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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*

Disertační práce/Doctoral thesis

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Abstract:

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 sRNAs 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 previously measured gene expression data, we selected several highly expressed sigma factors. Using mutant strains with HA-tagged sigma factors, 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 sigma factor, 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 SigR, SigB, and SigH; 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 factor, *cis*-asRNAs, mRNA, RACE, northern blot, RNase III

Abstrakt

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 RNA 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 SigR, SigB a SigH; 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 faktor, *cis*-asRNA, mRNA, RACE, Northern blot, RNáza III

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1 Introduction

Bacteria possess a wide variety of sophisticated regulatory mechanisms to control gene expression in response to changes in inner and outer environment. Transcriptional control plays a major role in the gene expression control, lying in promoter recognition, which is carried out by sigma factors. Sigma factor is a subunit of RNA polymerase and its selective binding to appropriate promoter initiates transcription. It recognizes promoter sequence, recruits RNA polymerase holoenzyme to the target promoter and ensures unwinding of the DNA duplex near the transcription start site (Gross *et al.*, 1998, Wosten, 1998). It is known that biological complexity is often correlated with regulatory complexity (Gruber & Gross, 2003, McAdams *et al.*, 2004). It is proven that bacteria living in highly variable conditions, or having a complex life cycle, possess a lot of genes with regulatory and signalling functions (McAdams *et al.*, 2004). So even the number of sigma factors in each organism correlates with the life cycle complexity. A high degree of developmental complexity in bacterial kingdom is represented by the genus of *Streptomyces*. *Streptomyces* are mycelial mostly soil-living organisms that undergo complex life cycle including spore germination, substrate and aerial mycelium formation and sporulation. They are also medicinally important due to their production of secondary metabolites including antibiotics. These organisms code in their genome 65 sigma factors, the highest number among bacteria (Bentley *et al.*, 2002). The principal and essential sigma factor HrdB regulates transcription of housekeeping genes in *Streptomyces coeliocolor* (Buttner et al., 1990, Shiina et al., 1991), the model organism among *Streptomyces*. The common approach to studying sigma factor function 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 (Buttner *et al.*, 1990). So we developed epitope tagging mutagenesis applied for *Streptomyces* coupled with ChIP-seq in order to identify HrdB regulon. Epitope tagging mutagenesis lies on attaching epitope tag (in our case HA tag) to the given sigma factor. Subsequent chromatin immunoprecipitation selectively isolates the given sigma factor crosslinked to promoter sequences of target genes. Next generation sequencing and bioinformatic tools enabled us to identify genes under regulation of the respective sigma factor. 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, because based on the previously measured microarray data during spore germination, they were found to be highly expressed and SigQ was the most highly expressed among them (Bobek *et al.*, 2014, Strakova *et al.*, 2014).

Another very effective regulation of gene expression is mediated through the acting of small regulatory RNAs affecting transcription, translation or mRNA stability. These can be *cis*-coded asRNAs sharing an extended complementarity and being coded near the target in an opposite direction and *trans*-encoded sRNAs sharing a limited complementarity and being coded far away from its target (Storz *et al.*, 2011). The small RNAs act by various mechanisms to influence a wide range of physiological responses (Waters & Storz, 2009, Storz *et al.*, 2011) and play a role in virulence (Murphy & Payne, 2007), quorum sensing (Baumgardt *et al.*, 2016) or stress conditions (Waters & Storz, 2009). Antisense sRNAs ensure a simple and efficient way of gene expression control due to their close proximity to their target and their capability to block ribosome binding site resulting in inhibition of translation. They can also affect transcription through transcription interference mechanism (Thomason & Storz, 2010). *Cis*-

antisense expression vary in bacteria from 13 % in *Bacillus subtilis* (Nicolas *et al.*, 2012) up to 49 % in *Staphylococcus aureus* (Lasa *et al.*, 2011).

Pairs of sRNAs - mRNAs are often subjected to degradation by RNases. Two main RNases – RNase E and RNase III are involved in the degradation process. RNase E, cleaving a single stranded RNA (ssRNA), is a part of a degradation machinery in *E. coli* (Carpousis *et al.*, 2009). Whereas RNase III degrades double stranded RNA (dsRNA) (MacRae & Doudna, 2007) and it was initially known to be associated with maturation of 16S and 23S rRNA (Gegenheimer & Apirion, 1981, Carpousis *et al.*, 2009). It occurs in increasing evidence that RNase III is coupled with the degradation of sense/antisense RNA pairs (Blomberg *et al.*, 1990, Gerdes *et al.*, 1992) which led us to search for novel asRNAs associated with RNase III in *Streptomyces coeliocolor* which have not yet been reported in this genus. It was also described that sigma factors themselves are regulated by sRNAs (Klein & Raina, 2017). 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 (Gogol *et al.*, 2011, Klein & Raina, 2015). 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 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, we have, interestingly identified three novel asRNAs against sigma factors SigB, SigH, and SigR and described their gene expression during the three different life stages including substrate mycelium formation, aerial hyphae formation and sporulation. According to our results RNase III is thought to create a complex with as-sigB/sigB mRNA and sigH mRNA.

1.1 Model organism *Streptomyces coelicolor*

Streptomyces coelicolor (Figure 1) is a Gram-positive, soil-living bacterium belonging to the group of *Actinobacteria,* order *Actinomycetales*. It is genetically best known among *Streptomyces* and therefore used as a model organism in laboratory experiments. This multicellular organism undergoes complex life cycle that is very unusual for bacteria. It includes differentiation into distinct growth forms (Kieser *et al.*, 2000). *Streptomyces coelicolor* is a very valuable organism to soil environment. It degrades insoluble organic materials such as lignocellulose and chitin including other organism's remains in order to obtain energy and thus it is integrated to the carbon cycle (Bibb, 1996, Bentley *et al.*, 2002).

It is also worth noting that actinomycetes produce a wide variety of the chemically diverse and biologically active metabolites (Berdy, 2005, Nett *et al.*, 2009). They produce more than 2/3 of clinically used antibiotics and nearly 80 % of them are produced by *Streptomyces spp.* (Kieser et al., 2000). *Streptomyces* produces not only antibiotics such as vancomycin, erythromycin, tetracycline (Watve *et al.*, 2001, Baltz, 2008) but also important antifungal (amphotericin B) (Caffrey *et al.*, 2008), anticancer (mitomycin C) (Olano *et al.*, 2009), antiparasitic (ivermectin) (Shiomi, 2004), and immunosuppressive (rapamycin) (Graziani, 2009) compounds. This finding makes them very important organisms in medicine.

Streptomyces coelicolor possesses a GC rich (72.1%) genome with linear chromosome in the size of 8.67 Mbp which is the largest completely sequenced bacterial genome (Bentley *et al.*, 2002). *Streptomyces coelicolor* has two plasmids: SCP1, which is linear 365 kb long, and circular SCP2 plasmid in the size of 31 kb. In our study we used strain *Streptomyces coelicolor*

M145 which is a prototrophic derivative of the wild-type strain A3(2) lacking these two plasmids (Bentley *et al.*, 2002).

Genome sequencing revealed that *Streptomyces coelicolor* A3(2) possesses 7825 predicted genes and more than 30 clusters for secondary metabolites production (Bentley *et al.*, 2002, Nett *et al.*, 2009). Among them, there are known or predicted secondary metabolites, for example antibiotics (actinorhodin, undecylprodigiosin, methylenomycin A, calcium dependent antibiotic), siderophores (coelichelin, coelibactin, desferrioxamines), pigments (tetrahydroxynaphtalene, grey spore pigment), lipids (hopanoids, eicosapentaenoic acid) and other molecules (geosmine, butyrolactones) with bioactive function (Bentley *et al.*, 2002, Nett *et al.*, 2009). However, most of the clusters, offering a potential to produce new compounds, are cryptic gene clusters that are expressed poorly or not under laboratory growth conditions (Bentley *et al.*, 2002, Baral *et al.*, 2018). Biosynthesis of secondary metabolites is under the complex regulatory control with pathway-specific regulators which are controlled by globally-acting transcription factors. These pleiotropic regulators are further regulated by environmental stimuli, morphological changes, stress conditions and therefore it is very difficult to activate these biosynthetic pathways to produce cryptic metabolites (Baral *et al.*, 2018). Activation of cryptic clusters can be triggered by several methods such as ribosomal engineering (Tanaka *et al.*, 2013), genetic engineering to increase metabolic flux through the biosynthetic pathway (Gomez-Escribano, 2012), co-cultivation with other organisms (Rateb, 2013), construction of eligible host organism for heterologous expression of cryptic metabolites (Baltz, 2010, Komatsu *et al.*, 2010), by constitutive expression of pathway specific regulator within the cryptic gene cluster (Laureti *et al.*, 2011) or by stress stimuli and chemicals (Yoon & Nodwell, 2014, Onaka, 2017). Given the fact that streptomycetes have a large number of cryptic gene clusters, they hide a huge potential to produce a novel biological compounds and thus it is a

major challenge for current research. For this reason it is also essential to clarify the regulatory systems in *Streptomyces* including the field of sigma factors, they regulons and regulators.

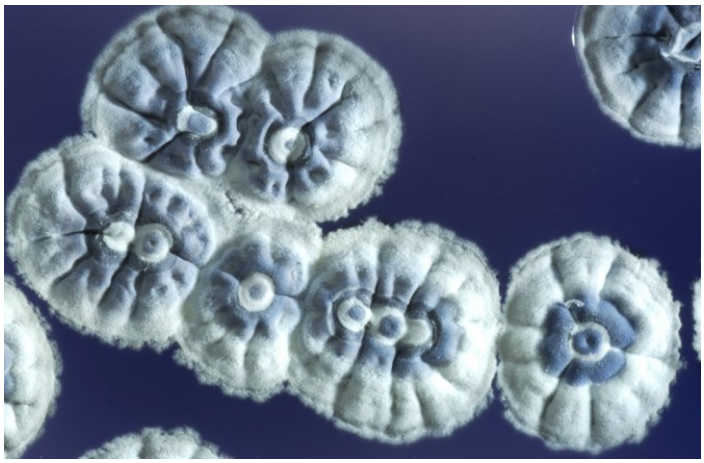


Figure 1. Colonies of *Streptomyces coelicolor* grown on solid medium. Fluffy surface of the colonies is caused by the aerial hyphae. Blue colour is caused by the secreted antibiotic actinorhodin. Adapted from John Innes centre web www.jic.ac.uk).

Life cycle

Streptomyces undergoes complex life cycle that is very similar to filamentous fungi in terms of forming of branching filaments (Kieser *et al.*, 2000). The growth of *Streptomyces* begins with **spore germination**, when the germ tubes appear. This life stage is represented by the transition from dormant state into active metabolism. The germination is initiated under favourable external conditions (water, nutrients, Ca^{2+}), when the water influx causes loss of hydrophobicity and heat resistance, swelling occurs. This process allows the cells to reactivate metabolism in several minutes (Kieser *et al.*, 2000, Bobek *et al.*, 2004, Bobek *et al.*, 2017).

In the next stage of the *Streptomyces* life cycle, germ tubes are differentiated into branching hyphae that give rise to **substrate mycelium**. Theses hyphal compartments are septated and each septum contains several copies of the chromosome. Vegetative hyphae penetrate into the medium and begin to produce secondary metabolites (Kieser *et al.*, 2000).

Afterwards **aerial hyphae** are formed in response to nutrient depletion. Part of the vegetative hyphae lyses and can be used as an alternative source of nutrients. In this life stage, the synthesis of antibiotics peaks in order to avoid the competitive organism. Aerial hyphae are then dissected by sporulation septa to form chains of **uninucleoid spores** (Kieser *et al.*, 2000). The whole *Streptomyces* life cycle is depicted in the Figure 2.

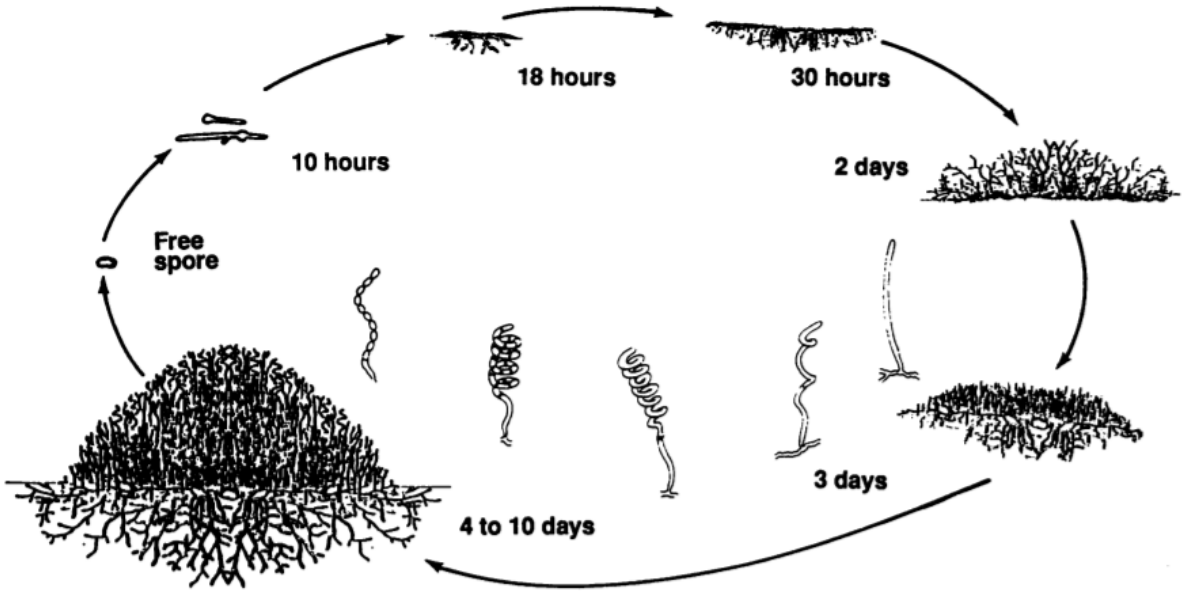


Figure 2. *Streptomyces* life cycle. The outer diagram shows mycelium development and the inner diagram is an expanded representation of hyphae in the developing aerial mycelium. After 2-4 days of growth, *Streptomyces* exhibit a fuzzy white surface of aerial mycelium. After 4-6 days, the white surface changes in gray when the long filament differentiate into chains of 50 or more uninucleoid spores. Adapted from (Schauer *et al.*, 1988).

1.2 Spore germination

Dormant spores of streptomycetes are the only haploid state in the development of this bacteria and their main function is to maintain the genetic material under unfavourable conditions and also to expand it into a new environment (Haiser *et al.*, 2009, Bobek *et al.*, 2017). Spore germination is accompanied by the increase in the size of the spores and decrease of phase

brightness with subsequent germ-tube appearance (Figure 3) (Ensign, 1978, Hardisson *et al.*, 1978, Susstrunk *et al.*, 1998).

Spores of *S. coelicolor* consist of several surface layers that make them highly hydrophobic and rigid. One of the surface layers, a rodlet layer, creates a mosaic of 8-10-nm-wide parallel rods (Wildermuth *et al.*, 1971, Smucker & Pfister, 1978, Claessen *et al.*, 2002).

Since germination is accompanied by the increased respiratory activity and ATP production, the initiation of germination is dependent on the capacity to produce energy (Eaton & Ensign, 1980). Spores contain intracellular nutrient and source of energy which is trehalose (Ranade & Vining, 1993). Trehalose is also responsible for the resistance properties of the spores, mainly for the protection against heat and desiccation (McBride & Ensign, 1987, McBride & Ensign, 1990). Mechanical disruption of the outer spore sheath, heat shock or addition of calcium ions to the medium can induce spore germination (Mikulik *et al.*, 1977, Stastna, 1977, Eaton & Ensign, 1980, Bobek *et al.*, 2017).

During spore germination, autoregulative inhibitor germicidin A is produced, which coordinates the germinating spores population and it helps to maintain some population of spore ungerminated as a reserve for the case of sudden unfavourable conditions (Petersen *et al.*, 1993, Aoki *et al.*, 2011, Cihak *et al.*, 2017).

Spore germination includes cell wall reconstruction and therefore it is dependent on the cell wall hydrolases. Two cell wall hydrolases RpfA and SwlA are involved in the process of spore germination. These two enzymes cause degradation of spore peptidoglycan during spore germination (Haiser *et al.*, 2009, Bobek *et al.*, 2017).

Cyclic AMP as a signalling molecule also plays an important role during spore germination (Susstrunk *et al.*, 1998). It was shown that mutation in cAMP-binding proteins reduces germination rate (Derouaux *et al.*, 2004). Mutation in cAMP receptor protein (CRP) resulted in

a decreased level of peptidoglycan hydrolase (SCO5466) important for the degradation of the spore wall during spore germination (Piette *et al.*, 2005).

Phases of spore germination

Spore germination consists of three stages: **darkening**, **swelling** and **germ tube emergence** (Figure 3) (Hardisson *et al.*, 1978). The **darkening** stage is characterized by decrease in absorbance and loss of refractility. The loss of refraction requires bivalent cations Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+} and Fe^{2+}. Spores undergo an uncoating and spore lysozyme-like hydrolases are reactivated. These enzymes cause lysis and facilitate the reconstruction of cell wall peptidoglycan (Hardisson *et al.*, 1978, Eaton & Ensign, 1980, Salas *et al.*, 1983).

Swelling stage is observed after 60 min of cultivation (Mikulik *et al.*, 1977). Continuous water influx resulting in an increasing size of spores is typical for the swelling stage. Although the cells contain trehalose as an inner source of energy, an input of exogenous carbon source is needed (Hardisson *et al.*, 1978). Diverse proteins and ribosomes are reactivated (Mikulik *et al.*, 1977, Cowan *et al.*, 2003). The spores have in this stage the highest cytochrome oxidase and catalase activities and respiratory quotient (Hardisson *et al.*, 1978).

The last stage, **germ tube emergence**, is observed after 120 – 140 min of cultivation and it is accompanied by the germ tubes appearance and a vegetative growth (Mikulik *et al.*, 1977). The central role of vegetative growth play a DivIVA protein localized at hyphal tips thus ensuring a new cell outgrowth (Flardh, 2003, Flardh *et al.*, 2012). The second important protein is a chaperon-like protein SsgA that helps the cell wall synthesizing components to find the position for the future synthesis of peptidoglycan (Noens *et al.*, 2007, Bobek *et al.*, 2017).

The process of germination differs among different *Streptomyces* species. For example *S. viridochromogens* and *S. granaticolor* are distinguished by robust germination with nearly

100 % of the spores germinating (Mikulik *et al.*, 1977, Bobek *et al.*, 2004, Xu & Vetsigian, 2017). *S. coelicolor* or *S. venezuelae* are characterized by a slow spore germination rate with some spores not germinating at all. It can be caused by the production of developmental inhibitors such as germicidins and hypnosins (Petersen *et al.*, 1993, Aoki *et al.*, 2007, Xu & Vetsigian, 2017). Spore germination of *S. coelicolor* is depicted in the Figure 3 and 4.

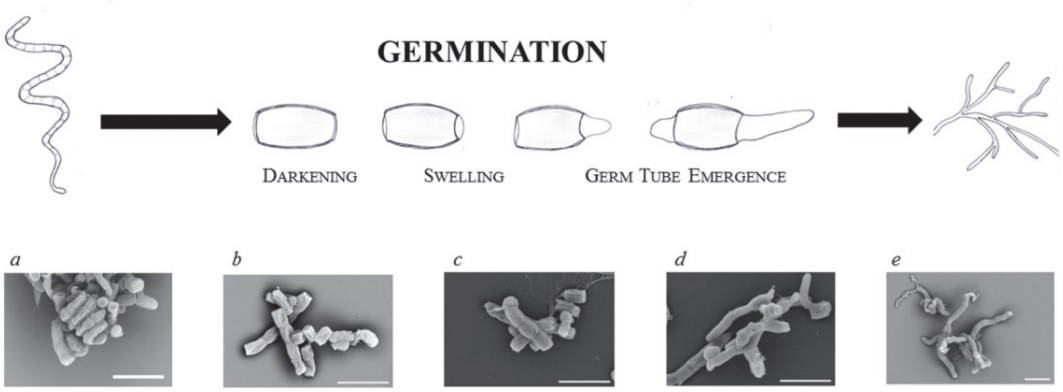


Figure 3. Streptomycete spore germination (a, b – dormant spores; c - e – germinating spores). Adapted from (Bobek *et al.*, 2017).

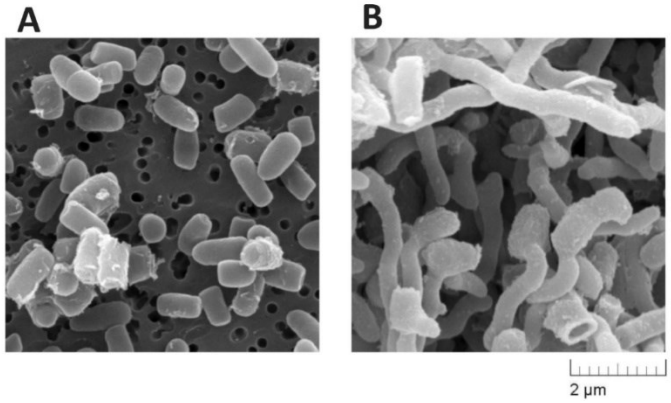


Figure 4. Spores of *S. coelicolor* observed using electron microscopy. A – dormant spores. B – germinating spores at the time of 5,5 hours of growth with visible germ tubes. Adapted from (Strakova *et al.*, 2013).

Dormant spores contain a pre-existing pool of mRNA and active ribosomes in order to ensure immediate translation of proteins at the beginning of spore germination (Mikulik *et al.*, 2002). RNA synthesis begins after 3 minutes of germination and protein synthesis starts at the 4th minute (Mikulik *et al.*, 1977). The hundreds of proteins are newly synthesized by de novo transcription and translation. Proteins for translation machinery and differentiation as well as chaperons are produced. Reactivation of translational systems occurs, resulting in massive proteome reconstitution accompanied by the rehydration, reactivation and refolding of aggregated proteins (Cowan *et al.*, 2003, Strakova *et al.*, 2013). Refolding of the proteins is ensured mainly by the chaperons GroEL, Trigger factor and DnaK. They also contribute to the reactivation of ribosomes. When ribosomes are active, it can trigger translation. This resulted in the synthesis of the enzymes of central carbon metabolism, amino acid and nucleotide biosynthesis (Bobek *et al.*, 2004, Strakova *et al.*, 2013).

Rapid proteosynthesis occurs in between 30 to 60 minutes of growth and the cells begin to communicate with outer environment. Proteins for energy metabolism, regulation and transport are produced. Master regulators such as BldD and Crp are expressed. Stress-related proteins, such as thioredoxin, also occur to maintain thiol homeostasis. Superoxide dismutase and catalase are also expressed to overcome redox stress (Strakova *et al.*, 2013, Strakova *et al.*, 2013).

It was also observed several important proteins necessary for cytoskeleton formation and cell division, such as DivIVA, FilP and also FtsZ, which is needed for germ tube emergence (Strakova *et al.*, 2013).

Transcriptomic and proteomic study were done systematically at 13 time points in 5,5 hours of germination. Figure 5 shows the expression profiles of each functional group. There are high peaks in the time point 1 hour of proteins connected with energy metabolism, regulation,

transport and binding which further decrease. In 1,5 hour there is a peak of functional group of membrane, lipoprotein and periplasmic proteins (Strakova *et al.*, 2013, Strakova *et al.*, 2013). Each functional group has own expression profile according to the need of the cells (Figure 5). The correlation between proteomic and transcriptomic data from the spore germination was found to be very low. Of the 247 genes/proteins, only 27.9 % were highly correlated. However, this finding is in agreement with other studies that were done in *Streptomyces* species showing a correlation of about one third of the expressed gene in the stationary phase (Vohradsky *et al.*, 2007, Jayapal *et al.*, 2008, de Sousa Abreu *et al.*, 2009). It is caused by the fact that not all mRNAs are directly translated into the proteins but undergo posttranscriptional regulation. Translational and posttranslational regulations also play a role in the variance between the proteomic and transcriptomic data (de Sousa Abreu *et al.*, 2009).

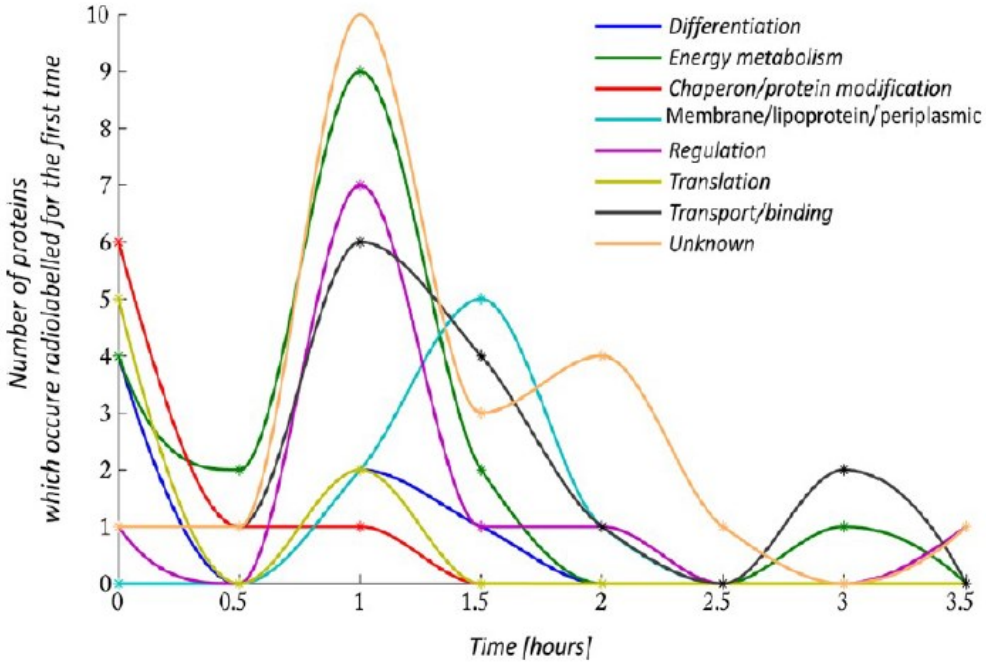


Figure 5. Expression profiles of *de novo* synthesized proteins within the measured time periods for the most occupied functional groups. Adapted from (Strakova *et al.*, 2013).

1.3 Transcription

Transcription is a process when DNA is transcribed to RNA. This process is ensured by RNA polymerase (RNAP). Bacterial RNA polymerase consists of six subunits – αI, αII, β, β', ω and σ (Burgess *et al.*, 1969, Travers & Burgess, 1969). Sigma factor (σ) is a specific dissociable subunit responsible for promoter recognition and is essential for the initiation of transcription (Wosten, 1998). Promoters are specific sequences on DNA where the transcription starts. They are upstream of the transcription start site (TSS) and are represented by -10 and -35 conserved regions (Browning & Busby, 2004).

Transcription is comprised of initiation, elongation and termination phases. The initiation phase is characterised by the association of a sigma factor with the RNAP core enzyme and the recognition of a promoter sequence by sigma factor. It is a multi-step process where the sigma factor bound to a promoter triggers a series of conformational changes in RNA polymerase and in the promoter region. Sigma factor plays the central role in this phase (Glyde *et al.*, 2018). Firstly, it recognizes the promoter elements and forms the closed complex (RPc). This is called isomerisation and it causes the opening of 13 bp from the -10 element creating the transcription bubble and an unstable open complex. Afterwards, the final stable open complex (RPo) is formed by melting a short region of DNA (Bae *et al.*, 2015). The +1 template strand base is situated in the active site of the RNA polymerase and the transcription can start (Kontur *et al.*, 2006, Kontur *et al.*, 2008, Gries *et al.*, 2010, Kontur *et al.*, 2010, Saecker *et al.*, 2011, Paget, 2015, Zuo & Steitz, 2015, Glyde *et al.*, 2018). Promoter escape occurs and sigma factor is released from the transcription complex via DNA-scrunching mechanism (Hsu, 2002, Kapanidis *et al.*, 2006) when the nascent RNA is 12-15 nt long, and the transcription proceeds to elongation phase (Mooney *et al.*, 2005, Saecker *et al.*, 2011). It was also shown that sigma

factor does not have to be released from the transcription complex, but it can remain associated with RNAP and can translocate with RNAP (Mukhopadhyay *et al.*, 2001, Mukhopadhyay *et al.*, 2003, Nickels *et al.*, 2004, Kapanidis *et al.*, 2005).

During this process, abortive transcription occurs very often, resulting in the slipping of the RNAP from the DNA, and incomplete transcripts are produced (van Hijum *et al.*, 2009). Usually, several abortive products are produced before the productive initiation occurs. The number and the length of these abortive products are a function of promoter sequence and conditions (Deuschle *et al.*, 1986, Hsu *et al.*, 2006, Saecker *et al.*, 2011). In the abortive initiation, RNA polymerase synthesizes short RNA transcript but does not escape from the promoter. Instead, RNA polymerase discharges RNA transcript, returns back to RPo and re-initiates transcription (Gralla *et al.*, 1980, Duchi *et al.*, 2016). In the productive pathway, RNA polymerase synthesizes 9 – 11 nts long RNA transcript and escapes from the promoter entering elongation (Mukhopadhyay *et al.*, 2001, Murakami & Darst, 2003, Duchi *et al.*, 2016).

Transcription elongation complex (TEC) is formed and RNAP core enzyme proceeds to elongation phase in which the nascent RNA is produced. RNA is transcribed from the template strand in 3'→5' direction generating the RNA molecule from 5'→3' (Belogurov & Artsimovitch, 2015). It follows the termination of transcription that can be accomplished by RNA stem-loop structure (intrinsic termination) or through the termination factor Rho (Ciampi, 2006, Washburn & Gottesman, 2015).

1.4 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 proteins stimulating transcription (Burgess *et al.*, 1969). 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 (Gross *et al.*, 1998, Wosten, 1998). 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 (Saecker *et al.*, 2011). RNA polymerase core enzyme must first interact with a σ subunit to form the holoenzyme (Wosten, 1998).

Bacterial sigma factors have from 20 to greater than 70 kDa (Helmann, 2001, Davis *et al.*, 2017). The number of sigma factors in each organism varies from 1 in *Mycoplasma sp*. over 17 in *Bacillus subtilis* to 65 in *Streptomyces coelicolor* (Mittenhuber, 2002, Gruber & Gross, 2003, Hahn *et al.*, 2003) and it correlates with the size of the genome and developmental complexity as shown in Figure 6.

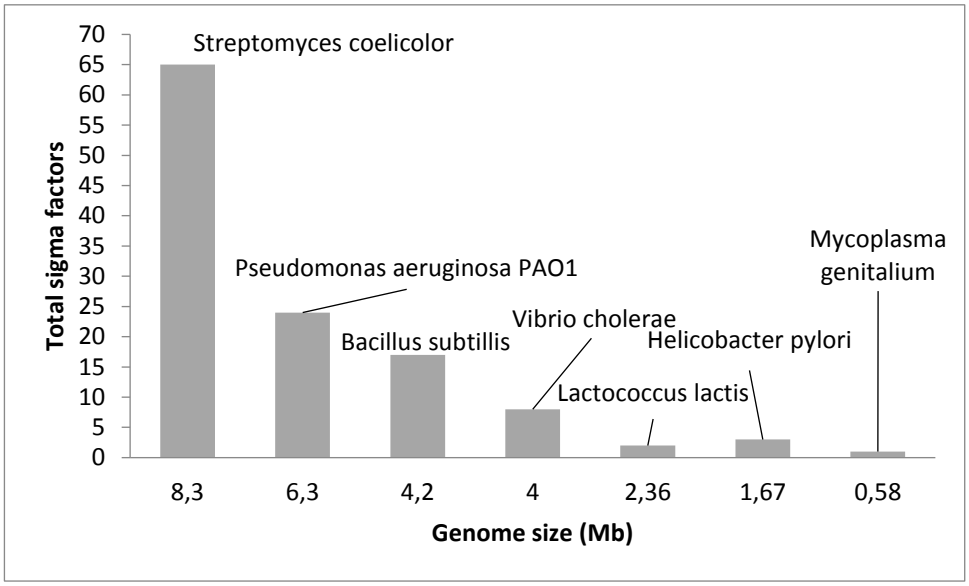


Figure 6. Number of sigma factors in diverse organisms (Mittenhuber, 2002)

Sigma factors are multidomain subunits with four conserved regions (σ_1, σ_2, σ_3, σ_4) (Figure 7), but not all sigma factors possess all four regions. All sigma factors possess the σ_2 and σ_4 domains containing the major RNAP- and promoter-binding determinants. Region 1.1 is present only in group 1 of sigmas (described below) and it serves as an autoinhibitory domain, masking DNA binding determinants in free σ^{70} (Gruber & Gross, 2003, Paget, 2015).

