MtrAB-LpqB: A conserved pathway regulating cell division in the phylum Actinobacteria?

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In loving memory of my Mum

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Abstract

Streptomyces are ubiquitous in soil and face a rapidly changing environment. Like other bacteria, they sense and respond to external stimuli via two component systems and *Streptomyces* species encode a particularly high number of these systems. One of these two component systems is called MtrAB-LpqB and it is highly conserved in the phylum Actinobacteria. Previous work in *Mycobacterium tuberculosis*, *Corynebacterium glutamicum* and *Streptomyces coelicolor* indicates that MtrAB-LpqB is involved in osmosensing and cell cycle progression. To investigate the function of MtrAB-LpqB I attempted to make single gene deletions in the new model organism *Streptomyces venezuelae*. I also performed chromatin immunoprecipitation and sequencing (ChIP-seq) against MtrA-3xFlag in *S. venezuelae* and *S. coelicolor* to identify the regulon of genes under its control. I present evidence that MtrA is essential in *S. venezuelae* whereas MtrB is dispensable. It was not possible to confirm deletion of *lpqB*. Deletion of *mtrB* activates MtrA and leads to the overproduction of cryptic secondary metabolite biosynthetic gene clusters (BGCs). The same effect was achieved by introducing a gain of function MtrA protein into the *S. venezuelae* wild-type strain. The cryptic BGCs are activated because MtrA binds to target genes spanning 85% of the BGCs in *S. venezuelae* and *S. coelicolor*. In *Streptomyces*, antibiotic production is linked to development and the MtrA regulon overlaps with the master regulator of development, BldD. The results presented here suggest that MtrAB senses external signals and modulates target gene expression to coordinate development with the production of secondary metabolites.

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1 Introduction

1.1 Actinobacteria

The phylum Actinobacteria is one of the largest taxonomic groups among bacteria (Ventura et al. 2007). Actinobacteria are Gram positive with a high G+C content in their DNA from 51% in some *Corynebacteria* to more than 71% in *Streptomyces* and *Frankia*. However *Tropheryma whipplei* is one exception among the sequenced Actinobacteria with a G+C content of less than 50% (Ventura et al. 2007). The phylum Actinobacteria is divided in six classes: Nitriliruptoria, Acidimicrobiia, Coriobacteriia, Thermoleophilia, Rubrobacteria and Actinobacteria, which is the largest class within the phylum (Goodfellow et al. 2012).

The phylum contains pathogens like *Mycobacterium tuberculosis* which infected 9.6 million people with Tuberculosis in 2014 and killed 1.5 million (http://www.who.int/mediacentre/factsheets/fs104/en/); *Mycobacterium leprae*, the causative agent of Hansen's disease (leprosy); *Nocardia spp*. which are human pathogens with low virulence infecting immunocompromised patients (Peleg et al. 2007), *T. whipplei*, an intracellular pathogen with a reduced genome causing Whipple's disease (Raoult et al. 2003), *Corynebacterium diphtheria* causing diphtheria (Sangal et al. 2012) and *Propionibacterium acnes* a facultative parasite causing acne (Bojar & Holland 2004). Furthermore, it contains *C. glutamicum* which is used industrially to produce amino acids, plant pathogens like *Leifsonia xyli subsp. xyli* (Monteiro-Vitorello et al. 2004), *Frankia spp.* which are nitrogen-fixing plant symbionts (Chaia et al. 2010), *Bifidobacterium* spp. beneficial bacteria which colonise the gastrointestinal tract (Leahy et al. 2005) and *Streptomyces spp.* which are ubiquitous in soil and a major source of commercially important antibiotics and numerous immunosuppressants, anti-helminthic and anti-cancer drugs (Bentley et al. 2002). All organisms mentioned above belong to the order Actinomycetales within the class Actinobacteria except *Bifidobacterium* spp which belong to the order Bifidobacteriales which is also in the class of Actinobacteria (Goodfellow et al. 2012).

1.2 *Streptomyces*

The genus *Streptomyces* includes some of the best studied prokaryotes due to their unique developmental life cycle and their important role in industrial antibiotic production. One of the natural habitats of *Streptomyces* species is the soil and they

comprise up to 20% of the soil microbial community (Schrempf 2006). With a saprophytic, obligate aerobic lifestyle *Streptomyces* species are well adapted to the soil environment and play a major role in the degradation of chitin, which is made available by the secretion of chitinase enzymes (Blaak & Schrempf 1995).

1.2.1. *Streptomyces* model organisms

Despite the vast amount of *Streptomyces* strains isolated so far, our knowledge of the molecular background of development and antibiotic production is obtained from mainly three model organisms: *S. coelicolor*, *S. griseus* and *S. venezuelae*. *S. coelicolor* was the first model organism to be adopted due to the pioneering work of David Hopwood (Hopwood 1999). *S. coelicolor* produces five antibiotics under laboratory conditions: the blue actinorhodin [ACT] (Bystrykh et al. 1996), the red undecylprodigiosin [RED] (Feitelson et al. 1985), the colourless calcium dependent antibiotic [CDA] (Hojati et al. 2002), the yellow pigmented coelimycin P1 [yCPK] (Gomez-Escribano et al. 2012; Gottelt et al. 2010) and the an unusual, plasmid encoded cyclopentanone antibiotic methylenomycin (O'Rourke et al. 2009). Most of the developmental regulatory genes were also identified and studied in this model organism and it was the first streptomycete to have its genome sequenced (Bentley et al. 2002). However, some developmental regulatory features have been studied in the streptomycin producing *S. griseus* (Chater & Horinouchi 2003). In recent years, a new model organism was established to study development: the chloramphenicol and jadomycin producing *S. venezuelae*, which sporulates in liquid culture (Schlimpert et al. 2016; Glazebrook et al. 1990) whereas *S. coelicolor* does not. This is an advantage to study these filamentous bacteria because it makes *Streptomyces* approachable for global genetic and transcriptional analysis such as chromatin immunoprecipitation and sequencing (ChIP-seq), (Pullan et al. 2011) and RNA sequencing (RNA-seq), (Munnoch et al. 2016).

1.3 Cell division and Differentiation in *Streptomyces*

1.3.1 The *Streptomyces* life cycle

Streptomyces grow in an unusual way compared to most other bacteria. In the early days of microbiology *Streptomyces* bacteria were misclassified as fungi because of their mycelial, fungal-like growth. The *Streptomyces* life cycle starts with a dormant, unicellular spore. *Streptomyces* species are non-motile (Kämpfer 2006) and the spores

allow distribution within the environment (see **Figure 1.1**). In nutrient rich conditions the spores start to germinate and grow as vegetative hyphae via apical growth. The vegetative hyphae form branches and the cell mass is called vegetative mycelium. When the nutrients are depleted in the vegetative hyphae part of the substrate mycelium undergoes apoptosis to release nutrients allowing the remaining vegetative hyphae to develop into aerial hyphae (Miguélez et al. 1999). The hyphae break through the water tension and grow up in the air to form the aerial hyphae that are not branched but rather form single hyphae containing multiple chromosomes (Elliot et al. 2008). In aerial hyphae, fundamental processes like chromosome replication, segregation, cell division and cell wall assembly change dramatically compared to vegetative growth and lead to the formation of long chains of spores containing a single chromosome.

