cis*-asRNAs corresponding to sigma factors SigH, SigB, and SigR; moreover, the last two are thought to mediate a complex formation with RNase III. Based on these data asRNA-sigma factor-regulon transcriptional control pathway can be suggested.

Key words: *Streptomyces*, sigma factors, *cis*-asRNAs, mRNA, RACE, northern blot, RNase III

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1 INTRODUCTION

Bacterial transcription is a process when DNA is transcribed to RNA. This process is ensured by RNA polymerase (RNAP) in which the most important component is sigma factor [1, 2]. Sigma factor is a subunit of RNA polymerase and its selective binding to the appropriate promoter initiates transcription. It recognizes promoter sequence, recruits RNA polymerase holoenzyme to the target promoter and ensures the unwinding of the DNA duplex near the transcription start site [3, 4]. The genome of the model organism *Streptomyces coelicolor* possesses astonishing 65 different sigma factors that govern its complex life cycle and help to adapt to variable environmental conditions [5]. So far, only about 20 sigma factors have been characterized in *Streptomyces coelicolor*. The common approach to studying the function of sigma factor is creating its deletion mutant and identifying which genes are affected. But this approach failed in the studying of the function of HrdB sigma factor in *Streptomyces*, because of the lethality of its deletion, so it was no longer examined [6]. HrdB is a principal and essential vegetative sigma factor in *Streptomyces* that is a functional homolog of σ^{70} in *E. coli* [7, 8] regulating the transcription of housekeeping genes. To overcome these obstacles, we developed epitope tagging mutagenesis applied for *Streptomyces* coupled with ChIP-seq in order to identify HrdB regulon. We employed this approach also for sigma factors SigQ, SigB, SigH, SigR, SigE, SigD and SCO1263 in order to clarify their function and identify their regulons in spore germination, since – according to the previously measured microarray data during this life stage – they were found to be highly expressed sigma factors and at that SigQ to be the most highly expressed among them [9, 10].

Sigma factors are regulated not only in the post-translational level through the acting of anti-sigma factors, 6S RNA, which binds σ^{70} of RNAP and inhibits transcription by competing with the promoter DNA [11, 12], or through the RbpA protein, which binds to HrdB and facilitates transcription from HrdB-dependent genes [13, 14] but it has been reported that sigma factors are also regulated by sRNAs [15-17]. These are for example sRNAs MicA, RybB, and SlrA (MicL) in *E. coli*, which are regulated by σ^{E} sigma factor in response to envelope stress and they simultaneously downregulate σ^{E} in a feedback mechanism [16, 17]. As we focused on studying sigma factors and their regulons, we wanted to know if the sigma factors in *Streptomyces coelicolor* are regulated by sRNAs as well. We selected 12 mRNAs of sigma

factors and subjected them to a potential asRNAs search using a combination of 5' and 3' RACE methods and Northern blot.

1.1 Sigma factors

Sigma factors play an important role in transcriptional regulation. The regulatory role of these proteins lies in selective promoter recognition and in coordinating transcription according to diverse stimuli such as stress, developmental stages, and changes in outer environment.

Sigma factors were first identified in 1969 as a protein stimulating transcription [1]. They possess three main functions: to recognize the promoter sequence, to recruit the RNA polymerase holoenzyme to the target promoter and to ensure the unwinding of the DNA duplex near the transcription start site [3, 4]. The core enzyme is capable of nonspecific binding to DNA and the initiation of RNA synthesis from DNA ends or nicks but the specific transcription from promoters is ensured by sigma factor [18]. RNA polymerase core enzyme must first interact with a σ subunit to form the holoenzyme [4]. Bacterial sigma factors have from 20 to greater than 70 kDa [19, 20].

My doctoral thesis focuses on sigma factors HrdB and SigQ.

1.1.1 HrdB

HrdB is a principal and essential sigma factor in *Streptomyces* responsible for the transcription of housekeeping genes whose deletion is lethal [6, 21]. It is the functional homolog of σ^{70} in *E. coli* [7, 8]. HrdB homologs are present in all *Streptomyces* species [22].

In *Streptomyces spp*., the principal sigma factor HrdB is involved not only in the transcription of housekeeping genes but also in morphological differentiation and secondary metabolism [23, 24]. HrdB in *S. coelicolor* affects secondary metabolism in two ways. First, it regulates the transcription of pathway-specific regulatory genes *actII-ORF4* and *redD* that control biosynthesis of antibiotics actinorhodin and undecylprodigiosin. Second, HrdB participates in the synthesis of precursors and energy through the regulation of primary metabolism genes and thereby connects the primary metabolism with secondary metabolism [23, 24]. Similarly, HrdB in *S. avermitilis* regulates the transcription of the pathway specific regulatory gene *aveR* to trigger the biosynthesis of avermectin [25]. The connection of HrdB with secondary metabolism was also confirmed experimentally. It was reported that the mutation in the 1.2 conserved region of HrdB resulted in antibiotic deficiency due to the reduced transcription of pathway-specific

regulators *actII-ORF4* and *redD* [26]. The presence of *hrdB* on a multicopy plasmid leads to the precocious overproduction of undecylprodigiosin [26].