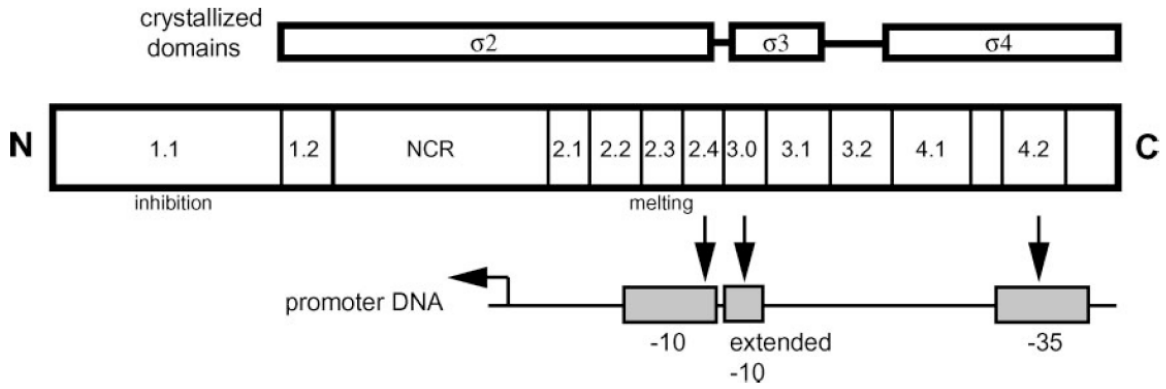


Figure 7. The schematic structure of σ^{70} and its interaction with promoter. Four conserved regions with non-conserved region (NCR) are indicated. Arrows point to the regions of sigma factor that bind the promoter. Regions

2.4 and 4.2 recognize the -10 and -35 elements of the promoter sequence. Region 3.0 recognizes the extended -10 element. Region 2.3 is responsible for DNA melting during transcription initiation (Paget, 2015). Adapted from (Gruber & Gross, 2003).

1.4.1 Promoter recognition

Bacterial promoters contain several motifs known as the -35 element, the extended -10 element, the -10 element, the discriminator region, the UP element (Figure 8) and the core recognition element (CRE). Only the -10 element is indispensable. Other elements may or may not be present. Each element is recognized specifically by RNAP and each has its own function from recognition over melting of DNA to the opening of the promoter so the whole process of initiation of transcription can be viewed as an orchestrated sequential process (Feklistov, 2013). The relative contribution of each element to the binding with RNAP varies from promoter to promoter (Browning & Busby, 2004) and the degree of similarity to the consensus sequences of each element determines the strength of a given promoter (Browning & Busby, 2016). Structural studies have shown that the -10 element and the discriminator element are both recognized as single-stranded (ssDNA) whereas other promoter elements (extended -10, -35 element and UP element) are recognized as double stranded DNA (dsDNA) (Feklistov & Darst, 2011, Zhang *et al.*, 2012, Bae *et al.*, 2015, Zuo & Steitz, 2015).

Sigma70-dependent promoters possess 2 highly conserved sequences consisting of -35 element and -10 element that characterize the promoter given and its selectivity (Gross *et al.*, 1998).

Region 4.2 of sigma factor specifically recognizes and binds to **-35** hexanucleotide sequence via multiple helix-turn-helix and major groove specific and non-specific interactions (Figure 8) (Gardella *et al.*, 1989, Campbell *et al.*, 2002, Lane & Darst, 2006). Consensus sequence of -35 element of primary sigma factor is TTGACA in *E. coli*, but it varies across the bacterial kingdom and according to the sigma group (Hawley & McClure, 1983). An interaction of β

subunit of RNAP with region 4 of sigma factor allows the region 4.2 to recognize and bind -35 element (Geszvain *et al.*, 2004).

Region 2.4 recognizes and binds the **-10** element on DNA (Figure 8) (Campbell *et al.*, 2002). This element is called Pribnow box after its discoverer (Pribnow, 1975). It is a highly conserved and essential bacterial promoter motif (Hook-Barnard & Hinton, 2007, Shultzaberger *et al.*, 2007). The consensus sequence of this element in *E. coli* is TATAAT (Hawley & McClure, 1983). The interaction of sigma factor with -10 element is critical for the melting of DNA, allowing the formation of transcription bubble. Region 2.3 also plays a role in DNA melting throughout the stacking interactions with -10 element (Helmann & Chamberlin, 1988, Feklistov & Darst, 2011).

There are two more important regions on DNA that are bound by RNA polymerase. It is the extended -10 element and the UP element. The **extended -10 element** is a conserved TGn motif immediately upstream of the -10 element. It had been presumed that promoters with this extended -10 element often lack the -35 element (Gross *et al.*, 1998) but it was shown that TGn motif plays an important role at promoters with weaker -35 elements (Campbell *et al.*, 2002) or longer spacers (Mitchell *et al.*, 2003). Extended -10 element increases the activity of RNAP through the interaction with region 3.0 of sigma factor (Figure 8) (Keilty & Rosenberg, 1987, Barne *et al.*, 1997).

The **UP element** is an AT rich 20 bp long region located between -40 and -60 bp on the DNA and is recognized by the C-terminal domains of the α subunits (αCTDs) (Figure 8) (Ross *et al.*, 1993, Gourse *et al.*, 2000). UP elements are capable of increasing the transcription from the rRNA promoters (Rao *et al.*, 1994, Estrem *et al.*, 1998) and comprises of two distinct subsites – proximal and distal, one for each αCTD. Promoters can have just one or both even though the distal element seems to function almost as well as the full UP element (Estrem *et al.*, 1999). UP

elements have been found in many bacterial and phage promoters and may function with different sigma factor-containing RNAPs (Newlands *et al.*, 1993, Ross *et al.*, 1993, Fredrick *et al.*, 1995).

Another important but non-conserved region is the **discriminator element** which is located between -10 element and the TSS. It interacts with region 1.2 of sigma factor and is involved in the regulation of open complex lifetime (Figure 8) (Haugen *et al.*, 2006, Haugen *et al.*, 2008, Zhang *et al.*, 2012, Zuo & Steitz, 2015). Most discriminator regions are 6 – 8 bases in length (Shimada *et al.*, 2014) and with increasing length from 6 to 8 the lifetime of open complexes decreases (Jeong & Kang, 1994, Liu & Turnbough, 1994, Lewis & Adhya, 2004).

Between the extended -10 element and -35 element there is a region called **spacer** which is a variable non-conserved sequence in the length of 16 – 18 bp (Figure 8) (Murakami *et al.*, 2002). The most common length of the spacer for σ^{70} promoters is 17 bp (Hawley & McClure, 1983, Mitchell *et al.*, 2003, Shimada *et al.*, 2014). The length and extent of the bending of the spacer influence the effect of region 1.1 of sigma factor on transcription initiation kinetics and the structure of the open complex (Hook-Barnard & Hinton, 2009). Sigma binding to -10 and -35 conserved region was suggested to produce a twist (or other deformation) in the spacer DNA that influences open promoter complex formation (Sztiller-Sikorska *et al.*, 2011).
In 2012, a novel element called core recognition element (CRE) was also identified. It is located between -4 and +2 on the non-template strand recognized by β subunit (Zhang *et al.*, 2012).

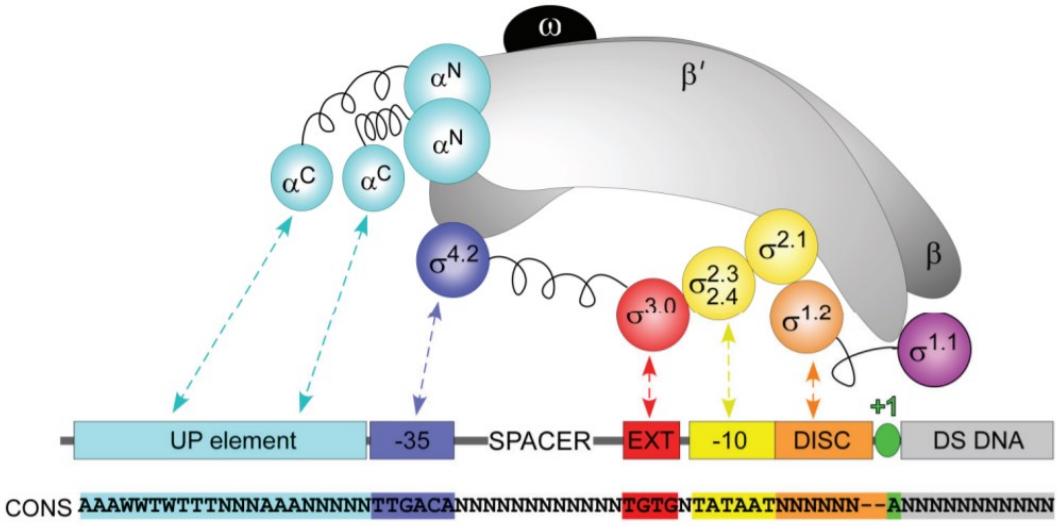


Figure 8. Interactions between the regions of σ^{70} RNAP and promoter regions. **RNAP**: α2 - cyan; β and β' - gray; ω - black. σ regions - as shown. **Promoter**: UP element - cyan; 35 element - blue; extended 10 - red; 10 element - yellow; discriminator - orange; transcription start site - green; DNA downstream of the transcription start site - gray. Linker regions in α and σ subunits are shown as springs. Adapted from (Ruff *et al.*, 2015).

1.4.2 Sigma factor families σ^{70} and σ^{54}

Sigma factors can be classified into σ^{70} and σ^{54} group according to the size and the promoter sequence they recognize. σ^{70} recognizes promoter sequence represented by -10 and -35 conserved regions and forms RPo spontaneously. It is responsible for transcription of housekeeping genes and is related to the principal σ factor in *Escherichia coli*, σ^{70}, which has a molecular mass of approximately 70 kDa (Browning & Busby, 2016, Glyde *et al.*, 2017, Glyde *et al.*, 2018). σ^{54} controls genes related with stress, heat shock, membrane stress and nutrient starvation (Buck *et al.*, 2000). σ^{54} recognizes and binds promoter region -12 and -24 and needs an activator (enhancer) protein and ATP hydrolysis to form RPo formation (Rappas *et al.*, 2007, Glyde *et al.*, 2017, Glyde *et al.*, 2018). The name is derived from the molecular mass which is in *E. coli* 54 kDa (Buck & Cannon, 1992).

Although bacteria possess multiple members of the σ^{70} family, they usually have no more than one member of σ^{54} (Buck *et al.*, 2000). Some organisms contain both σ^{70} and σ^{54} family of sigma factors, whereas others possess only σ^{70} family sigmas. σ^{54} and its enhancers have never been reported in high-GC Gram-positive bacteria, including *Streptomyces coelicolor* (Buck *et al.*, 2000, Studholme & Buck, 2000, Paget, 2015, Zhang & Buck, 2015).

1.4.3 Classification of sigma factors

In bacteria, primary sigma factors play essential role by ensuring the transcription of housekeeping genes. Alternative sigma factors are responsible for the transcription of diverse genes according to environmental conditions, life stages, stress responses, morphological development and other stimuli in inner and outer environment (Helmann, 2001, Feklistov *et al.*, 2014). They are further classified into four major phylogenetically and structurally distinct groups. In group 1, there are primary sigma factors and group 2 – 4 consist of alternative sigma factors with specialized functions. The groups differ from each other by the presence or absence of four conserved regions (σ_1, σ_2, σ_3, σ_4) (Figure 9), their members' essentiality, structure, size and function (Paget, 2015).

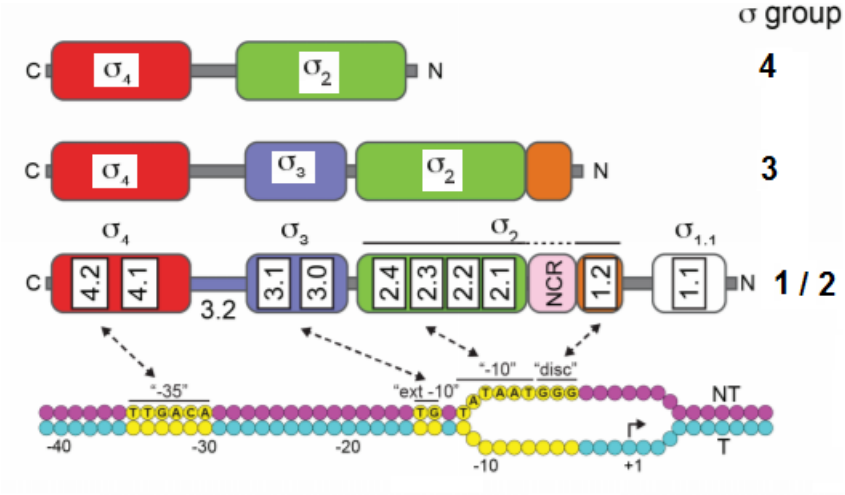


Figure 9. The domain organization of sigma factors groups 1 – 4. Adapted from (Paget, 2015).

Group 1 consists of essential primary sigma factors. They are presented in all known bacteria and are responsible for transcription of genes essential for cell survival. They contain all four conserved domains and a non-conserved region (NCR) (Helmann, 2002). The primary sigma factor in *E. coli* is encoded by *rpoD* gene and is known as σ^{70} (σ^{D}), in *Mycobacterium spp.* it is known as MysA/SigA (Hurst-Hess *et al.*, 2019), in *Streptomyces spp.* as HrdB (σ^{hrdB}) (Tanaka *et al.*, 1988) and in *Bacillus subtilis* and other gram-positive bacteria as SigA (σ^{A}) (Helmann & Chamberlin, 1988, Gruber & Bryant, 1997). The consensus sequence (TTGACA and TATAAT) of the promoter recognized by the primary sigma factor in *E. coli* and *B. subtilis* seems to be similar in many bacteria (Helmann, 1995, Patek *et al.*, 1996).

Group 2 is not essential for bacterial growth (Lonetto *et al.*, 1992). The structure of these sigma factors lacks region 1.1 but is very similar to the primary sigma factors in the amino acid sequence suggesting that both groups have extensive overlap in promoter recognition (Wosten, 1998, Helmann, 2002, Sun *et al.*, 2017). Sigma factors from group 2 participate in the regulation

of adaptation to stress associated with the stationary phase. In *E. coli* and other enteric bacteria, σ^S (*RpoS*) is responsible for general stress response and mainly for gene regulation during the entry into stationary phase (Hengge-Aronis, 1999). In Gram-negative cyanobacteria it is SigB and SigC that are important for circadian responses during photosynthetic growth (Tsinoremas *et al.*, 1996, Gruber & Bryant, 1997). The members of this group in high-GC Gram-positive bacteria are MysB in *Mycobacterium spp*., SigB in *Corynebacterium glutamicum,* and finally HrdA, HrdC, and HrdD in *Streptomyces spp.* (Gruber & Bryant, 1997, Kang *et al.*, 1997, Wosten, 1998, Helmann, 2002).

Group 3 lacks σ_1 domain. Members of this group are involved in adaptive responses including flagellum biosynthesis, heat shock response, general stress and sporulation. This group is significantly smaller in size than group 1 and 2 – typically 25 to 35 kDa (Wosten, 1998, Helmann, 2002, Paget & Helmann, 2003, Paget, 2015, Sun *et al.*, 2017). *E. coli* σ^{28} (FliA) and other close related sigma factors responsible for flagellum biosynthesis in all motile Gram-negative and Gram-positive bacteria belong to this group (Chen & Helmann, 1992). In *B. subtilis* it is for example SigB responsible for general stress response and four related sigma factors SigF, SigE, SigG, SigK regulating endospore formation (Hecker *et al.*, 2007, Nannapaneni *et al.*, 2012, Paget, 2015). Group 3 sigma factors is represented in *Streptomyces coelicolor* with sigma factor WhiG responsible for spore formation (Ryding *et al.*, 1998, Ainsa *et al.*, 1999, Kaiser & Stoddard, 2011), and nine alternative sigma factors (SigB, SigF, SigG, SigH, SigI, SigK, SigL, SigM and SigN) called SigB-like sigma factors, that are homologs of *Bacillus subtilis* SigB (Cho *et al.*, 2001). SigB in *Streptomyces* controls multiple stress response, morphological differentiation and secondary metabolism (Lee *et al.*, 2005, Facey *et al.*, 2009).

Group 4 is known as the extracytoplasmic function (ECF) sigma factors. They lack $\sigma_{1.1}$ and σ_3 domains and also the first helix of $\sigma_{R1.2}$ so they are the most minimal sigma factors (Haugen *et al.*, 2008, Zhang *et al.*, 2012). Due to very different amino acid sequences and structure they were first not identified as sigma factors. Their function is responding to environmental changes through the cell wall integrity. This is the largest and the most diverse group among sigma factors. It includes sigma factors responsible for cell envelope stress response, iron transport, oxidative stress and the general stress response (Staron *et al.*, 2009, Paget, 2015). ECF sigma factor is usually co-transcribed with a transmembrane anti-sigma factor with an extracytoplasmic sensory domain and an intracellular inhibitory domain (Helmann, 2002). Sigma factor AlgU of *Pseudomonas aeruginosa* responsible for alginate biosynthesis, and FecI of *E. coli* and PbrA of *Pseudomonas fluorescens*, both regulating iron uptake, belong to this group (Angerer *et al.*, 1995, Sexton *et al.*, 1996). *B. subtilis* contains five ECF sigma factors – SigV, SigW, SigX, SigY, and SigZ. It is known only the function of SigX which is required for the overcoming of high temperatures (Huang *et al.*, 1997, Kunst *et al.*, 1997, Sorokin *et al.*, 1997). The *Streptomyces coelicolor* genome encodes 51 ECF sigma factors out of 65 sigma factors reflecting their complex habitat and developmental cycle (Helmann, 2002, Sun *et al.*, 2017). They are responsible for stress response and cell wall integrity. Sigma factors SigR regulating thiol-oxidative stress, SigE controlling cell envelope and BldN required for aerial mycelium formation are well-known (Paget M. S. B, 2002, Sun *et al.*, 2017).

Streptomycetes sigma factors are listed in Table 1.

Table 1. Sorting of sigma factors in *Streptomyces coelicolor*

Group of sigma factor	Function	Representants	Size (kDa)
Group 1	**Essential** **Regulation of transcription of housekeeping genes**	**HrdB**	40 - 70
Group 2	- Non-essential - Regulation of growth	**HrdA, HrdC, HrdD**	37 - 43
Group 3	- Stress response - Sporulation - Morphological differentiation	**SigB**, **SigF**, SigG, **SigH**, **SigI**, SigK, SigL, SigM, SigN, **WhiG**	25 - 35
Group 4	**ECF (extracytoplasmatic function) sigma factors** - Regulation of cell wall integrity -Stress response - Regulation of aerial hyphae formation	BldN, SigA, **SigE**, **SigR**, SigT, SigU,etc.	20 - 25

1.4.4 Regulation of sigma factors

A wide variety of mechanisms regulates the action and availability of the sigma factors. They can be controlled at the level of transcription, translation or protein turnover (Paget, 2015). Action of sigma factors might be regulated by the competition between sigma factors for the limited pool of RNAP core enzyme. It was found that *E. coli* σ^{70} , the major sigma factor, has 16 times higher binding affinity to RNAP core than SigS (Maeda *et al.*, 2000), which could be – besides other things – caused by the number of multiple contact interfaces with RNAP core of σ^{70} unlike SigS (Sharp *et al.*, 1999). It is also proposed that growth related changes in transcription pattern, resulting in different sigma subunit binding affinities, could be caused by the specific intracellular conditions or additional factors (Ishihama, 1997, Ishihama, 1999, Kim

et al., 2004). For example the increase of glutamate (Ding *et al.*, 1995), trehalose (Kusano & Ishihama, 1997) or polyphosphate (Kusano & Ishihama, 1997) can stimulate the preference of σ^S over σ^{70}. Also the regulatory nucleotides such as cAMP, ppGpp and AppppA might influence the transcription by different RNAP holoenzymes in different ways (DiRusso & Nystrom, 1998).

Another way to regulate sigma factors is the synthesis of alternative sigma factors as inactive pro-proteins carrying inhibitory leader peptide that must be cleaved in order to activate the sigma factor by controlled proteolysis (Chandrangsu, 2014). These are e. g. SigE and SigK, *Bacillus subtilis* sporulation σ factors (LaBell *et al.*, 1987, Lu *et al.*, 1990).

Sigma factors are also regulated by positive-acting regulatory proteins. A typical example is σ^{54} requiring for its action an enhancer protein (Wigneshweraraj *et al.*, 2008).

1.4.4.1 Anti-sigma factors

Alternative sigma factors, primarily ECF sigma factors, are commonly controlled by anti-sigma factors. They sequester sigma factors and prevent binding to RNAP core enzyme by blocking the key RNAP core binding determinants (σ_2, σ_4) in order to stabilise them and inhibit the transcription from given promoter (Helmann, 1999, Paget, 2015). Anti-sigma factors are often co-transcribed with sigma factor gene, because they are encoded within the same operon. Thus the stochiometric level of given anti-sigma factor is maintained (Ho & Ellermeier, 2012, Paget, 2015).

Anti-sigma factors are often membrane associated proteins sensing the environmental conditions. They are comprised from extra-cytoplasmic domain and an intracellular inhibitory domain (Helmann, 2002). Environmental stimuli cause the release of sigma factor from the

association with anti-sigma factor, resulting in the activation of the transcription of related genes to overcome emerging conditions (Paget, 2015).

A broad range of mechanisms leading to the release of sigma factors in response to environmental signals occurs such as **partner-switching**, **direct sensing** and **regulated proteolysis** (Paget, 2015).

Partner switching

Partner-switching mechanism is a common strategy of cells to modulate sigma factor activity which was extensively studied for the group 3 sigma factors SigB and SigF in *B. subtilis* (Helmann, 1999) (Figure 10). Even though SigF regulates sporulation (Stragier & Losick, 1996) and SigB controls stress response (Hecker & Volker, 1998), they are together with their regulators, paralogs.

SigB in unstressed cells is sequestered by anti-sigma factor RsbW. Stress stimuli causes dephosphorylation of anti-anti-sigma factor RsbV resulting in the association of RsbW and RsbV and a simultaneous release of SigB that directs transcription of stress related genes (Hecker *et al.*, 2007).

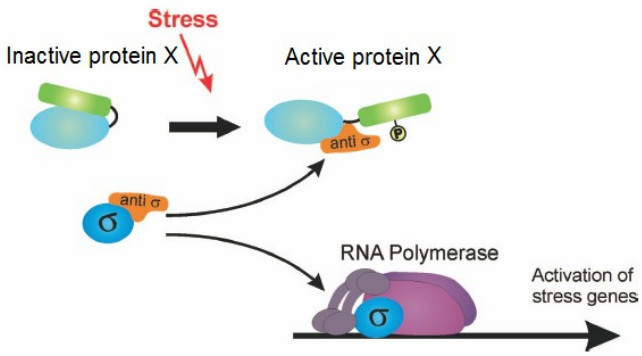


Figure 10. Model of partner-switching mechanism controlling general stress response. In unstressed cells protein X is inactive and an ECF sigma factor is inhibited by the anti-sigma factor. In response to stress Protein X is

phosphorylated and bind to anti-sigma factor, thus releasing σ to allow them to associate with RNAP core to transcribe stress related genes. Adapted from (Francez-Charlot *et al.*, 2009).

Regulated proteolysis

This mechanism, found in all kingdoms (including human) of sigma factor activation, lies in proteolytic cascade called regulated intramembrane proteolysis (RIP) pathway. It causes the release of sigma factor from the inactive membrane-associated sigma/anti-sigma complex and thereby allowing it to initiate transcription (Brown *et al.*, 2000, Heinrich & Wiegert, 2009, Joshi *et al.*, 2019).

Environmental stimuli (such as stress) are sensed by the anti-sigma factor causing the activation of associated site-1 protease. Subsequent proteolysis of anti-sigma factor by site-2 protease and subsequently by cytoplasmic protease follows, resulting in the release of sigma factor from its binding which activates transcription of stress related genes (Figure 11) (Heinrich & Wiegert, 2009). Site-1 and site-2 peptidases belongs to the important intramembrane cleaving proteases (I-CLiPS) catalysing the cleavage of transmembrane domains of substrate proteins (Weihofen & Martoglio, 2003, Erez *et al.*, 2009).

RIP plays a major role in a broad range of bacterial transmembrane signalling processes such as stress response (Schobel *et al.*, 2004, Ades, 2008), sporulation (Rudner *et al.*, 1999, Yu & Kroos, 2000), cell division (Bramkamp *et al.*, 2006), cell cycle regulation (Chen *et al.*, 2005), quorum sensing (Stevenson *et al.*, 2007), pheromone and toxin production (An *et al.*, 1999, Matson & DiRita, 2005), biofilm formation (Qiu *et al.*, 2007, Heinrich *et al.*, 2008) and virulence of pathogens (Urban, 2009).

Among Gram-positive bacteria, this pathway was described in *B. subtilis* for σ^W and its anti-sigma factors RsiW. In response to a signal elicited by the antimicrobial peptides or other

agents, RsiW is cleaved by the site-1 protease PrsW and subsequently by the site-2 protease RasP (Schobel *et al.*, 2004, Ellermeier & Losick, 2006).

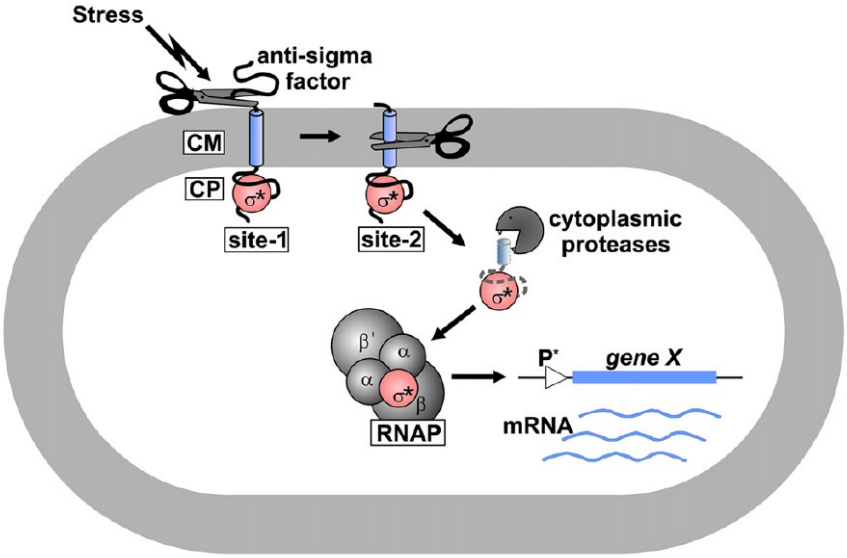


Figure 11. Model of the RIP pathway. Site-1 and site-2 are proteases. CM – cytoplasmic membrane, CP – cytoplasm. Adapted from (Heinrich & Wiegert, 2009).

Direct sensing

The environmental signal can be sensed directly by the anti-sigma factor leading to the conformational change resulting in the release of sigma factor from the association with anti-sigma factor (Paget, 2015). This system was described for the first time in *Streptomyces coeliocolor* for SigR-RsrA system responsible for the oxidative stress response (Figure 12) (Kang *et al.*, 1999). Sigma factor SigR is normally sequestered by binding anti-sigma factor RsrA located in the cell envelope. Upon oxidative stress RsrA is oxidised resulting in release of SigR, which directs transcription of its own operon (*sigR-rsrA*) and the thioredoxin reductase genes (*trxBA*). The induction of the thioredoxin system leads to reduction of RsrA and rebinding to SigR returning the cell to the balance state (Kang *et al.*, 1999).

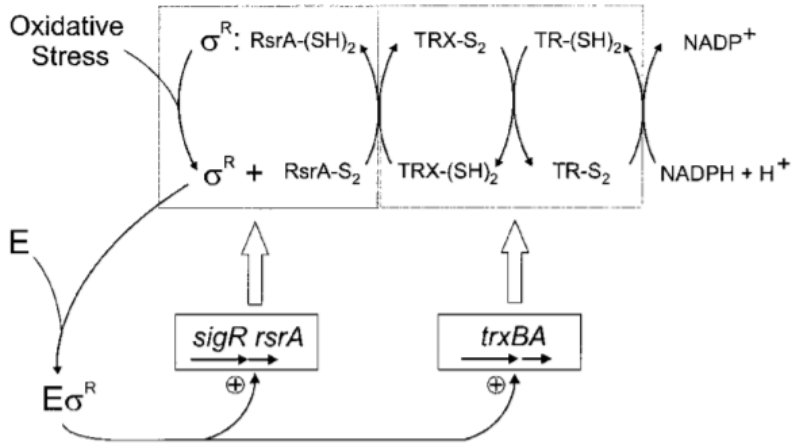


Figure 12. Model for a feedback regulatory loop of oxidative stress response mediated by σR. Adapted from (Kang *et al.*, 1999).

1.4.4.2 RbpA factor

RNA polymerase **b**inding **p**rotein A (RbpA) was discovered in *Streptomyces coelicolor* and is highly conserved among actinomycetes including *M. tuberculosis and M. smegmatis* (Paget *et al.*, 2001, Newell *et al.*, 2006). It is responsible for rifampicin resistance of RNAP in vitro and for basal levels of rifampicin resistance in vivo and it stimulates the transcription of HrdB dependent ribosomal promoters in *Streptomyces coelicolor* (Newell *et al.*, 2006, Dey *et al.*, 2010). Even though RbpA mutants grow slowly, they are viable and thus RbpA is not essential for the growth in *Streptomyces coelicolor* (Newell *et al.*, 2006). Tabib-Salazar et al. described the direct binding between RbpA protein and principle sigma factor HrdB and group 2 sigma factor HrdA. HrdB and RbpA interact via σ_2 domain of HrdB and C-terminal region of RbpA. The authors suggested that RbpA plays a key role in sigma cycle possibly acting as a chaperon-like protein that helps in the formation of active holoenzyme during potential stress conditions (Tabib-Salazar *et al.*, 2013).

1.4.4.3 6S RNA

Another way of sigma regulation is via 6S RNA. This small non-coding RNA was first discovered in *E coli*. in 1967 (Hindley, 1967) and its structure and function have been identified further (Brownlee, 1971). It is highly abundant among bacteria (Barrick *et al.*, 2005, Wehner *et al.*, 2014) including *Streptomyces coeliocolor* (Panek et al., 2008, Mikulik et al., 2014).

6S RNA binds σ^{70} of RNAP and inhibits transcription by competing with promoter DNA (Wassarman & Storz, 2000, Cavanagh & Wassarman, 2014). Region 4.2 of σ^{70} seems to be essential for its binding; it is also responsible for binding to -35 element of promoter DNA (Cavanagh *et al.*, 2008, Klocko & Wassarman, 2009).

6S RNA folds into closely related structures that have been conserved across various bacterial species. The structure consists of a single-stranded central bulge within a highly double-stranded molecule and thus it mimics a DNA template in an open promoter complex (RPo) (Figure 13) (Wassarman, 2002, Barrick *et al.*, 2005).

6S RNA binds to σ^{70} when the cells enter the stationary phase of growth and leads to a downregulation of σ^{70} -dependent transcription (Trotochaud & Wassarman, 2005). About 90 % of the 6S RNA in the cell associate with σ^{70} during exponential and stationary phase in *E. coli* (Wassarman & Storz, 2000).

6S RNA not only resembles an open promoter complex but it can be used as a template for $E\sigma^{70}$ to synthesize pRNA (14-20 nts RNA product) (Wassarman & Saecker, 2006, Gildehaus *et al.*, 2007). Synthesis of pRNA leads to the release of 6S RNA from the association with RNAP (Wassarman & Saecker, 2006). It is suggested that pRNA synthesis occurs during the outgrowth from stationary phase and serves as a liberation of RNAP from 6S RNA in response to nutrient availability (Wassarman & Saecker, 2006).