1.3.2. Cell division in *Streptomyces*

Due to the unique development in *Streptomyces* species, sporulation differs from cell division in rod shaped bacteria. Sporulation is not essential for survival in *Streptomyces* thus cell division genes that are essential in unicellular bacteria (*ftsZ*, *ftsI*, *ftsW*, *ftsE*, *ftsX*, *ftsL*, *ftsQ* and *divIC*) can be deleted in *Streptomyces* bacteria (Jakimowicz and van Wezel 2012). The bacterial tubulin homologue FtsZ plays a central role in cell division and *Streptomyces* species are the only bacteria known so far in which FtsZ is dispensable (McCormick et al. 1994). Due to their filamentous growth, *Streptomyces* strains use FtsZ in the formation of vegetative cross walls and sporulation septa. During vegetative growth, low levels of FtsZ form occasional cross wall. In aerial hyphae at the onset of sporulation the expression of FtsZ is upregulated and up to 100 cell division septa are formed which lead to the separation of aerial hypha into single spores. The distinct levels of FtsZ expression are achieved by three promoters regulating FtsZ. While *fts3p* is important for vegetative growth, *ftsZ2p* is upregulated in sporogenic aerial hyphae, which coincides with the onset of sporulation (Flärdh et al., 2000).

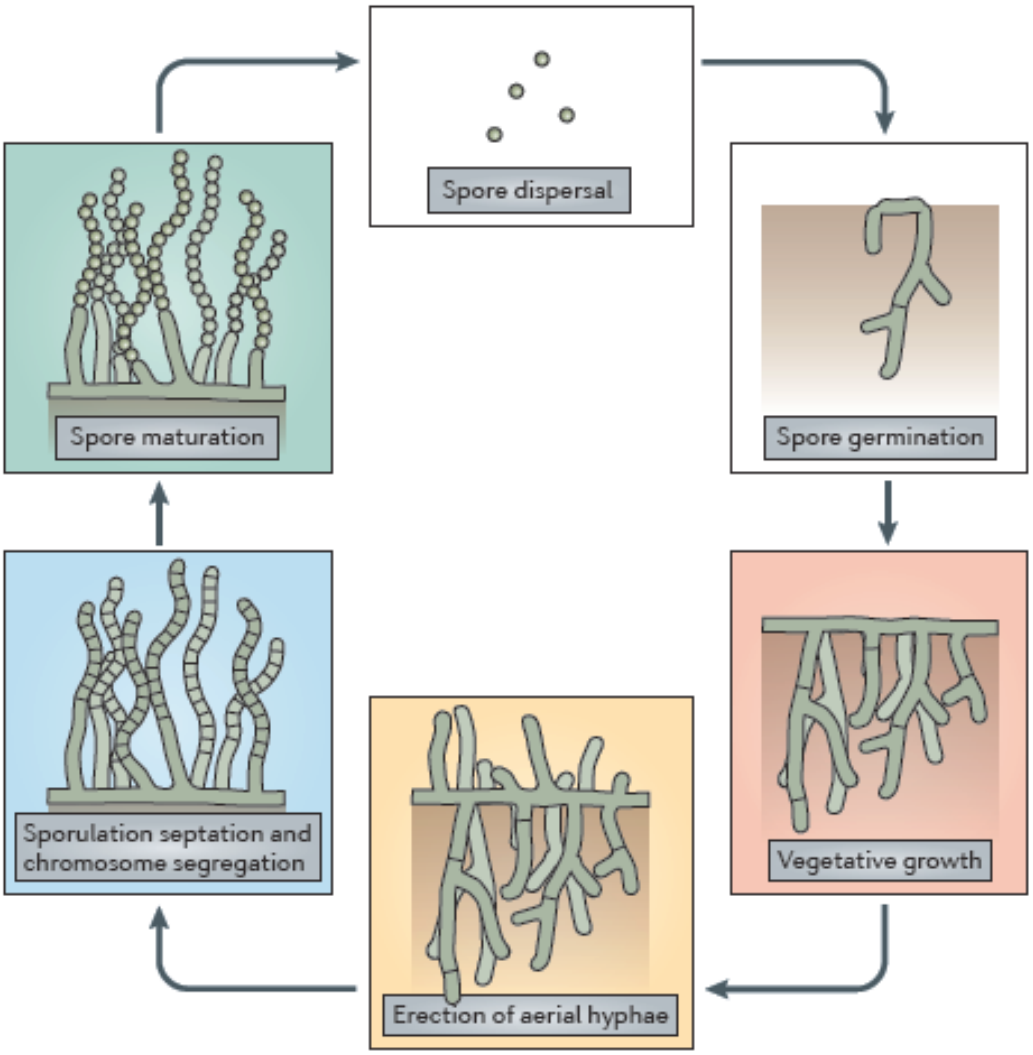

Figure 1.1 Life cycle of *Streptomyces*. Under nutrient rich conditions spores germinate and form germination tubes which grow into the soil or media and form the vegetative hyphae. Their differentiation can be induced by environmental conditions, nutrient availability, metabolism and extra-cellular signalling which causes the aerial hyphae to break through the surface tension and grow into the air. Then aerial hyphae differentiate into a long chain of pre-spore compartments. The genome replicates and multiple copies are distributed evenly in spores by the chromosome segregation machinery. The pre-spores contain a single copy of the genome and develop into mature spores. Reprinted (adapted) with permission from (Bush et al. 2015) Copyright (2017) Nature Reviews Microbiology.

The formation of sporulation septa is controlled by SsgA-like proteins (SALPs). SsgA and SsgB are essential for sporulation-specific cell division in *Streptomyces coelicolor*. After the increased expression of *ftsZ* at the onset of sporulation SsgA is the first protein to localise at the septation sites followed by the localisation of SsgB, which recruits FtsZ to the septation site (Willemse et al. 2011). SsgC–G are responsible for correct DNA segregation/condensation (SsgC), spore wall synthesis (SsgD), autolytic spore separation (SsgE, SsgF) or exact septum localization (SsgG) (Noens et al. 2005). The SALP proteins are not the only proteins involved in the formation of the sporulation

septa. There are around a dozen other essential proteins which are subsequently recruited to the divisome via protein–protein and protein–cell envelope interactions (Haeusser & Margolin 2016). One of the proteins involved in cell wall synthesis and remodelling is the division-specific transpeptidase FtsI (Penicillin Binding Protein [PBP] 3) (Bennett et al. 2009).

1.3.3 Polar growth in *Streptomyces* species

Rod shaped bacteria like *Escherichia coli* and *Bacillus subtilis* grow by inserting new peptidoglycan throughout the lateral wall and DNA replication and cell division are tightly linked. In contrast, *Streptomyces* species (Holmes et al. 2013) as well as other non-filamentous actinomycetes like *Mycobacterium* (Joyce et al. 2012) and *Corynebacterium* species, grow by tip extension (Letek et al. 2008; Daniel & Errington 2003) and in *Streptomyces* species chromosome replication is independent of cell division. In streptomycetes hyphal tip extension is performed by a multiprotein complex called the polarisome (or tip organizing centre [TIPOC]). The first protein to localise at the growing tips is the essential positional marker protein, DivIVA (Flärdh 2003). Together with DivIVA, the *Streptomyces* cytoskeletal element Scy forms the anchor for the filament protein FilP (Holmes et al. 2013) and the penicillin binding protein FtsI which contribute to the structure of the polarisome. Tip extension and chromosome segregation are linked by interactions between the chromosome positioning protein ParA, the DNA-binding protein that organizes the segregation of nucleoprotein complexes and Scy (Ditkowski et al. 2013; Donczew et al. 2016).