1.1.2 SigQ

Not much known is about SigQ sigma factor. We know that SigQ is directly regulated by afsQ1/afsQ2 which is a two-component system and a pleiotropic regulator of antibiotic biosynthesis and morphological differentiation in a glutamate-based growth conditions [27]. Deletion of SigQ caused increased levels of actinorhodin, undecylprodigiosin and calcium-dependent antibiotic and led to a delayed formation of aerial mycelium in the glutamate-based minimal medium. These results suggest that SigQ together with afsQ1/afsQ2 contribute to the regulation of antibiotic biosynthesis [28]. It was shown that SigQ is highly expressed during spore germination [10]. Our hypothesis of its enormous expression during spore germination was that it negatively regulates pathway-specific regulators of antibiotic production ActII-ORF4, RedD, and CdaR during spore germination.

1.2 sRNAs

The other important regulators of gene expression are sRNAs. Bacterial small RNAs are widespread and functionally heterogeneous short RNA transcripts usually in the size of ~50 – 500 nucleotides [29]. They are commonly encoded in intergenic regions, within the genes or in the 5' or 3' untranslated regions (UTRs) [29-32] possessing own promoters which can be induced by stress conditions, environmental and developmental changes [33-35].

Bacterial sRNAs can be classified into two groups: ***Cis*-encoded sRNAs** and ***trans*-encoded sRNAs** [34]. *Cis*-encoded sRNAs are encoded in *cis* on the DNA strand opposite the target mRNA and share extended regions of complete complementarity with their target [36]. Therefore they are called *cis*-antisense sRNAs. They are highly structured (one to four stem-loops), mostly untranslated [37], and have been found first mainly in plasmids, phages and transposons [38]. Antisense RNA can bind 5'-end or 3'-end, the middle or the entire transcript encoded by the gene opposite to the asRNA gene. Base pairing between the asRNA and the target mRNA in one site can possibly influence another site of the mRNA. The transcription of the genes coded for asRNA and mRNA may be synchronized or may not [39].

Trans-encoded sRNAs are situated in another chromosomal location, usually far away from the target, and show only partial complementarity (7 – 12 nts) to their target mRNA [35, 40, 41].

Due to the limited complementarity, an Hfq chaperone is needed in several bacteria for the base pairing between sRNA and target mRNA [35, 42]. It has to facilitate RNA-RNA interactions between the sRNA and target mRNA [43, 44]. Although *trans*-encoded sRNAs in Gram-negative bacteria require chaperone Hfq for its action [44-48], it has never been reported in Gram-positive bacteria except for *Listeria monocytogenes* [49]. Hfq protein or its homologue has also never been identified in *Streptomyces* [50]. However, it was suggested that there might be another protein that fulfils the function of Hfq in Gram-positive bacteria such as CsrA in *Bacillus subtilis. Trans*-encoded sRNAs are responsible for the regulation of translation and/or for the stability of target mRNAs [43, 51]. Each single *trans*-encoded sRNA can typically base-pair with multiple mRNAs. The possibility for multiple base pairing interactions is enabled by more limited contacts of the given sRNAs with their target mRNAs [35, 51].

It is known that sigma factors create a regulatory feedback loop together with sRNAs to regulate stress responses in *E. coli* or in *Salmonella enterica* [15]. The regulation by sRNAs is achieved by reducing the translation of sigma factors, leading to the dampening of elevated stress responses or altered metabolic pathways [17]. Several sRNAs have been reported to regulate sigma factors. The best examples of such regulation are sRNAs MicA, RybB, and SlrA (MicL) in *E. coli*, which are regulated by σ^E sigma factor in response to envelope stress and they simultaneously downregulate σ^E in a feedback mechanism [16, 17]. Another stress related sigma factor σ^S is in *E. coli* positively regulated by sRNAs ArcZ, RprA [52], DsrA [53] and negatively regulated by SdsR and OxyS [15, 54]. In *Salmonella enterica*, sRNA SdsR regulates general stress sigma factor σ^S and simultaneously SdsR sRNA is contained in σ^S regulon [55].

sRNAs in Streptomyces were intensively studied. Several systematic approaches were carried out to identify globally novel sRNAs in *Streptomyces* [56-60]. Using deep sequencing method, bioinformatic approaches, and RNA sequencing, around 105 sRNAs have been identified. Some of them were experimentally verified by RT-PCR or Northern blots [56, 58, 60]. However, the function of the vast majority of these sRNAs is unknown.

2 HYPOTHESIS AND AIMS

The aim of this thesis was to analyse gene expression control in *S. coelicolor* provided on the transcriptional and post-transcriptional level by sigma factors and asRNAs, respectively.