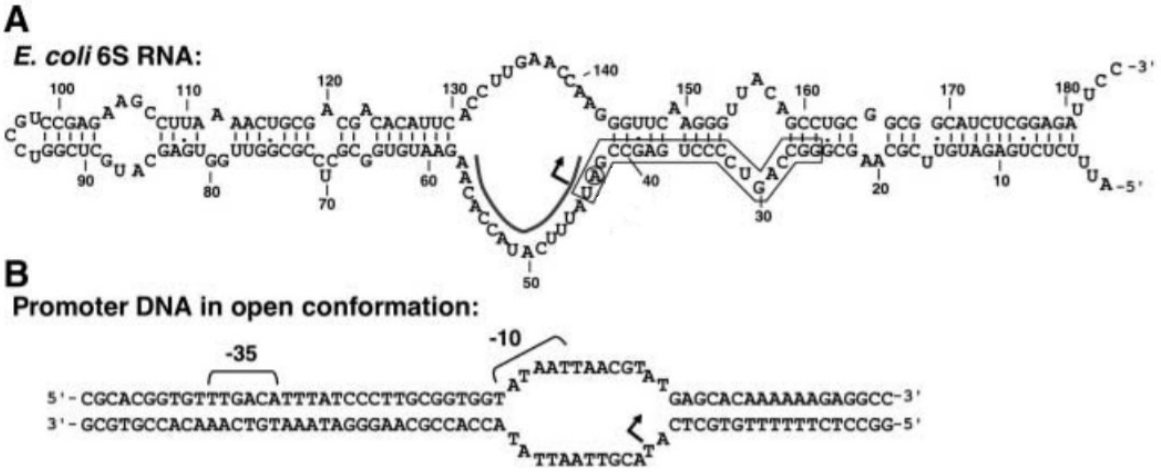


Figure 13. A) Schematic of secondary structure of *E. coli* 6S RNA. The sequence in 6S RNA complementary to the longest pRNA is boxed. B) Promoter DNA in open conformation. Adapted from (Wassarman & Saecker, 2006).

1.4.5 Sigma factors in *Streptomyces*

Sigma factors are a part of the metabolic pathways connected with primary and secondary metabolism; they participate in signal transduction systems and other complex regulatory mechanisms in the cells. Only a minor part of the 65 sigma factors in *Streptomyces* has been studied. These studies are based on the studying of deletion mutant of given sigma factors and only several systematic studies with identification of sigma regulons have been carried out.

Group 1 sigma factors contain HrdB sigma factor, whereas group 2 sigma factors are composed of HrdA, HrdC and HrdD sigma factors. Group 3 sigma factors are called sigB-like sigma factors, consisting of 10 sigma factors - SigB, SigF, SigG, SigH, SigI, SigK, SigL,SigM, SigN, WhiG. Finally, group 4 ECF sigma factors is comprised of 51 sigma factors. So far, only about 20 sigma factors have been characterized in *Streptomyces coelicolor*. We focused our interest on those sigma factors that are indispensable (HrdB) or were shown to be highly expressed during germination and early vegetative growth.

1.4.5.1 HrdB

HrdB is a principal and essential sigma factor in *Streptomyces* responsible for the transcription of housekeeping genes whose deletion is lethal (Buttner *et al.*, 1990, Shiina *et al.*, 1991). It is the functional homolog of σ^{70} in *E. coli* (Tanaka et al., 1988, Buttner, 1989). HrdB homologs are present in all *Streptomyces* species (Takahashi *et al.*, 1988).

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 (Fujii *et al.*, 1996, Sun *et al.*, 2017). 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 (Fujii *et al.*, 1996, Sun *et al.*, 2017). Similarly, HrdB in *S. avermitilis* regulates the transcription of the pathway specific regulatory gene *aveR* to trigger the biosynthesis of avermectin (Zhuo *et al.*, 2010). 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* (Aigle *et al.*, 2000). The presence of *hrdB* on a multicopy plasmid leads to the precocious overproduction of undecylprodigiosin (Aigle *et al.*, 2000)

Due to the connection of primary and secondary metabolism provided by HrdB, genetic manipulation of the HrdB has been used for enhancing antibiotic production. Genetic engineering was conducted by two different methods. In the first method, the site-mutated hrdB gene was introduced into the avermectin-high producing strain *S. avermitilis* 3-115, where it caused by 50 % higher levels of avermectin B1 production (Zhuo *et al.*, 2010). The second

method is based on the replacement of the native promoter of antibiotic biosynthetic genes or pathway-specific regulatory genes by the *hrdB* promoter to increase the levels of antibiotic biosynthesis, as the strength of the *hrdB* promoter is greater than other used *Streptomyces* promoters. The example is a significant increase of nikkomycin production by the replacement of the promoter of pathway-specific regulator *sanG* by *hrdB* promoter in *S. ansochromogenes* TH322 (Du *et al.*, 2013, Sun *et al.*, 2017).

HrdB has several known promoter binding sites that it binds. They were identified mostly by in vitro transcription or by S1 nuclease mapping. These genes are listed in Table 2. The proposed consensus sequence for HrdB is TTGACN-16 to 18 bp-TAGA(Pu)T (Strohl, 1992). The 2.4 and 4.2 regions (responsible for promoter recognition) of other *Streptomyces* sigma factors HrdA, HrdC and HrdD are identical or highly similar in amino acid sequence to HrdB, thus suggesting that they recognize similar promoter sequences (Buttner, 1989, Lonetto *et al.*, 1992). While HrdB is an essential sigma factor, the other three sigma factors HrdA, HrdC and HrdD are dispensable for the growth and their biological function is still unknown (Buttner *et al.*, 1990).

Table 2. Known HrdB dependent promoters

SCO number	Gene name	Protein name	Discovered by
SCO0561	*furA*	Fe regulatory protein	(Hahn *et al.*, 2000)
SCO0598	*sigB*	sigma factor SigB	(Cho *et al.*, 2001)
SCO1321	*tuf3*	elongation factor TU-3	(van Wezel *et al.*, 1995)
SCO1429	*chiD*	chitinase D	(Delic *et al.*, 1992, Saito *et al.*, 2000)
SCO2026	*gltB*	glutamate synthase	(Brown *et al.*, 1992)
SCO2082	*ftsZ*	cell division protein	(Flardh *et al.*, 2000)
SCO3471	*dagA*	extracellular agarase	(Brown *et al.*, 1992, Kang *et al.*, 1997)

SCO5003	*chiA*	chitinase A	(Saito *et al.*, 2000)
SCO5376	*chiC*	chitinase C	(Delic *et al.*, 1992, Saito *et al.*, 2000)
SCO5673	*chiB*	chitinase B	(Saito *et al.*, 2000)
SCO7263	*chiF*	chitinase F	(Saito *et al.*, 2000)
SCOr09	*rrnD*	rRNA operon D	(Baylis & Bibb, 1988, Kang *et al.*, 1997)
SCOr13	*rrnA*	rRNA operon A	(van Wezel *et al.*, 1994)
SCO5877	*redD*	transcriptional regulator	(Fujii *et al.*, 1996)
SCO5085	*actII-ORF4*	actinorhodin cluster activator protein	(Fujii *et al.*, 1996)

HrdB itself possesses two promoters. The first one, *p1* is indeed the primary promoter in the absence of stress and the second one, *p2* is about 50 nts downstream of the p1 and is connected with stress (Buttner *et al.*, 1990). *P2* was found to be SigR-dependent (Buttner *et al.*, 1990, Kim *et al.*, 2012). Diamide stress caused the decrease of the *p1* transcripts and increase of the *p2* SigR dependent-transcripts, thus suggesting that SigR is responsible for the maintaining the level and activity of HrdB under thiol-oxidative stress conditions (Kim *et al.*, 2012).

1.4.5.2 SigB

SigB sigma factor has been extensively studied among *Streptomyces*. It regulates morphological differentiation and secondary metabolism throughout the regulation of related genes such as *dpsA, whiB, redH* and *redZ*. *DpsA* gene is required for spore maturation, *whiB* is responsible for aerial hyphae formation, *redH* coding for phosphoenolpyruvate-utilizing enzyme, and *redZ* encodes a pathway-specific regulator for actinorhodin (RED)(Lee *et al.*, 2005, Facey *et al.*, 2009, Facey *et al.*, 2011). SigB controls the concentration of ppGpp synthesis, an important signalling molecule, influencing the regulation of genes needed for aerial hyphae formation. SigB also controls pathway-specific regulators (*cdaR*, *actII-ORF*)

(Hesketh *et al.*, 2007) by regulating relA protein, coding for ppGpp synthetase (Lee *et al.*, 2004). In addition, SigB governs the osmotic and oxidative stress response. It regulates the genes responsible for osmoadaptation, such as *osaB* (Figure 14) (Fernandez Martinez *et al.*, 2009), and activates gene expression of other two sigma factors, SigL and SigM (which play a role in sporulation) in a hierarchical order, and as well as itself during osmotic stress (Lee *et al.*, 2005). Salt induced SigB-dependent sigma factors were SigH, SigI, SigJ, HrdA, HrdB, HrdC, HrdD, SigE, SigR, SigT, SCO3613, SCO4409, SCO4866, SCO7104 (Lee *et al.*, 2005). The activation of gene expression of multiple sigma factors in response to stress supports the hypothesis about the existence of much more complex regulatory network (Karoonuthaisiri *et al.*, 2005). SigB further regulates oxidative stress response genes inducing the biosynthesis of mycothiol (major thiol buffer in actinomycetes), thioredoxin, cysteine and catalase (Cho *et al.*, 2001, Lee *et al.*, 2005). Finally, SigB regulates also the synthesis of cold shock proteins suggesting that it participates in cold shock response (Lee *et al.*, 2005).

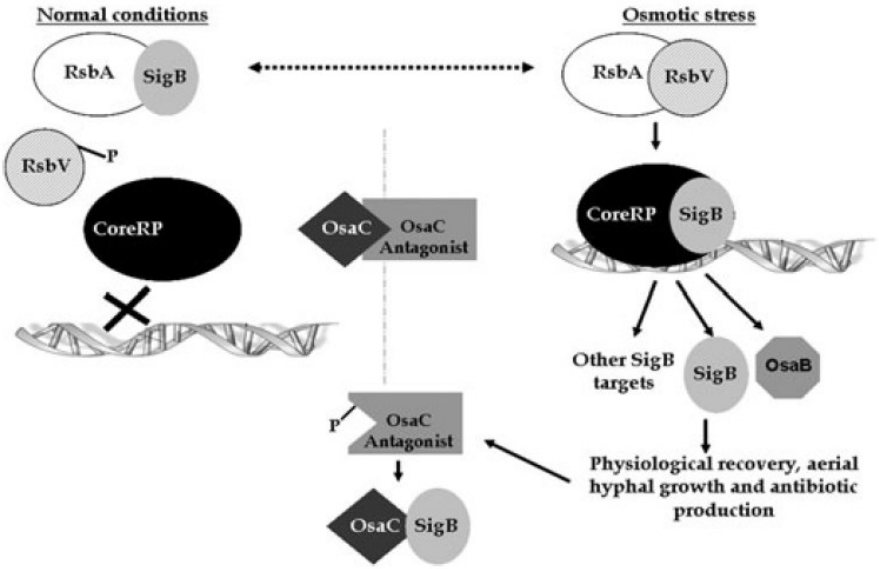


Figure 14. Under normal conditions SigB is sequestered by the anti-sigma factor RsbA. After the osmotic stress anti-anti-sigma factor RsbV is dephosphorylated and bound to anti-sigma factor RsbA leading to release of SigB resulting in activation of transcription of stress related genes including *osaB*. Physiological recovery of from osmotic stress results in activation of kinase domain of OsaC that than phosphorylates a predicted OsaC antagonist, releasing bound OsaC that can than associate with SigB, preventing continued expression of the sigB regulon (RsbA – anti-sigma factor, RsbV – anti-anti-sigma factor). Adapted from (Fernandez Martinez *et al.*, 2009).

1.4.5.3 SigH

SigH belongs to the SigB-like sigma factors and regulates osmotic stress response and morphological differentiation, in which influences septation of aerial hyphae (Kormanec *et al.*, 2000, Kelemen *et al.*, 2001, Sevcikova *et al.*, 2001, Viollier *et al.*, 2003).

SigH is regulated by its anti-sigma factor PrsH/UshX and anti-anti-sigma factor BldG by partner switching-like mechanism (Sevcikova *et al.*, 2001, Sevcikova & Kormanec, 2002, Viollier *et al.*, 2003, Viollier *et al.*, 2003, Sevcikova *et al.*, 2010). In unstressed conditions, SigH is sequestered by the anti-sigma factor UshX. After osmotic stress, BldG is activated by

dephosphorylation with an unknown phosphatase and interacts with UshX releasing SigH to activate stress related genes. Dephosphorylated BldG also interacts with the ApgA anti-sigma factor, resulting in the release of another SigB homologue, to activate its stress related regulon (Figure 15) (Sevcikova *et al.*, 2010).

It was also shown that SigH is posttranslationally regulated. SigH is expressed as three primary translational products – SigH-σ^{37}, SigH- σ^{51}, SigH- σ^{52}. While SigH- $\sigma^{51/52}$ as the primary gene products were present at the early stages of growth and their level decreased in later stages of growth, the levels of SigH-σ^{37} and two proteolytic derivatives of SigH- $\sigma^{51/52}$ (with size of 34 and 38 kDa) were increased. The occurrence of these isoforms of SigH is correlated with the developmental stage and differentiation (Viollier *et al.*, 2003).

SigH sigma factor regulates the transcription of *gltB* – glutamate synthase which plays a role in osmotic stress response (Kormanec & Sevcikova, 2002). It also regulates the transcription of SsgB, a regulator of aerial hyphae septation (Kormanec & Sevcikova, 2002, Keijser *et al.*, 2003, Sevcikova & Kormanec, 2003), and the transcription of *sigJ* sigma factor gene (Mazurakova *et al.*, 2006).

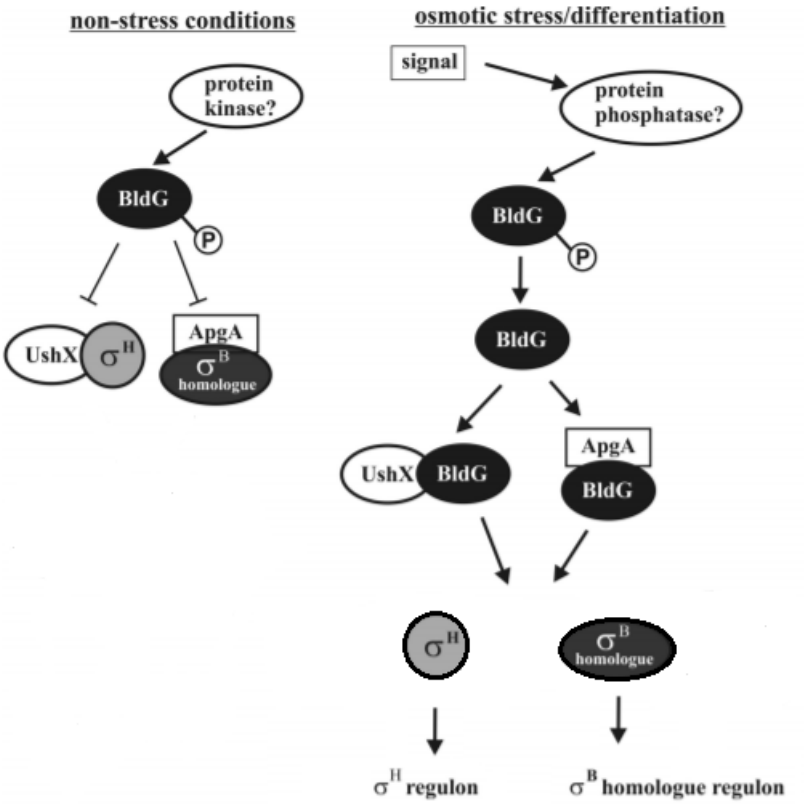


Figure 15. Model of stress response of sigH and sigB homologue. In unstressed conditions sigH is sequestered by anti-sigma factor UshX. After osmotic stress induction, anti-anti-sigma factor BldG is dephosphorylated and interact with UshX releasing of SigH to activate stress related genes. Here is also depicted another pathway of activation of sigB homologue regulon. Dephosphorylated BldG interact also with the anti-sigma factor ApgA resulting in release of SigB homologue to activated its regulon. Adapted from (Sevcikova *et al.*, 2010).

1.4.5.4 SigE

SigE is one of the 51 ECF sigma factors in *Streptomyces coelicolor*. It is required for normal cell envelope integrity participating in cell envelope stress response (Hutchings *et al.*, 2006, Tran *et al.*, 2019). This stress response is mediated in contribution of two-component system consisting of CseB (response regulator) and CseC (transmembrane sensor histidine kinase) (Figure 16), so there is no anti-sigma factor involved in the SigE-dependent signal transduction

system unlike in *E. coli*. or in other ECF sigma factors (Hutchings *et al.*, 2006) where the sigma factor is bound to an anti-sigma factor as a transmembrane protein which monitors cell envelope stress throughout its sensor domain (Mascher, 2013, Tran *et al.*, 2019).

Recently, it was published SigE regulon identified by ChIP-seq containing over 50 genes with cell-envelope related function and many other targets participating in signal transduction systems and regulation, including sigma factor HrdD (Tran *et al.*, 2019). SigE governs the transcription of HrdD at two different promoters: hrdDp1 and hrdDp2 (Kang *et al.*, 1997, Paget *et al.*, 1999). Both promoters were induced under Mg^{2+} deficiency suggesting that the intracellular activity of SigE is increased when the cell wall is altered (Paget *et al.*, 1999). Within the cell envelope related genes there are genes involved in peptidoglycan assembly, cell wall teichoic acid deposition, lateral cell wall synthesis and sporulation, membrane modification and maintenance of integrity, such as penicillin binding proteins (PBPs), LCP proteins (responsible for the attachment of wall teichoic acid and capsular polysaccharide to the peptidoglycan), MreB (actin homolog), WhiB (essential for the initiation of sporulation septation), L,D-transpeptidases, PspA protein homologue, etc. These results suggest SigE to be a major regulator of cell envelope stress response (Tran *et al.*, 2019).

They also identified SigE consensus promoter sequence. However, it was found that the majority of the promoters tested by S1 nuclease mapping were only partially dependent on SigE and most of these promoters were vancomycin-inducible in the SigE-mutant. This finding suggests that there are additional ECF sigma factors that recognize these promoters (Tran *et al.*, 2019). This phenomenon was described in several bacterial species for example in *B. subtillis*, where SigM, SigW and SigX can transcribe genes from the same promoters (Kingston *et al.*, 2013, Mascher, 2013) or in *S. coelicolor* where SigR-dependent promoters still remain active

in SigR null mutant during oxidative stress thus suggesting a contribution of other ECF sigma factors that ensure stress response (Paget *et al.*, 2001, Kim *et al.*, 2012).

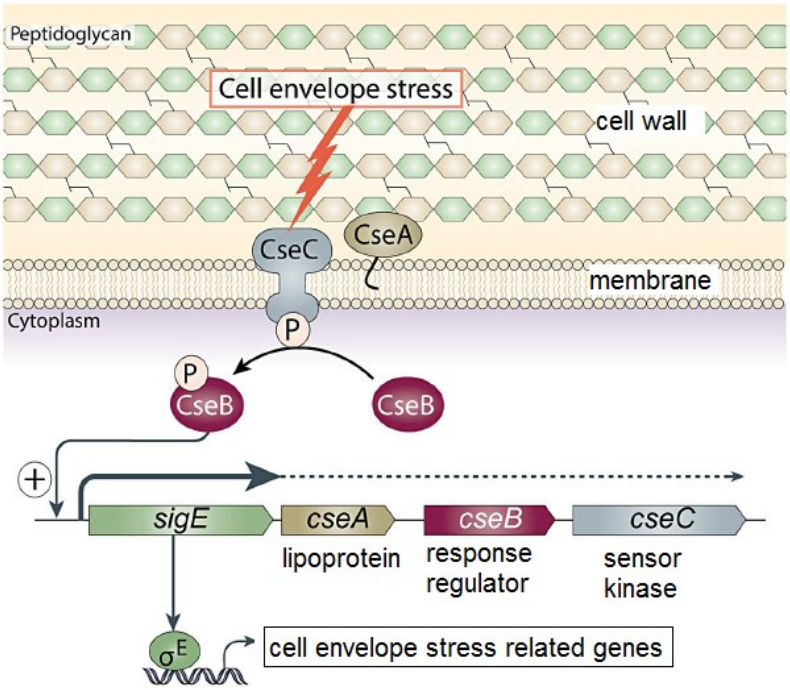


Figure 16. Model for the σ^E cell envelope stress response. Expression of the gene encoding σE (*sigE*) is regulated at the level of transcription by the CseB/CseC two-component signal transduction system. In response to signals originating in the cell envelope when it is under stress, the sensor kinase, CseC, becomes autophosphorylated and transfers this phosphate to the response regulator, CseB. Phospho-CseB activates the promoter of the *sigE* operon, and σE is recruited by core RNA polymerase to transcribe its regulon. Note that >90% transcription from the *sigE* promoter terminates just downstream of *sigE* and that the promoter of the *sigE* gene itself is not a σE target. CseA is a lipoprotein localised to the extracytoplasmic face of the cell membrane and loss of the CseA results in upregulation of the *sigE* promoter. Adapted from (Tran *et al.*, 2019).

1.4.5.5 SigR

SigR is an ECF sigma factor responsible for oxidative stress response via inducing the thioredoxin system in cooperation with its anti-sigma factor (Paget *et al.*, 1998, Kang *et al.*,

1999). SigR directly regulates genes responsible for maintaining redox balance as well as thiol homeostasis and thus protects the cells against oxidation changes (Kallifidas *et al.*, 2010, Park *et al.*, 2019). Bacterial cytoplasm is maintained as a reducing environment. Due to the absence of glutathione in actinomycetes, which is the major thiol-disulfide redox buffer, the reducing environment is ensured by mycothiol (Newton *et al.*, 1996) and thioredoxin system (Aharonowitz *et al.*, 1993, Cohen *et al.*, 1993). The activation of this system is triggered by SigR-RsrA in response to oxidative stress as depicted in Figure 17 (Paget *et al.*, 1998).

SigR gene has two promoters. In the absence of oxidative stress, SigR is expressed from the first promoter. After the exposure to oxidative stress, SigR is released from the binding with RsrA and activates its own expression from the second promoter. It was shown that the first promoter has a highly unusual GTC start codon leading to another level of regulation in which SigR translation is inhibited by translation initiation factor 3 (IF3). IF3 functions in bacteria as the repressor of translation from noncanonical start codons by destabilizing 30S initiation complexes (Petrelli *et al.*, 2001). Changing the GTC start codon to canonical start codon (ATG, GTG, TTG) results in an overproduction of SigR in relative to anti-sigma factor RsrA, leading to an unregulated and constitutive expression of the SigR regulon. Even if SigR and RsrA are in the same operon, RsrA has its own promoter for its independent expression. Therefore, in the absence of oxidative stress, SigR is buffered with the higher amount of anti-sigma factor RsrA (Feeney *et al.*, 2017).

Kim et al. identified SigR regulon by ChIP-chip procedure. They revealed more than 163 target genes under SigR control transcribed from 108 promoters with the consensus sequence GGAAY-N18-19-GTT. SigR regulon includes genes responsible for thiol homeostasis (*trxAB, trxC, trxA4, mrxA*, …), sulphur metabolism, ribosome modulation (*rpmJ, rpmG3*, …), guanine nucleotide metabolism, protein degradation, transcriptional regulators (*sigR-rsrA, ndgR, hrdB,*

hrdD, rbpA), energy metabolism, DNA repair and recombination, protection against UV and thiol-reactive damages, cofactor metabolism and lipid metabolism. They have also shown that SigR participates on the activity and expression level of the housekeeping sigma factor HrdB during thiol-oxidative stress (Kim *et al.*, 2012).

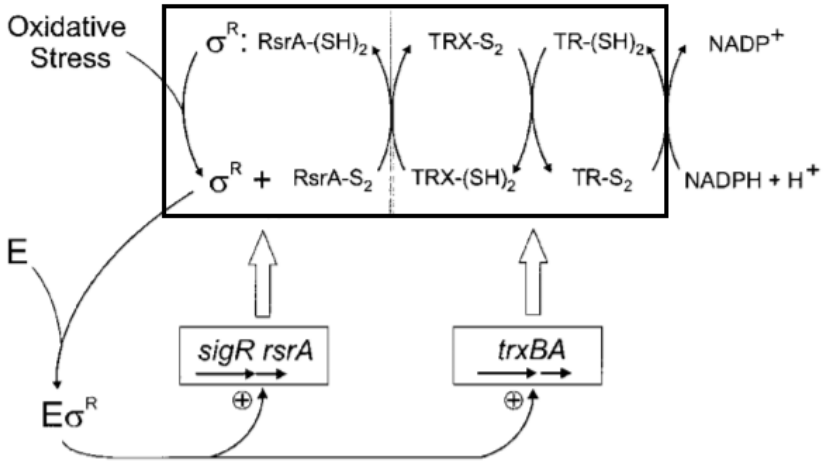


Figure 17. Model for a feedback regulatory loop that modulates expression of the thioredoxin system in response to oxidative stress. Under unstressed conditions, σ^R is sequestered by binding to the reduced form of RsrA [RsrA-(SH)2]. Upon oxidative stress, RsrA is inactivated by the formation of intramolecular disulfide bond(s) (RsrA-S2), releasing σ^R. σ^R then binds core RNA polymerase and directs transcription of its own operon (sigR-rsrA) and the thioredoxin (TRX)/thioredoxin reductase (TR) genes (trxBA). The induction of the thioredoxin system shifts the intracellular thiol–disulfide balance and reduces RsrA to its active state in which it rebinds σ^R, thereby returning the system to the pre-stimulus state. Adapted from (Kang *et al.*, 1999).

1.4.5.6 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 (Wang

et al., 2013). 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 (Shu *et al.*, 2009).

According to the study of Bobek and his colleagues of gene expression changes in spore germination, SigQ was found to be the most highly expressed (>18x) between the time interval 0 – 30 min of growth. Dormant spores also contain high levels of SigQ sigma factor. In the rehydration period, in the initial 10 min of growth, its expression was slightly decreased before its highest increase (Bobek *et al.*, 2014). 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.

1.5 Small RNAs

Bacterial small RNAs are widespread and functionally heterogeneous short RNA transcripts usually in the size of ~50 – 500 nucleotides (Gottesman & Storz, 2011). They are commonly encoded in intergenic regions, within the genes or in the 5' or 3' untranslated regions (UTRs) (Gottesman & Storz, 2011, Miyakoshi *et al.*, 2015, Heidrich *et al.*, 2017, Ren *et al.*, 2017) possessing own promoters which can be induced by stress conditions, environmental and developmental changes (Waters & Storz, 2009, Storz *et al.*, 2011, Heueis *et al.*, 2014). sRNAs also play an important role during virulence, such as in *Shigella dysenteriae* (Murphy & Payne, 2007), or in quorum sensing, such as in *Vibrio harvei* or *Vibrio cholerae* (Lenz *et al.*, 2004) or in *Sinorhizobium melliloti* (Baumgardt *et al.*, 2016), or influence the life cycle differentiation in *Chlamydia trachomatis* (Grieshaber *et al.*, 2006, Papenfort & Vogel, 2009).

Many sRNAs regulate their target mRNAs through more or less limited base pairing. This base pairing between sRNAs and target mRNAs leads to an inhibition or activation of translation, changes the mRNA stability and affects the target gene expression via a variety of mechanisms (Beisel & Storz, 2010, Barquist & Vogel, 2015, Updegrove *et al.*, 2015). Due to its size, one might think that longer sRNA has a longer region for base pairing, but it is not true. This region is called seed region and is responsible for the recognizing of the target mRNA and binding to them. Most seed sequences are less than 20 nts long and well conserved (Bandyra *et al.*, 2012, Carrier *et al.*, 2018).

Bacterial mRNAs are not the only targets of sRNAs. It is also known that number of sRNAs bind to cellular proteins to modulate their activity. For example CsrB-like sRNA binds to CsrA/RsmA proteins or 6S RNA interacts with RNA polymerase to regulate gene expression (Babitzke & Romeo, 2007, Wassarman, 2007, Lapouge *et al.*, 2008)

Although they are referred to as non-coding, because of lacking open reading frame (ORF), there are some exceptions. Three sRNAs are known to have a dual function: RNAIII and *psm-mec* RNA in *S. aureus,* and SgrS in *E. coli*. RNA III controls various virulence factors and encodes the short 26 amino acid hemolytic peptide δ-hemolysin (Bronesky *et al.*, 2016). The *psm-mec* regulatory RNA interacts with the virulence-associated *agrA* mRNA inhibiting its translation and encodes a small peptide PSM-mec with proinflammatory properties (Kaito *et al.*, 2013). Finally, SgrS sRNA regulates multiple target mRNA and encodes a small 43 amino acid protein SgrT (Lloyd *et al.*, 2017).

Bacterial sRNAs can be classified into two groups: ***Cis*-encoded sRNAs** and ***trans*-encoded sRNAs** (Storz *et al.*, 2011).

1.5.1 *Cis*-encoded antisense sRNAs

These regulatory RNAs are encoded in *cis* on the DNA strand opposite the target mRNA and share extended regions of complete complementarity with their target (Georg & Hess, 2018). Therefore they are called *cis*-antisense sRNAs. They are highly structured (one to four stem-loops), mostly untranslated (Brantl, 2007), and have been found first mainly in plasmids, phages and transposons (Brantl, 2002).

Antisense RNAs are usually between 100 – 300 nts long, but many asRNAs are much longer, ranging from 700 – 3500 nts. Antisense RNAs with the size of 7000 nts were found in *Prochlorococcus* sp. strain MED4, overlapping 14 genes of a ribosomal protein operon, protecting them from RNase E degradation during phage infection (Stazic *et al.*, 2011).

The secondary structure of the efficient asRNA is created by the 5 – 8 GC rich loops and stems are often interrupted by bulges to prevent dsRNase degradation and facilitate melting during the interaction between asRNA and its target mRNA (Hjalt & Wagner, 1992, Hjalt & Wagner, 1995).

In the last decades, a lot of asRNAs have been found in bacterial genomes – in *E.coli, B. subtilis, Synechocystis species, Streptomyces coelicolor* etc. (Brantl, 2002, Wagner *et al.*, 2002, Brantl, 2007, Setinova *et al.*, 2017). Within the first documented and characterised asRNAs there were micF RNA inhibiting the gene expression of membrane porin ompF (Aiba *et al.*, 1987), RNA I controlling ColE1 replication (Itoh & Tomizawa, 1980) and the OOP asRNA of bacteriophage λ (Krinke & Wulff, 1987). The occurrence of asRNAs is becoming widespread. They are documented in all three domains of life – in Bacteria, Archaea as well as Eukaryota (Georg & Hess, 2011). The occurrence of *cis*-antisense sRNAs varies between the different genus of bacteria from 13 % in *Bacillus subtilis* (Nicolas *et al.*, 2012), 27 % in *Synechocystic* PCC6803

(Mitschke *et al.*, 2011), 30 % in *Anabaena spp.* PCC7120 (Mitschke *et al.*, 2011), and 46 % in *Helicobacter pylori* (Sharma *et al.*, 2010) to 49 % in *Staphylococcus aureus* (Lasa *et al.*, 2011).

1.5.1.1 Mechanism of action

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 (Thomason & Storz, 2010). *Cis*-encoded asRNAs may impact transcription, translation or mRNA stability. They are described below in the Figure 18 and separately described in the Figure 19A in the comparison with *trans*-encoded sRNAs (Brantl, 2007, Gerdes & Wagner, 2007, Fozo *et al.*, 2008).

Transcription interference and attenuation

Antisense RNAs affect the transcription of given gene by two different ways – transcription interference and transcription attenuation.

Transcription interference is characterized as the situation when transcription from one promoter is inhibited by the transcription from a second promoter present in *cis* (Figure 18A) (Shearwin *et al.*, 2005). Transcription interference does not involve base pairing and does not occur when the asRNA is provided in *trans* (Thomason & Storz, 2010).