1.3.4 Regulation of the *Streptomyces* life cycle

Each stage of development is visible in a typical *Streptomyces* colony grown on solid agar medium. Vegetative growth results in clear, shiny colonies; aerial hyphae result in a white, almost furry appearance; while the onset of sporulation results in cell division and the production of an associated spore pigment (grey and green for *S. coelicolor* and *S. venezuelae*, respectively). The first developmental genes discovered in *Streptomyces* species were the bald (*bld*) and white (*whi*) genes due to the appearance of the mutants. Bald mutants are unable to form aerial hyphae and thus the colonies stay clear with no visible white aerial hyphae. On the other hand white mutants stay white and are unable to form spores and the associated spore pigment (Chater 2001). Intensive study of these

genes revealed that most of the *bld* and *whi* genes encode global master regulators essential for the switch from vegetative to aerial hyphal growth and aerial growth to sporulation (Flärdh & Buttner 2009; McCormick & Flärdh 2012). The insights of the regulatory network of development described in the following section are pieced together from work in the three model organisms *S. venezuelae*, *S. coelicolor* and *S. griseus*, as described above. The overall mechanism of development is likely to be similar in these distantly related model organisms but it is possible that some details of the regulatory network differ (Chater & Horinouchi 2003).

The secondary messenger c-di-GMP is ubiquitous in bacteria (Hengge 2009). However in *Streptomyces* c-di-GMP has the unique role acting as a signal to control multicellular development (Tran et al. 2011; Hull et al. 2012; Tschowri et al. 2014). Diguanylate cyclases (DGCs) containing GGDEF domains synthesise c-di-GMP from two molecules of GTP and phosphodiesterases (PDEs) containing EAL domains or HD-GYP domains degrade c-di-GMP (Hengge 2009; Schirmer & Jenal 2009). The overproduction of DGCs and therefore high levels of c-di-GMP prevent the formation of aerial hyphae and overproduction of PDEs which lead to decreased levels of c-di-GMP causes early sporulation (Tschowri et al. 2014). It has recently been shown that c-di-GMP controls development via the global regulator BldD (Tschowri et al. 2014). c-di-GMP binds to BldD which then represses two classes of target genes: approximately 170 sporulation genes (Elliot et al. 2001; Hengst et al. 2010) including *ftsZ* and associated cell division genes. Repressing these target genes inhibits sporulation.

BldD regulates the expression of FtsZ, which is the central protein of the divisome and forms a ring like structure and is upregulated during sporulation (Flärdh & Buttner 2009; Flärdh et al. 2000; Hengst et al. 2010). The septum forming proteins SsgA and SsgB are also regulated by BldD. The two proteins are actinomycete-specific and are responsible for the assembly of the FtsZ ring at the cell division sites (Willemse et al. 2011). Another direct target of BldD is the *smeA*–*sffA* operon. SmeA is a small membrane protein, which targets the putative DNA translocase SffA which is involved in chromosome segregation into spores (Ausmees et al. 2007). Additionally the gene encoding SspA, a lipoprotein which influences the shape of spores, is direct target of BldD (Tzanis et al. 2014).

Streptomyces form a hydrophobic sheath to break the surface tension and erect aerial hyphae. The hydrophobic sheath contains rodlins, chaplins and SapB (Claessen et

al. 2003; Elliot et al. 2008). The expression of rodlins and chaplins is regulated via σ^{BdlN} and the anti-sigma factor RsbN (Bibb et al. 2012) whereas the expression of SapB is regulated via AdpA and BldA (Bush et al. 2015). σ^{BldN} belongs to the extracytoplasmic function (ECF) subfamily of RNA polymerase sigma factors and activates the expression of the *bldN* as well as the genes encoding rodlins and chaplins. *bldN* expression is repressed by BldD-(c-di-GMP). However, σ^{BdlN} activity is also repressed by its anti-sigma factor RsbN which is regulated by an external unknown signal. Therefore the rodlins and chaplins are only expressed when *bldN* is derepressed and σ^{BldN} is released from RsbN by the external signal (Bush et al. 2015), see **Figure 1.2.**

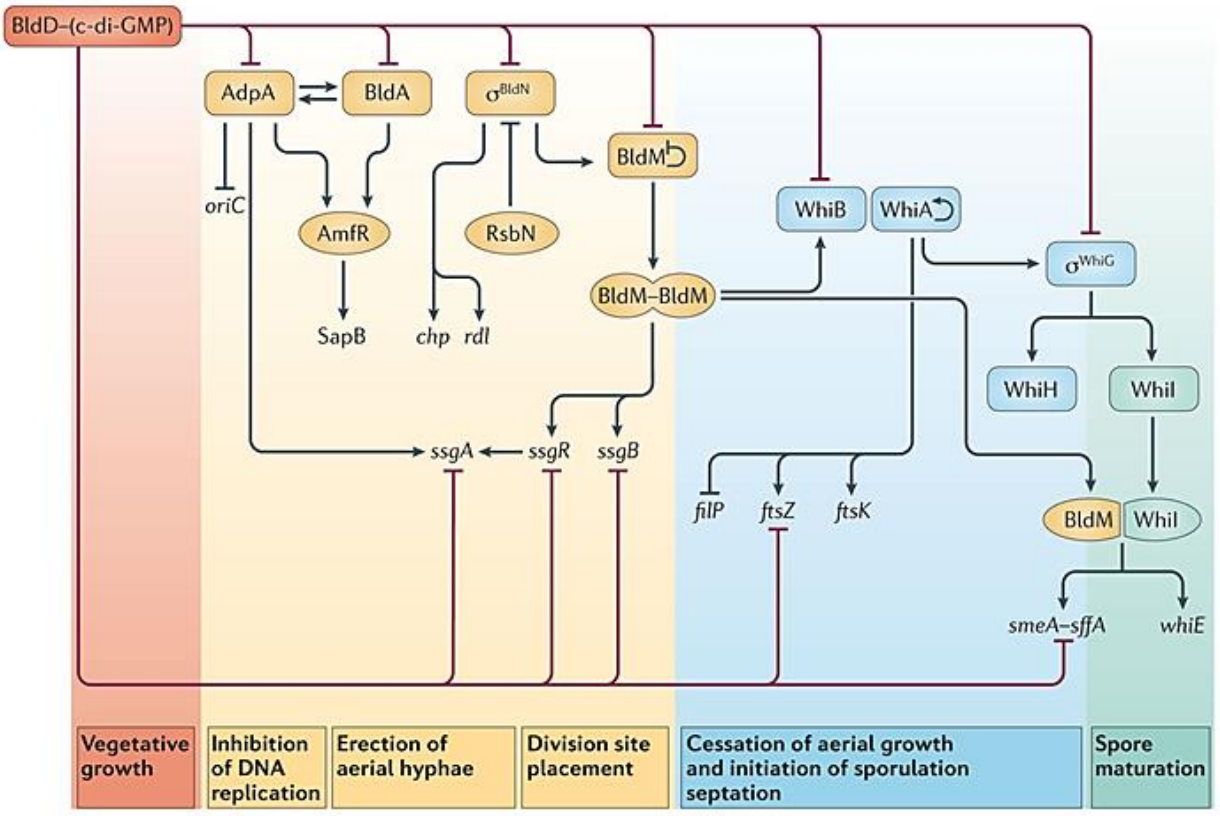

Figure 1.2 Regulatory network of the master regulator BldD in development. BldD–(c-di-GMP) represses a large global regulon of sporulation genes during vegetative growth for description of single genes see text. Arrows indicate activation, and bars indicate repression. Reprinted (adapted) with permission from (Bush et al. 2015) Copyright (2017) Nature Reviews Microbiology.

The expression of SapB is regulated via the master developmental regulator AdpA and the transfer RNA (tRNA) BldA. AdpA is a transcriptional regulator and controls a large regulon of sporulation genes (Higo & Horinouchi 2011). Additionally AdpA controls chromosome replication during development by binding to the origin of

replication (*oriC*) and blocking access for the DNA replication initiation protein DnaA (Wolański et al. 2012). BldA is the only tRNA which can translate TTA codons. AdpA contains a TTA codon which leads to a unique positive feedback loop in which AdpA activates transcription of *bldA* which is itself required for expression of AdpA (Higo & Horinouchi 2011). This mechanism functions at two target genes, *adpA* and *ramR* which regulate the expression of SapB (Bush et al. 2015; O'Connor & Nodwell 2005).