The particular goals are as follows:

1. To create mutants with an epitope tag of those sigma factors that are highly expressed during spore germination (SigB, SigD, SigE, SigH, SigR, SigQ and SCO1263) and a principal sigma factor HrdB during vegetative growth phase (Publication 1, Manuscript 2)
2. To perform chromatin immunoprecipitation and next generation sequencing (ChIP-seq) to reveal HrdB/SigQ sigma factor-specific binding sites on DNA to identify their regulons (Publication 1, Manuscript 2)
3. To conduct kinetic modelling of the identified genes in HrdB/SigQ regulons to prove the possibility of regulatory influence (in collaboration with Laboratory of bioinformatics, Institute of Microbiology of the CAS (Publication 1, Manuscript 2)
4. To test, whether expression of the sigma factors in question include an antisense transcript-mediated control (Publication 3)
5. To characterize expression profile of the novel sRNAs by northern blot in wt and *rnc* strain (RNase III deletion strain) during three life stages of growth – substrate mycelium formation, aerial hyphae formation and sporulation (Publication 3)

3 MATERIALS AND METHODS

3.1 Methods used for identification of sigma regulons

Strains and growth conditions

Strains used in our work are *S. coelicolor* M145, *E. coli* K-12 MG1655 [61] and derivatives from GM2929 [62]. *E. coli* BW25113/pIJ790 has λ Red recombination system under the control of arabinose inducible promoter and this strain was used to propagate *S. coelicolor* cosmid. *E. coli* ET12567/pUZ8002 is methylation-deficient strain for intergeneric conjugation with *S. coelicolor*. For the preparation of the epitope tagged mutant strain, *S. coelicolor* was cultivated on solid agar plates with MS medium (2% (w/v) mannitol, 2% (w/v) soya flour, 2% (w/v) bacterial agar in tap water) or DNA medium (2,3% (w/v) Difco nutrient agar) [63]. Apramycin (50 ug/ml), chloramphenicol (25 ug/ml), kanamycin (50 ug/ml) or nalidixic acid (25 ug/ml) was added to the media when needed. The list of genetic material used is given in Table 1.

Epitope tagging mutagenesis

Primers containing the HA tag were chemically synthesized. The nucleic acid sequence of the HA tag (YPYDVPDYA) was optimized for the codon usage in *S. coelicolor* (TAC CCG TAC GAT GTG CCG GAT TAC GCG). A gene cassette containing FRT flanking regions, apramycin resistance marker and oriT was amplified from plasmid pIJ773 as described (50), cut by EcoRI and HindIII restriction enzymes and used as a PCR template. The PCR was performed to a final volume of 50 ul containing 2,5 U iProof DNA polymerase (Bio-Rad), 5 µl GC buffer, 2,5 µl 100% DMSO, 1 µl 10mM dNTPs (50 uM each), 0,5 µl template DNA (50 ng pIJ773 cut by EcoRI and HindIII, gel purified) and 0,5 ul each primer for mutagenesis (50 pmol each, SigQ_HAtag_left, SigQ_HAtag_right). PCR cycle conditions were 10 cycles with 45 sec denaturation at 98°C, 45 sec annealing at 50°C, 90 sec extension at 72°C, 15 cycles with 45 sec denaturation at 98°C, 45 sec annealing at 50°C, 90 sec extension at 72°C. PCR product was then gel purified.

Purified PCR product was then electroporated into *E. coli* BW25113/pIJ790 containing *S. coelicolor* cosmid 2StK8. The cells were then cultivated at 37°C for 1 hour in 1 ml LB. The culture was centrifuged 15 sec, 10.000 g and spread onto LB agar with apramycin (50 µg/ml). The cosmid with inserted cassette was then transformed into methylation-deficient *E. coli* ET12567/pUZ8002 and the resulting strain was conjugated with *S. coelicolor* M145 [64]. Final mutants were selected on MS medium containing apramycin, kanamycin and nalidixic acid [63]. Double cross-over exconjugants (kanamycin sensitive, apramycin resistant) were selected. Chromosomal DNA was then isolated and cassette integration into the chromosome was confirmed by PCR and sequencing.