Antisense RNA can influence the transcription of the target mRNA by attenuation whereby transcription of the opposite strand is prematurely terminated (Figure 18B). In some cases base-pairing between asRNA and target mRNA can enable the formation of termination structure (Thomason & Storz, 2010).

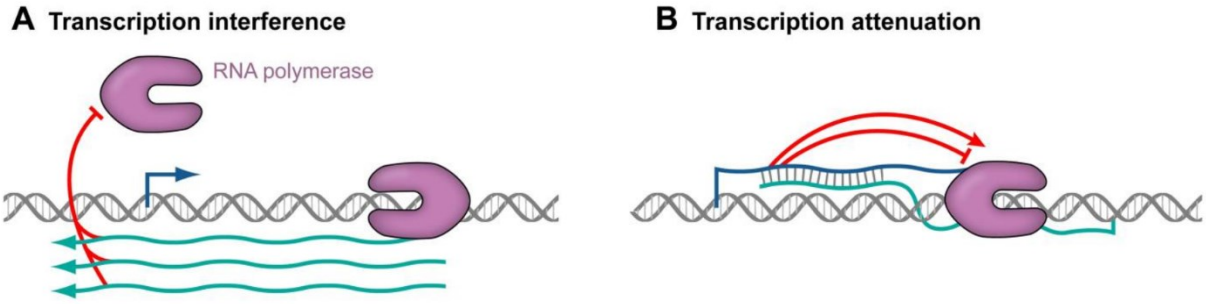


Figure 18. Mechanism of action of asRNAs in transcription. Antisense RNAs can induce transcription interference (A), where transcription from one promoter blocks transcription from a second promoter by preventing RNA polymerase from either binding or extending a transcript encoded on the opposite strand. In transcription attenuation (B), base pairing of the antisense RNA to the target RNA causes changes in the target RNA structure ultimately affecting transcription termination. Adapted from (Thomason & Storz, 2010).

Target mRNA degradation/stabilisation

A common mechanism of action is targeting the given mRNA to degradation by base pairing with them. In bacteria, two major endoribonucleases are associated with asRNA inducing mRNA cleavage (Thomason & Storz, 2010): RNase III and RNase E. RNase III cleaves dsRNA and has been initially found to be related with the maturation of 16S and 23S rRNA (Gegenheimer & Apirion, 1981, Carpousis *et al.*, 2009); later, it was found to play a role in asRNA mediated regulation (Blomberg *et al.*, 1990, Gerdes *et al.*, 1992). RNase E cleaves ssRNA and is a part of degradosome cooperating with Hfq protein in *E. coli* (Carpousis *et al.*, 2009).

Base pairing of asRNA with target mRNA can also alter the structure in order to protect mRNA from the cleavage by RNases and thus influence mRNA stability (Thomason & Storz, 2010).

Affecting the translation

Antisense RNAs can base-pair with ribosome binding site (RBS) and thereby cause blocking translation of target mRNA. Nevertheless, the binding of asRNA to RBS can destabilize target mRNA and subject it to degradation by RNases (Lee & Groisman, 2010). Antisense RNAs could also positively or negatively influence the translation by the indirect mechanism. Antisense RNA can modify the mRNA structure at a RBS by binding to the site far away from them (Thomason & Storz, 2010).

1.5.2 *Trans*-encoded sRNAs

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 (Waters & Storz, 2009, Bandyra *et al.*, 2012, Carrier *et al.*, 2018). Due to the limited complementarity, an Hfq chaperone is needed in several bacteria for the base pairing between sRNA and target mRNA (Brennan & Link, 2007, Waters & Storz, 2009). It has to facilitate RNA-RNA interactions between the sRNA and the target mRNA (Valentin-Hansen *et al.*, 2004, Aiba, 2007). The hexameric Hfq ring may ensure the melting of inhibitory secondary structure of the RNAs (Maki *et al.*, 2008). Hfq also contributes to the regulation of sRNAs levels because it protects sRNAs from degradation in the absence of base pairing with mRNAs (Valentin-Hansen *et al.*, 2004, Aiba, 2007, Brennan & Link, 2007). Hfq may also serve to recruit degradation machinery including RNase E to degrade RNAs (Waters & Storz, 2009, Morita & Aiba, 2011). Although *trans*-encoded sRNAs in Gram-negative bacteria require chaperone Hfq for its action (Valentin-Hansen *et al.*, 2004, Bohn *et al.*, 2007, Jousselin *et al.*, 2009, Vogel & Luisi, 2011, Kavita *et al.*, 2018), it has never been reported in Gram-positive bacteria except for *Listeria monocytogenes* (Nielsen *et al.*, 2010). Hfq protein or its homologue has also never been

identified in *Streptomyces* (D'Alia *et al.*, 2010). 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* where further global approach experiments should be done to clarify whether its role in the promotion of sRNA/mRNA binding is important only for the case of SR1/*ahrC* or also in other cases (Muller *et al.*, 2019). Recently, a new RNA chaperone ProQ, acting in Gram-negative bacteria, was discovered (Attaiech *et al.*, 2016, Smirnov *et al.*, 2016). ProQ promotes the binding between sRNA RaiZ and *hu-α* mRNA to inhibit its translation (Smirnov *et al.*, 2017). Further RNA chaperone, identified previously, is FinO encoded in F and R1 plasmids, enhancing the interaction of antisense RNA FinP and its target mRNA *traJ* (Jerome et al., 1999, Mark Glover et al., 2015).

Trans-encoded sRNAs are responsible for the regulation of translation and/or for the stability of target mRNAs (Gottesman, 2005, Aiba, 2007). The regulation is in many cases negative (Gottesman, 2005, Aiba, 2007). The interaction between the sRNA and its target mRNA usually results in translational inhibition, mRNA degradation, or both (Figure 19B). These sRNAs primarily bind to the 5'UTR of the target mRNAs and most often block the ribosome-binding site (RBS) (Waters & Storz, 2009). Nevertheless, there are some sRNAs such as GcvB or RyhB in *E. coli* acting far upstream of the RBS (Sharma *et al.*, 2007, Vecerek *et al.*, 2007). However, sRNAs can also activate the expression of their target mRNAs. It is carried out by an anti-antisense mechanism, whereby base pairing of the sRNA to the target mRNA causes a disruption of an inhibitory secondary structure of the target mRNA, resulting in an uncovering of the ribosome-binding site to allow the translation (Figure 19B) (Gottesman, 2005, Waters & Storz, 2009, Beisel & Storz, 2010). Examples of such regulation are RyhB or DsrA in *E. coli*, both acting as repressors and activators of gene expression (Majdalani *et al.*, 1998, Prevost *et al.*, 2007, Frohlich & Vogel, 2009).

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. The regions of potential base pairing between *trans*-encoded sRNAs and target mRNA typically include the region of 10 – 25 nucleotides. Yet, it was found that only a core of the nucleotides seems to be critical for the regulation (Gottesman, 2005, Waters & Storz, 2009). For example, for the binding between SgrS sRNA and *ptsG* mRNA, six nucleotides seem to be important (Kawamoto *et al.*, 2006).

Most of *trans*-encoded sRNAs in *E. coli* are in contrast to *cis*-encoded sRNAs, expressed under specific conditions. They can be induced by oxidative stress (OxyR-activated OxyS), outer membrane stress (σ^E-induced MicA and RyhB), changes in glucose concentration (CRP-repressed Spot42 and CRP-activated CyaR), and low iron conditions (Fur-repressed RyhB) (Urbanowski *et al.*, 2000, Gottesman, 2005, Gorke & Vogel, 2008, Johansen *et al.*, 2008, De Lay & Gottesman, 2009).

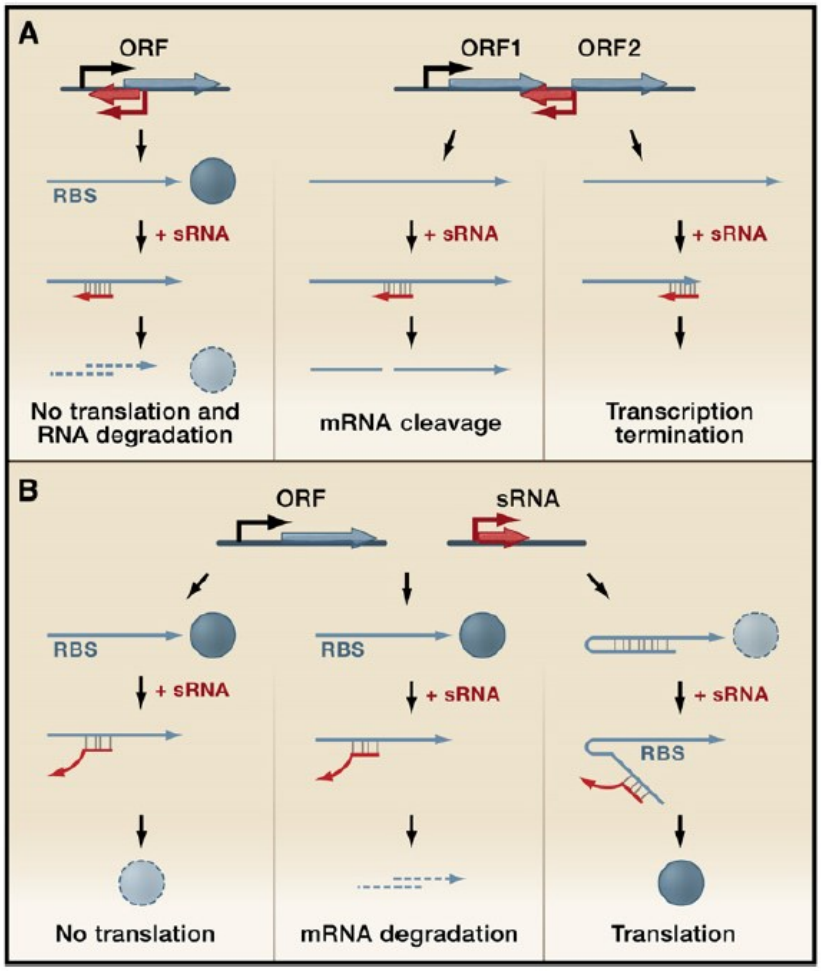


Figure 19. A) **Mechanism of action of *cis*-encoded sRNAs.** Cis-encoded sRNAs are in red, their target mRNA is in blue. **Left panel** – sRNA encoded in 5' UTR of the target mRNA bind to the target mRNA in ribosome binding site (RBS) and inhibit translation resulting in RNA degradation. **Right panels** – sRNA encoded opposite to the sequence separating two genes in an operon in the first case bind to the target mRNA recruiting RNA degradation machinery resulting in mRNA cleavage and in the second case bind to the target mRNA resulting in the termination of transcription B) **Mechanism of action of *trans*-encoded antisense sRNAs.** *Trans*-encoded sRNAs, located separately from their target genes, are in red, their target mRNA is in blue. **Left panel** - *trans*-encoded sRNA can act negatively by binding to the 5'UTR, blocking RBS and inhibit translation. **Middle panel** – *trans*-encodes sRNA bind to the target mRNA resulting in recruiting RNases and RNA degradation. **Right panel** – example of positive acting of trans-encoded sRNA when the base pairing between sRNA and target mRNA causes opening of inhibitory secondary structure resulting in accessing the RBS and translation. Adapted from (Waters & Storz, 2009).

1.5.3 sRNAs in *Streptomyces*

Several systematic approaches were carried out to identify globally novel sRNAs in *Streptomyces* (Panek *et al.*, 2008, Swiercz *et al.*, 2008, Vockenhuber *et al.*, 2011).

The first genome-wide search for novel sRNAs was done by our lab. BLAST search of conserved intergenic regions between *S. coelicolor* and *S. avermitilis* was performed and afterwards co-localized Rho-independent terminators were identified. 32 novel sRNAs were predicted and out of them 20 were examined by microarray analysis. 9 of them were experimentally verified by RT-PCR. The study included also the structural and functional analysis of predicted sRNAs using RNAz revealing that nearly all (29 out of the 32) of the predicted sRNAs possess strongly conserved secondary structure; functional feature was identified only for one sRNA, suggesting that it is 6S RNA (Panek *et al.*, 2008).

The next systematic approach was performed by Elliot and co-workers. They combined BLAST and sRNA finder to identify 114 IGR containing possible sRNAs. Twenty IGRs were further examined by Northern blot analysis from the samples grown in rich and minimal medium during vegetative growth, aerial mycelium formation and sporulation. Only six potential sRNAs were successfully verified exhibiting some degree of medium specificity, amongst which three were exclusively expressed under one culture condition which is slightly unusual in *S. coeliocolor*. Finally, they cloned sRNAs to further identify them; they identified further three sRNAs and they observed their expression in developmental mutants. Interestingly, only two of the identified sRNAs had been found in the results of our lab (Swiercz *et al.*, 2008).

The first deep sequencing approach, performed by Suess lab using the 454 technology, followed. They analysed the transcriptome at the end of the exponential phase of growth, when streptomycetes start to produce secondary metabolites. They identified 63 non-coding RNAs,

including 29 cis-encoded sRNAs, and the expression of 11 sRNAs was confirmed by northern blot. Nine of them exhibited strong growth-phase dependency (Vockenhuber *et al.*, 2011).

Another systematic approach was done by Horinouchi lab. They used bioinformatic analysis to identify 54 sRNAs in *S. griseus* which are conserved also in *S. coelicolor* and *S. avermitilis*. 17 sRNAs were experimentally validated by northern blot and RT-PCR. Seven of them were transcribed in growth-phase dependent manner. They performed also phenotypic analysis of sRNAs deletion mutants but no obvious phenotype was observed. Interestingly, seven sRNAs were significantly affected by a mutation of AdpA (encoding the central transcriptional regulator of the A-factor regulatory cascade participating in the regulation of morphological differentiation and secondary metabolism in *S. griseus*) (Tezuka *et al.*, 2009).

Another whole-genome approach lying in comparative RNA sequencing analysis of three different *Streptomyces* species (*S. coeliocolor*, *S. avermitilis*, *S. venezuelae*) was performed by Elliot lab. All strains were cultivated in the same conditions and RNA was isolated at three different developmental stages, subsequently pooled and sequenced. Hundreds of novel cis-antisense sRNAs and intergenic sRNAs were identified; 99 asRNAs were detected in *S. coelicolor*, 79 asRNAs were detected in *S. venezuelae* and new 59 asRNAs were detected in *S. avermitilis*. The vast majority of these asRNAs were species specific. Only 11 of them were conserved in all *Streptomyces* species (Moody *et al.*, 2013).

Small RNAs in *Streptomyces coelicolor* are named with the abbreviation **scr** according to ***S****treptomyces* ***c****oelicolor* **R**NA, followed by the SCO gene number of the downstream protein coding gene (Swiercz *et al.*, 2008).

Scr5239 is one of the best characterized sRNA in *Streptomyces*. It is 159 nt long and highly structured and is encoded between SCO5238 (TetR family regulator with unknown function) and SCO5239 (highly conserved histidine kinase). It can be found *in silico* in 19 of the 31

Streptomyces genomes (Vockenhuber *et al.*, 2011). This sRNA represses expression of *dagA* gene coded for extracellular agarase which participates in the degradation of agar by direct base pairing between 33 nt and 52 downstream of the ribosome-binding site. In 2014, the second target of scr5239 – *metE* was discovered. *MetE* gene coded for methionine synthase, playing a role in methionine biosynthesis. The regulation of this second target mRNA is also negative. The sRNA scr5239 is constitutively expressed in a basal level throughout the whole developmental cycle, decreased only in stationary phase under nitrogen-limiting conditions, and its expression level is also dependent on the availability of different amino acids such as glutamine, glutamate and methionine (Vockenhuber & Suess, 2012, Vockenhuber *et al.*, 2015). The second sRNA whose target is known, is cnc2198.1. It is 88 nt long asRNA that represses the expression of *glnA* coded for glutamine synthetase. Overexpression of the cnc2198.1 resulted in a 40 % reduction of GlnA (D'Alia *et al.*, 2010).

Elliot lab was trying to identify the biological function of scr4677. They found out that it is a *cis*-antisense sRNA negatively affecting SCO4676 coded for regulatory protein influencing actinorhodin production under specific growth conditions. But it was not fully described and further experiments need to be conducted (Hindra *et al.*, 2014).

2 The aims of the thesis

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 Experimental methods

3.1 Methods used for identification of sigma regulons

3.1.1 Strains and growth conditions

Strains used in our work are S. coelicolor M145, E. coli K-12 MG1655 (Datsenko & Wanner, 2000) and derivatives from GM2929 (MacNeil et al., 1992). 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) (Kieser et al., 2000). 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 3.

Table 3. Strains, plasmids and oligonucleotides used in this study

Strains	Genotype/comments	Reference/source
S. coelicolor		
M145 A3(2) WT	SCP1-, SCP2-	(Bentley *et al.*, 2002)
M145 A3(2) *sigQ*-HA	HA tagged *sigQ* mutant strain:: *apr oriT* cassette	This study
M145 A3(2) *hrdB*-HA	HA tagged *hrdB* mutant strain:: *apr oriT* cassette	This study
E. coli		
ET12567	*dam, dcm, hsdM, hsdS, hsdR, cat, tet*	(Flett *et al.*, 1997), (MacNeil *et al.*, 1992)
BW25113	K-12 derivative; Δ(*araD-araB*)*567* Δ *lacZ4787*(::*rrnB-4*) *lacIp4000*(*lacI*^{Q}), l-*rpoS369*(*Am*) *rph-1*	(Datsenko & Wanner, 2000)

Plasmids/cosmids		
pIJ773	P1-FRT-oriT-aac(3)IV-FRT-P2 (plasmid template for amplification of the *apr oriT* cassette for 'Redirect' PCRtargeting)	(Gust *et al.*, 2003)
pIJ790	λ-RED (gam, bet, exo), cat, araC, rep101^{ts}	(Gust *et al.*, 2003)
pUZ8002	tra, neo, RP4	(Paget *et al.*, 1999)
St5B8	carb, kan	Cosmid library University of Wales, Swansea
2StK8	carb, kan	Cosmid library University of Wales, Swansea
2StK8 *sigQ*-HA	carb, kan, HA tagged *sigQ* mutant strain:: *apr oriT* cassette	This study
St5B8 *hrdB*-HA	carb, kan, HA tagged *hrdB* mutant strain:: *apr oriT* cassette	This study
Oligonucleotides		
SigQ_HA_left	GGCCGTGACCAGGAGGACGCCTTCGGCGAACTGGTCGC CTACCCGTACGACGTCCCGGACTACGCCTGAGAAGTTC CCGCCAGCCTCGC	
SigQ_HA_right	AGCCCCGCCCCCTCGGGCCGGTCCCCCCGGCCCGTGCC TGGAATAGGAACTTATGAGC	
HrdB_HA_up	CACCCCTCGCGCTCGCAGGTGCTGCGCGACTACCTCGA CTACCCATACGACGTCCCAGACTACGCTTAGATTCCGG GGATCCGTCGACC	
HrdB_HA_down	CGTCTGGTCGTACCGCCGGTCCGTACGGTCGGCTACGA CTGTAGGCTGGAGCTGCTTC	

3.1.1.1 Spore germination

The details regarding *S. coelicolor* M145 spore cultivation and growth were published in work by (Strakova *et al.*, 2013). Briefly, matured spores were harvested after 14 days of growth in 28°C on solid agar plates (0.4% (w/v) yeast extract, 1% (w/v) malt extract, 0.4% (w/v) glucose, 2.5% (w/v) bacterial agar, pH 7.2) overlaid with cellophane discs. For the germination, spores were activated by mechanical disruption of the outer coat and 10 min heat shocked in 50°C to boost synchronicity. The germination course proceeded in liquid AM medium (20 amino acids at 0.2 mM, 20 mM KH_2PO_4, 30 mM Na_2HPO_4, 2% (w/v) glucose, 0.05% (w/v) $MgCl_2$, 0.5 mM $CaCl_2$, 7 mM KCl, and a mixture of nucleic acids, each 0.01% (w/v)) at 37°C. Spores were cultivated to the 30th min when the SigQ reaches the highest expression level during germination, as was found in work (Strakova *et al.*, 2013).

3.1.1.2 Vegetative growth

For ChIP-seq analysis, spores stocks were prepared by harvesting them from agar plates grown for 10 days. Following the procedure described in Nieselt *et al*. (Nieselt *et al.*, 2010), we inoculated thawed spore stock (2,5*10^9/ml CFU—typically 3 ml) to 100 ml 2YT medium (bacto tryptone, 16 g/l; bacto yeast extract, 10 g/l; NaCl, 5 g/l) to 500 ml Erlenmeyer's flask (customised to enable aeration) and germinated at 30°C for 5 h. The germinated spores were harvested by centrifugation (3200 × *g*, 15°C, 5 min) and resuspended in 5 ml ion-free water. Then they were inoculated into an 1.8 l Na-glutamate medium (Na-glutamate, 61 g/l; glucose monohydrate, 44 g/l; $MgSO_4$, 2.0 mM; Na_2HPO_4, 2.3 mM; KH_2PO_4, 2.3 mM) supplemented with 8 ml/l of trace element solution ($ZnSO_4$ x 7 H_2O, 0.1 g/l; $FeSO_4$ × 7 H_2O, 0.1 g/l; $MnCl_2$ × 4 H_2O, 0.1 g/l, $CaCl_2$ × 6 H_2O,0,1 g/l, NaCl 0,1 g/l) (Kieser *et al.*, 2000) and 5.6 ml/l of TMS1 ($FeSO_4$ × 7H_2O, 5 g/l; $CuSO_4$ × 5 H_2O, 390 mg/l; $ZnSO_4$ × 7 H_2O, 440 mg/l; $MnSO_4$ × H_2O, 150 mg/l; Na_2MoO_4 × 2 H_2O, 10 mg/l; $CoCl_2$ × 6 H_2O, 20 mg/l; HCl, 50ml/l). Cultivation was performed at 30°C at 250 RPM and pH of 7 was maintained during growth. Samples for the ChIP-seq analysis were collected after 22 h (equivalent to exponential phase) of growth (Nieselt *et al*., 2010).

3.1.2 Construction of epitope-tagged mutant strains

To avoid the construction of a deletion strain supplemented by the epitope-tagged gene on a separate plasmid, we modified the mutagenesis procedure (Gust *et al.*, 2004) in order to insert the epitope tag directly to the gene within its native site on the chromosome. 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 (Gust *et al.*, 2004), cut by EcoRI and HindIII restriction enzymes and used as a PCR template. The PCR was performed to a final volume of 50 µl containing 2,5 U iProof DNA polymerase (Bio-Rad), 10 µl 5xGC buffer, 2,5 µl 100% DMSO, 1 µl 10mM dNTPs (200 µM, 50 µM each), 0,5 µl template DNA (50 ng pIJ773 cut by EcoRI and HindIII, gel purified) and 0,5 µl each primer for mutagenesis (50 pmol each). 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 (carrying *sigQ* gene) and cosmid St5B8 (carrying *hrdB* gene). 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 (Flett *et al.*, 1997). Final mutants were selected on MS medium containing apramycin, kanamycin and nalidixic acid (Kieser *et al.*, 2000). 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.

3.1.3 ChIP-seq

ChIP-seq (Chromatin immunoprecipitation coupled with next generation sequencing (NGS)) is a widespread method for global mapping of DNA binding sites of specific DNA-binding proteins to determine the regulation of gene expression by the respective protein of interest. We used this method for identifying sigma factor's bound DNA sequences (promoters) to know which genes are regulated with principal sigma factor HrdB during vegetative growth and SigQ during spore germination.

Cells are cultivated as needed and then treated by formaldehyde to crosslink the DNA associated proteins with DNA. DNA is then isolated from the cells and sonicated to fragment the DNA. Then, immunoprecipitation of isolated and fragmented DNA follows using specific antibody against the protein of interest. The antibodies are coupled to agarose, sepharose or magnetic beads. Immunoprecipitated complexes are washed to remove non-specifically bound DNA and then reverse crosslinked to unbound the specific antibody from the protein. Proteins bound to the DNA are removed by treating with proteinase K.

Then, next generation sequencing of isolated DNA fragments is performed. ChIP-seq data that represent specific sequences bound by target protein are then analysed by computational methods to identify these sequences. ChIP-seq method is depicted in the Figure 20.

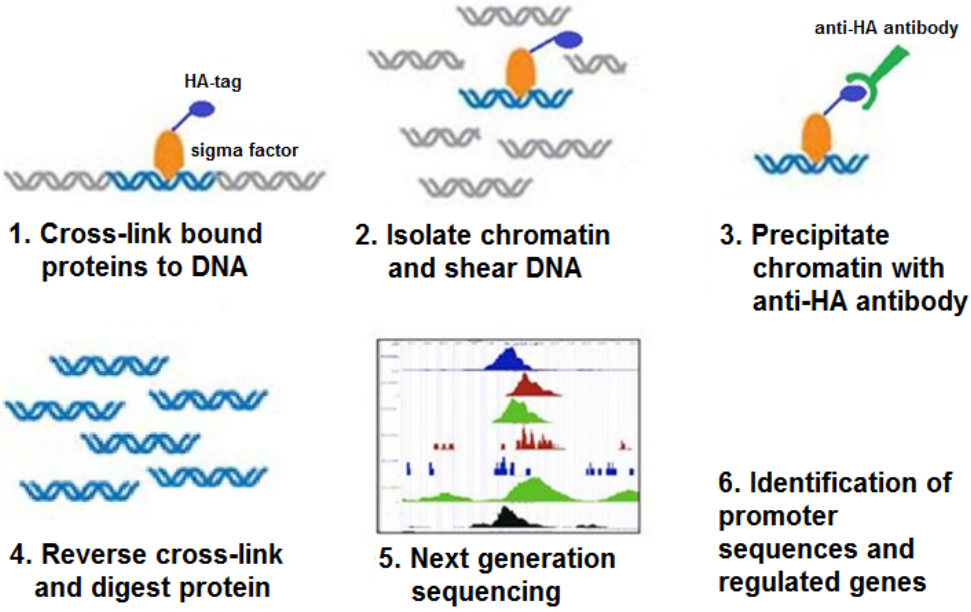


Figure 20. Overview of the ChIP-seq method. Modified picture is adapted from (Shah, 2009)

3.1.3.1 Chromatin immunoprecipitation of SigQ-bound DNA

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 phosphate buffered 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, 4µl/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 µl (2 µg) 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 µg IgG mouse serum (Sigma) was added to the cell extract. 20 µl protein A/G PLUS agarose was added to the non-specific control and 20 µl 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, pH 8,5; 0,5 M LiCl, 1% Triton X 100, 1% sodium deoxycholate, twice with Lysis buffer RIPA (Santa Cruz) and twice with TE buffer (10 mM Tris-HCl, pH 8,0; 1 mM EDTA). Sample was resuspended in 30 µl 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 µg 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. The negative control was the wild-type strain of *S. coelicolor* that was processed in the same way. Samples and controls were prepared in triplicates.

Sequence analysis of SigQ-bound DNA

Next generation sequencing was performed on the Illumina MiSeq platform in a paired-end 2x350 bp run in the Laboratory of environmental microbiology of the ASCR (doc. Baldrian). Illumina reads were trimmed by removing adapters and leading/trailing low quality or N bases

with Trimmomatic (v 0.36) (Bolger *et al.*, 2014). Quality control of the reads were checked using FastQC and Qualimap (v2.2)(Okonechnikov *et al.*, 2016).

Data analysis of SigQ-bound DNA

(performed in collaboration with Ing. J. Vohradský, CSc. and his colleagues Mgr. M. Modrák, Ph.D. and Ing. M. Schwarz)

Filtered reads were mapped to the *S. coelicolor* reference genome (GenBank: NC_003888) with Gsnap program (Wu & Nacu, 2010). The SAM files from three replicates and three controls were assembled and analysed using MACS v1.4 to detect read enriched regions in the genome (Feng *et al.*, 2012). Enriched regions were selected for further study based on p-value ≤ 0.05. Target genes were identified according to peak summits in ≤ 300 bp upstream of an annotated start codon of the respective gene. Operon categorization was done using operon prediction approach published in (Castro-Melchor *et al.*, 2010). For the visualization of the reads we used Tablet (1.15.09.1)(Milne *et al.*, 2010).

3.1.3.2 Chromatin immunoprecipitation of HrdB-bound DNA

ChIP-seq analysis was done as was described previously with minor modifications (Spencer *et al.*, 2003). Briefly, after 22 h of growth, cells were incubated for 30 min with 1% formaldehyde (CH_2O 36,5 %–38 % Sigma Aldrich). Crosslink reactions were stopped with prechilled 2M glycine for 5 min at room temperature. Cells were harvested by centrifugation at 4°C and washed five times with 1 × phosphate-buffered saline (PBS). The pellet was stored at −80°C prior further processing. Subsequently, the pellet was resuspended in RIPA buffer (SDS, 0.1%;

sodium deoxycholate, 0.5%; Triton X-100, 0.5%; 1 mM; NaCl, 150 mM; Tris, pH 8, 50 mM; Proteases inhibitor-Complete, Mini, ethylenediaminetetraacetic acid (EDTA)-free, Roche, 10nM) and sonicated 6 × 15 s, amplitude 0.5, on ice (Hielscher sonicator, UP 200S). The DNA fragments lengths were determined by agarose gel electrophoresis and were shown to be in the desired range of 200–500 bp. The cell lysate was centrifuged at 4°C, 20 000 × g, 20 min. Supernatant was collected and precleared for 2 h at 4°C by pre-equilibrated Protein-G Plus Agarose (Santa Cruz sc2002) in RIPA buffer. The concentration of proteins was measured and 2 mg of total proteins in lysate were added to 2 µg of anti-HA high affinity antibody (Roche, 11867423001) and incubated for 16 h at 4°C. Protein-G Plus Agarose was added and incubated for 6 h at 4°C. Samples were washed three times with RIPA buffer, four times with WASH buffer (Triton X-100, 0.5%; Sodium deoxycholate, 0.4%; LiCl, 0.5 M; Tris, pH 8.5, 100 mM), twice with RIPA buffer a second time, twice with TE buffer (EDTA, 10 mM; Tris, pH 8, 10 mM). Subsequently, elution was achieved using Elution buffer (SDS, 1%; EDTA, 10 mM; Tris, pH 8, 50 mM). Decrosslink reactions were done by addition of 200 mM NaCl and Proteinase K at 60°C overnight. Finally, DNA was purified by NucleoSpin gDNA Clean-up (Macherey-Nagel). The negative control was the wild-type strain of *S. coelicolor* that was processed in the same way. Samples and controls were prepared in triplicates.

Sequence analysis of HrdB-bound DNA

Libraries were prepared with NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (NEB #E7645), and all material was used as input. The adaptors were diluted 1:30 times, and 17 PCR cycles were performed. Libraries were measured with Qubit DNA High sensitivity assay, and afterward loaded on Agilent Bioanalyzer 2100 with DNA 1000 kit. Samples were pooled equimolar before sequencing, and the pool was measured with Qubit DNA high sensitivity kit.

The pool was clustered on cBot. Before clustering samples were diluted and denatured following Illumina recommendations (cBot system guide). Samples were diluted to 3.5 nM as final concentration for clustering. Libraries were sequenced on HiSeq 3000/4000 (HiSeq 4000 system guide). Run was performer with a 50 cycles kit, and the mode was 50 bp single read (51 bp read 1+ 7 bp index read). The samples were sequenced at EMBL-Gene Core, Germany.

Data analysis of HrdB-bound DNA

(performed in collaboration with Ing. J. Vohradský, CSc. and his colleagues Mgr. M. Modrák, Ph.D. and Ing. M. Schwarz)

Raw ChIP-seq data were processed in Chipster (https://chipster.csc.fi/) ran on the server of the Metacentrum (https://www.metacentrum.cz/). Three samples and two controls fastq data files were analyzed (ChIP-seq data have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6926). Quality control of the sequences was done individually for each file using FastQC. According to the report, data were trimmed left by 5 nt. Sample files and control files were concatenated for further processing. Genome sequence annotation file (.gtf) and the sequence file (fasta) were downloaded from Ensembl database (ftp://ftp.ensemblgenomes.org/pub/bacteria/release-37/fasta/bacteria_0_collection/streptomyces_coelicolor_a3_2_/dna/). The data were aligned using Bowtie2 algorithm. Peaks in the aligned sequence were identified using MACS2. Peaks were mapped to the *S. coelicolor* reference genome (Gene Bank: NC 003888) and were inspected in Genome browser Tablet (Milne *et al.*, 2013, Milne *et al.*, 2016). Corresponding genes and their operons were identified by selecting statistically significant peaks ($P < 0.05$) with fold enrichment ≥ 2. Target genes were identified according to peak summits in ≤ 300 bp

upstream of an annotated start codon of the respective gene. Operon categorization was done using operon prediction approach published in (Castro-Melchor *et al.*, 2010). Altogether 2147 genes were identified.