Other regulators have not been connected to the regulation of the developmental network yet including BldC, which is an unusual MerR-like regulator without an effector domain (Hunt et al. 2005). The target genes of this regulator are not known but *bldC* is a direct target of the phosphate metabolism regulating response regulator (RR), PhoP (Allenby et al. 2012). The anti-anti-sigma factor BldG is involved in the regulation of development and antibiotic production in response to osmotic stress and dephosphorylated BldG represses the anti-sigma factor / sigma factor pairs ApgA / σ^B, UshX / σ^H, RsfA / σ^F (Mingyar et al. 2014).

It is not only *bld* and *whi* genes that are involved in the regulation of development. Several genes involved in general metabolism have also been shown to have a role in development such as *citA* and *clpP1*. *citA* encodes a citrate synthase, the first enzyme in the tricarboxylic acid (TCA) metabolism and a Δ*citA* mutant in *S. coelicolor* displays a bald phenotype and antibiotic production is blocked (Viollier et al. 2001). Citrate synthases are regulated by several global nutrient sensory regulators like GlnR and DasR, see **Figure 1.3** (Liao et al. 2014), which is a good example of the linkage of nutrient availability and development. Additionally, proteases are important to remove regulatory proteins during development. *clpP1* is the catalytic subunit of an ATP-dependent protease and a Δ*clpP1* mutant has a bald phenotype (Crecy-Lagard et al. 1999). *clpP1* is under the direct control of the master regulator AdpA (Wolański et al. 2012).

WhiA and WhiB regulate the transition from aerial hyphae to spores. WhiB and other WhiB-like (Wbl) proteins have a [4Fe–4S] iron–sulphur cluster coordinated by four invariant cysteines (Crack et al. 2009; Crack et al. 2011) which is highly unusual and can only be found in Actinobacteria. The *S. venezuelae* genome contains eight Wbl proteins (Munnoch 2015). WhiB and WhiD are involved in sporulation (Bush et al. 2013; Flärdh et al. 1999; Aínsa et al. 2000), WblC activates multi-drug resistance in *M. tuberculosis* (Morris et al. 2005), WblA is involved in very early sporulation and WblE appears to be essential (Fowler-Goldsworthy et al. 2011). During vegetative growth WhiB is repressed

by BldD-(c-diGMP) and at the onset of sporulation WhiB is activated by homodimeric BldM. WhiB and WhiA have the same white phenotype and WhiB appears to act as a partner protein for WhiA (Bush et al. 2016). During sporulation WhiA can act as a repressor or activator. WhiA activates expression of components of the divisome like FtsZ, the DNA tranlocase FtsK and the putative lipid II flippase FtsW. To stop hyphal tip extension WhiA directly represses the expression of the polarisome component FilP (Bush et al. 2013).

In addition to the regulation of components of the divisome, WhiA also directly activates the RNA polymerase sigma factor σ^{WhiG} which in turn regulates the expression of late sporulation genes like WhiH and WhiI. WhiH is a GntR family transcription factor. The exact role of WhiH is unclear, however Δ*whiH* form only occasional sporulation septa which leads to the formation of compartments containing multiple copies of the genome and is crucial for spore formation (Flärdh et al. 1999). WhiI is an orphan response regulator which lacks a typical phosphorylation pocket (Aínsa et al. 1999; Ryding et al. 1998) and does not bind to DNA as a homodimer. BldM has a set of targets for the BldM homodimer and a different set of target genes for the BldM-WhiI heterodimer and WhiI by itself does not activate or repress genes. The heterodimer BldM-WhiI activates late stage sporulation genes like the *smeA-sffA* operon and *whiE*, which encodes an enzyme involved in spore pigment formation. The heterodimer formation provides a mechanism of temporal regulation of sporulation (Al-Bassam et al. 2014).

1.4 Two component systems

As soil inhabitants *Streptomyces* must survive in a rapidly changing and highly variable environment. Bacteria can sense an external stimulus from the environment and adjust the internal cellular processes via two component systems (TCS). These were first described in the late 1980s and since then they have been found in bacteria, archaea and some eukaryotic organisms. It is noteworthy that TCS are predominant in bacteria. Other classes of organisms which use TCS to sense external stimuli are unicellular eukaryotes, fungi and plants (Stock et al. 2000). The absence of TCSs in humans or animals makes them an ideal target for antibiotics. *Streptomyces* species have a high number of TCS, for example the *S. coelicolor* genome encodes at least 84 histidine kinases (HK) and 80 response regulator (RR). 67 of these RR and HK genes are adjacent to each other and thus predicted to form TCS (Hutchings et al. 2004). There are three different classes of TCS.

The classical TCS in which a transmembrane HK senses the signal and transfers a phosphate group to its cognate RR. The second class are hybrid TCS in which HK and RR are fused together which means the whole TCS complex is membrane bound. The third class is the phosphorelay which includes phosphotransferases that transfer the phosphoryl group from the sensor kinases to the ultimate target via multiple phosphotransfer steps (Groisman 2016).

Since MtrAB-LpqB belongs to the classical TCS the following section describes the general features of this system. In a classical TCS the transmembrane HK autophosphorylates using ATP at a conserved histidine residue due to sensing of an external stimulus or the state of the membrane. The phosphate group is then transferred to a conserved aspartate in the cognate RR. HKs contain two domains: a variable extracellular sensor domain spanning the membrane and a highly-conserved kinase core located inside of the cell (Park et al. 1998). The kinase core contains a dimerization domain (HAMP and HisKA in MtrB) and a catalytic domain which contains the ATP binding site (Tanaka et al. 1998).

The majority of RRs consist of two domains: a conserved N-terminal regulatory domain and a variable DNA binding domain. RR can be divided in three major families OmpR, NarL and NtrC depending on the type of DNA-binding domain. MtrA belongs to the OmpR family (Friedland et al. 2007). The majority of RRs belonging to this superfamily have DNA-binding activity and can activate or repress transcription of target genes (Stock et al. 2000). OmpR-family RRs interact with DNA via a conserved recognition helix which interacts with the major groove of DNA and flanking loops (Stock et al. 2000), see blue recognition helix in **Figure 1.5**.

1.4.1 Regulation of Two Component Systems (TCS)

The activity of TCS systems is tightly regulated both at the expression level and phosphorylation state to ensure an appropriate response to the sensed stimulus. In most TCS systems the phosphorylated RR functions as the active form because the DNA binding affinity is increased upon phosphorylation (Gao & Stock 2015; Purushotham et al. 2015). RR typically function as homodimers and phosphorylation promotes dimerization (Barbieri et al. 2013; Boudes et al. 2014).

All HKs autophosphorylate and most HKs are bifunctional proteins that can also act as phosphatases to control the phosphorylation state of the cognate RR (Groisman 2016). The HK kinase domain interacts with the RR via a recognition surface which is highly conserved (Laub & Goulian 2007) whereas the signals sensed by each HK and the regulons controlled by each RR differ widely due to highly variable input and output domains (Mascher et al. 2006).

When a TCS senses a stimulus the amount of phosphorylated RR increases in the cell. The stimulus promotes the autophosphorylation of a HK as well as inhibiting the phosphatase activity. In the latter case the amount of phosphorylated RR would further increase because some RRs can be phosphorylated by small phosphate donors like acetyl phosphate or phosphoramidate (McCleary & Stock 1994; Friedland et al. 2007). Also, a stimulus may alter the transcription or activity of additional proteins which in turn can modify the kinase or phosphatase activities of individual HKs.