ChIP-seq

The epitope-tagged streptomycete spores were grown in 80 ml AM medium at 37°C. After 30 min of cultivation, spores were crosslinked by adding formaldehyde (final 1%) at room temperature for 10 min. Then 125 mM glycine was added and culture was incubated at room temperature for other 5 min. Spores were centrifuged at 6000 g, 5 min at 4°C, washed three times with 50 ml ice cold phosphatebuffered saline (pH 7,4) and freezed in liquid nitrogen. The washed spores were resuspended in 2 ml lysis buffer (50 mM Tris-HCl, pH8; 150 mM sodium chloride, 1% Triton X100, 0,5% sodium deoxycholate, 0,1% SDS, 1mM PMSF, protease inhibitors (Roche, 4ul/ml) with zirconium beads (1g/1.5ml tube) and disrupted in FastPrep-24 machine (Biomedicals) at 4°C, speed 5,5, 12x45 sec. The lysate was then centrifuged at 14000g for 10 min in 4°C. 20 ul protein A/G PLUS-agarose (Santa Cruz) was preequilibrated in RIPA buffer (Santa Cruz) 10 ul (2 ug) of mouse monoclonal anti-HA antibody (clone F-7, Santa Cruz) was added to the pre-equilibrated Protein A/G PLUS-agarose and incubated at 4°C for 3 hours on rotator. For the non-specific control and also for the sample, 1 ug IgG mouse serum (Sigma) was added to the cell extract. 20 ul protein A/G PLUS agarose was added to the non-specific control and 20 ul protein A/G PLUS agarose with bound anti-HA antibody was added to the sample. Both were incubated overnight at 4°C on rotator. Sample was treated three times with Lysis buffer RIPA (Santa Cruz), four-times with wash buffer (100 mM Tris-HCl, pH8,5; 0,5 M LiCl, 1% Triton X100,

1% sodium deoxycholate, twice with Lysis buffer RIPA (Santa Cruz) and twice with TE buffer (10 mM Tris-HCl, pH8,0; 1 mM EDTA). Sample was resuspended in 30 ul elution buffer (10 mM Tris-Cl, pH 8,0; 10 mM EDTA, and 1% SDS) and incubated for 15 min at 65°C. Then NaCl (200 mM final) was added to the sample and incubated for 30 min at 65 °C for reverse cross-linking. Beads were removed by centrifugation at 14.000 g for 5 min. Proteins were removed from the sample by incubation with 10 ug of Proteinase K (Roche) for 3 hours at 65 °C. The sample was then purified by NucleoSpin gDNA Clean-up (MACHEREY-NAGEL). The DNA size was checked by Bioanalyzer system (Agilent) if the fragments were between 75-1000 bp with median size around 250 bp. If not, than additional sonication was included. Samples and controls were prepared in triplicates.

Western blotting

Streptomycete cells were cultivated 48 hours in 2YT medium at 30°C. When needed, 30 minutes prior to the termination of cultivation, the medium was supplemented by 5M NaCl or 30mM $(NH_4)_2SO_4$ to induce *sigQ* expression. For the protein extracts, cells were resuspended in lysis buffer with zirconium beads and disrupted in FastPrep-24 machine (Biomedicals) at 4°C, speed 5,5, 12x45 sec. The lysate was then centrifuged at 14.000g for 20 min at 4°C. Concentration of proteins was measured using Pierce BCA Protein assay kit (Thermo Scientific). Load buffer (1:4) was then added to the lysates or immunoprecipitated samples and boiled for 4 min. Samples were separated on SDS-PAGE (NuPAGE BisTris Mini Gels, MES SDS Running buffer (Novex), 200 V, 35 min) and then transferred to the PVDF membrane (blotting buffer 48 mM Tris, 39 mM glycine, 0.0375 % SDS, 20 % methanol, 360 mA, 30 min). The anti-HA antibody conjugated to peroxidase (High Affinity Anti-HA-Peroxidase, 3F10, Roche) was used to probe HA-tagged sigma factor and detected by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

3.2 Methods used for identification of novel *cis*-asRNAs

Bacterial strains and cultivation

In this study we used the *Streptomyces coelicolor* wild-type (wt) strain M145 [63] and its RNase III-deletion strain derivative [rnc, M145 rnc::aac(3)IV] [65]. 10^8 spores were inoculated on solid R2YE medium [63] covered by cellophane disc and cultivated at 29°C. Cells were collected after 24, 48, and 72 h of cultivation, where each time point represented a substrate mycelium formation, aerial mycelium formation and sporulation respectively .

RNA isolation

Total RNA was isolated using a TRIzol method [66]. Harvested cells were immediately submerged in TRIzol reagent (Ambion) on ice (1 ml of TRIzol per 50 cm^2 of culture dish surface area). Five glass beads (3mm in diameter) were added to the cell suspension. The cells were disrupted using a Minilys homogenizer (Precellys) twice for 2 min at 3,000 rpm and twice for 2 min at 4,000 rpm, cooled on ice between the cycles. The samples were subsequently centrifuged for 2 min at 10,000 g and purified in TRIzol/chloroform (5:1) and chloroform. Samples were incubated in isopropanol at -20°C overnight and centrifuged for 30 min at 10,000 g, for RNA precipitation. Precipitated RNA was washed in ethanol and resuspended in 30 µl of RNase-free water. Residual DNA in the RNA samples was removed by DNase I treatment (Ambion). Typically, a concentration between 1 and 3.5µg/µl was obtained. RNA quality was checked on a 1 % agarose gel.

5‘ and 3‘ RACE

5’ and 3’ RACE (Rapid Amplification of cDNA ends) is a technique for obtaining the full length of an RNA-transcript. Using RACE we produce cDNA copy of the RNA sequence of interest. This method includes RT-PCR (reverse transcription followed by PCR) followed by cloning into a sequencing vector and sequencing of the obtained transcript fragment. Using RACE we can map wide variety of RNA molecules, including sRNAs. We used commercially available kit from Invitrogen.