3.1.4 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 14.000 g 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).

3.1.5 Statistical analysis, data processing, kinetic modelling

(Performed by Ing. J. Vohradský, CSc. and his colleagues Mgr. M. Modrák, Ph.D. and Ing. M. Schwarz)

3.1.5.1 SigQ

DNA microarray data processing and analysis

Gene expression data were processed as in our previous paper (Strakova *et al.*, 2013). The data preprocessing steps are repeated here to make clear how the values used for the analysis in this article were obtained. RNA quality control and gene expression levels were performed by Oxford Gene Technology (Oxford, UK) using Agilent DNA microarrays covering the entire *S. coelicolor* genome and the standard Bacterial RNA amplification protocol for two-channel assays by OGT.

The data were normalized using LOWESS and filtered for background and flag information (from Agilent documentation) in the GeneSpring software to obtain genes that were expressed significantly above background and to avoid side effects of possible cross hybridization. These methods reduced the number of entities on a single array from 43888 to 25312, which finally represented the outcome for 7115 genes out of 7825. The data discussed in this publication have been deposited in the NCBI Gene Expression Omnibus and are accessible using the GEO Series accession number GSE44415 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE44415).

Array normalization

The experiment included 37 arrays from 13 distinct time points during *S. coelicolor* germination. The arrays shared a common reference in the red channel (Cy5), which consisted of a mixture of RNA samples from all examined time points. The distributions of Log2Ratio

values (Log2Ratio = log2 (Sample (Cy3)/Reference (Cy5))) for all samples were scattered around a common mean and all had similar variance. Therefore, the distributions for each array were centered so that the medians and the median absolute deviations of all the array distributions were equal. To eliminate array outliers, we filtered out the 0.02 quantile of the least and the most intensive Log2Ratio values. Normalized Log2Ratios were exponentiated to return the values to the original. The outliers among gene replicates at individual time points were filtered using the Q-test (for 3-9 inputs) and the Pierce test (for > 10 inputs).

Statistical analysis and kinetic modelling

We build upon our previous work on an ordinary differential equation (ODE) model for gene regulation, inspired by the neural network formalism (Vohradsky, 2001). In this model the synthesis of new mRNA for a gene *z* controlled by set of *m* regulators $y_1,..,y_m$ (genes or any other regulatory influence) is determined by sigmoid function $f(\rho(t))$ of the regulatory input

$$\rho(t) = \sum_{j=1..m} w_j y_j(t) + b\rho(t) = \sum_{j=1..m} w_j y_j(t) + b$$

Here w_j is the relative weight of regulator yj, b is bias (inversely related to the regulatory influence that saturates the synthesis of the mRNA). In our case, f is the logistic function f(x) = 1/(1 + e-x). The transcript level of z is then governed by the ODE:

(1) $$\frac{dz}{dt} = k_1 f(\rho) - k_2 z$$

where k_1 is related to the maximal level of mRNA synthesis and k_2 represents the decay rate of the mRNA. Both k_1 and k_2 must be positive. The complete set of parameters for this model is β

$= (k_1, k_2, b, w_1, \ldots, w_m)$. Given N samples from a time series of gene expression taken at time points $t_1, \ldots, t_N$ the

inference task can be formalized as finding $\hat{\beta}\hat{\beta}$ that minimizes squared error with regularization:

$$\hat{\beta} = \underset{\beta}{\operatorname{argmin}} \left[\sum_{i=1}^{N} \left(\hat{z}_\beta(t_i) - z(t_i) \right)^2 + r(\beta) \right] \quad (2)$$

Here z is the observed expression profile, $\hat{z}_\beta\hat{z}_\beta$ the solution to (Eq. 1) given the parameter values β and the observed expression of $y_1,..,y_m$, and $r(\beta)$ is the regularization term. The regularization term represents a prior probability distribution over β that gives preference to biologically interpretable values for β. Assuming Gaussian noise in the expression data, (Eq. 2) is the MAP estimate of β.

Our approach is similar to the Inferelator algorithm (Bonneau *et al.*, 2006), although there are important differences: the Inferelator does not model decay (k_2) – it assumes decay is always one. Further, Inferelator minimizes the error of the predicted derivative of the expression profile, while we minimize the prediction error for the actual expression profile.

Since the expression data is noisy, we have smoothed the data prior to computation with a piecewise cubic spline with 6 knots (the best number of knots was determined empirically). By smoothing we get more robust results with respect to low frequency phenomena, but sacrifice our ability to discover high frequency changes and regulations (oscillations with frequency comparable to the measurement interval are mostly suppressed). Further our experiments with fitting raw data or tight interpolations of the data (e.g. B-spline with knots at all measurement points) have had little success in fitting even the profiles that were highly correlated, due to the

amplified noise in the data. Approaches to GRN inference using smoothed data have been described in the literature (Berrones, 2016).

Further advantage of smoothing is that it lets us subsample the fitted curve at arbitrary resolution. We have subsampled the profiles at 1-minute time steps (360 points) which allowed us to integrate Eq. 1 accurately with the computationally cheap Euler method, making evaluation of the error function fast and easy to implement in OpenCL.

We minimized Eq. 2 by simulated annealing. For each gene and candidate regulator set we execute 128 runs with different initial parameter values. To increase speed, the inference algorithm was implemented for parallel execution on both CPU and GPU architectures with OpenCL. On a mid-tier GPU (NVidia GTX 960), the system found optimal parameters for 300 gene-regulator pairs in 11 seconds.

For the task at hand, we have first tested single regulator models for all genes in the SigQ regulon. Since SigQ has three major peaks in the collected data, and it is plausible that those peaks correspond to different phases of germination with possibly different regulatory interactions. We have therefore also tried to fit the single regulator models on subset of the data containing only one or two of the peaks.

3.1.5.2 HrdB

Modelling gene expression profiles

To model the possible regulatory effects of HrdB we used the Genexpi tool and associated workflow (Modrak & Vohradsky, 2018) with minor additions. In particular, the expression of all genes was first smoothed with a B-spline and the smoothed expression of putative targets was modelled as an ordinary differential equation (ODE). A maximum a-posteriori estimate of

the parameters of the ODE was determined with simulated annealing. We tried to fit three models of increasing complexity to the time series of the target gene expression. After each model was applied, the results were filtered by fit quality. Only genes that did not fit sufficiently well with a simpler model were tested with a more complex model. The three models were—the constant synthesis model, direct model and cooperative model. The constant synthesis model assumes that the synthesis rate of the target is constant over time:

$$\frac{dx}{dt} = k_1 - k_2 x \qquad (1)$$

Here, x is the expression of the target gene as a function of time, $k1$ and $k2$ are mRNA synthesis and degradation rate constants. When a gene is fit by the constant synthesis model, it does not necessarily mean that it is not regulated by HrdB, only that its synthesis is not affected by concentrations changes of HrdB observed in the experiment. The direct model assumes that the target gene is regulated solely by HrdB:

$$\frac{dx}{dt} = k_1 \frac{1}{1 + e^{-(wy + b)}} - k_2 x \qquad (2)$$

Here, y is the smoothed expression of the regulator (HrdB) as a function of time, $k1$ is related to the maximum possible synthesis, w is the regulatory weight, b is bias (inversely related to the regulatory influence that saturates the synthesis of the mRNA) and $k2$ is mRNA decay rate constant. For further details on those two models and the optimization procedure, see (Modrak & Vohradsky, 2018). The cooperative model assumes that the target is regulated by a complex of HrdB and RbpA. This is a slight extension of the direct model and it is a novel contribution of this paper. The model follows Equation 2, but the HrdB + RbpA complex is considered as a

regulator (*y*). The concentration of the complex is in turn determined by the concentrations of its constituents and an equilibrium constant

$$q = \frac{y_A y_B}{y_{AB}} = \frac{[Hrd\,B][Rbp\,A]}{[Hrd\,B - Rbp\,A]} \qquad (3)$$

Solving for *yAB*we get

$$y_{AB} = y = \frac{1}{2}\left(y_A + y_B + q - \sqrt{(y_A - y_B)^2 + 2q\,(y_A + y_B) + q^2}\right) \quad (4)$$

In practice, this means that *yAB*is interpolated between 0 (for $q \to \infty$) and *min(yA, yB*) (for $q \to 0$) and it is used as a regulator (y) in Equation 2. The equilibrium constant *q* becomes another parameter of the ODE that is optimized jointly with the other parameters of Equation 2. Source code for fitting all of the models is available at https://github.com/cas-bioinf/genexpi.

Binding motif search

The binding motif search was based on known transcription start sites (TSS) for *S. coelicolor* published by Jeong *et al.* (Jeong *et al.*, 2016).

Promoter region location

For the genes that were assigned to individual ChIP-seq peaks we found those for which the TSS was known from the Jeong's paper (1723 sites). From this set, we selected only genes with TSS assigned to the category 'primary'. Final set contained 1048 TSS, (filtered TSS).

Motif discovery

Based on known genome loci of the filtered TSS we extracted two locations, 20 bp upstream to 0 relative to each TSS for −10 region and −40 to −25 bp upstream relative to −35 region. The motif search was performer with MEME software (http://meme-suite.org/) using two approaches. Method 1 (same as Jeong's *et al*. used) uses MEME with -dna -oops parameters, meaning that one motif site from each sequence (here promoter region) contribute to resulting motif. Final motif is then obtained by filtering for motif sites with P-value < 0.05. Method 2 uses MEME with -dna -zoops parameters, meaning that 0 or 1 motif sites are expected per sequence and only motif sites with P-value < 0.05 contribute to final motif. Discovered motifs were compared with the published ones found by Jeong *et al*. The motif positions numbering was relative to TSS described in the Jeong's paper, and motifs shown are drawn from aligned sequences.

Presence of G or GG immediately prior to located motif

According to literature (Zhu *et al.*, 2017), the presence of G or GG motif prior to −10 region stabilizes the binding of the transcription factor holoenzyme. We therefore inspected the −14 and −13 regions for occurrence of GG or G. As a source data we took locations for motifs with P < 0.05 as found with MEME by the method 2 (see above) (954 sites). Then we considered our motif to be TANNNT and we extracted exactly 2 nucleotides ahead of that motif (referenced as −14 and −13). Then we grouped the motif sites by presence of G nucleotide into three groups (GG—both positions contain G, G—one of positions contains G (but not both), noG—no position contain G). The expected frequency of dinucleotides GG was calculated as fGG = count(GG)/(length(genome)-1), where count (GG) means number of all occurrences of GG dinucleotide in genome. The expected number of sites having GG randomly was N=

fGG*number of sites, where fGG is frequency of dinucleotide GG in genome, and number of sites represent number of sites analyzed.

3.2 Methods used for identification of novel cis-antisense sRNAs

3.2.1 Bacterial strains and cultivation

In this study we used the *Streptomyces coelicolor* wild-type (wt) strain M145 (Kieser *et al.*, 2000) and its RNase III-deletion strain derivative [*rnc*, M145 *rnc::aac(3)IV*] (Sello & Buttner, 2008). 10^8 spores were inoculated on solid R2YE medium (Kieser *et al.*, 2000) 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.

3.2.2 RNA isolation

Total RNA was isolated using a TRIzol method (Van Dessel *et al.*, 2004). 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.

3.2.3 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/) (Untergasser *et al.*, 2012) (Table 4).

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.

Table 4. List of primers used in our study

Primer name	Sequence 5'-3'
RACEhrdA2465inner	GCGGTACCTGAGTGCACGCGT
RACEhrdB5820inner	GTCGGCCAGCACATCCCGT
RACEhrdC0895inner	GGACGGAGGAAACGACATGGC
RACEhrdD3202inner	CAAGGGAGCACGCATGGCAA
RACEsigB0600inner	GGGAGGGCACATCATGACGAC
RACEsigD4769inner	GACCGATGCGTGACGACGAT
RACEsigE3356inner	GGAAGGCGGTTGACATGGGC
RACEsigF4035inner	GCCAGTACTGCGCCTCAAGCA
RACEsigH5243inner	CGACGTGAGGGACGGGGAC
RACEsigI3068inner	ATCATGTCACCCCGGCTCGA
RACEsigR5216inner	GGAGGAGGTGGGTCCGGTCACT
RACEwhiG5621inner	GACGAATGCCCCAGCACACCT
RACEhrdA2465outer	TGGCGGCGATGTGTCGTACC
RACEhrdB5820outer	CAACGTTCCGAGAGGTTGTTCG
RACEhrdC0895outer	GTCCGGAAGCCCGTGATCC
RACEhrdD3202outer	CCACCCGCTGAACCACCATC
RACEsigB0600outer	GTGACAACGACGACCGATCCA

RACEsigD4769outer	ACGGCCGCAAGGCACCAT
RACEsigE3356outer	CCACCGTCGGAGTACGGGGAT
RACEsigF4035outer	GATCACGAGGTGGAGTTGACCGT
RACEsigH5243outer	CGATCAGCCTCTACAAACAGCGC
RACEsigI3068outer	CGTATGCCGAGAACGATGGAGGA
RACEsigR5216outer	CGGTCTCGGTTTCACGACGAC
RACEwhiG5621outer	ACATCACGGCAGAACGGCACTA
3RACEsigB0600inner	TGGATCGGTCGTCGTTGTCACCT
3RACEsigH5243inner	CCGCGCTGTTTGTAGAGGCTGAT
3RACEsigR5216inner	CCGGTCTCACTCGAATCGGAGGAT
3RACEsigB0600outer	GCGGTCGTCGTCGTCATGATGT
3RACEsigH5243outer	CCGTCCCCGTCCCTCACGT
3RACEsigR5216outer	CAGTGACCGGACCCACCTCCTC
NB_sigB	CAGCTCACCGTGCTGGAGGAG
NB_sigH	GAGGGCACACGTTCTCCGAAC
NB_sigR	TCGACCAGATGTACTCGGCCG

3.2.4 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

Streptomyces are soil-living, secondary metabolite-producing bacteria with complex life cycle which includes developmental life stages such as substrate mycelium and subsequent aerial hyphae formation and sporulation (Chater, 1993). This developmental programme, together with changing environmental conditions, leads to the formation of a complex regulatory network responding by fine-tuning of gene expression which results in the modulation of metabolic pathways and morphological processes in the cells. This complex regulatory network is mediated throughout the action of specific set of genes that are transcribed by 65 different sigma factors (Bentley *et al.*, 2002). Each sigma factor possesses its own sigma regulon containing a set of genes that it regulates (Paget, 2015). A huge number of sigma factors in *Streptomyces* has unknown regulons and without this important knowledge it is impossible to understand all these complex regulatory networks and metabolic processes in the cells. So far, only about 20 sigma factors have been characterized in *Streptomyces coelicolor*. Our previous investigations focused on the identification of proteome and transcriptome during spore germination in *Streptomyces coelicolor*. We have found several sigma factors in this life stage with unknown regulons that were highly expressed. We wanted to know why are they highly expressed and what is their role in spore germination. So we tried to identify the sigma regulons of several highly expressed sigma factors in spore germination and of the most important vegetative sigma factor HrdB. The most highly expressed among the highly expressed sigma factors in spore germination was sigma factor SigQ, on which we focused first; we characterized its regulon by ChIP-seq. The second one was the principal and essential sigma factor HrdB which is important in the vegetative phase of growth and whose deletion is lethal

for the cells. Although it is a housekeeping sigma factor, its regulon was unknown so far. We identified its regulon by ChIP-seq in the vegetative phase of growth.

ChIP seq data of both sigma factors HrdB and SigQ were complemented with kinetic modelling to see the expression profiles of target genes and thus verify the mode of action of regulatory function of SigQ and HrdB. These results are published in the article Šmídová, K.; Ziková, A.; Pospíšil, J.; Schwarz, M.; Bobek, J.; Vohradský, J. DNA mapping and kinetic modeling of the HrdB regulon in *Streptomyces coelicolor*. *Nucleic Acids Res.* 2019, *47* (2), 621–633. and in the article 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.*

Sigma factors as transcriptional regulators are further regulated by anti-sigma factors via different mechanisms (Helmann, 1999, Paget, 2015) and by non-coding 6S RNA that binds σ^{70} of RNAP and inhibit transcription by competing with promoter DNA (Wassarman & Storz, 2000, Cavanagh & Wassarman, 2014); specifically sigma factor HrdB is regulated by RbpA protein that functions as a chaperon and facilitates transcription from HrdB-dependent promoters (Newell *et al.*, 2006, Tabib-Salazar *et al.*, 2013). In the times of increasing evidence of regulations throughout sRNAs and with respect to published papers about the regulation of sigma factors by sRNAs in *E. coli* and *Salmonella enterica* (Klein & Raina, 2015), we addressed the question whether sigma factors in *Streptomyces* could be also regulated by sRNAs. We selected 12 sigma factors and we found out that three of them (SigB, SigH, SigR) possess asRNAs. These results partially contribute to the published paper: 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.

4.1 Preparation of tagged sigma factors by insertional mutagenesis

Our previous investigations described gene expression during spore germination (Bobek *et al.*, 2014). During this life stage, we observed a high activity of sigma factors HrdD, SigR, SigE, SigD, and SigH in the initial 30 mins of germination. Nevertheless, the absolutely highest induction (18x) exhibited SigQ sigma factor. The other sigma factors, such as SigB or SCO1263, also exhibit significantly increased gene expression profiles, suggesting for their importance in spore germination (Bobek *et al.*, 2014). The first step of our procedure leading to identification of sigma factor regulons was the tagging of selected sigma factors with HA tag by epitope tag insertional mutagenesis. For further analysis we prepared epitope tagged mutant strains with following tagged sigma factors: SigB, SigH, SigR, SigE, SigD, SCO1263, SigQ and vegetative sigma factor HrdB. This method of adding epitope tag to the gene of interest was first developed by (Kim *et al.*, 2012). The epitope tag insertional mutagenesis is based on PCR targeting system (Gust *et al.*, 2003) which lies on gene replacement. But instead of gene replacement we added the HA-epitope tag sequence to a sigma factor gene in the native site in the genome. Our PCR-based tagging strategy begins with an amplification of the HA tag sequence that is followed by an apramycin resistance cassette and an oriT site, all that flanked by FRT sites (Figure 21-3). The entire DNA fragment is inserted into the cosmid by the electroporation into *E. coli* BW25113/pIJ790 containing the cosmid (carrying the gene of interest), followed by a subsequent λ-Red-mediated recombination. The cosmid with an inserted HA tag is then transformed into methylation-deficient *E.coli* ET12567/pUZ8002 and conjugated to *S. coelicolor* M145 (Gust *et al.*, 2003, Kim *et al.*, 2012) (Figure 21). The correct insertion of HA tag into the sigma factor gene before its stop codon was validated by sequencing of PCR product and then by western blotting of tagged sigma factor (Figure 22). Since the HA-

tagged SigQ sigma factor was undetectable by standard Western blotting control from spore germination phase, we tried to detect it after induction of its gene expression (Figure 23) (details below). The remaining tagged sigma factors (SigE and SCO1263) have not yet been checked by western blot.

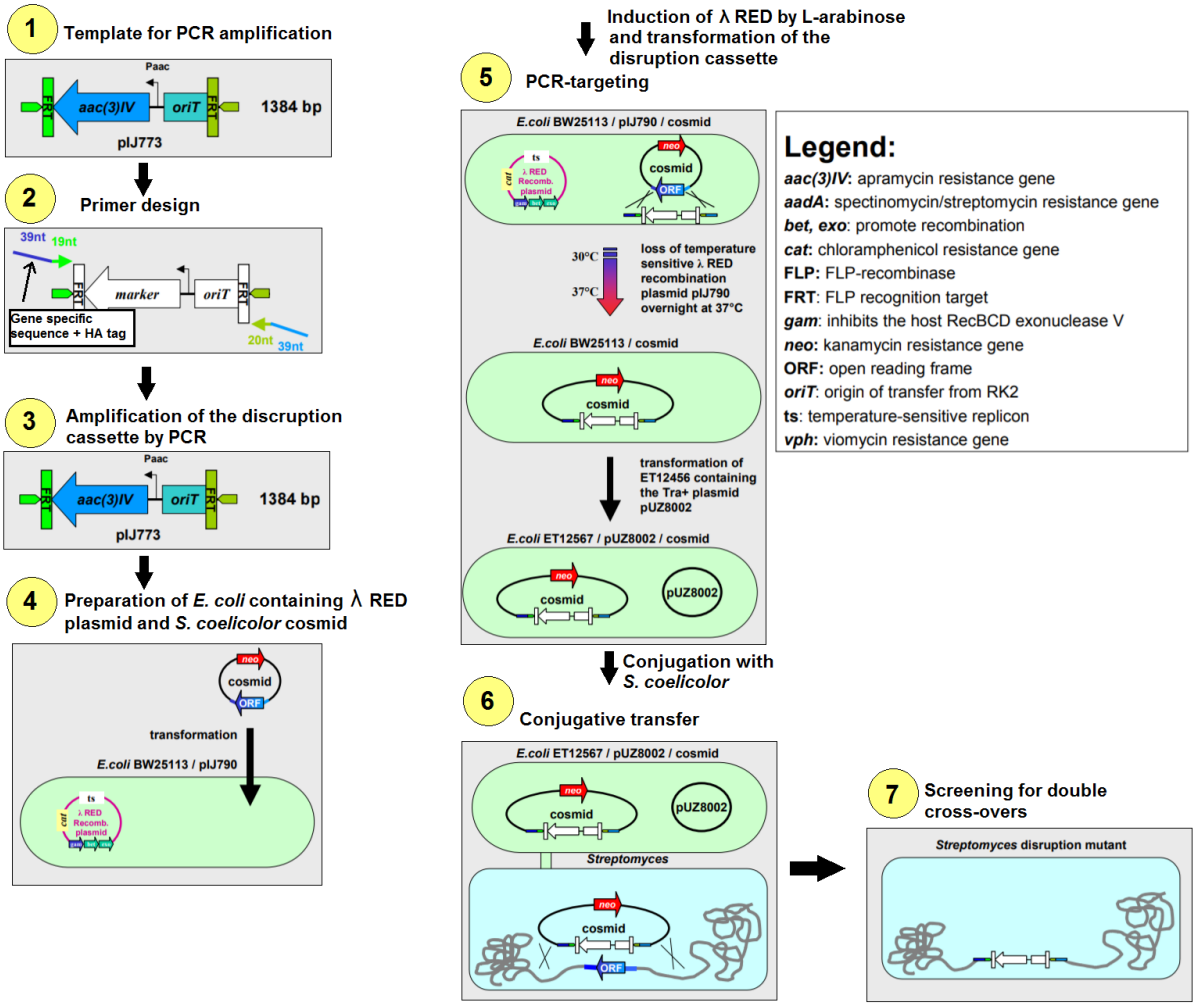


Figure 21. PCR targeting system in *Streptomyces* – described in the text. Part of gene specific sequence with HA tag sequence is incorporated as a part of the cassette before the FRT site. After the insertion into the cosmid, it becomes a consistent part of the target gene before the STOP codon. Adapted from (Gust *et al.*, 2003).

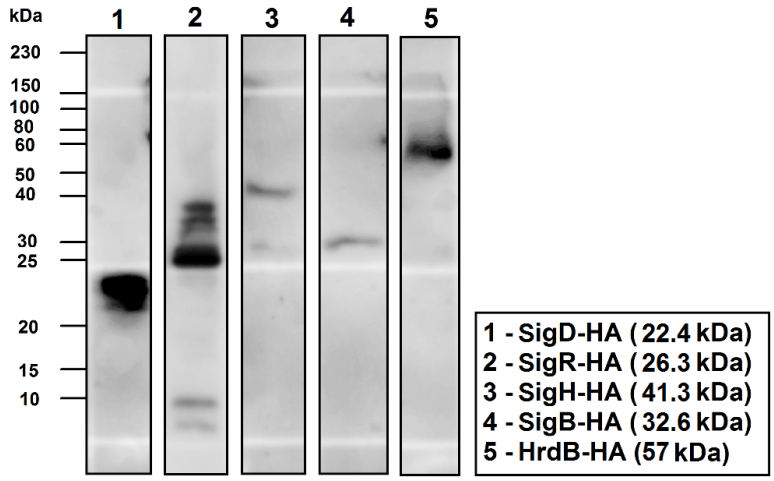


Figure 22. Western blot of HA-tagged sigma factors SigD, SigR, SigH, SigB, HrdB with their molecular weights (SigD (SCO4769) – 22.4 kDa (21.3 kDa + 1.1 kDa HA tag), SigR – 26.3 kDa (25.2 kDa + 1.1 kDa HA tag), SigH – 41.3 kDa (40.2 + 1.1), SigB – 32.6 kDa (31.5 + 1.1), HrdB – 57 kDa (55.9 + 1.1).

SigQ induction

The SigQ-HA strain was cultivated for 48h because the SigQ from germination was undetectable by western blot. The original wild type strain lacking the HA-tag was used as a negative control. The cell-free protein extract from the SigQ-HA strain revealed a band of approximately 23.6 kDa (corresponding to 22.5 kDa of the SigQ protein + 1.1 kDa of the HA tag). To see whether the expression of SigQ is induced by osmotic or nitrogen stress, the cultivation medium was supplemented for the last 30 minutes by 0.5M NaCl or 30mM $(NH_4)_2SO_4$ (Figure 23, lane 2 or 3, resp.). After this, the SigQ level highly increased in the presence of ammonium sulphate and increased even more in NaCl. These data suggest that the SigQ sigma factor is another alternative sigma factor of streptomycetes that controls cellular osmotic stress responses. As the osmotic stress is concomitant to germination as was suggested previously (Strakova *et al.*, 2013), the proposed role of SigQ in the osmotic stress response may explain the 18x increase in its expression during germination.

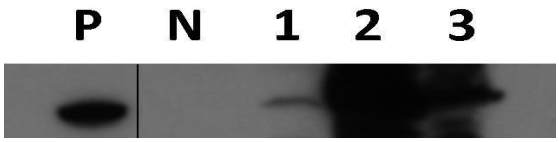


Figure 23. SigQ-induction western blot. In order to prove eligibility of the method, western blot was performed using anti-HA antibody. Cells were cultivated 48h in 2YT medium. P - precipitated SigQ-HA protein induced by NaCl (0,5 M final) as a positive control of SigQ localization; N - protein extract from the wild type strain as a negative control; 1 - protein extract from the SigQ-HA strain; 2 - protein extract from the SigQ-HA strain induced by NaCl (0.5M final); 3 - protein extract from the SigQ-HA strain induced by $(NH_4)_2SO_4$ (30mM final).

4.2 Identification and modelling of the SigQ regulon in spore germination

The previous investigations of our lab described gene expression during spore germination (Strakova *et al.*, 2013, Strakova *et al.*, 2013, Bobek *et al.*, 2014, Strakova *et al.*, 2014). Therefore, based on these measured data, Bobek and his colleagues created a model of gene expression changes, which revealed the highest induction (18x) of sigma factor SigQ (Bobek *et al.*, 2014). In this study, we focused on the SigQ regulon. 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, as described in Methods. 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 (Strakova *et al.*, 2013, Strakova *et al.*, 2013).

4.2.1 Identification of SigQ binding regions by ChIP-seq

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.

4.2.2 Regulatory function of SigQ from kinetic modelling

(performed by Ing. J. Vohradský, CSc. and his colleagues)

Previously measured microarray data in 13 time points through the initial 5.5 hours of growth during spore germination (Strakova *et al.*, 2013, Strakova *et al.*, 2014) were used for the modelling of gene expression kinetics of the target genes and SigQ. This analysis revealed a possibility of regulatory influence of SigQ on the identified target genes and also the mode of its action. Out of the original number (326 genes), 25 expression curves had either very low response to the expression pattern of SigQ or were too flat; among them, 3 gene expression profiles were not identified in the expression time series dataset. These genes have been discarded from the analysis. The remaining 301 genes found by ChIP-seq and operon analysis were subjected to the kinetic modelling. Another 74 genes were discarded from our dataset due to the inconsistency within the operon.

4.2.2.1 Target gene expression profile clustering

Expression profiles of the selected genes were normalized and the normalized profiles were clustered (Figure 24). The clustering showed three principal trends: 1. profiles that were at some interval correlated with the expression profile of *sigQ* (CL1, 6, 10), 2. profiles inversely correlated with the *sigQ* profile (CL3, 4, 5, 9), suggesting that, for a large part of the SigQ regulon, SigQ acts as a repressor, 3. profiles that were at some interval correlated with the expression profile of *sigQ* and at some profile were correlated inversely or were not correlated (e.g. CL6, 10 was positively correlated with *sigQ* profile after ca 120 min., or CL3 was negatively correlated with the *sigQ* profile consistently during the first ca 150 minutes. Some, as CL8, were not correlated with *sigQ* at all). According to these principal trends, our modelling results were classified into three categories: 'Positive' – regulated gene expression profile was successfully modelled with SigQ as a regulator in whole measured interval, 'Negative' – regulated gene expression profile was inverse, suggesting that sigQ acts as a repressor, and 'Inconsistent' – SigQ could not model the given profile or the modelling results were inconsistent, i.e. their segments were modelled with SigQ as both a positive and a negative regulator, therefore such gene expression profiles were considered as not controlled by SigQ. 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 (category 'Inconsistent').

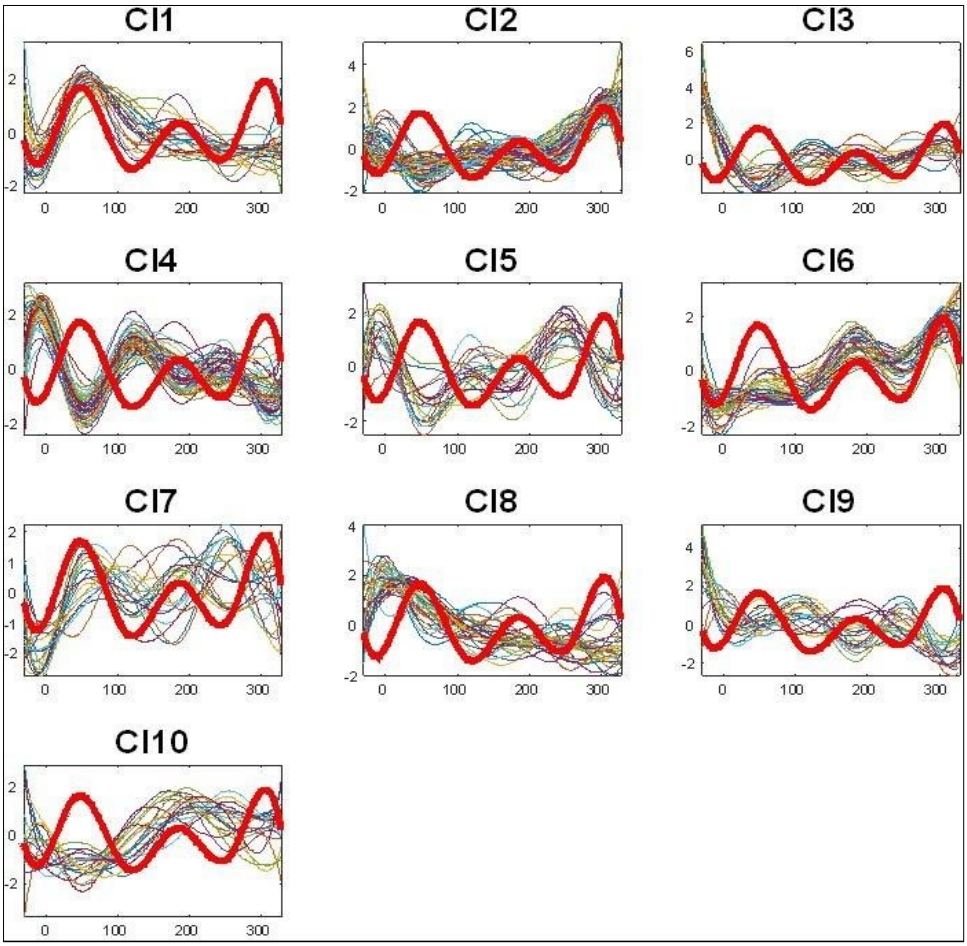


Figure 24. Gene expression profiles of the SigQ regulon clustered according to similarity of their expression profiles with superimposed profile of *sigQ* (red). Adapted from (Smidova, Manuscript)

4.2.3 Positive and negative regulation of SigQ regulon

Clustered expression profiles of target genes that were inversely correlated with the *sigQ* profile (CL 3, 4, 5, 9) were labelled as negatively regulated by SigQ, whereas expression profiles that were at some interval correlated with the expression profile of *sigQ* (CL 1, 6, 10) were labelled as positively regulated by SigQ. Negative regulation in our results means that when the expression profile of *sigQ* increases, the expression profile of target gene decreases – and vice versa. We observed this trend in the entire interval measured and also at certain segments only.