In order to react to a stimulus appropriately most TCS are transcriptionally autoregulated. Most TCS are encoded in operons and they must be transcribed at a basal level to be able to sense the HK inducing stimulus which means that most of the TCS that have been studied in detail are transcribed from two promoters. One promoter is weak and constitutive (Miyashiro & Goulian 2008) and the second promoter is autoregulated and is responsible for the response to the signal. The location of the two promoters can vary between TCS (Groisman 2016).

1.5 Antibiotic production in *Streptomyces*

Streptomyces are known for their production of antibiotics. Most of the antibiotics we use today are derived from this genus. *Streptomyces* genomes contain at least 20 or more biosynthetic gene clusters (BGC), (Niu et al. 2016) encoding antibiotics or other secondary metabolites. Antibiotics are defined as molecules which inhibit or prevent the growth and / or proliferation of bacteria whereas secondary metabolites are defined as molecules that have a specific function in the producing organism and are not directly involved in normal growth and development (Hopwood 2007).

The regulation of antibiotic production is complex and tightly linked to development. One link between antibiotic production and development is the master regulator AdpA which was first discovered in *S. griseus* (Ohnishi et al. 1999) and

originally named BldH in *S. coelicolor* (Takano et al. 2003). As mentioned above, AdpA regulates the developmental cycle but also plays a key role in production of secondary metabolites. In *S. griseus* the signalling molecule γ-butyrolactone A-factor is sensed by the ArpA repressor. When the concentration of A-factor reaches a threshold (Ohnishi et al. 2005), ArpA is released from the *adpA* promoter region and AdpA is expressed. AdpA then targets genes involved in secondary metabolism and differentiation in *S. coelicolor* and *S. griseus* (Ohnishi et al. 2005; Horinouchi 2007). In general, secondary metabolite BGCs include cluster situated regulators (CSRs) which regulate the expression of the secondary metabolite and which can be also regulated by AdpA.

Streptomyces differentiate in response to external or internal signals (Bush et al. 2015). These signals are recognised by membrane bound sensors which sense the availability of nutrients like N-Acetylglucosamine (GlcNAc), glucose or xylose. A majority of environmental changes are sensed by TCS and the nitrogen-sensing AfsQ1/Q2 (Shu et al. 2009) and the phosphate-sensing PhoRP (Martín 2004) are involved in the regulation of antibiotic production (**Figure 1.3**).

Most environmental stimuli that trigger antibiotic production target CSRs. One well studied example of BGC regulation by a CSR is the production of actinorhodin by *S. coelicolor*. The *act* gene cluster contains five transcriptional units. The expression is regulated by the OmpR-like CSR ActII-4. Almost all stimuli leading to increased actinorhodin production are mediated via ActII-4 (Liu et al. 2013) which is in turn regulated by at least eight known regulatory proteins (**Figure 1.3**): the xylose operon repressor ROK7B7 (SCO6008) (Heo et al. 2008), DasR which mediates the global response to N-acetylglucosamine (GlcNAc) (Rigali et al. 2008), AtrA which is a transcriptional activator binding to targets associated with metabolism of acetyl coenzyme A [acetyl-CoA], an ACT precursor (Uguru et al. 2005; Nothaft et al. 2010), the RRs of the TCSs DraR and AfsQ1 responding to nitrogen access (Wang et al. 2013; Yu et al. 2012), the global repressor of antibiotic synthesis AbsA2 (Sheeler et al. 2005; Uguru et al. 2005), the global regulator of DNA damage response LexA (Iqbal et al. 2012) and the global developmental regulator AdpA (Ohnishi et al. 2005). The export of actinorhodin is regulated by *actR*, which is also situated within the Act BGC. ActR is a TetR-like protein that represses the adjacent *actA* operon. Both *actII-4* and *actR* contain a TTA codon which makes the two regulators dependent on BldA (Fernández-Moreno et al. 1991).

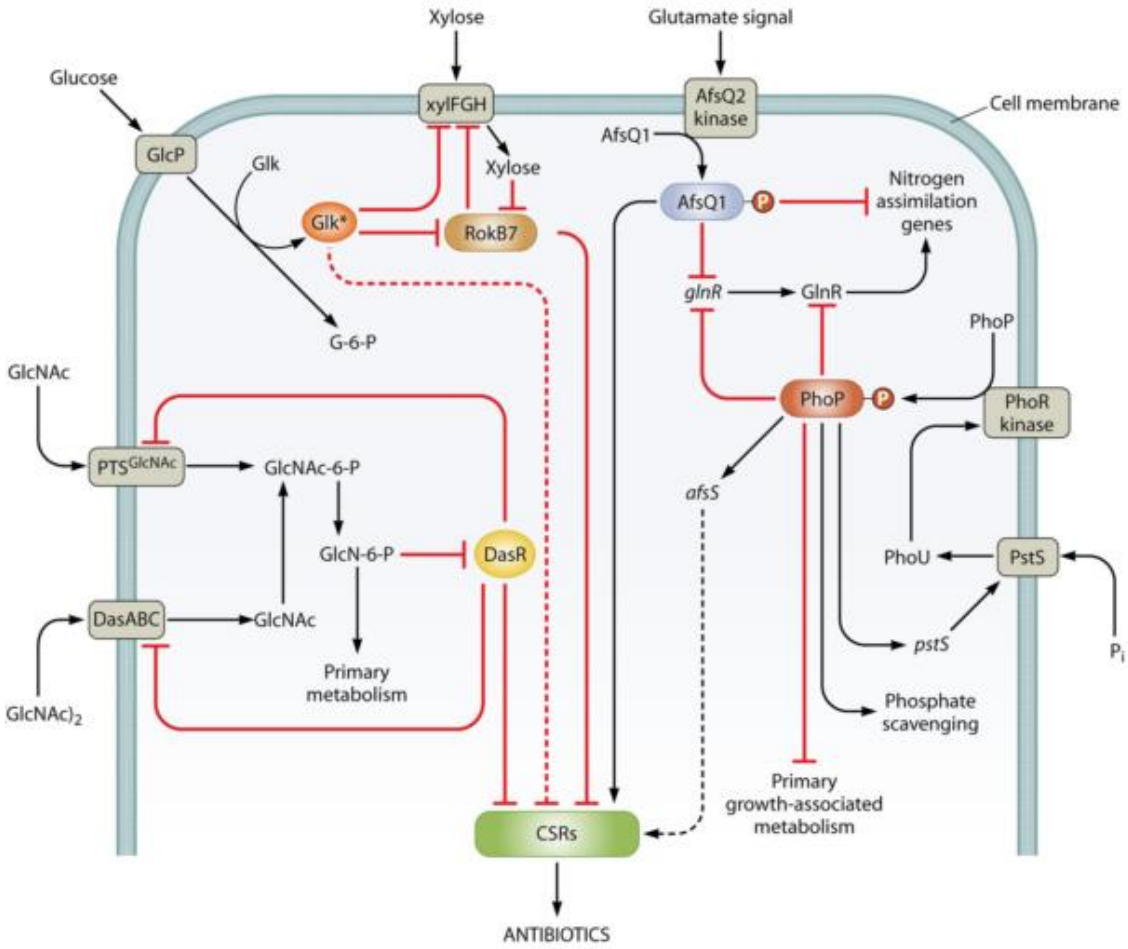

Figure 1.3 Nutrient-sensing regulators of antibiotic production in *S. coelicolor* and their cross talk. Nutrient availability is sensed by membrane bound sensor kinases or transport of nutrients. The signal of availability of carbon, nitrogen, and phosphate are transferred to global regulators (circled) which then regulate central metabolic genes and cluster situated regulator (CSR) genes. The global regulators control both central metabolic genes and CSR genes, either directly (solid lines) or through unknown routes (dotted lines). Arrows indicate activation, and bars indicate repression. AfsQ1/2 and PhoRP are TCS. Reprinted (adapted) with permission from (Liu et al. 2013) Copyright (2017) Microbiology and Molecular Biology Reviews.