In the first step, we designed specific primers for potential RNA transcript of interest. We assumed that an asRNAs bind to the RBS (ribosome binding site) of the target mRNA, so we designed primer that covers RBS and start codon of a cognate mRNA but in opposite direction. All of the primers were designed using the Primer3 software (http://sourceforge.net/projects/primer3/) [67].

RNA samples were isolated after 24 h, 48 h, 72h of cultivation of both *wt* and *rnc* strains. Antisense RNA expressions were tested by FirstChoice RLM-RACE Kit (Invitrogen) following the manufacturer's instructions with the several exceptions:

1. Because the uncapped 5' ends of bacterial RNAs are sensitive to the CIP (calf intestinal phosphatase) enzyme dephosphorylation, the treatment was omitted from the 5' RACE procedure.

2. A gene-specific primer was used instead of random decamers in the 5' RACE.

3. The PrimeScript (Takara, 100 units per 10 µl of reaction mixture) reverse transcriptase was always included in the experiment. Negative control lacking the enzyme was always attached to the experiment.

4. The reverse transcription proceeded at 42°C for 45min and 48°C for 10min.

5. Preceding the 3' RACE, total RNA samples were polyadenylated using 5 units of Poly(A) Polymerase I (New England Biolabs) and ATP, according the manufacturer's protocol. Final PCR products were separated on a 1,2% agarose gel. Products that were found in samples but absent in negative controls were excised and purified using the Qiagen MiniElute PCR purification kit. The purified products were cloned into the TOPO vector using the TOPO TA Cloning kit (Invitrogen) and transformed into *E. coli* One Shot TOP10F' competent cells (Invitrogen). Plasmids containing the cDNA inserts were isolated using the QIAprep Miniprep kit, and sequenced to map 5' and 3' ends of RNAs transcripts.

Northern blot analysis

30 µg of RNA samples were denatured for 10 min at 70°C in RNA loading buffer (95% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 10mM EDTA) and separated in a 1% agarose gel containing formaldehyde using the NorthernMax Kit (Ambion). Separated samples were transferred onto positively charged nylon membranes (ZetaProbe, Bio-Rad) by electroblotting at 240mA for 45min. The nylon membrane was crosslinked by UV and by baking in 80°C, 2 hours. Oligonucleotides were radioactively labeled on their 5′ ends by g-32P-ATP using T4 polynucleotide kinase (Thermo Scientific) and purified (QIAquick Nucleotide Removal Kit, Qiagen). Hybridization was performed in ULTRAhyb hybridization buffer (Ambion) overnight at 37–42°C. The membranes were then washed twice with 2 x SSC, 0.1 % SDS (NorthernMax kit) at room temperature and once with 0.1x SSC, 0.1 % SDS (NorthernMax kit) at 42 °C. The membranes were dried and exposed in a BAS cassette on the imaging plate (Fuji-Film) for 4 days. The signals were visualized using a Phosphorimager FX (Bio-Rad) and quantified using QuantityOne analysis software (Bio-Rad), where the signals were standardized proportionally to the 5S RNA levels. Each northern blot was performed at least twice with samples from separate cultivations in the same conditions.

4 RESULTS AND DISCUSSION

4.1 Identification and modelling of the SigQ regulon in spore germination

In our study, we focused on sigma factor SigQ, because according to our lab's previous results, it was the most highly expressed sigma factor in spore germination [10]. We identified its regulon to uncover its role during this life stage. For this reason, we developed an epitope-tag insertional mutagenesis approach which allowed us to attach HA tag to the sigma factor in its native site in the genome and to subject the tagged SigQ protein to ChIP-Seq analysis. The ChIP-seq results were complemented with the kinetic analysis of gene and operon expression, which were found to be under the SigQ control, using the previously measured time series of gene expression during germination [68, 69].

SigQ regulon was established in the 5.5 hours of growth during spore germination in an amino acid rich medium. These cultivation conditions were used based on the conditions in which we had measured gene expression time series previously, as was published in [10, 68, 69]. Altogether, we detected 130 SigQ-binding regions (0.05 significant, fold enrichment ≥ 2) by ChIP-seq approach. These regions were mapped to the genome of *Streptomyces coelicolor* and we identified 260 genes falling into the criterion of location in ≤ 300 nts upstream of the annotated start codon, including genes that were encoded in both reverse and forward directions in the same region. Then we complemented this set of genes with the genes located in the same operons in the right direction and we finally identified 208 operons consisting of 326 protein-coding genes and three tRNAs to be in SigQ regulon.