This negative regulation of the target genes by SigQ leads to the suggestion that SigQ acts also as a repressor. This is the first time when sigma factor is suggested to be a repressor. It is important finding as the negative regulation was revealed in almost one half of target genes. This theme will be discussed in the chapter Discussion.

4.2.4 Functional classification of the SigQ regulon

The genes of the SigQ regulon were further characterized by a classification into functional groups according to The Sanger Institute database or KEGG database (http://www.genome.jp/kegg/pathway.html) (Kanehisa & Goto, 2000) (Appendix 1). 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 (Figure 25). We identified SigQ bound regulatory regions that included ribosomal proteins, dehydrogenases, ABC transporters, aminotransferases, ATP/GTP binding proteins, cold shock proteins, components of cytochrome, helicases, elongation proteins, transcriptional regulators, hydrolases, hypothetical proteins, chaperonins, integral membrane transport proteins, lipoproteins, oxidoreductases, regulatory proteins, sigma factors, sugar transporters, amino acid synthases, two component system proteins, and transmembrane proteins.

The most abundant group were 1.3.1 Chaperones with 13.3 % genes from all genes in this group (2 genes out of 15 genes in total), then 3.1.0 Amino acid biosynthesis group with 10.6 % (13 genes out of 123 in total) and group 1.8.1. Differentiation and sporulation with 9 % (1 gene out of 11 in total). Other groups were represented only in a minority from 1 to 5 % (Figure 25). List of genes and their functional and pathway annotations, found to be controlled by SigQ is in Appendix 1.

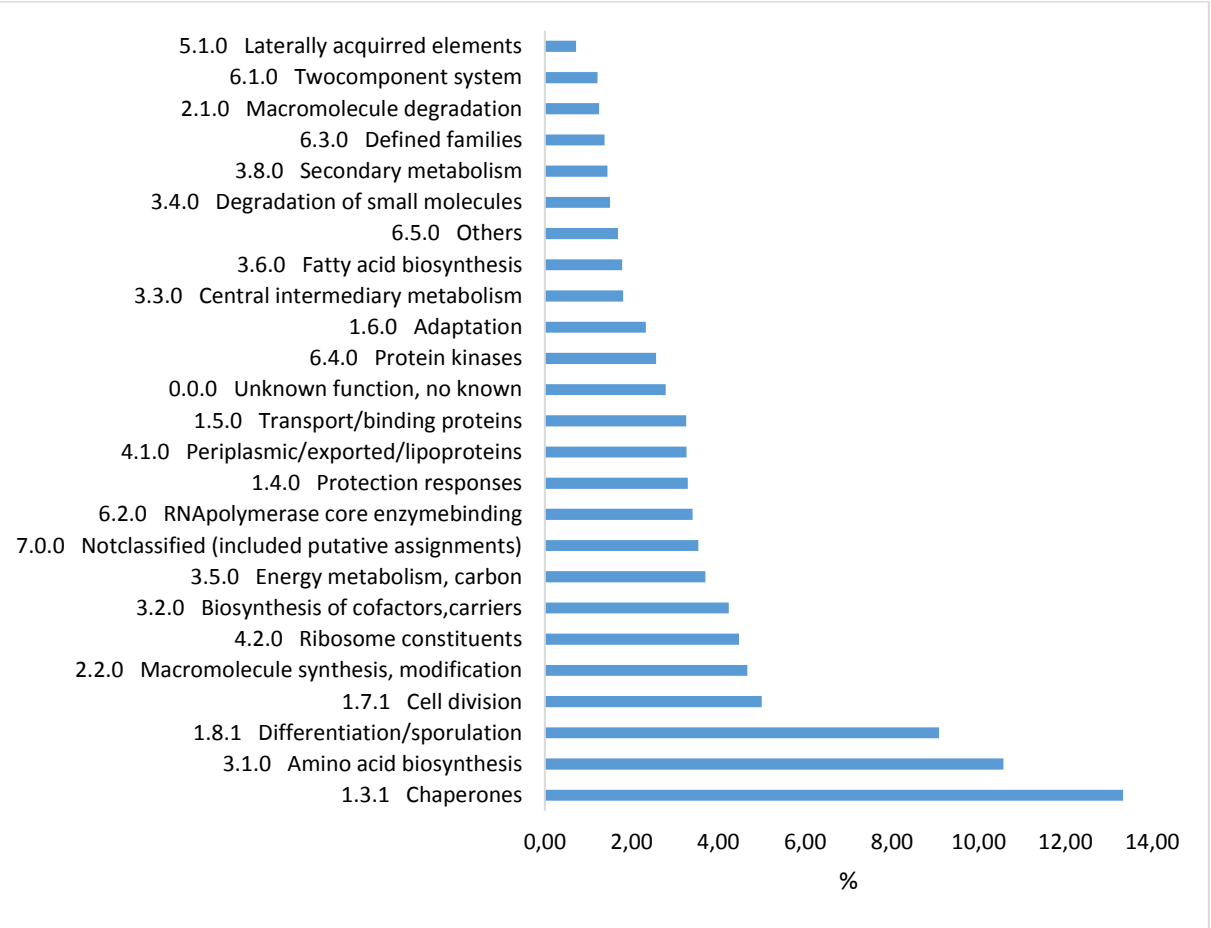


Figure 25. Proportional representation of gene ontologies in SigQ regulon (each group represent a percentual proportion of genes in SigQ regulon from all genes in each gene ontology group). This graph includes all genes from HrdB regulon that were used for kinetic modelling.

4.2.4.1 Genes positively regulated by SigQ

We identified 90 genes falling into a positive regulation pattern, where the SigQ played a role as an activator of gene expression. Most of the genes in this group were formed by unknown (18 genes) and not classified genes (6 genes). The second largest group was formed by genes from group 4.1.0 Periplasmic/exported/lipoproteins (17 genes), namely hypothetical proteins (SCO2067, SCO2095, SCO2096, SCO2347, SCO2622, SCO2960, SCO2978, SCO4471,

SCO4472, SCO4474, SCO5540, SCO5623, SCO5628, and SCO5751) and alkaline phosphatase (SCO2068), small membrane protein (SCO1634) and lipoprotein (SCO3107).

The third largest group was formed by genes of group 3.1.0 Amino acid biosynthesis (11 genes) formed by glutamine synthetase (SCO2241, SCO2198), glutamate synthase (SCO2025) and threonine synthase (SCO5355), followed by imidazole glycerol phosphate synthase genes (SCO2048, SCO2051), and imidazole glycerol phosphate dehydratase (SCO2052). The rest formed the genes: phosphoribosyl isomerase A (SCO2050), histidinol-phosphate aminotransferase (SCO2053), diaminopimelate decarboxylase (SCO5353) and homoserine dehydrogenase (SCO5354).

Nine genes were assigned to group of 2.2.0 Macromolecule synthesis, modification, formed by peptidylprolyl cis-trans isomerase (SCO1638), leucyl-tRNA synthetase (SCO2571), DEAD/DEAH box helicase (SCO3732), SCO4318, elongation factor Tu (SCO4662), tRNA (guanine-N(1)-)-methyltransferase (SCO5594), elongation factor Ts (SCO5625), ribosome recycling factor (SCO5627), and peptidyl-prolyl cis-trans isomerase (SCO7510).

Four genes were assigned to the group of 3.2.0 Biosynthesis of cofactors, carriers, namely molybdenum cofactor biosynthesis protein A (SCO1821), cytochrome biogenesis-like protein (SCO4473, SCO4475) and polyprenyl diphosphate synthase (SCO4583). Another group was formed by the genes of 3.5.0 Energy metabolism, carbon with 4 genes: dihydrolipoamide dehydrogenase (SCO2180), dihydrolipoamide succinyltransferase (SCO2181), type II citrate synthase (SCO2736) and 6-phosphogluconate dehydrogenase (SCO6658).

Group of 1.5.0 Transport/binding proteins is comprised of 3 genes: 2 ABC transporters (SCO3947 and SCO5392) and transport system kinase (SCO5400). Three genes were involved in the group of 3.4.0 Degradation of small molecules (rhamnose kinase (SCO814), acetyl-CoA acetyltransferase (SCO5399) and tagatose 6-phosphate kinase (SCO5848)). Group of 4.2.0

Ribosome constituents is comprised of 3 genes: 30S ribosomal protein S16 (SCO5591), 30S ribosomal protein S2 (SCO5624) and 16S rRNA-processing protein RimM (SCO5593).

Two genes were in group of 3.8.0 Secondary metabolism (subtilisin-like protease SCO1824, AgaS protein SCO5849) and in group of 6.5.0. Others, including regulatory proteins, regulatory protein SCO2094 and regulatory protein SCO6169.

The remaining functional groups included only one gene: 1.4.0 Protection responses (penicillin acylase SCO3184), 1.6.0 Adaptation (acetyltransferase SCO1864), 1.8.1 Differentiation/sporulation (SCO1772), 3.3.0 Central intermediary metabolism (uridylate kinase SCO5626), 3.6.0 Fatty acid biosynthesis (3-hydroxybutyryl-CoA dehydrogenase SCO1591), 5.1.0 Laterally acquired elements (SCO5902), 6.2.0 RNA polymerase core enzyme binding (SCO3548) and 1.3.1 Chaperones (GroES – SCO4761).

4.2.4.2 Metabolic pathways positively regulated by SigQ

Only 29 genes out of 90 positively regulated genes were identified within the genes annotated in KEGG pathways database. We identified two major positively regulated pathways: amino acid metabolism and carbohydrate metabolism.

Amino acid metabolism

The largest metabolic pathway positively regulated by SigQ was the Amino acid metabolism (28 genes). It is probably caused by the cultivation in amino acid rich medium. From this finding we can deduce that one of the main functions of SigQ during spore germination might be a positive regulation of amino acid metabolism, mainly metabolism of histidine, glycine, serine, threonine.

Carbohydrate metabolism

The second largest pathway potentially controlled by SigQ (21 genes) was Carbohydrate metabolism. Mainly glyoxylate and dicarboxylate metabolism (6 genes), citrate cycle and propanoate metabolism (3 genes) and others like pyruvate metabolism, butanoate metabolism, glycolysis/gluconeogenesis, pentose phosphate pathway, pentose and glucuronate interconversion, fructose and mannose metabolism, and galactose metabolism.

Other positively regulated metabolic pathways were: pathways of Energy metabolism, Signal transduction, Biosynthesis of secondary metabolites, Lipid metabolism, Metabolism of cofactors and vitamins, Ribosome, Xenobiotics biodegradation and metabolism, Membrane transport, Metabolism of terpenoids and polyketides and in small numbers Folding, sorting and degradation pathway, Metabolism of other amino acids and Nucleotide metabolism

4.2.4.3 Genes negatively regulated by SigQ

We found 110 genes negatively regulated by SigQ at least in one of the three intervals defined in Methods, where SigQ probably played a role as a repressor of gene expression. Altogether 46 genes fell to the non-annotated groups of unknown function or not classified (0.0.0 and 7.0.0). The largest annotated group was that of 4.1.0. Periplasmic/exported/lipoproteins (22 genes), most of them formed by hypothetical proteins (SCO1590, SCO1823, SCO2199, SCO2348, SCO2454, SCO2572, SCO2621, SCO4002, SCO4128, SCO4129, SCO4511, SCO4515, SCO4610, SCO4995, SCO5204, SCO5461, SCO5823, SCO6573, SCO7191, SCO7224), neuraminidase (SCO0033) and D-alanine-D-lactate ligase (SCO3595).

17 genes were assigned to the group of 1.5.0 Transport/binding, including the genes of ABC transporter (SCO2258), ABC transporter ATP-binding protein (SCO4963, SCO6062), ABC transporter permease (SCO6063, SCO6064), integral membrane and transmembrane transport

proteins (SCO1822, SCO2979, SCO4964, SCO5827), integral membrane permease (SCO0067) and sugar transport proteins (SCO1056, SCO1058, SCO6229). Th remaining genes were L-lactate permease (SCO3029), solute binding protein (SCO6569), ammonium transporter (SCO5583) and BCCT family transporter (SCO6739).

Five genes were assigned to group of 6.3.0. Defined families, namely LuxR family- (SCO6334), GntR family- (SCO3264), MarR family- (SCO7727) and TetR family transcriptional regulators (SCO1699, SCO1702). Three genes fell into groups of 2.1.0. Macromolecular degradation (protease SCO2920, D-alanine:D-alanine dipeptidase SCO3596, and heat shock protein HtpX SCO4609) and 3.5.0. Energy metabolism (cytochrome oxidase subunit II SCO3946, D-lactate dehydrogenase SCO3594, and glyceraldehyde 3phosphate dehydrogenase SCO7511).

Two genes were found in the groups of 3.1.0 Amino acid biosynthesis (methylmalonic acid semialdehyde dehydrogenase SCO2726, and cysteine synthase SCO0992), 6.1.0 Two component system (two component sensor SCO5824 and two-component sensor kinase SCO7089), 6.2.0 RNA polymerase core enzyme binding (ECF sigma factors SCO4996 and sigma factor SCO7192), 6.5.0 Others (nitrogen regulatory protein P-II SCO 5584 and DNA binding protein SCO6571), 3.8.0 Secondary metabolism (transcriptional regulator cdaR SCO3217, which was previously found to be regulated by SigQ in contribution with AfsQ1/AfsQ2 (Shu *et al.*, 2009)) and hydrolase SCO0878.

Single genes were assigned to groups of 2.2.0 Macromolecule synthesis, modification (helicase SCO3550, 1.3.1 Chaperones (SCO5210), 3.2.0 Biosynthesis of cofactors, carriers ($NAD^{(+)}$ synthase (glutamine hydrolysing), SCO2238), and 3.3.0 Central intermediary metabolism (membrane-bound proton translocating pyrophosphatase, SCO3547).

4.2.4.4 Metabolic pathways negatively regulated by SigQ

19 genes out of 110 negatively regulated genes were assigned to KEGG pathways. The major negatively regulated pathways were membrane transport, signal transduction, carbohydrate metabolism and energy metabolism.

Membrane transport

The group of Membrane transport is composed of around 5 genes. There were genes coded for ABC transporters: SCO2258 (ABC transporter), SCO2979 (integral membrane transport protein), SCO6062 (ABC transporter ATP binding protein), SCO6063 (ABC transporter permease), and SCO6569 (solute binding protein).

Signal transduction

The second most influenced pathway was signal transduction with the same percentage of incident genes as the membrane transport (5 genes). This group comprised genes from the two-component system pathway, including genes SCO3594 (*dldh*, *vanH*, D-lactate dehydrogenase), SCO3595 (*ddlA2*, *vanA*, Dalanine-D-lactate dehydrogenase), SCO3596 (*vanX*, D-alanine:D-alanine dipeptidase), coming from one operon. Then, genes SCO3946 (*cydB*, cytochrome oxidase subunit II) and SCO5584 (*glnK*, nitrogen regulatory protein P-II).

Carbohydrate metabolism

Four genes from the carbohydrate metabolism pathway were negatively regulated by SigQ: SCO0462 (glyceraldehyde 3-phosphate dehydrogenase) from the Glycolysis/gluconeogenesis, SCO0951 (methylmalonic acid semialdehyde dehydrogenase) falling into Propanoate

metabolism group, SCO1031 (methylmalonic acid semialdehyde dehydrogenase) and SCO1022 (hypothetical protein) from the Inositol phosphate pathway.

Energy metabolism

From the energy metabolism group, we detected four genes as repressed by SigQ. SCO1096 (membrane-bound proton-translocating pyrophosphatase) and SCO1086 (cytochrome oxidase subunit II) from the Oxidative phosphorylation pathway, SCO1106 (oxidoreductase) assigned to Methane metabolism and SCO1188 (cysteine synthase) from Sulphur metabolism. Other SigQ-negatively regulated metabolic pathways were Amino acid metabolism, Drug resistance, Metabolism of cofactors and vitamins, Biosynthesis of other secondary metabolites, Cell motility, Metabolism of other amino acids, Xenobiotics biodegradation and metabolism.

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

The vegetative phase of growth begins when hyphal tubes begins to branch and substrate mycelium emerges (Kieser *et al.*, 2000). The main sigma factor that plays a pivotal role in this life stage is HrdB. Not much is known about it. It is a homologue of *E. coli* σ^{70} sigma factor (Brown *et al.*, 1992) and it is a housekeeping sigma factor that belongs to the Group 1 of sigma factors (Sun *et al.*, 2017). HrdB was studied in the past using deletion studies, but when it was found that its deletion is lethal for the cells, it was examined no more (Buttner *et al.*, 1990). We decided to reveal its regulon by ChIP-seq method in the vegetative phase of growth. The ChIP-seq results were further complemented with gene expression kinetic analysis applying the data from Gene expression omnibus (GEO) published by Nieselt *et al.* (Nieselt *et al.*, 2010). We

used this data for the evaluation of the gene expression kinetics as discovered by ChIP-seq and this approach helped us to find out and confirm if HrdB regulates identified target genes.

It was discovered that RbpA protein facilitates the transcription from HrdB-dependent promoters (Tabib-Salazar *et al.*, 2013), so we included RbpA protein to our gene expression kinetic modelling as well.

4.3.1 Identification of HrdB binding regions by ChIP-seq

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 annotated start codon. Genes that were encoded in both reverse and forward directions in the same region are also included. Than 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.

4.3.2 Kinetic modelling of the HrdB regulon

(performed by Ing. J. Vohradský, CSc. and his colleagues)

The identified genes were searched in gene expression database published by (Nieselt *et al.*, 2010) which is available in GEO under accession number GSE18489 and their gene expression profiles were compared with the gene expression profile of HrdB. Gene expression

measurements consist of 32 time points without replicates (20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 46, 48, 50, 52, 54, 56, 58, 60 h). The data of individual time points were normalized in order to have the same mean of the distribution of expression values. The variance of the expression profiles is very high also because Nieselt were ran the experiments without replicates. In order to find the trend in expression, the time series were splined as defined in Lundgren's procedure (Lundgren, 2010). Of the 2147 genes found by the ChIP-seq analysis results, gene expression time series were found in the Nieselt's dataset for 2137 genes (Figure 26; 27A).

The published papers (Newell *et al.*, 2006, Tabib-Salazar *et al.*, 2013) suggested the important function of RbpA protein during the initiation of transcription (see section 1.4.4 in Introduction). The complex HrdB-RbpA stimulates the transcription from HrdB-dependent promoters(Tabib-Salazar *et al.*, 2013). Thus, we examined genes under the control of HrdB, identified by ChIP-seq, to see if there could be any improvement in the fitting when the RbpA protein is added.

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 HrdB–RbpA complex at all (121 genes). For the modelling, we used Genexpi tool and associated workflow with minor additions (Modrak & Vohradsky, 2018).

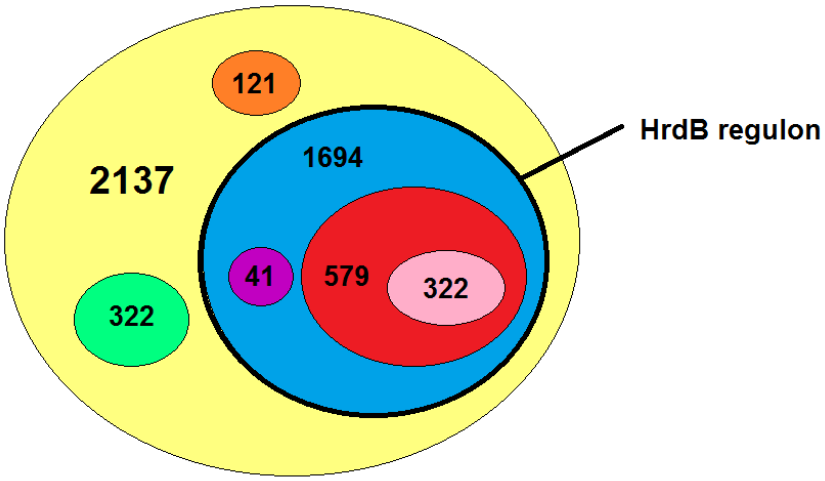


Figure 26. Schematic representation of genes distributed according to modelling results. 2137 genes were identified by ChIP-seq analysis (yellow), 322 genes have constitutive rate of expression (green). 121 genes could not be modelled at all (orange). 1694 genes were modelled with HrdB or a HrdB-RbpA complex. 41 genes can be modelled by the HrdB-RbpA complex only. 579 genes gave 10 % better fit with the complex HrdB-RbpA as a regulator and 322 genes gave 20 % better fit with with the complex HrdB-RbpA as a regulator. Adapted from (Smidova *et al.*, 2019).

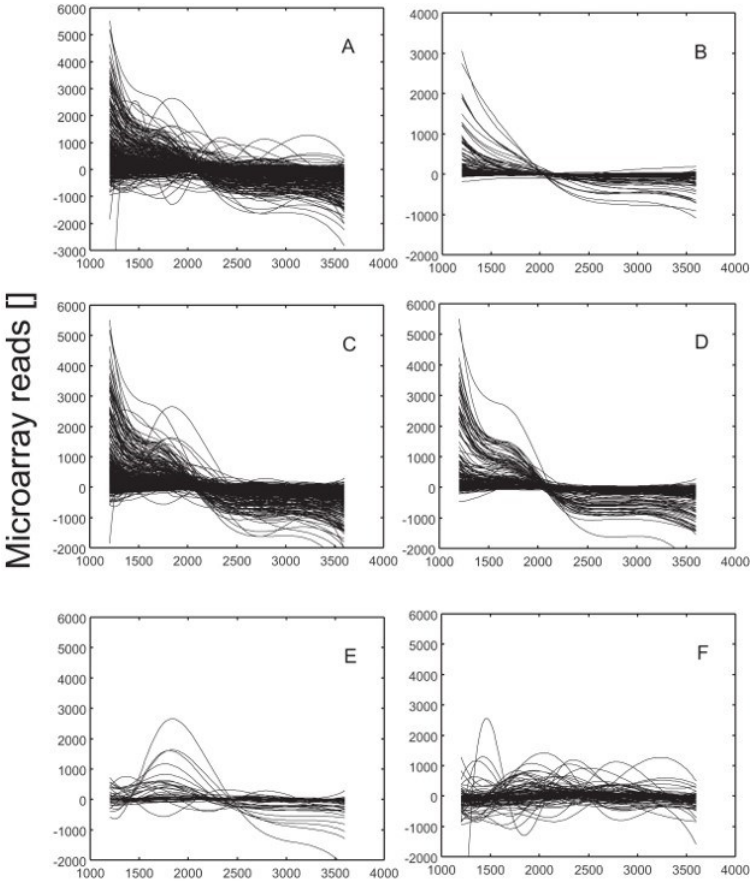


Figure 27. Splined expression profiles of genes selected by various models. (A) All genes of the HrdB regulon as found by ChIP-seq, (B) genes with constant rate of transcription, (C) genes modeled with HrdB or HrdB–RbpA complex, (D) genes modeled 20% better with HrdB–RbpA complex than with HrdB alone, (E) genes modeled exclusively by the complex HrdB–RbpA, (F) genes not modeled by any of the models used. The data were obtained from GEO, accession number GSE18489. Adapted from (Smidova *et al.*, 2019)

2137 genes identified in the HrdB regulon were successfully modelled and divided in several groups according to the mode of regulation by HrdB. Their schematic representation is in Figure 26. The group of genes with constitutive rate of expression consists of 322 genes whose mRNA expression profiles is independent on the expression profile of the sigma factor HrdB or the complex HrdB-RbpA (Figure 26 green, 27B). So there is no dependence on HrdB sigma factor to influence the expression of genes in this group. An unspecific mixture of genes was in this

group. There is also 121 genes that have not been fitted by any of the used models (Figure 26 orange; 27F). The proportional classification exhibits the most represented group as secondary metabolism (30 genes of total 277). Most of these genes were from the polyketide synthase pathway.

The next and the most important group is a group of genes controlled by HrdB or the complex HrdB-RbpA, which is further called as 'HrdB regulon' and is discussed in Discussion. There is 1694 genes that were modelled by HrdB or the complex of HrdB-RbpA (Figure 26 blue; 27C). This group contains also 41 genes that could be fitted only with the HrdB-RbpA complex (Figure 26 purple; 27E), 579 genes that were modelled by 10 % better using the complex HrdB-RbpA (Figure 26 red) and a group of 322 genes that were by 20 % better fitted with the complex HrdB-RbpA (Figure 26 pink; 27D). In this group, we identified mostly genes from the groups of Chromosomal replication, Adaptation, Energy metabolism, Nucleotide biosynthesis and Ribosome constituents (38 of 67 total), including the majority of ribosomal proteins that seem to be most important (Appendix 2). Mentioned 41 genes that were found to be controlled exclusively by the complex HrdB-RbpA are a mixture of diverse genes. Detailed proportional classification analysis did not reveal any specific group of genes that would be most represented. There are hypothetical proteins, several proteins from energy metabolism such as nitrate reductase subunits, electron transport protein, membrane proteins and others.

4.3.3 Functional classification of the HrdB regulon

In the HrdB regulon there are genes that were fitted by the models with HrdB or with the complex HrdB-RbpA. Altogether 1694 genes were subjected to proportional classification and

we identified the most abundant groups of gene ontologies (Figure 28). The list of genes in HrdB regulon is in Appendix 2.

From the proportional representation (Figure 28) we can see that 76,7 % of genes from Nucleotide biosynthesis, 70 % of genes from Ribosome constituents group and almost 63 % of genes from Chromosome replication group are regulated by HrdB. Other groups are regulated from 10 – 30 % by HrdB which is also quite high amount. There are Energy and Carbon metabolism, Amino acid biosynthesis, Macromolecule synthesis and modification, Biosynthesis of cofactors and carriers, Central intermediary metabolism, Protein kinases, Cell division, Chaperones, Periplasmic/exported lipoproteins, Protection responses, Not classified proteins and proteins with unknown function etc. Detailed description of genes from selected functional groups that were most abundant and are essential for the cell living are summarized in the following paragraphs.

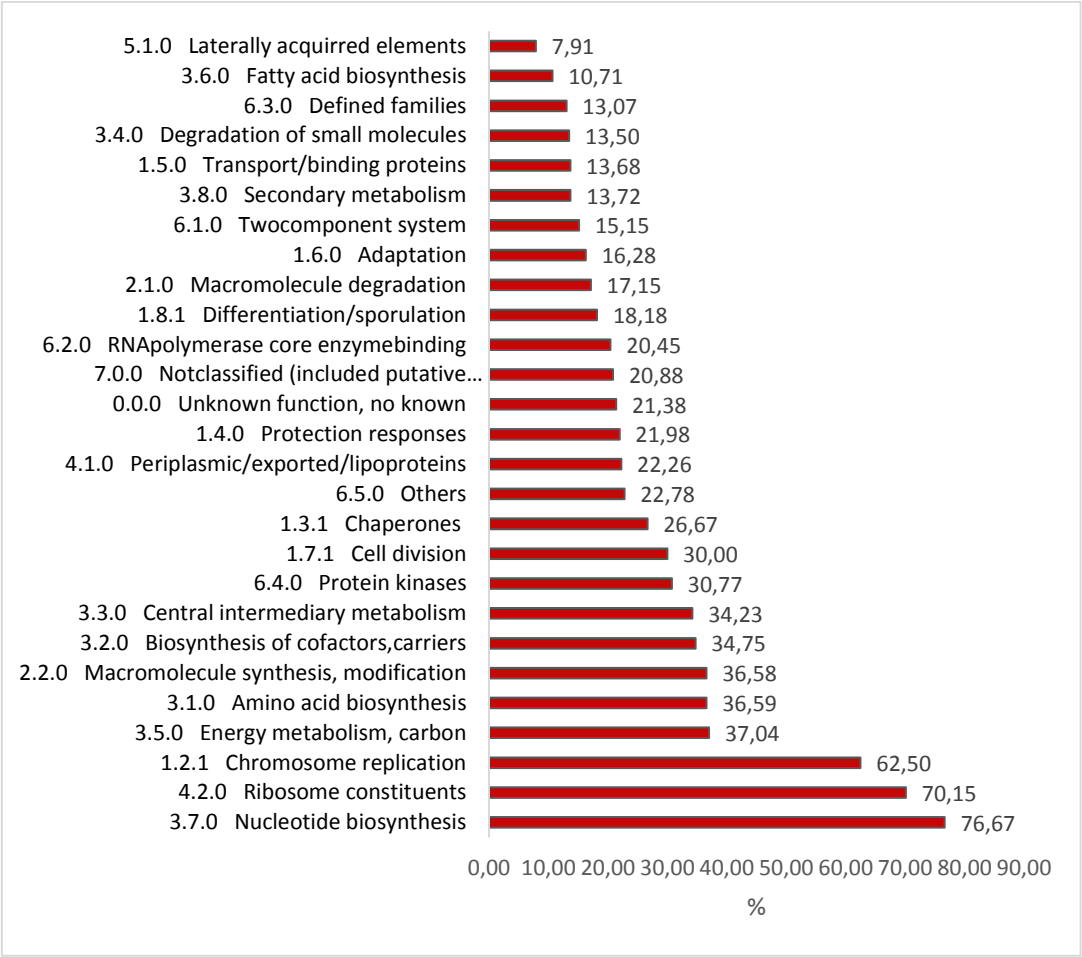


Figure 28. Proportional representation of gene ontologies in HrdB regulon (each group represent a percentual proportion of genes in HrdB regulon from all genes in each gene ontology group).

4.3.3.1 Nucleotide biosynthesis

This group contains 2 subgroups: Purine biosynthesis genes and Pyrimidine biosynthesis genes. We have identified almost 77 % of all genes from this group in HrdB regulon and it was the most abundant group at all. In the purine biosynthesis subgroup we identified 17 genes (from 21): *prsA* (SCO0782), *purB* (SCO1254), *guaB2* (SCO1461), *purE* (SCO3059), *purK* (SCO3060), *prsA2* (SCO3123), *purA* (SCO3629), SCO3677, *purQ* (SCO4078), *purL* (SCO4079), *purF* (SCO4086), *purM* (SCO4087), *adk* (SCO723), *guaB* (SCO4770), SCO4771,

purN (SCO4813), *purH* (SCO4814). In the 'pyrimidine biosynthesis' subgroup we found six genes (from 9) - *pyrB* (SCO1487), *carB* (SCO1483), *pyrAA* (SCO1484), *pyrC* (SCO1486), *pyrF* (SCO1481), and *cmk* (SCO1760).

4.3.3.2 Ribosome constituents and translation

Based on our results, we found out that HrdB regulates 70 % of all ribosome constituents coded in the *Streptomyces* genome.

Within this group we identified in the HrdB regulon genes encoding essential proteins for translation such as elongation factors (Tu (SCO1321, SCO4662), P (SCO1491), Ts (SCO5625), G (SCO1528, SCO4661)), translation initiation factor (IF-1 (SCO4725), IF-3 (SCO1600), signal peptidases (SCO5596-SCO5599), ribosome-binding factor A (RbfA) (SCO5708) needed for efficient processing of 16S rRNA and for maturation of the 30S ribosomal subunit, signal recognition particle protein (SCO5586) (in the 'others' group we also identified srp RNA consisting of 4,5S rRNA), peptidyl-prolyl cis-trans isomerase (SCO1510), peptidyl-prolyl isomerase (SCO5939)), 16S rRNA-processing protein RimM (SCO5593), tRNA pseudouridine synthase B (SCO5709) and pseudouridine synthase (SCO1768).