Undecylprodigiosins are regulated in *S. coelicolor* by a cascade of two CSRs. RedZ activates the expression of *redD* which directly activates biosynthesis of the RED operons (White & Bibb 1997). Similarly to *actII-4*, *redZ* is repressed by AbsA2~P (Sheeler et al. 2005; Uguru et al. 2005) and responds to GlcNAc via DasR (Rigali et al. 2008) and external glutamate via AfsQ1 (Wang et al. 2013). The *redZ* gene also contains a TTA codon which makes it BldA dependent (White & Bibb 1997).

The most studied BGCs in *S. venezuelae* are those encoding chloramphenicol and jadomycin. The chloramphenicol cluster contains 14 structural genes (SVEN15_0880-0893) and a recent study (Fernández-Martínez et al. 2014) showed that three genes (SVEN15_0877-79) upstream of the known gene cluster are also part of the chloramphenicol BGC and that SVEN15_0877 (*cmlR*) is a CSR. Chloramphenicol

production in a Δ*cmlR* mutant was abolished (Fernández-Martínez et al. 2014). However in *S. venezuelae* cultures grown in the standard rich medium MYM the *cmlR* promoter seems to be inactive and thus does not play a crucial role in regulation of the chloramphenicol BGC (Sekurova et al. 2016). It was shown that CSRs in the jadomycin cluster play a major role in chloramphenicol regulation (Xu et al. 2010). The jadomycin cluster contains four CSRs. JadR1 activates the biosynthesis of jadomycin and is dependent on jadomycin concentration wherein low levels of jadomycin activate JadR1 and high levels repress the biosynthesis of jadomycin (Wang et al. 2009). JadR1 also binds to promoter regions in the chloramphenicol BGC and represses chloramphenicol production (**Figure 1.4**). Adjacent to *jadR1* is the divergently transcribed *jadR2* which inhibits expression of *jadR1*. Additionally, the transcription of *jadR1* is repressed by JadR*. The repressors JadR2 and JadR* act synergistically to repress the transcription of *jadR1* (Zhang et al. 2013). JadR2 and JadR* repression of JadR1 leads to biosynthesis of chloramphenicol (Xu et al. 2010). JadR3 is activated by γ-butyrolactone SVB1 which then stimulates transcription of *jadR1* while repressing *jadR2*.

The regulatory network of jadomycin and chloramphenicol is complex and a recent study has shown that external stresses like ethanol shock can influence the biosynthesis of jadomycin and chloramphenicol (Sekurova et al. 2016).

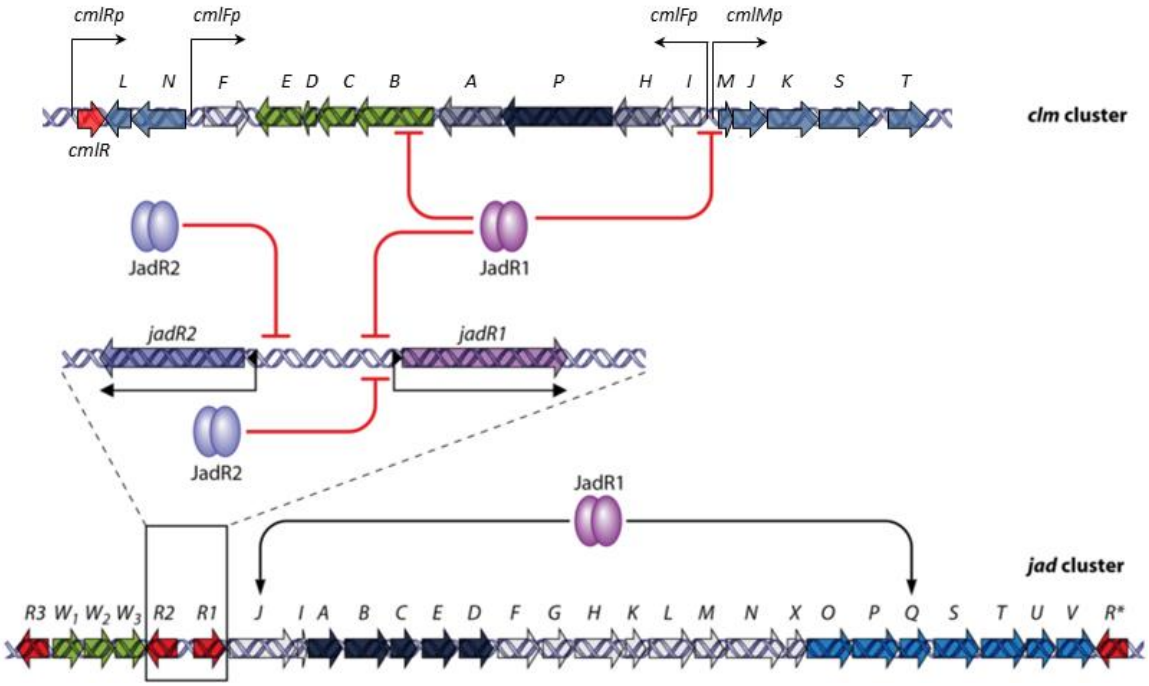

Figure 1.4 Overview of the cross-talk regulation of chloramphenicol and jadomycin in *S. venezuelae*. JadR2 directly represses the transcription of *jadR1* and also binds chloramphenicol (Cm) and jadomycin. The cluster-situated regulator JadR1 activates the biosynthesis of jadomycin B by activating the transcription of biosynthetic structural genes. JadR1 also represses the production of Cm by binding to the promoters of the structural genes, (Sekurova et al. 2016). Reprinted (adapted) with permission from (Liu et al. 2013) Copyright (2017) Microbiology and Molecular Biology Reviews.

1.6 The role of MtrAB-LpqB in Actinobacteria

The MtrAB-LpqB operon is present in most Actinobacteria. However detailed studies of this three-component system have only been undertaken in Mycobacteria and *Corynebacterium glutamicum*.

1.6.1 MtrAB-LpqB in Mycobacteria

The MtrAB-LpqB TCS is best investigated in Mycobacteria due to its involvement in antibiotic susceptibility (Cangelosi et al. 2006), cell wall synthesis (Nguyen et al. 2010) and cell cycle progression (Fol et al. 2006). MtrA was first identified in *M. tuberculosis* (Mycobacterium tuberculosis regulator A) as homologue of *Pseudomonas aeruginosa* PhoB by DNA hybridisation (Via et al. 1996). MtrAB-LpqB in Mycobacteria are encoded by an operon which is conserved throughout Actinobacteria. The RR gene *mtrA* is located upstream of the sensor kinase gene *mtrB* and a lipoprotein encoded by *lpqB*. The role of MtrA as an RR was confirmed by phosphorylating MtrA via the non-cognate HK CheA (Via et al. 1996) and MtrA directly interacts with MtrB

and DNA (Li et al. 2010). MtrB has been demonstrated to be a typical HK due to its ability to autophosphorylate and then phosphotransfer to MtrA which requires Mg^{2+} (Al Zayer et al. 2011).