Kinetic modelling revealed a possibility of a regulatory influence of SigQ on the identified target genes and also the mode of its action. We got altogether 109 genes that could be negatively regulated by SigQ and 90 that could be positively regulated. 28 gene expression profiles could not be modelled by SigQ and they were categorized as 'Inconsistent'. The rest of the genes were excluded from our results because of inconsistency within the operon, or they had very low response to SigQ.

The genes of the SigQ regulon were further characterized by a classification into functional groups according to The Sanger Institute database. Altogether 227 genes were subjected to gene proportional classification (we have compared genes from the respective group in SigQ regulon and all the genes from the respective group) and we identified the most abundant groups of gene ontologies. The most abundant group were Chaperones with 13.3 % genes from all genes in this group (2 genes out of 13 in total), then Amino acid biosynthesis group with 10.6 % (13

genes out of 123 in total) and group Differentiation and sporulation with 9 % (1 gene out of 11 in total). Other groups were represented only in a minority from 1 to 5 %. A detailed investigation of SigQ regulon revealed that SigQ is an important regulator of nitrogen metabolism, cell wall reconstruction, secondary metabolism and participates in osmotic stress response accompanying spore germination.

4.2 Identification and modelling of the HrdB regulon in vegetative growth phase

The second sigma factor, on which we focused was HrdB. It is a major vegetative sigma factor and is supposed to be a functional homolog of σ^{70} in *E. coli* [21]. In 1990 it was found that HrdB is essential for the cells as its deletion is lethal [6]. Due to the lethality of its deletion it has not been further studied so far. Because of the lethality of sigma factor HrdB and its importance during vegetative phase of growth, we decided to identify its regulon to know which genes are regulated by this sigma factor.

First we had to tagged the sigma factor by HA tag by insertional mutagenesis as in case of SigQ. HrdB regulon was identified by ChIP-seq method in the exponential stage of growth in 22 hours. The ChIP-seq results were coupled with the gene expression time series performed by Nieselt *et al.* [70] in order to examine the gene expression kinetics of the identified genes during the time interval between 20 hours and 60 hours of growth and to verify the possibility of the regulation of identified genes by HrdB. Altogether we detected 1245 HrdB-binding regions (0.05 significant, fold enrichment ≥ 2) by ChIP-seq approach. These regions were mapped to the genome of *Streptomyces coelicolor* and we identified 1599 genes falling into the criterion of location in ≤ 300 nts upstream of the annotated start codon. Genes that were encoded in both reverse and forward directions in the same region are also included. Subsequently we complemented this set of genes with genes located in the same operons in the right direction and we finally identified 337 operons consisting of 2137 protein-coding genes, 75 small RNAs (previously discovered), 62 tRNAs, 6 rRNAs and 3 miscellaneous RNAs to be in HrdB regulon.

We included to our kinetic analysis RbpA protein, as it was reported that it facilitates transcription of HrdB dependent genes [13, 14] suggested the important function of RbpA protein during the initiation of transcription. The expression profiles of 2137 genes were modelled using three different models: constitutive rate of expression (322 genes), expression

controlled by HrdB or the complex HrdB-RbpA (1694 genes) and expression not controlled by HrdB or the complex HrdB-RbpA at all (121 genes).

All genes were further characterized by a classification into functional groups according to The Sanger Institute database and 1694 genes that were controlled by HrdB or by the complex HrdB-RbpA were subjected to gene proportional classification. We identified the most abundant groups of gene ontologies. In HrdB regulon, we have identified genes that constitute 77 % of group Nucleotide biosynthesis, 70 % of genes of group Ribosome constituents or 63 % of group Chromosome replication. Other genes from HrdB regulon constitute from 30 % to 10 % of respective groups, suggesting for a wide regulatory extent of HrdB. Detailed analysis revealed that HrdB regulates 70 % of all ribosome constituents and all ribosomal RNAs coded in the *Streptomyces* genome. In HrdB regulon, 75 sRNAs out of 105 reported in *Streptomyces* were also identified.

From the ChIP-seq results of HrdB regulon, we can conclude that HrdB regulates essential processes in the cells ensuring gene expression, energy metabolism, biosynthesis of important macromolecules needed for the cell structure and growth including regulation of morphological differentiation through the influence of pleiotropic regulators, implying that HrdB as a major regulator of these systems, is an essential vegetative sigma factor; without it cells can't survive. Also the number of the genes from a given pathway, identified in HrdB regulon, confirms the essentiality of HrdB during the vegetative phase of growth.

4.3 Experimental search for novel *cis*-antisense sRNAs

As we focused on studying sigma factors and their regulons, we wanted to know if sigma factors in *Streptomyces coelicolor* are regulated by sRNAs as well. We have chosen opposite way of searching for sRNAs. The common approach for the searching of sRNAs lies in using a combination of a sequencing approach with computational approach to search the possible sRNAs. But we have selected the target at first and then we have tried to find sRNAs. We assumed that the potential sRNAs are coded opposite the target mRNA, act against 5' ends of the target transcripts and base pair with the ribosome binding site (RBS) and start codon. These sRNAs are called asRNAs. In order to find asRNAs to given mRNAs, we performed 5' and 3' RACE analysis to map the 5' and 3' end of the antisense transcripts. The primers for RACE experiments were designed to cover the RBS and START codon of the target mRNA in an opposite direction.