4.3.3.3 Chromosome replication

Within the group of Chromosome replication we identified in HrdB regulon *dnaE*—DNA polymerase III subunit alpha (SCO2064), *dnaN*—DNA polymerase III subunit beta (SCO3878), *dnaZ*—DNA polymerase III subunit gamma and tau (SCO4067), *dnaA*—chromosomal replication initiation factor (SCO3879) and *dnaB*—replicative DNA helicase (SCO3911).

Other DNA replication proteins identified in HrdB regulon belongs to the group of 2.2.3 DNA replication, repair, restriction modification system, where 21 genes out of total 85 (around 25%) were in HrdB regulon. These are genes such as DNA helicases (SCO1167, SCO3550, SCO4092, SCO5815), DNA topoisomerases (I-SCO3543, IV subunit beta SCO5822), subunits of DNA gyrase (A - SCO3873, B - SCO3874), and DNA ligase SCO6707.

4.3.3.4 Transcription

In the HrdB regulon, almost 26 genes were revealed to play a role in the transcription. These were: RNA polymerases subunits, transcription termination factor Rho, transcription elongation factor NusA and GreA, transcription antitermination protein NusB and mainly sigma factors including WhiG, SigK, HrdD, SigE, SCO5147, BldN, SigI, SigQ and anti-sigma factor RsbA and its antagonist RsbB.

4.3.3.5 Cell division

In HrdB regulon we identified also genes from cell division machinery. These were genes coded for FtsZ, FtsI, FtsE, FtsH-like protein, cell division trigger factor SCO2620, cell division protein FtsX, Sfr protein and septum determining protein SCO4531.

4.3.3.6 sRNAs, tRNAs, rRNAs

Our ChIP-seq experiments and kinetic modelling revealed 75 sRNAs to be under the HrdB control out of 105 known sRNAs (Panek *et al.*, 2008, Moody *et al.*, 2013). These included T-box leader scr2076 and three miscellaneous RNAs—srp RNA (signal recognition particle RNA), ssrA gene for tmRNA and rnpB (probable ribonuclease P RNA).

We also found 62 tRNAs within the HrdB regulon out of 63 tRNAs in *Streptomyces* genome (Bentley *et al.*, 2002).

At last, we identified all six sets of ribosomal RNA gene clusters in the streptomycetes genome (Bentley *et al.*, 2002) within the HrdB regulon.

4.3.4 Promoter region binding motif search

Performed by Ing. M. Schwarz

4.3.4.1 Analysis of -10 and -35 region

First we analysed the -10 and -35 regions upstream of the genes found in HrdB regulon with the known transcription start sites (TSS). The identified binding motifs were compared with the general promoters found by transcriptional analysis by (Jeong *et al.*, 2016). The comparison of the motifs found by method 1 and 2 with the motif published by (Jeong *et al.*, 2016) is in Figure 29. Details of the methods are in the article (Smidova *et al.*, 2019).

The motif found in the -10 region is very similar to the motif found by Jeong *et al.*, whereas the motif in -35 region is not clear and seems to be similar only when the method 1 was used. According to our comparison of extracted sequences for -10 regions from Fimo of HrdB dependent genes and HrdB-independent genes, we found out that the occurrence of the motif was quite similar suggesting that the -10 motif is quite general and not absolutely specific for HrdB.

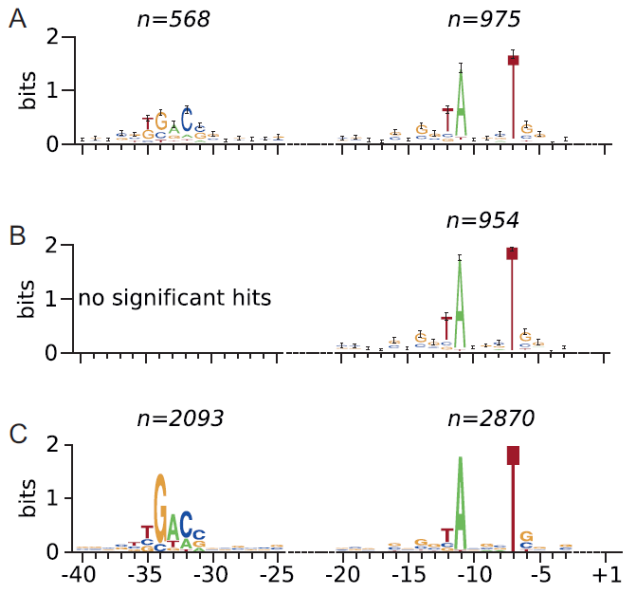


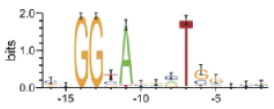
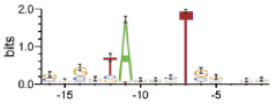
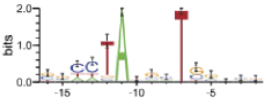
Figure 29. Comparison of identified binding motifs (A, B) with published motif (C) by (Jeong *et al.*, 2016). (A) Motifs identified with method 1, the motif in -10 region (right) was identified at 93 % sequences. (B) Motif for -10 region obtained (right) with method 2, the best motif for -35 region was present only at 4 % sequences (not shown). (C) Image adopted from (Jeong *et al.*, 2016). The -10 motif is reported in 80 % and the – 35 motif in 59 % of sequences. The motifs were drawn with Weblogo version 3.6.0. Adapted from (Smidova *et al.*, 2019).

4.3.4.2 Analysis of -13 and -14 region

The -35 region seems to be low-conserved in HrdB regulon according to our data. It is quite common that promoters lack – 35 region. And these promoters with lacking -35 regions often possess extended -10 regions (Kumar *et al.*, 1993, Kuznedelov *et al.*, 2002). Also the GC rich *Actinomycete*, *Mycobacterium tuberculosis* possesses the majority of promoters with lacking -35 regions (Cortes *et al.*, 2013). The importance of guanosines in -13 and -14 regions was shown as well (Zhu *et al.*, 2017). Interestingly, crystallographic studies of mycobacterial transcription initiation complex revealed the importance of RbpA protein that facilitates the binding between RNAP and the extended -10 region, especially -13 and -14 regions (Hubin *et*

al., 2017). So, based on these findings, we performed statistical analysis of the occurrence of guanosines mono-and di-nucleotides in the positions -13 and -14 of the HrdB-dependent promoters. We inspected the sites in the motifs identified with method 2 and divided them into three groups according to presence of GG, G or no G at the position -13 and -14 (Table 5). We also included T at position -12 because it exhibited an interesting finding with a potential importance. We also compared the frequency of occurrence of GG in the whole genome and in our 954 analysed sites and subjected it to Fischer test. We can conclude that the occurrence of GG sites in the regions -13 and -14 is non-random and may play a role in the transcription initiation from the HrdB-dependent promoters.

Table 5. Occurrence of guanosine mono- and dinucleotides in the -14 and -13 regions. N number of sites with GG, G and no G at −14 −13 positions of 954 total. Horizontal axis in logos was taken relative to TSS. Error bars in logos indicate an approximate Bayesian 95% confidence interval. T at −12—number of sites with T at −12 position, the percentages were calculated relative to N. The motifs were drawn with Weblogo version 3.6.0. Adapted from (Smidova *et al.*, 2019).

	GG	only G	no G
N	221 (23%)	514 (54%)	219 (23%)
Logo bits			
T at -12	114 (52%)	363 (71%)	183 (84%)

4.4 Experimental search for novel cis-antisense sRNAs

sRNAs in bacteria are a widespread molecules that act by diverse mechanisms to balance their gene expression in response to changes in environment, morphological and developmental changes. They affect transcription or translation of genes associated with these changes (Dutta & Srivastava, 2018). Also the sigma factors themselves are regulated by sRNAs (Klein & Raina, 2017). It is known that sigma factors together with sRNAs create a regulatory feedback loop to regulate stress responses in *E. coli* or in *Salmonella enterica* (Klein & Raina, 2017). The regulation by sRNAs is achieved by reducing the translation of these regulators resulting in the dampening of elevated stress responses or altered metabolic pathways (Klein & Raina, 2015). 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 (Gogol *et al.*, 2011, Klein & Raina, 2015). Another stress related sigma factor σ^S is in *E. coli* positively regulated by sRNAs ArcZ, RprA (Majdalani *et al.*, 2001), DsrA (Majdalani *et al.*, 1998) and negatively regulated by SdsR and OxyS (Zhang *et al.*, 1998, Klein & Raina, 2017). In *Salmonella enterica*, sRNA SdsR regulates general stress sigma factor σ^S and simultaneously SdsR sRNA is contained in σ^S regulon (Frohlich *et al.*, 2016).

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 have chosen opposite way of searching for sRNAs. The common approach for the searching of sRNAs lies in using a sequencing approach combined with computational approach. 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, thus influencing their translation. 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. So the primers for RACE experiments were designed to cover the RBS and START codon of the target mRNA in an opposite direction (Figure 30). 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 selection of sigma factors to our experiments is based on their importance (connection with stress, morphological differentiation) or because their function is 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 (Blomberg *et al.*, 1990, Gerdes *et al.*, 1992); 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 30; 31).

The expression level of as-sigB in wt strain decreased in 48 hours of growth and in 72 hours of growth increased again (Figure 31); in *rnc* strain it kept continuously increasing during the time of growth to the same value as in wt. The expression level of as-sigH kept continuously increasing with the time of growth in wt strain, whereas in *rnc* strain it increased in 48 hours of growth and then slightly decreased (Figure 31). The expression level of as-sigR has the same

profile in wt strain as well as in *rnc* strain. In 48 hours of growth it decreased and in 72 hours of growth it increased (Figure 31). The expression levels of cognate mRNAs of all sigma factors reached their maximum in 48 hours of growth in *rnc* strain, whereas in wt strain the expression levels of *sigH* mRNA and *sigB* mRNA were almost constant during the time of growth and the expression level of *sigR* mRNA was the highest in 48 hours of growth. When we compare the expression profiles of as-sigR and *sigR* mRNA, we can see that there is an evident negative influence of as-sigR on *sigR* mRNA (Figure 31). When as-sigR decreased in 48 hours of growth, *sigR* mRNA increased; when as-sigR increased in 72 hours of growth, *sigR* mRNA decreased. Thus in case of as-sigR, we can see the direct effect of this *cis*-asRNAs on the expression level of target *sigR* mRNA suggesting for the negative regulation of SigR by as-sigR.

It is obvious that these novel asRNAs are also associated with stress related sigma factors SigB, SigH, SigR as was reported previously in *E. coli* and *Salmonella enterica*, where the sRNAs found were targeted against stress related sigma factors (Frohlich *et al.*, 2016, Klein & Raina, 2017), suggesting that the regulation of stress related sigma factors by sRNAs could be more widespread.

The data presented in this chapter contribute to the published paper: 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., where the set of identified asRNAs were used as a control set. Raw northern blot images, quantification of the Northern blot signals is in Appendix 3.

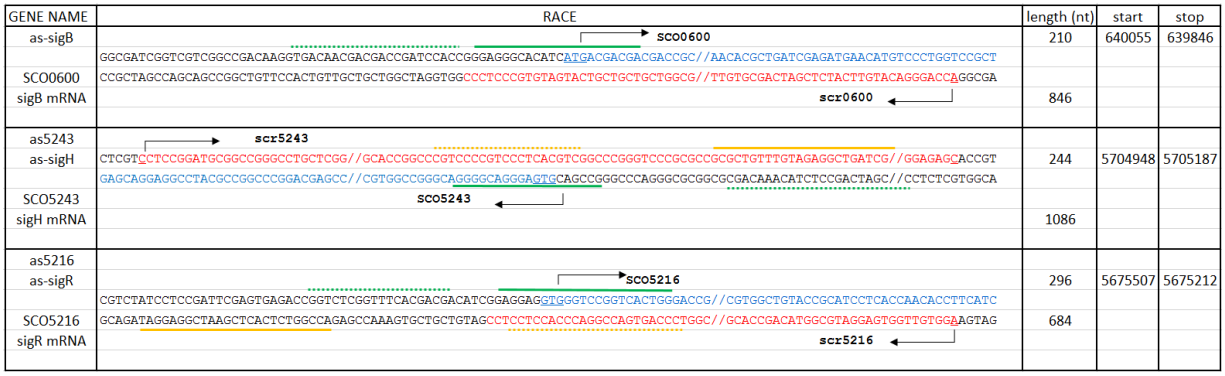


Figure 30. Genomic arrangement of novel asRNAs of sigma factors SigB, SigH, SigR (sequence of asRNA – red, sequence of mRNA – blue). Transcriptional start sites are indicated by arrows. Full green line represents 5' RACE inner primers, dotted green line represents 5' RACE outer primers. Full orange line represents 3' RACE inner primers, dotted orange line represents 3' RACE outer primers.

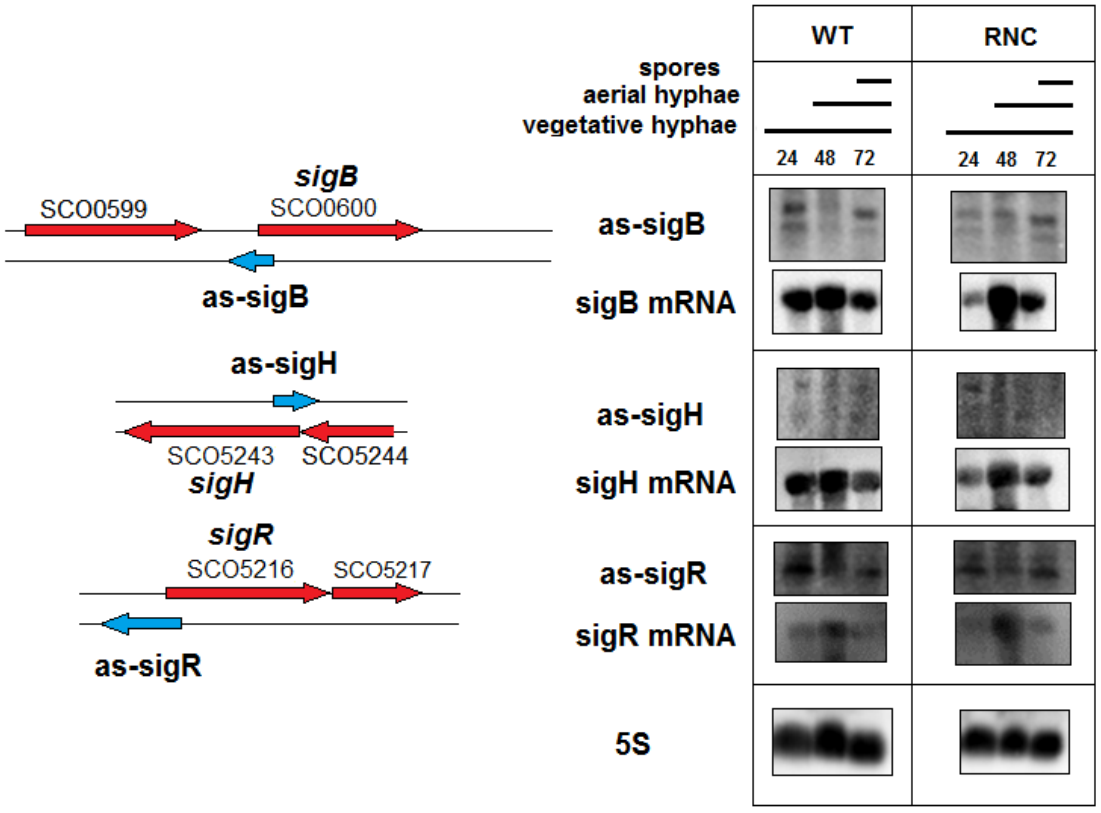


Figure 31. Genomic arrangement (left) and Northern blots (right) of identified novel asRNAs of sigma factors sigB, sigH and sigR in *S. coelicolor* M145 wt strain and *rnc*-mutant strain with 5S RNA as a loading control. Genes are in red and asRNAs are in blue.

5 Discussion

This thesis focuses on the identification and mapping of gene expression regulators during the spore germination and vegetative growth in bacterium *Streptomyces coelicolor*. We are engaged in the study of sigma factors as the main transcriptional regulators. Principally, we focused on the characterization of their regulons and regulators. The characterization of the sigma regulons is of high importance because it tells us more about the function and the role of sigma factors in the given growth phase and helps us understand better gene expression control mediated through each specific sigma factor. Being potent transcriptional regulators requires their own expression and/or functional control, which has been found to be provided herein by sRNAs, more or less specific regulators playing a role in transcriptional and translational control.

5.1 Identification of sigma regulons

As was shown in our previous lab's results (Bobek *et al.*, 2014), spore germination exhibits an importance of ECF sigma factors expression. Our lab has revealed about 10 different sigma factors that are known to control various stress responses and whose fast expression changes suggest their importance during germination (Bobek *et al.*, 2014). From these data, we suggested that the process of germination evokes both osmotic (due to the water influx) and oxidative stress responses (Strakova *et al.*, 2013). The SigQ is one of the early expressed regulators whose expression is the highest one among all the sigma factors expressed in spore germination. In addition, we have found the intracellular SigQ level to be highly increased by the osmotic stress (0.5 M NaCl) (Figure 23). Based on these investigations, SigQ is proposed to be one of the major regulators acting in spore germination on which we have further focused.

The second sigma factor which we dealt with in our work is HrdB sigma factor. It is a primary sigma factor and is supposed to be a functional homolog of σ^{70} in *E. coli* (Shiina et al., 1991). Of the four *hrd* genes (*hrdA, hrdB, hrdC, hrdD*), it is the only one that is present in all *Streptomyces* species (Takahashi *et al.*, 1988). In 1990 it was found that HrdB is essential for the cells as its deletion is lethal (Buttner *et al.*, 1990). Due to the lethality of its deletion it has not been further studied so far.

We decided to reveal the sigma regulons of these two sigma factors – HrdB and SigQ by ChIP-seq method in order to know which genes are regulated by these sigma factors. To perform the ChIP-seq experiment, a tagged sigma factor is needed. We developed a unique method for epitope tagging by insertional mutagenesis in *Streptomyces* that is derived from the PCR targeting gene replacement system invented by Gust and his colleagues (Gust *et al.*, 2003). We used *S. coelicolor* cosmids (Redenbach *et al.*, 1996) as the as the source of DNA homologous to the DNA in the genome. An antibiotic resistance cassette selectable in *E. coli* and *Streptomyces* with an attached HA tag was inserted into this cosmid, thanks to the λ-Red recombination system in *E. coli*. The origin of a transfer (oriT, RPK2) site in the cassette enabled the conjugal transfer of the PCR-targeted cosmid from *E. coli* into *S. coelicolor,* generating exconjugants with the attached HA tag in the desired sigma factor. This method also includes the elimination of the resistance cassette by FLP-recombinase-mediated-site-specific recombination but we were unsuccessful with this step (Cherepanov & Wackernagel, 1995). We didn't observe any obvious changes due to the unremoved cassette from the chromosome. Details of this method is described in Results – 4.1 Preparation of tagged sigma factors by insertional mutagenesis).

The insertion-based method used here is novel within the field of streptomycetes in that point of view that the epitope tag is attached directly to the gene of interest within the chromosome.

A similar approach was previously, but independently, performed by Kim and his colleagues. They attached c-myc tags to several global transcription factors with tandem arrangement to amplify the tagging system. They also proved the validity of this system by ChIP-seq (Kim *et al.*, 2012).

In our work, a 27 bp sequence coding the HA-tag was inserted within the sigma factor gene in its native site in the chromosome. The mutagenesis procedure followed the REDIRECT technology protocol (http://streptomyces.org.uk/redirect/protocol_V1_4.pdf) with several modifications (see Methods) leading to an addition of the HA-tag to the selected sigma factor (Gust *et al.*, 2003). Our Western blot of a tagged sigma factor approved the competence of the method (Figure 22). As the amino acid sequence of the tag was very short (9 amino acids), no influence on the protein function and folding had been expected and none has been revealed.

We focused on those sigma factors that exhibit fast gene expression changes and high levels of gene expression during spore germination according to our previous investigations (Bobek *et al.*, 2014). These are SigB, SigH, SigR, SigE, SigD, SCO1263, and SigQ. In addition, we also included a primary sigma factor HrdB to our experiments. All these sigma factors were successfully tagged and verified by Western blot (Figure 22), except for SCO1263 and SigE, which were successfully tagged and verified only by PCR. So far, ChIP-seq has been performed on sigma factors SigQ and HrdB.

HrdB regulon was identified 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.* (Nieselt *et al.*, 2010) in order to examine the gene expression kinetics of the identified genes during the time interval between 20 hours and 60 hours of growth. The cultivation conditions were the same as those in the experiments of Nieselt *et al.* (Nieselt *et al.*, 2010).

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 in accordance with the conditions in which we had measured gene expression time series previously, as was published in (Strakova *et al.*, 2013, Strakova *et al.*, 2013, Bobek *et al.*, 2014). ChIP-seq results were also coupled with these gene expression time series to perform gene expression kinetics of the identified genes during the first 5.5 hours of spore germination.

5.2 Comparison of identified regulons

In SigQ regulon, we have identified 326 protein-coding genes and three tRNAs, whereas in HrdB regulon, we have identified 2137 protein-coding genes, 75 small RNAs (previously discovered), 62 tRNAs, 6 rRNAs and 3 miscellaneous RNAs (Appendix 2). HrdB regulon is much wider and diverse compared to the SigQ regulon. It is because HrdB is a major vegetative sigma factor that regulates essential pathways needed for the cell living such as nucleotide biosynthesis, transcription, translation and replication components, energy metabolism, amino acid biosynthesis, central intermediary metabolism etc.. Besides this, SigQ acts in spore germination as a regulator of only several specific events such as nitrogen metabolism, and participates in osmotic stress response and in cell wall reconstruction within the cell remodelling that accompanies spore germination (Bobek *et al.*, 2017). These statements also confirm the representation of a group of genes in each sigma regulon. HrdB regulon contains genes that represent tens of percent of given metabolic pathways, for example, 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 (Figure 28). Other genes from HrdB regulon constitute from 30 % to 10 % of respective groups, whereas genes

from SigQ regulon constitute only units of percent of respective groups. It is probably caused by the specificity of sigma factor, which correlates with the size of the regulon and with the amount of genes of respective groups, regulated by studied sigma factor. The most represented group is Chaperones, whose 13 % of genes were found in SigQ regulon. The other most represented groups were Amino acid biosynthesis with 11 % and Differentiation/Sporulation with 9 % (Figure 25).

5.3 The role of SigQ during spore germination

Our data clarify the enormous SigQ expression during spore germination, as was measured previously (Bobek *et al.*, 2014). Spore germination is a process when the cells awake its metabolism and it is accompanied by a cell wall reconstruction with the final stage of germ tube emergence. According to the previous results, this process is associated with stress (Strakova *et al.*, 2013). SigQ plays a major role in the events during spore germination. As our data indicate, SigQ probably participates in the regulation of osmotic stress, in the cell wall remodelling in connection with cell division, and is involved in nitrogen metabolism.

Although the most represented group was Chaperones with 13 % genes found in SigQ regulon from all genes in this group (Figure 25), there were only 2 genes in SigQ regulon out of 13 in total encoded in *Streptomyces* genome. One of them SCO4761 coding for GroES chaperon, which has been found to be positively regulated and the second one, SCO5210 that was negatively regulated. Chaperones are proteins that protect other proteins from damaging stress by preventing protein aggregation and misfolding and assist in the repair of protein that has been damaged by stresses that accompany spore germination (Lund, 2001, Henderson *et al.*,

2006, Strakova *et al.*, 2013). During spore germination, also reactivation of translational systems occurs, leading to the reactivation and refolding of aggregated proteins (Cowan *et al.*, 2003, Strakova *et al.*, 2013). Refolding of the proteins is ensured mainly by the chaperons. They also contribute to the reactivation of ribosomes needed for the translation (Bobek *et al.*, 2004, Strakova *et al.*, 2013).

The second most represented group was Amino acid metabolism with 11% of genes from this group that could be related with the cultivation in amino acid rich medium. There were 13 genes out of 123 in total; out of them 11 were positively regulated and 2 was negatively (exhibit inverse expression profiles compared to the *sigQ*). Among the positively regulated, there were proteins belonging to the nitrogen metabolism group which is discussed below.

The third most abundant group in SigQ regulon was group of Differentiation/Sporulation with 9 % genes from this group. There were 1 gene out of 11 in total. It is partitioning or sporulation protein SCO1772, which is probably associated with cell division that massively occurs during spore germination.

We have identified in SigQ regulon several key genes that are associated with nitrogen metabolism. These are heat-stable glutamine synthetase glnA (SCO2198) (Hillemann *et al.*, 1993), a key enzyme in nitrogen metabolism in all bacteria responsible for a conversion from glutamate and ammonia to glutamine (Merrick & Edwards, 1995), glutamine synthetase glnA2 (SCO2241), nitrogen regulatory protein GlnK (SCO5584), ammonium transporter AmtB (SCO5583) and GltD (SCO2025). GltD coded for a glutamate synthase responsible for the production of glutamate from L-glutamine (Merrick & Edwards, 1995). It is a little bit confusing to produce glutamate, when it is in the medium, but the active transcription of this enzyme catalysing the production of glutamate does not necessarily mean that glutamate is really produced and GltD can have another regulatory role that is unknown. However, the

accumulation of glutamate has been proved in *E. coli* and *Salmonella typhimurum* as a response to osmotic stress (Csonka *et al.*, 1994, Saroja & Gowrishankar, 1996). Likewise our conditions during spore germination are connected with osmotic stress (Strakova *et al.*, 2013), and thus the production of glutamate could be connected with osmotic stress response in *Streptomyces coelicolor* as well.

It is also important to note that the spores of *S. coelicolor* for this ChIP-seq experiments were cultivated in an amino acid rich medium. These cultivation conditions could result in an enormous expression of SigQ, and indeed, SigQ protein was also clearly detectable during the vegetative phase of growth of *S. coelicolor* after ammonium stress in SMM and R2YT media (Nieselt *et al.*, 2010) or in 2YT medium (Figure 23), while the SigQ protein was only slightly detectable without stress on our western blots (Figure 23). On the contrary, osmotic stress is an concomitant event of spore germination (Strakova *et al.*, 2013), which would contribute to the enormous SigQ expression or could be the only aspect of such enormous SigQ expression. One way or another, thanks to these conditions and the choice of the specific growth phase, we have revealed the nitrogen metabolism dependence on SigQ.

The other important role of SigQ which we have revealed via the ChIP-seq results is osmotic stress response regulation. In SigQ regulon, we have identified *EctABCD* operon (SCO1864-SCO1867). But genes *ectB* (SCO1865), *ectC* (SCO1866) and *ectD* (SCO1867) were excluded from our results due to the inconsistency within the operon. This set of enzymes (EctABCD) catalyses a biochemical reaction from L-aspartate-gamma-semialdehyde to (hydroxy-) ectoine (Kuhlmann *et al.*, 2008). The ectoine is a protective substance that helps cells to survive high salinity or temperature conditions (Bursy *et al.*, 2008, Kol *et al.*, 2010). Due to the consequence that germination evokes an osmotic stress (Strakova *et al.*, 2013), the role of the produced ectoine is probably being as a stress protectant (Bursy *et al.*, 2008).

The third important regulatory role of SigQ revealed in our results is the regulation of cell wall formation and cell division during spore germination. Cell remodelling accompanies spore germination and includes reconstruction and growth of the cells. Peptidoglycan biosynthesis and other components are needed for the cell wall composition. Penicillin binding proteins (PBP) with various enzymatic activities, such as D-alanine carboxypeptidases, peptidoglycan transpeptidases, and peptidoglycan endopeptidases, are essential for the process (Spratt, 1977). In SigQ regulon, we have identified two penicillin binding proteins: SCO3156 and SCO3157. They are transpeptidases that catalyse crosslinking between two adjacent glycan chains (Spratt, 1975, Ogawara, 2015). Also the cell division protein FtsI was found to be a member of SigQ regulon. It belongs to the penicillin-binding proteins as it contains transpeptidase domain (Ogawara, 2015). Although it is needed for an efficient cell division, it could also participate in peptidoglycan synthesis (Spratt, 1975).

Cell wall biogenesis is also connected with the action of glycoproteins as defects in these proteins resulted in significantly retarded growth and increased sensitivity to cell-wall targeting antibiotics (Keenan *et al.*, 2019). Several of these proteins were revealed in SigQ regulon. These are transglutaminase/protease-like membrane protein SCO2096, penicillin acylase SCO3184, integral membrane protein SCO5204 and membrane protein SCO5751. These proteins are predicted to be responsible for participating in cell wall biosynthesis or in maintaining membrane integrity (Keenan *et al.*, 2019).

The results mentioned above suggest that SigQ governs (among others) the nitrogen metabolism, cell wall reconstruction and is probably associated with osmotic stress. Taking into consideration that the cultivation of the spores was carried out in an amino-acid-rich medium, one may argue that such an enormous SigQ expression appeared due to these cultivation conditions. But when we also consider the fact that SigQ was clearly detectable after

ammonium or high salt stress (Figure 23) and the fact that spore germination itself is accompanied by the stress conditions, it is evident that there is a direct link between the expression of SigQ during spore germination rather than between SigQ expression and cultivation in amino-acid rich metabolism. Also the occurrence of osmotic stress protectants, such as ectoin and chaperons, in SigQ regulon together with its high induction by NaCl suggests that SigQ is a regulator of osmoprotection.

As reported previously, SigQ together with two component system genes afsQ1 and afsQ2 is associated with the regulation of antibiotic biosynthesis through the regulation of pathway-specific genes *actII-orf4*, *cdaR*, and *redD (Shu et al., 2009)*. Interestingly, *cdaR*, which is responsible for calcium dependent antibiotic biosynthesis (Ryding *et al.*, 2002), was found to be present in SigQ regulon. CdaR was found to be negatively regulated by SigQ, supporting the hypothesis that beside the above mentioned roles of SigQ in spore germination, it probably also contributes to the negative regulation of the antibiotic biosynthesis during spore germination.

Our measured data of SigQ regulon have not been published yet, because when our colleague, bioinformatician Jiří Vohradský, CSc., performed a test when he interchanged the data from sample and negative control, conducted the same kinetic analysis and compared the distribution of correlation coefficients of the identified genes and *sigQ* profile and all expressed genes and *sigQ* profile, he found out that the ChIP-seq results seem more likely to be a random choice rather than a specific SigQ regulon. We assumed that it was probably due to the badly chosen parameters of the next generation sequencing with an insufficient depth. But when we sent the same samples to the NGS service in Heidelberg, we got almost the same data. Another reason, why we are tentative about the publication, is associated with the negative regulation of the identified genes by SigQ. Sigma factor is in general an activator of transcription and this was

the first time, when we encountered the possibility that the regulation could be also negative. This statement of negative regulation is based only on kinetic modelling data that are derived from gene expression levels during the growth of *Streptomyces*. We took into consideration the entire time interval (300 min of growth), but when we take a look at each gene separately, we can see that SigQ profile is inverse or slightly inverse only at some shorter intervals. It is also important to note that we coupled the transcriptomic data and ChIP-seq data. Not each promoter binding leads to the transcription and not all the transcripts are translated into a protein. Expression profiles of *sigQ* and identified genes come out from the mRNA levels during the 5.5 hours of growth and the mRNA abundancy can be different from the protein abundancy. Thus the protein expression profile of SigQ can be different from its mRNA expression profile. It was reported previously that the correlations of proteomic and transcriptomic data vary widely across organisms, and are often surprisingly low, the squared Pearson's correlation coefficient (R^2) is in the range of 0.20 to 0.46 (de Sousa Abreu *et al.*, 2009). The variability between the proteomic and transcriptomic data is caused by the posttranscriptional processes, regulation of translation, protein degradation, and by the errors during experiments as well (de Sousa Abreu *et al.*, 2009). The studies of *S. coelicolor* described that 30 % of the genes exhibited significantly divergent patterns, of which almost one third showed opposing trends (Vohradsky *et al.*, 2007, Jayapal *et al.*, 2008, Strakova *et al.*, 2013). In addition, ChIP-seq method is not a strand-specific method, one peak might be allocated to two genes and thus many genes can be false positive. Also the operon prediction incorporation to our results carries some inaccuracies.