Comparison of amino acid sequences suggested that MtrA belongs to the OmpR family with a characteristic winged helix-turn-helix DNA binding motif in the effector domain. This was confirmed by solving the crystal structure of the non-phosphorylated, inactive form of MtrA from *M. tuberculosis*. MtrA contains two domains, an N-terminal regulatory domain exhibiting the classic α/β fold observed in all RRs and a C-terminal effector domain, see **Figure 1.5** (Friedland et al. 2007).

The MtrAB-LpqB system is one of two essential TCS in *Mycobacteria* (Griffin et al. 2011; Haydel et al. 2012; Zahrt & Deretic 2000). However, the HK MtrB can be deleted in *M. tuberculosis*, *Mycobacterium avium* and *Mycobacterium smegmatis* (Zahrt & Deretic 2000; Cangelosi et al. 2006; Plocinska et al. 2012) suggesting that the HK is not responsible for the essentiality of the TCS. Small molecule phosphodonors like acetyl phosphate and phosphoramidate can phosphorylate MtrA(TB) (Friedland et al. 2007) which indicates that MtrA can be phosphorylated independently of the cognate HK.

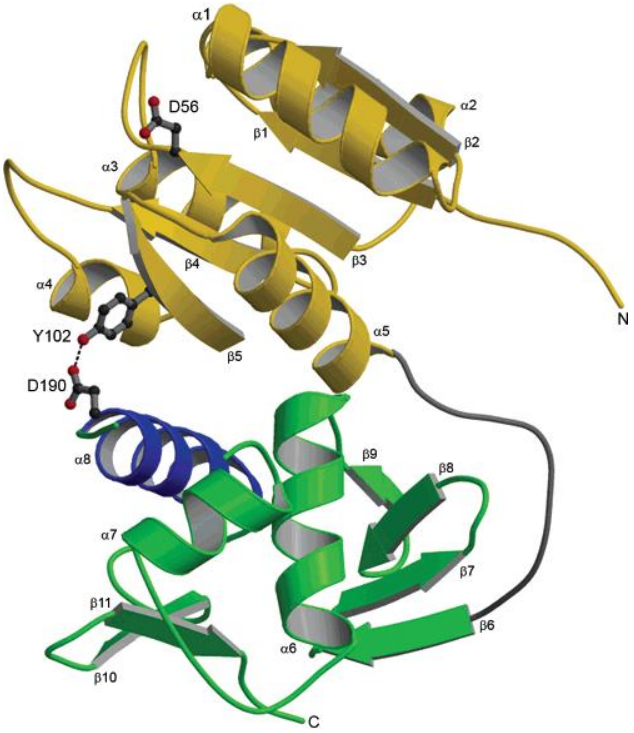

Figure 1.5 Crystal structure of MtrA(TB). The regulatory domain is shown in gold, and the DNA-binding domain is shown in green with the recognition helix, α8, highlighted in blue. The side chains of Asp 56, the site of phosphorylation, and Tyr 102 and Asp 190 that form an inter-domain hydrogen bond are shown in ball-and- stick format with the hydrogen bond depicted by a dashed line. Reprinted (adapted) with permission from (Friedland et al. 2007) Copyright (2017) American Chemical Society.

Additionally, MtrA(TB) binds DNA in a non-phosphorylated state although with a much lower affinity (Rajagopalan et al. 2010). These findings indicate that optimal function of MtrAB is dependent on the ratio of phosphorylated MtrA to non-phosphorylated MtrA and that MtrB regulates the state of phosphorylation of MtrA (Fol et al. 2006). This is coherent with the phenotypes in the deletion mutants of Δ*mtrB* in *M. avium* and *M. smegmatis*. The Δ*mtrB* mutant cells in *M. avium* are fivefold more sensitive to penicillin, ciprofloxacin and clarithromycin. Additionally, the *M. avium* Δ*mtrB* mutant exhibited cell envelope defects, resulting in increased permeability, and an elongated cell shape. The mutant strain also showed a loss in virulence reflected by the inability to survive in macrophages (Cangelosi et al. 2006). In the *M. smegmatis* Δ*mtrB* mutant a cell wall defect was observed which leads to increased susceptibility to lysozyme. And the mutant cells are filamentous, with increased cell clumping and defective septum formation and cell division (Plocinska et al. 2012).

Little is known of the signals and regulation which modulate the MtrAB TCS. However, it was shown that *mtrAB-lpqB* expression in *M. tuberculosis* is controlled by the RNA polymerase sigma factor, sigma C (Sun et al. 2004). Furthermore, the accessory protein LpqB interacts with the extracellular domain of MtrB to modulate its activity. An *lpqB* mutant in *M. smegmatis* shows pleiotropic phenotype caused by cell wall changes which include increased cellular aggregation and loss of biofilm formation, as well as changes in motility and cell morphology during growth. Interestingly the cell morphology of the *lpqB* mutant is similar to the hyphal growth and polyploidism observed in *Streptomyces* species (Nguyen et al. 2010). These phenotypes can be reversed by expressing constitutively active MtrA which leads to the conclusion that LpqB has to interact with MtrB for normal cell wall maintenance and growth (Nguyen et al. 2010). Additionally, the interaction of LpqB with MtrB affects the phosphotransfer to MtrA and subsequent downstream effects on the regulon, including changes in the expression of the DNA replication initiation regulator *dnaA* (Nguyen et al. 2010). MtrA was shown to bind to the *dnaA* promoter region and positively regulate the expression of *dnaA* in a phosphorylation dependent manner (Fol et al. 2006). Another finding strongly suggests a role for *M. tuberculosis* MtrB in the regulation of cell division: MtrB localises to the cell division septa and cell poles in an FtsZ-dependent manner (Plocinska et al. 2012) and interacts with the divisome components FtsI and Wag31 (Plocinska et al. 2014) which are the homologs of PBP3 and DivIVA, respectively, in *S. coelicolor*.

The above findings show that MtrAB-LpqB is involved in regulating cell cycle progression and cell wall maintenance in Mycobacteria. Furthermore, several studies have shown that MtrAB-LpqB is involved in virulence in Mycobacteria. MtrA was upregulated in multi drug resistant *M. tuberculosis* strains and expression of MtrA could be induced by the anti-tuberculosis drugs isoniazid and rifampicin (Zhou et al. 2015). Additionally a DNA microarray analysis showed that MtrA was more highly expressed in two clinical drug resistant isolates in response to the three anti-tuberculosis drugs isoniazid, capreomycin and rifampicin (Yu et al. 2015). Interestingly, the expression of MtrAB differs between virulent and avirulent strains. In *Mycobacterium bovis* BGG the system is induced after infection into macrophages (Via et al. 1996; Zahrt & Deretic 2000), but it is constitutively expressed in *M. tuberculosis*, where MtrA is essential (Zahrt & Deretic 2000).

The complete regulon of MtrA in *Mycobacteria* has not been defined but several direct targets in addition to *dnaA* have been found. The expression of the major secreted immunodominant antigen Ag85B, encoded by *fbpB* is increased in an MtrA overexpression strain of *M. tuberculosis* (Rajagopalan et al. 2010). The *fbpB* binding site consisting of two direct repeats of GTCACAgcg (**Figure 1.6**) can be also found in the *oriC*, suggesting a key role of MtrA in chromosomal replication (Rajagopalan et al. 2010). A study in *M. smegmatis* using unphosphorylated MtrA suggests that MtrA may bind to an alternative binding site CAGGCCG (**Figure 1.6**) which can be found in 420 Mycobacterial genes (Li et al. 2010). Another confirmed target is the essential cell wall hydrolase *ripA* in *M. smegmatis* (Plocinska et al. 2012).