We applied this method to uncover the potential asRNAs against twelve selected sigma factors HrdA, HrdB, HrdC, HrdD, SigB, SigD, SigE, SigF, SigH, SigI, SigR and WhiG. The sigma factors were selected according to their importance (connection with stress, morphological differentiation) or because their function was not much known. The transcripts were amplified, cloned to the vector and sequenced. We confirmed our results by Northern blot. As we know, the duplex of base paired sRNA-mRNA was reported to be subjected to RNase III cleavage [71, 72]; we decided to measure the expression profiles of novel asRNAs and their target mRNAs in RNase III deletion mutant strain (*rnc*) to see the influence of RNase III on these expression levels. RNA for Northern blots were isolated from the cells (wt strain and *rnc* mutant strain lacking RNase III enzyme) grown in the three different life stages represented by vegetative hyphae, aerial hyphae and spores, after 24 h, 48 h and 72 h, respectively.

Out of the 12 sigma factors, 3 of them (SigB (SCO0600), SigH (SCO5243), SigR (SCO5216)) possess antisense transcripts. These are 210 nts long as-sigB, as-sigH which is 244 nts long and as-sigR which has 296 nts (Figure 3).

In our results, we observed several possible mechanism of action of RNase III. The first one, which was also our hypothesis is based on the base pairing of asRNA and target mRNA resulting in the generation of a duplex that is subjected to RNase III cleavage as was described in [73]. This was observed in the case of sigB mRNA/as-sigB in the 48 and 72 hours of growth (Figure 3), when there is a stronger bands in *rnc* strain compared to the wt strain indicating that in this life stage, when aerial mycelium emerges, RNase III is required for the degradation of the duplex as-sigB/sigB mRNA. When RNase III is absent, no cleavage of these transcripts occurs and we may observe an accumulation of these transcripts represented by stronger bands in *rnc* strain.

The second possible mechanism is based on the cleavage of the target mRNA leading to the opening of ribosome binding site resulting in the activation of translation, as mRNA is able to form a secondary structure with stem-loops that includes double stranded regions. It was the case of *sigR* mRNA in the 48 hours of growth (without the accumulation of as-sigR), where in *rnc* strain there is a stronger band than in wt strain. It was described in *E. coli*, where the RNase III cleavage of adhE mRNA leads to the activation of translation [74].

The next possible mechanism of RNase III lies in a stabilisation of the target transcript due to the ribosomal protection of the RNase III-processed mRNAs from ribonucleases [75, 76]. We observed this possible mechanism in the case of as-sigB/*sigB* mRNA expression in the 24 hours

of growth (Figure 3), where a reduction of the as-sigB/*sigB* mRNA level in *rnc* strain can be seen compared to the wt. This suggests that RNase III is required for the transcription and/or stability and/or translation of this transcript, because when it is absent, it results in the decreased levels of both transcripts due to the degradation of these transcripts by ribonucleases [76].

It has also been shown that RNase III may bind a subset of RNAs and modulate their stability via a non-catalytic mechanism [73] and this could be the reason for the reduced levels of mRNA/asRNA transcripts. There is also an obvious reduction of *sigH* mRNA level in the 24, 48 and 72 hours of growth in *rnc* strain (Figure 3), suggesting the impact of RNase III onto the transcription and/or stability of *sigH* mRNA, where RNase III is needed for these events.

No effect of RNase III to our transcripts were observed in the case of as-sigH, it seems that the transcript levels are the same as in wt and *rnc* strains in the 24, 48, and 72 hours of growth. Also in the case of as-sigR and sigR mRNA expression, there are more or less similar expression levels of the transcripts in wt and in *rnc* strains indicating no effect of RNase III on the transcription or the stability of these transcripts. This was likewise reported in the previous studies in *S. coelicolor* [77] and in a study in *B. subtillis* [78] where they found no effect or a little effect of RNase III on the levels of sRNAs or antisense transcript.