Additional experiments should be performed to verify the negative regulation of SigQ. ChIP-seq experiments with cells carrying the deleted *sigQ* gene would be interesting as well as SigQ protein level measurement during the first 5.5 hours of growth.

5.4 The role of HrdB during vegetative growth

We have identified HrdB regulon by ChIP-seq method and coupled the results with a gene expression dataset from several time points during the vegetative phase of growth performed by Nieselt *et al.* (Nieselt *et al.*, 2010). This coupling helped us to see whether HrdB can regulate a respective gene identified by ChIP-seq. Based on this comparison, we divided the results to different ways of regulation, including 'no regulation' (even though the gene was identified by ChIP-seq) which means – simply put – that the expression profile of HrdB is different from the expression profile of the regulated genes. Our modelling included also a RbpA protein that binds HrdB and was shown to enhance the transcription initiation (Hu *et al.*, 2012, Tabib-Salazar *et al.*, 2013). We also compared kinetic models with RbpA and without RbpA to see the differences. RbpA is needed mainly in the late stages of vegetative growth when HrdB expression slowly decreases (Figure 32). Therefore, the expression profiles of genes from late stages of growth, when they reach higher levels of expression, are better modelled with the contribution of RbpA. The model fidelity increased for 322 genes out of the 1694 that were found to be controlled by HrdB when we included RbpA to our modelling, and for 579 genes when the less stringent criterion was used. We assume RbpA to be a substantial protein in HrdB-regulated genes, affirming the previously established findings in *Streptomyces* (Newell *et al.*, 2006, Tabib-Salazar *et al.*, 2013).

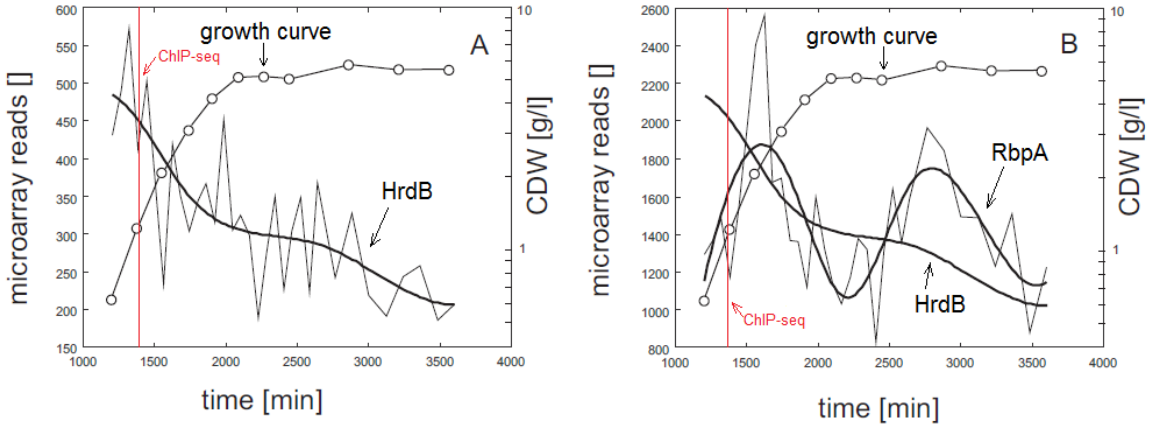


Figure 32. Expression profile of HrdB (A) and RbpA (B). Splined and measured together with the growth curve. The data were obtained from GEO, accession number GSE18489 and (Nieselt *et al.*, 2010)

The most represented group in HrdB regulon was group of Nucleotide biosynthesis with 77 % genes (Figure 28). There were 23 genes out of 30 in total. Among them, there were genes coded for enzymes needed for the biosynthesis of purines and pyrimidines that are important structural components of DNA, RNA, which are essential biomolecules in all life forms on the Earth. Nucleotides play a central role in metabolism. They provide chemical energy throughout ATP, GTP, CTP and UTP, needed for cell division and all enzymatic reactions in the cells. They are also part of cofactors (coenzyme A, FAD, FMN, NAD, NADP+) and participate in cell signalling (cAMP, cGMP) (Alberts, 2002).

The next most represented group in HrdB regulon was group of Ribosome constituents with 70 % genes. We have identified 47 genes out of 67 in total, consisting of mainly 30S and 50S ribosomal proteins that together with rRNA form ribosomal subunits involved in the process of translation (Owen *et al.*, 2007). A central role of ribosomal proteins is to stabilize the rRNA (Moore & Steitz, 2003) and to mediate the many interactions during translation (Brodersen & Nissen, 2005). Not all ribosomal proteins are essential. For example *Bacillus subtilis* possesses 57 genes encoding ribosomal proteins and out of them, 22 proteins have been shown to be

nonessential, at least for cell proliferation (Akanuma *et al.*, 2012). In *E. coli*, 9 ribosomal proteins out of 54 are nonessential for cell survival (Shoji *et al.*, 2011).

The last most abundant group was Chromosome replication with 63 % of genes from this group. There were 5 genes out of 8 in total; among them, DNA polymerase subunits, replicative DNA helicase DnaB, and initiation protein DnaA. All of them are needed for chromosome replication, mainly for initiation and elongation phase (Plachetka *et al.*, 2019). Chromosome replication is a key process for cell reproduction in all organisms which must be tightly regulated to prevent the loss of energy and to ensure that the DNA is completely replicated once and once per cell cycle (Boye *et al.*, 2000, Plachetka *et al.*, 2019).

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 vegetative phase of growth.

From the identified genes in HrdB regulon, there were a lot of genes associated with morphological development as well as secondary metabolism. These were mainly *bld*, *whi* and *wbl* (**W**hi**B**-**l**ike) genes including a key regulator of morphological development AdpA. HrdB regulates morphological differentiation through the regulation of the major pleiotropic regulators, implying that HrdB controls the main players in the regulation of cell morphology, development, primary and secondary metabolism. Within the HrdB regulon, we have identified BldB, BldC, BldD, BldN (sigma factor), BldH/AdpA, WhiB, WhiG (sigma factor), WblE, WblH, WblA. *Bld* genes are mostly transcription factors with pleiotropic effect on vegetative

cell growth and development including regulation of antibiotics biosynthesis (Merrick, 1976, Champness, 1988, Pope *et al.*, 1996). Whi genes (*whiG* and *whiB*) found in HrdB regulon are early sporulation genes required for a proper sporulation septation (Chater, 1993, Kelemen *et al.*, 1996). These are not the only genes, found in HrdB regulon that are associated with the sporulation. This was a big question for us, because *Streptomyces coelicolor* does not sporulate in any liquid medium (Manteca *et al.*, 2010), not even in 22 hours of growth. This finding was thus a little confusing and shall be discussed below in more detail. *Wbl* genes are exclusively found in *Actinobacteria* (Soliveri *et al.*, 2000, Bush, 2018). From the three genes, found in HrdB regulon, only *wblA* has been studied in more depth and it seems to be a pleiotropic down-regulator of antibiotic biosynthesis among *Streptomyces* (Kang *et al.*, 2007, Noh *et al.*, 2010, Rabyk *et al.*, 2011, Nah *et al.*, 2012), it plays an important role in aerial mycelium formation and functions in oxidative stress response among actinobacteria, such as *C. glutamicum* and *S. coelicolor* (Kim *et al.*, 2012). Other important developmental regulators found in HrdB regulon were AdpA which is a major regulator of morphological differentiation in *Streptomyces* (Vujaklija *et al.*, 1991, Vujaklija *et al.*, 1993).

We have revealed in HrdB regulon other genes linked with morphological differentiation that are responsible for crosswall formation, cell division and hyphae branching during the vegetative phase of growth. These are mainly FtsZ, required for septation (Flardh, 2003, McCormick, 2009), FtsI, needed for peptidoglycan synthesis, FtsX, FtsE, FtsH-like cell division protein (SCO3404), FtsW, and FtsQ. FtsW, FtsI and FtsQ are a part of the prokaryotic cell division cell wall (DCW) gene cluster and are needed for sporulation septation but not during vegetative septation (Mistry *et al.*, 2008). FtsW together with FtsI are both required for Z-ring formation during sporulation septation (Mistry *et al.*, 2008). FtsX and FtsE are located in one operon showing a similarity to ABC transporters, with FtsE corresponding to the ATP-

binding component interacting with FtsX as a membrane component (Higgins, 1992, Noens, 2007). FtsZ, is structurally and biochemically very similar to eukaryotic tubulins (Lowe & Amos, 1998, Nogales *et al.*, 1998) and is essential for cell division (Lutkenhaus & Addinall, 1997, Rothfield *et al.*, 1999). FtsZ is needed for two developmentally distinct types of cell division in *Streptomyces coelicolor*: infrequent cross wall formation in vegetative mycelium and septation of the apical compartments of aerial hyphae resulting in the formation of unigenomic spores (McCormick *et al.*, 1994).

In HrdB regulon, we have also found important proteins that are associated with cell wall synthesis that accompanies hyphae branching to remodel the cell morphology needed during branching of vegetative hyphae. These are mainly penicillin binding protein PBP2 (SCO2608), rod shape-determining proteins MreB, MreC and MreD, secreted penicillin binding protein SCO2897, transpeptidase SCO3580, glycosyl transferase SCO3672, D-alanyl-D-alanine carboxypeptidase SCO4847, transferase SCO5365, muramoyl-pentapeptide carboxypeptidase SCO5467, D-alanyl-alanine synthetase A SCO5560, and UDP-N-acetylglucosamine transferase SCO5998. MreB is a protein structurally similar to actin (van den Ent *et al.*, 2001) that forms cytoskeleton in many rod-shaped bacteria (Jones *et al.*, 2001), and recently was found to be also under the SigE control (Tran *et al.*, 2019). It is highly conserved and is involved in cell shape determination and chromosome segregation in cell division (Mazza *et al.*, 2006). Peptidoglycan synthesis has a pivotal role in determining cell shape and it is important step in the remodelling the cell morphology during the vegetative growth (Cabeen *et al.*, 2009). It is directed by the distinct elements of bacterial cytoskeleton like MreB, representing actin-like protein and FtsZ representing tubulin-like protein (Typas *et al.*, 2011). MreB protein ensures the insertion of peptidoglycan into multiple sites in the lateral wall of the cell and later, FtsZ is required for cell division (Typas *et al.*, 2011). For the peptidoglycan synthesis,

glycosyltransferases and transpeptidases are needed. Glycosyltransferases are responsible for the polymerization of glycan chains and transpeptidase, also called penicillin-binding proteins (PBPs), crosslink the peptides (Suginaka *et al.*, 1972, Vollmer & Bertsche, 2008). In *Streptomyces*, PBPs are likely to be required during the life cycle; some of them are necessary for growth by apical extension during vegetative growth and they are necessary mainly at the earliest stage of sporulation to form sporogenic hyphae, septum formation, and spore maturation (Gray *et al.*, 1990, Miguelez *et al.*, 1992, Hao & Kendrick, 1998). The genome of *Streptomyces coelicolor* possesses 13 PBPs, out of which 4 were identified in HrdB regulon. These are secreted penicillin-binding protein SCO2897, transpeptidase SCO3580, FtsI (SCO2090), and PBP2 SCO2608 (Ogawara, 2015).

Although the cells of *S. coeliocolor* for ChIP-seq experiments were cultivated for 22 hours in a liquid medium, where no aerial mycelium formation and sporulation occurs (Manteca *et al.*, 2010), we identified in HrdB regulon the already mentioned *bld* and *whi* genes and other additional genes that participate in the regulation of aerial mycelium formation and sporulation. It was reported that several streptomycetes strains are able to sporulate in liquid cultures, such as *S. venezuelae* (Glazebrook *et al.*, 1990) or *S. griseus* (Kendrick & Ensign, 1983). Sporulation in liquid cultures was also observed in other Streptomyces species including *Streptomyces coelicolor* – after phosphate nutritional downshift (Koepsel & Ensign, 1984, Daza *et al.*, 1989, Novella *et al.*, 1992, Rueda *et al.*, 2001, Ohnishi *et al.*, 2002). Nevertheless, our data are consistent with the proteomic and transcriptomic studies that have been reported previously, which showed that many developmental genes linked with sporulation or aerial mycelium formation, including *bldN* and *whiH*, were expressed also in 24 or 72 hours of growth of cultivation in the same liquid medium (Nieselt *et al.*, 2010). Nieselt *et al.* observed that these developmental genes increased as though the cells prepare for differentiation, but their

expression stopped around 33 hours so that differentiation did not occur (Nieselt *et al.*, 2010). In a liquid medium, streptomycete cells first form a compartmentalized mycelium that differentiates into a specialized multinucleated vegetative mycelium MII with sporadic septa (second mycelium) after a programmed cell death (PCD) (Manteca *et al.*, 2008). This MII mycelium produces secondary metabolites and also expresses sporulation related genes (Manteca *et al.*, 2008). Other transcriptomic analyses of *S. coeliocolor* grown in submerged culture reported that several *whi* and *wbl* genes, such as *wblA, whiG, whiH* and *whiJ* that regulates early stages of sporulation, were expressed (Yague *et al.*, 2014), which is also in agreement with our results. This finding leads to the suggestion that these sporulation or aerial mycelium-associated genes could have its own specific role during the vegetative growth in a liquid medium, where no sporulation and aerial mycelium formation occur.

Beside above mentioned important processes, we have found in HrdB regulon also set of important ECF sigma factors (SigE, SCO5147) needed for the maintenance of cell envelope integrity (Hutchings *et al.*, 2006, Tran *et al.*, 2019) (Huang *et al.*, 2005) and sigma factors, which regulates morphological differentiation including antibiotic production (WhiG, BldN, SigK, HrdD) (Kelemen *et al.*, 1996) (Bibb *et al.*, 2000) (Mao *et al.*, 2009) (Fujii *et al.*, 1996), participate in the osmotic sensory system and osmotic stress response (SigI, SigQ) (Viollier *et al.*, 2003, Homerova *et al.*, 2012), anti-sigma factors (RsbA), and anti-anti-sigma factors (RsbB). HrdD is very closely related to HrdB in its amino acid sequence and promoter specificity (Buttner *et al.*, 1990). They have almost identical 2.4 and 4.2 regions, which are responsible for the promoter recognition and binding to it (Helmann & Chamberlin, 1988, Buttner *et al.*, 1990), indicating their overlapping or identical promoter specificities (Buttner & Lewis, 1992).

According to our ChIP-seq results, HrdB is also associated with the regulation of energy metabolism, which is an essential process in bacteria. It ensures the formation of ATP by oxidation of organic substrates, so it is not surprising that we have identified several genes regulating this process in HrdB regulon. This process includes TCA cycle, glycolysis, pentose phosphate pathway coupled with electron transport and ATP-proton motive force leading to the generation of energy in the form of ATP. This energy is utilized for growth, chemical reactions and other essential processes in the cell. It plays a central role in bacteria (Madigan & Brock, 2012). HrdB regulates nearly 50 % of all genes from energy metabolism, encoded in the *Streptomyces* genome. 56 % of the genes from glycolysis are under the control of HrdB, such as 6-phosphofructokinase, triosephosphate isomerase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase.
65 % of the genes from TCA cycle was found in HrdB regulon. They are key enzymes including citrate synthase, succinyl-CoA synthetase, succinate dehydrogenases and others. Among others, citrate synthase CitA (SCO2736) has been previously shown to be crucial for maintaining the physiological balance of the cells. Its proper function is linked with cellular differentiation as its mutant loses abilities to form aerial mycelium and to produce antibiotics when grown on glucose (Viollier *et al.*, 2001).

Furthermore, HrdB participates also in the regulation of 46.5 % of the genes from electron transport. These are mainly NADH dehydrogenases and their subunits. ATPases are essential for energy metabolism as they convert proton motive force to ATP that is needed for the living of the cells (Ward, 2015). HrdB regulates key components of this energy generator in *S. coelicolor*.

Energy metabolism in bacteria is also tightly regulated by several regulatory proteins. In *Streptomyces*, it is well known redox sensing transcriptional repressor Rex (SCO3320), found

in HrdB regulon. Rex responds to the cellular NADH/NAD^+ levels (Brekasis & Paget, 2003, Liu *et al.*, 2017) and represses the transcription of several respiratory genes including two of them – *cydCD* (SCO3947) and *hemD* (SCO3317)), also found in HrdB regulon (Brekasis & Paget, 2003, Liu *et al.*, 2017). Rex is also considered to be an essential regulator of aerobic metabolism, because it reflects oxygen quantity by the intracellular ratio of NADH to NAD^+ (Brekasis & Paget, 2003, Gyan *et al.*, 2006, Pagels *et al.*, 2010, Bitoun *et al.*, 2012). When the NADH/NAD^+ ratio is low, Rex represses the transcription of genes involved in NAD^+ regeneration. Besides that, it was described that Rex also governs morphological differentiation and regulates avermectin production in *S. avermitilis*, suggesting its major role as a repressor among *Streptomyces* (Liu *et al.*, 2017). Not only Rex gene, but also Rex regulated operons *cydABCD* and *rex-hemACD* are under the control of HrdB that strengthens the influence of Rex repressor and highlights the importance of HrdB in the regulation of energy metabolism with respect to oxygen quantity in the environment.

HrdB has several known promoter binding sites (Table 2). Out of the 15 that were reported previously, only 5 of them were identified in HrdB regulon. These are *tuf3, gltB, ftsZ, rrnD, rrnA* coded for transcription elongation factor TU-3, glutamate synthase, cell division protein, rRNA operon D, rRNA operon A, respectively. The other 10 genes that weren't identified in our HrdB regulon may not be transcribed by HrdB in vivo or they could be transcribed by HrdB in different growth conditions, in another growth phase or in other specific conditions.

5.5 asRNAs of sigma factors

Small regulatory RNAs in *Streptomyces* are widespread. It was confirmed by several systematic studies that have revealed hundreds of novel sRNAs (Panek *et al.*, 2008, Swiercz *et al.*, 2008,

Vockenhuber *et al.*, 2011, Gatewood *et al.*, 2012) but the regulatory role of most of them remains unknown. The conservation of sRNAs in this strain varies from abundance in all streptomycete genomes to being present exclusively in *S. coelicolor* (Vockenhuber *et al.*, 2011). They are in a vast majority (about 80 %) located in the core region of a linear chromosome with a lesser amount in the arm regions (Vockenhuber *et al.*, 2011).

The role of sRNAs has been studied more extensively in *E. coli* or in *Salmonella* (Frohlich et al., 2012, Guo et al., 2014, Porcheron et al., 2014, Kim et al., 2019), whereas only a few sRNAs have been experimentally characterized in *Streptomyces*. These are scr4677, which influences the actinorhodin production under specific growth conditions (Hindra *et al.*, 2014) and scr3097, which impacts the expression of *rpfA* (a muralytic enzyme required for establishing and exiting dormancy) post-transcriptionally (St-Onge & Elliot, 2017). Other examples are antisense RNA cnc2198.1, regulating glutamine synthase *glnA* (D'Alia *et al.*, 2010) and scr5239, required for the regulation of *metE* (methionine synthase), *dagA* (agarose) (Vockenhuber *et al.*, 2011, Vockenhuber & Suess, 2012, Vockenhuber *et al.*, 2015), and phosphoenolpyruvate carboxykinase (PEPCK) (Engel *et al.*, 2019).

Our study revealed three novel as-RNAs coded opposite to sigma factors SigB, SigH and SigR, probably acting against these sigma factors. We observed that the expression of these asRNAs and their target mRNAs varied depending on the growth stage in wt strain and RNase III deletion strain (*rnc*) (Figure 31). This set of asRNAs has served as a control set since they were not affected by RNase III in a study of Gatewood *et al.* (Gatewood et al., 2012). However, we tried to find out if there might be some effect of RNase III deletion on the expression of identified asRNAs and their target mRNAs. Our hypothesis was that base pairing of asRNA and target mRNA results in the generation of a duplex that is subjected to RNase III cleavage as was described in (Nicholson, 2011). Based on our results, we observed this mechanism of

action of RNase III marginally and, for example only in the respective growth phase, not throughout the entire growth cycle (Figure 31). If there is this mechanism of RNase III, in *rnc* strain (RNase III deletion strain) there will be stronger bands of both asRNA and mRNA transcripts compared to the wt that should indicate the accumulation of these transcripts as a result of an absent RNase III cleavage. This was the case of sigB mRNA/as-sigB in the 48 and 72 hours of growth (Figure 31), 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. This cleavage of the newly formed duplex can't be subjected to degradation but can be regulated in a positive way to open the ribosome binding site (RBS) of the target mRNA and activate translation. Without the given asRNA, no translation of the target transcript occurs. In *E. coli*, it was reported that agrB antisense RNA binds to the dinQ mRNA resulting in a formation of a specific secondary structure including a double strand region that is subjected to RNase III cleavage, leading to the opening of the RBS and the activation of translation of dinQ mRNA (Kristiansen *et al.*, 2016). This is an example of a positive regulation of target transcript by asRNA. Should we want to know the influence of RNase III onto the translation of our transcripts, we would need to observe the protein levels of the regulated sigma factors in wt and *rnc* strains. The mRNA itself should be also a potential target of RNase III, due to the ability to form a secondary structure with stem-loops that includes double stranded regions. It was observed in 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. AdhE mRNA forms a secondary structure in which the RBS is blocked. Due to the

RNase III cleavage, this secondary structure is destroyed resulting in the opening of RBS for the subsequent translation. RNase III deletion leads to the accumulation of this adhE mRNA (Aristarkhov *et al.*, 1996).

Contrary to this hypothesis about the formation of RNA duplex between asRNA and the target mRNA that is subjected to RNase III cleavage, there is another mechanism that is typical for RNase III. It is a stabilisation of the target transcript due to the ribosomal protection of the RNase III-processed mRNAs from ribonucleases (Sim *et al.*, 2010, Lee *et al.*, 2019). It was observed in the case of as-sigB/*sigB* mRNA expression in the 24 hours of growth (Figure 31), 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 (Sim *et al.*, 2010). It has also been shown that RNase III may bind a subset of RNAs and modulate their stability via a non-catalytic mechanism (Nicholson, 2011) 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 31), suggesting the impact of RNase III onto the transcription and/or stability of *sigH* mRNA, where RNase III is needed for these events.

In the case of as-sigH, the bands in Northern blot are not well-visible, and it seems that the transcript levels are the same 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* (Hindra *et al.*, 2014) and in a study in *B. subtillis* (Durand *et al.*, 2012)

where they found no effect or a little effect of RNase III on the levels of sRNAs or antisense transcript.

RNase III ensures the processing of dsRNAs, which is an essential step in the maturation and decay of non-coding RNAs in bacteria (Nicholson, 2014). It can act by two mechanisms. The first one, catalytic mechanism, ensures the catalytic cleavage of dsRNAs and results in destabilizing of sRNAs/mRNAs, and the second one, non-catalytic mechanism, ensures only the binding of RNase III to dsRNA without the cleavage, so RNase III serves as a dsRNA-binding protein resulting in the stabilizing of sRNAs/mRNAs (Blaszczyk *et al.*, 2004, Gan *et al.*, 2005, Ji, 2006, Nicholson, 2011). Both of these mechanisms are thought to be included in our results but whether RNase III acts directly or indirectly on RNA transcripts in our study remains to be determined.

Important findings were reported by Durand *et al.* about the base pairing of the sense/antisense RNAs and their degradation (Durand *et al.*, 2012). They studied the effect of three depleted RNases – RNase III, RNase J1 and RNase Y – on the abundance of asRNAs in *B. subtillis*. These RNases are essential and are thought to be involved in mRNA decay, where RNase J1 is 5'-3' specific exonuclease (Mathy *et al.*, 2007), RNase Y ensures endonucleolytic cleavage (Shahbabian *et al.*, 2009) and RNase III is a double-strand specific enzyme (Oguro *et al.*, 1998). They found that only 5 % of the studied asRNAs were affected by the depletion of RNase III, whereas by the depletion of RNase J1, which is a single-strand specific RNase, 10 % of asRNAs were affected, and 17 % were affected by the depletion of RNase Y (Durand *et al.*, 2012). It is a little surprising that single strand specific RNases have greater effect on asRNAs than double-strand specific RNase III. Extended duplexes of sense and antisense RNAs might be formed less likely than expected. In the regulation of R1 plasmid replication, it was described that the antisense RNA *copA* and its target *copT* forms a four-way helical junction rather than extended

duplex (Kolb *et al.*, 2000). A similar conformation was reported between antisense RNA *inc* and its target *repZ* of plasmid Col1b-P9 (Kolb *et al.*, 2001). These findings suggest that some single-stranded regions in the duplexes of sense/antisense transcripts could occur more likely than extended duplexes (Durand *et al.*, 2012). What could also be involved in our results is a specific asRNA/mRNA conformation that includes single-stranded regions and therefore these transcripts are not affected by the deletion of RNase III, as in the case of as-sigR/*sigR* mRNA (Figure 31).

Cis-asRNAs represent an effective way of gene expression control with minor space requirements in their genome (Georg & Hess, 2011). As our results showed, *cis*-asRNAs are connected with the regulation of 25 % of the tested sigma factor genes (SigB, SigH, SigR). Possibly, it is not a random phenomenon that all these sigma factors are associated with stress response in *S. coelicolor*. SigB regulates oxidative and osmotic stress response (Fernandez Martinez *et al.*, 2009), SigH is a SigB-like sigma factor responsible for osmotic stress response (Viollier *et al.*, 2003) and SigR regulates oxidative stress through thioredoxin system (Paget *et al.*, 1998, Kang *et al.*, 1999). They are the main stress related sigma factors in *Streptomyces*. We tested the expression levels in wt and RNase III deletion strain to examine the hypothesis about the RNase III cleavage of duplex asRNA/mRNA. Unlike the claim of this hypothesis, we observed that RNase III acts by the stabilisation of our transcripts or had no influence on these transcripts. It was also reported that the enzymatic activity of *E. coli* RNase III is regulated via stress induced by an entry into stationary phase and temperature and osmotic changes (Kim *et al.*, 2008, Sim *et al.*, 2010, Kavalchuk *et al.*, 2012, Lim & Lee, 2015, Lee *et al.*, 2019), supporting the fact that asRNA mediated regulation of sigma factors in contribution with RNase III was identified just for stress related sigma factors SigB, SigH and SigR in *S. coelicolor*.

It is known that RNase III is a pleiotropic regulator of antibiotic biosynthesis in *S. coelicolor* (Adamidis & Champness, 1992). The deletion of the gene encoding RNase III (*rnc* gene, also termed as *absB*) results in a decrease of production of all four antibiotics (actinorhodin, undecylprodigiosin, calcium dependent antibiotic, methylenomycin) (Adamidis & Champness, 1992) that is thought to be caused by the regulation of pathway-specific regulators such as *actII-orf4*, *cdaR* and *redD*, which were defective in RNase III deletion mutant (Adamidis & Champness, 1992, Aceti & Champness, 1998), but it is not known, whether RNase III directly cleaves the transcripts of these pathway-specific regulators or whether it influences their expression by another mechanism. The reduction of antibiotic production in *rnc* strain could be influenced by the accumulated *sigB* mRNA in 48 hours of growth, which is not able to be translated because of the absent RNase III enzyme needed for the activation of translation (Aristarkhov *et al.*, 1996), as mentioned above. The reduction of antibiotic production could also be caused by the decreased level of *sigB* mRNA in 24 hours of growth or *sigH* mRNA in 24/48 hours of growth, where – if there is no RNase III enzyme, which stabilizes the transcript and thus protects them from the ribonucleases – these mRNAs are more labile and prone to be degraded by ribonucleases (Sim *et al.*, 2010, Lee *et al.*, 2019). It is possible that SigB or SigH might be involved in the antibiotic production in *S. coelicolor*. Interestingly, it was reported that SigB regulates antibiotic production throughout the regulation of *redH* and *redZ* genes (Lee *et al.*, 2005, Facey *et al.*, 2009, Facey *et al.*, 2011) and SigB also controls pathway-specific regulators *cdaR*, *actII-ORF* (Hesketh *et al.*, 2007) by regulating RelA protein, encoding ppGpp synthetase (Lee *et al.*, 2004). Regulation of antibiotic production by SigH has not been described.

Our work focused on the identification of sigma factors, their regulons and RNA regulators. We developed a complex approach involving the combination of ChIP-seq data and computational modelling enabling us to identify sigma regulons and simultaneously verify the expression of the target genes throughout the respective life stage. First, we identified a regulon of SigQ in spore germination revealing its role as an osmotic stress response regulator, regulator of cell wall reconstruction, secondary metabolism and nitrogen metabolism. The second identified regulon was the regulon of the major vegetative sigma factor HrdB that elucidated its essentiality through the regulation of essential metabolic events in primary metabolism and gene expression machinery, and revealed its role as a regulator of morphological differentiation, cell division, cell wall synthesis and energy metabolism. The last pillar of our work was to identify regulators of sigma factors – sRNAs, which was confirmed by the identification of 3 *cis*-asRNAs of stress related sigma factors SigB, SigH and SigR out of the 12 ones tested in our experiments. We can also conclude that RNase III is thought to be connected with the processing and stability of these transcripts. All these findings significantly broaden the knowledges about sigma regulations and reveal the importance of asRNAs in these regulations in *S. coelicolor*.

6 Conclusions

For the study of sigma factors, an HA tag was inserted directly into the genome. This allowed the ChIP-seq technique to proceed without the construction of plasmid supplemented gene deletions. Using this 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 ECF 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 modelling of the gene expression time series using SigQ as a regulator revealed a 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 spore 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 stress related 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 after 24 hours, 48 hours

and 72 hours of growth which correspond to vegetative growth, aerial mycelium formation and sporulation respectively. 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 sigma factors in *Streptomyces*.

7 Abbreviations

asRNA – antisense RNA

ATP – adenosine triphosphate

α-CTD – C-terminal domain of α subunit

bp – base pair

CRE – core recognition element

CRP – cAMP receptor protein

ChIP-Seq – chromatin immunoprecipitation with next generation sequencing

dsDNA – double-stranded DNA

ECF – extracytoplasmic function

IGR – intergenic region

ncRNA – non-coding RNA

NCR – non-conserved region of sigma factor

NGS – next generation sequencing

PCR – polymerase chain reaction

RbpA – RNA polymerase binding protein A

RBS – ribosome binding sites

RNAP – RNA polymerase

RNA-Seq – RNA sequencing

RT-PCR – reverse transcription polymerase chain reaction

RACE – Rapid amplification of cDNA ends¨

RPo – open promoter complex

Scr – *Streptomyces coelicolor* RNA

sRNA – small RNA

ssDNA –single-stranded DNA

TSS – transcription start site

UTR – untranslated region

8 List of publications

8.1 Publications related to the thesis

1. **Šmídová, K.**; Ziková, A.; Pospíšil, J.; Schwarz, M.; Bobek, J.; Vohradský, J. DNA mapping and kinetic modeling of the HrdB regulon in *Streptomyces coelicolor*. *Nucleic Acids Res.* **2019**, *47* (2), 621–633.
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. 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.
4. 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.

8.2 Publications not related to the thesis

1. Baumgardt, K.; **Šmídová, K.**; Rahn, H.; Lochnit, G.; Robledo, M.; Evguenieva-Hackenberg, E. The stress-related, rhizobial small RNA RcsR1 destabilizes the autoinducer synthase encoding mRNA sinI in *Sinorhizobium meliloti*. *RNA Biology* **2016**, *13* (5), 486–499.
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

9 Appendices

9.1 Appendix 1 – List of SigQ regulon

SigQ regulon is available in this link https://uloz.to/file/B5GAZcAsMRaN/sigq-regulon-xlsx.

9.2 Appendix 2 – List of HrdB regulon

HrdB regulon is available in this link https://academic.oup.com/nar/article/47/2/621/5146190#supplementary-data.

Supplementary file 1 contains fit comparisons and functional classification of each category (see list func hrdB or hrdBrbpA). In Supplementary file 2 there is a list of sRNAs, tRNAs and rRNAs found in HrdB regulon.

9.3 Appendix 3 – Additional files to novel asRNAs

Raw Northern blot images, quantification of the Northern blot signals are available in this link https://www.frontiersin.org/articles/10.3389/fmicb.2017.02693/full#supplementary-material

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