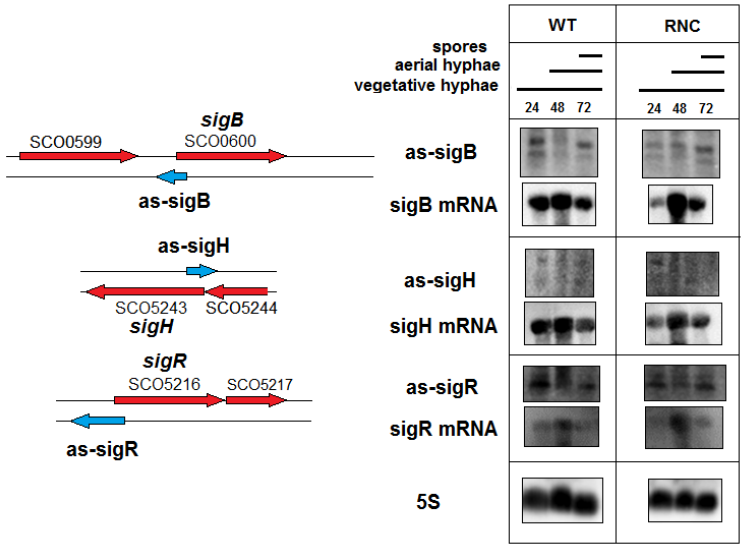

Figure 3. Genomic arrangement (left) and Northern blots (right) of identified novel asRNAs of sigma factors sigB, sigH and sigR in *S. coelicolor* M145 WT strain and *rnc*-mutant strain with 5S RNA as a loading control. Genes are in red and asRNAs are in blue.

5 CONCLUSIONS

In this thesis I have focused on sigma factors, their regulons and regulators. Firstly, we have successfully tagged selected sigma factors with HA tag using by us developed epitope tagging mutagenesis. Using ChIP-seq method, the genome-wide binding of SigQ and HrdB was analysed. The combination of epitope tagging based ChIP-seq analysis with kinetic modelling of gene expression enabled us to identify genes and operons potentially controlled by sigma factor SigQ, which was shown to be largely and divergently expressed during germination, and principal and essential vegetative sigma factor HrdB during vegetative growth.

The kinetic modelling enabled us to verify the possible regulatory influence of sigma factors HrdB and SigQ on the identified genes. In case of SigQ we described different mode of its action on the genes under its control. Surprisingly, we found that SigQ acted not only to initiate transcription, but mostly as a repressor, which, in some parts or throughout the whole course of germination, suppressed expression of the genes whose promoters it bounds. The functional classification of SigQ regulon revealed the function of SigQ to be a regulator of nitrogen metabolism, osmotic stress response and vegetative cell wall reconstruction during germination. The identification of HrdB regulon finally clarifies its essentiality in the vegetative phase of growth and reveals its function as a major regulator of overall gene expression through the control of transcription of individual components of replication, transcription and translation machineries. Furthermore, it regulates key processes such as morphological differentiation, energy metabolism, central intermediary metabolism, and cell wall reconstruction during the vegetative phase of growth.

Further, we have successfully identified three novel *cis*-asRNAs of sigma factors SigB, SigH, SigR using 5' and 3' RACE experiments, and verified its expression by northern blots in WT and RNase III deletion strain in three different life stages – vegetative growth, aerial mycelium formation and sporulation. All three asRNAs are located in an opposite strain of respective sigma factors and cover RBS and start codon. As-sigB has 210 nts, as-sigH is 244 nts long and as-sigR has 296 nts. As-sigB and as-sigH were found to be affected by RNase III – unlike as-sigR not.

All these findings expand the knowledge about sigma regulons of two important sigma factors – SigQ in spore germination and HrdB in the vegetative phase of growth – and about sRNAs-mediated regulation of gene expression in *Streptomyces*.

6 LIST OF PUBLICATIONS

6.1 Publications related to the thesis

1. **Šmídová, K.**; Ziková, A.; Pospíšil, J.; Schwarz, M. DNA mapping and kinetic modeling of the HrdB regulon in *Streptomyces coelicolor*. *Nucleic Acids Res.* **2019**, *47* (2), 621–633. **IF(2019) 11.140**
2. **Šmídová, K.**; Bobek, J.; Ziková, A.; Černý, M.; Schwarz, M.; Vohradský, J. Systems level identification and kinetic modelling of SigQ-mediated control of germination in *Streptomyces coelicolor*. *Manuscript.*
3. Bobek, J.; **Šmídová, K.**; Čihák, M. A Waking Review: Old and Novel Insights into the Spore Germination in *Streptomyces*. *Frontiers in Microbiology* **2017**, *8*, 1–12. **IF(2019) 4.190**
4. Šetinová, D.; **Šmídová, K**.; Pohl, P.; Musić, I.; Bobek, J. RNase III-Binding-mRNAs Revealed Novel Complementary Transcripts in *Streptomyces*. *Frontiers in Microbiology* **2018**, *8*, 1–12. **IF(2019) 4.190**

6.2 Publications not related to the thesis

1. Baumgardt, K.; **Šmídová, K.** The stress-related, rhizobial small RNA RcsR1 destabilizes the autoinducer synthase encoding mRNA sinI in *Sinorhizobium meliloti*. *RNA Biology* **2016**, *13* (5), 486–499. **IF(2019) 4.200**
2. Čihák, M.; Kameník, Z.; **Šmídová, K.**; Bergman, N.; Benada, O.; Kofroňová, O.; Petříčková, K.; Bobek, J. Secondary Metabolites Produced during the Germination of *Streptomyces coelicolor*. *Frontiers in Microbiology* **2017**, *8*, 1–13 **IF(2019) 4.190**

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