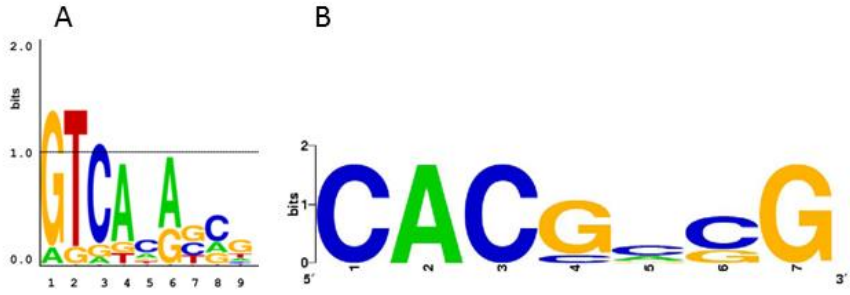

Figure 1.6 MtrA binding sites in Mycobacteria. **A** Sequence logo of seven *oriC* binding sites and the *fbpB* promoter region in *M. tuberculosis*. All binding sites were confirmed by footprinting. Reprinted (adapted) with permission from (Rajagopalan et al. 2010). Copyright (2017) The Journal of Bacterial Chemistry. **B** Sequence logo in Mycobacteria, the *dnaA* promoter as well as the promoter of Rv0341 (Isoniazid inducible gene IniB), Rv0574 (hypothetical protein), and Rv3476 (dicarboxylate transporter KgtP) and in *C. glutamicum*, the promoter of *mepA* (a secreted metallopeptidase) and *proP* (uptake carrier for proline/ectoine). All binding site were confirmed by electrophoretic mobility shift assay [EMSA] (Li et al. 2010), open access journal.

1.6.2 MtrAB-LpqB in *Corynebacterium glutamicum*

C. glutamicum is a non-pathogenic soil bacterium and has industrial importance because it is used to produce the amino acids L-glutamate and L-lysine. In 2004 Möker et al. deleted *mtrAB* and found that the mutant cells were elongated, segmented and sometimes showed an irregular cell division septum. They were also more susceptible to penicillin and vancomycin and more resistant to ethambutol (Möker et al. 2004). A DNA microarray experiment showed that MtrA activates and represses genes. Three genes were upregulated in the mutant background: *lpqB*, which lies downstream of *mtrB*; *mepA* a secreted metallopeptidase; *ppmA* a membrane-bound protease modulator. A further eight genes were repressed in the mutant background: *mtrA* and *mtrB*, the uptake carriers *proP* and *betP* for proline/ectoine and betaine respectively and four genes encoding hypothetical proteins. This was the first data suggesting that MtrAB-LpqB is involved in osmoprotection and cell wall biosynthesis since MtrA regulates three out of four uptake systems for compatible solutes (*proP*, *betP* and *lcoP*), the mechanosensitive channel *mscL* and the extracytoplasmatic metallopeptidase MepA which might modulate peptides already incorporated in the peptidoglycan sacculus or lipid II peptides that will be incorporated.

In a later publication *mtrA* and *mtrB* were deleted separately (Brocker & Bott 2006). The phenotypes are similar to the Δ*mtrAB* mutant but the cells were not as elongated as in the double mutant. The antibiotic susceptibility of the Δ*mtrA* and Δ*mtrB* mutants is similar to the double mutant with the exception that the Δ*mtrB* mutant is sensitive to ethambutol. However, the authors do not show any data for complementation of the single mutants which makes the non-essentiality of MtrA in *C. glutamicum* doubtful because the deletion of *mtrA* might have introduced secondary mutations. Interestingly, MtrA is essential in pathogenic *C. pseudotuberculosis* (Hassan et al. 2014) but it remains unknown as to whether MtrA has the same function in the closely related but environmentally different strains. Nevertheless the *C. glutamicum* Δ*mtrA* mutant was used for ChIP-on-chip (chromatin immunoprecipitation combined with DNA microarray) to find direct targets of MtrA. These experiments suggest that there are at least four different targets of MtrA in *C. glutamicum*: *mepA, nlpC, betP* and *proP*. MepA and NlcP are putative cell wall peptidases and directly repressed by MtrA whereas ProP and BetP which are both carriers for compatible solutes are directly activated by MtrA (Brocker & Bott 2006; Brocker et al. 2011).

The next question addressed in *C. glutamicum* concerned the signal sensed by MtrB. Due to the direct targets of MtrA involved in osmotic stress response it seems likely that MtrB acts as an osmosensor. It is crucial for bacteria to sense the environmental osmolality and counteract osmotic changes but it is very difficult to measure the effects of osmolality *in vivo* in a cell because the environmental osmolality leads to many internal and external changes in the cell. This includes the concentrations of specific solutes, ionic strength, internal osmolality and the physical state of the membrane. Bacteria use two mechanisms to cope with osmotic stress. Upon osmotic stress bacteria increase the concentration of compatible solutes (e. g. proline, glutamate and ectoine) by uptake or *de novo* synthesis to increase to turgor pressure (Csonka 1989). Additionally, bacteria can alter the membrane lipid composition by changing the ratio of phospholipids and fatty acids (Russell et al. 1995). To investigate MtrB under controlled condition *in vitro* the HK was reconstituted in proteoliposomes in an inside-out orientation (Möker et al. 2007). In this artificial set up, MtrB is fully functional and transfers the phosphoryl group to MtrA and also dephosphorylates MtrA in the presence of ADP. It is interesting that phosphorylated MtrA seems to be highly stable with a 30% dephosphorylation rate in 60 minutes. It is a common theme for osmosensors to sense either ionic strength, a solute or K^+ on the cytoplasmatic side of the membrane as indicator for hypertonicity (Rübenhagen et al. 2001; Jung et al. 2001; Jung et al. 2000; van der Heide et al. 2001; Culham et al. 2003). That is why monovalent cations were tested to see if they could stimulate MtrB autophosphorylation. Among other monovalent cations, K^+ stimulated the autophosphorylation of MtrB but at the same time K^+ also stimulated DcuS which senses C4-dicarboxylates and is not involved in osmosensing. The authors concluded that MtrB does not sense the internal cation concentration as osmotic stress signal and that the K^+-dependent activation seems to be a non-specific feature of HKs.

However it was shown that MtrB could be activated by various solutes, sugars, amino acids and polyethylene glycol (Möker et al. 2007). The authors speculate that MtrB is unlikely to be activated by binding a specific osmolyte. They hypothesise that MtrB detects osmotic stress in the cytoplasmic region of the protein via the modulation of the hydration state of the protein, which is induced via preferential and / or steric exclusion of osmolytes from the protein surface. This is coherent with the data that MtrB needs to be integrated in a membrane to be active and that the extracellular loop domain is dispensable for activation by osmotic stress.

To define the MtrA targets further in *C. glutamicum* the promoter regions of the confirmed targets of MtrA were analysed with the MEME software (Brocker et al. 2011) and then confirmed by electrophoretic mobility shift assay (EMSA). In *C. glutamicum* MtrA either directly or indirectly regulates 24 genes which have transport functions or are proteins predicted to be proteases / peptidases, proteins with other predicted function (stress protein of unknown function, resuscitation promoting factor, putative acetyltransferase, putative glutaredoxin, NAD synthetase, putative membrane-bound protease modulator) putative membrane proteins of unknown function and proteins with unknown function.

MtrA represses or activates genes depending on the MtrA binding site in relation to the transcriptional start site. If MtrA activates a gene it binds upstream of the -35 region and can promote binding of the RNA polymerase to the DNA and therefore stimulate initiation of transcription. On the other hand, if MtrA binds in the vicinity of the -10 region it can prevent binding of the RNA polymerase and thus prevent initiation of transcription. The predicted binding site is a 19 bp consensus site with an 8 bp direct repeat separated by three variable base pairs shown in **Figure 1.7**.

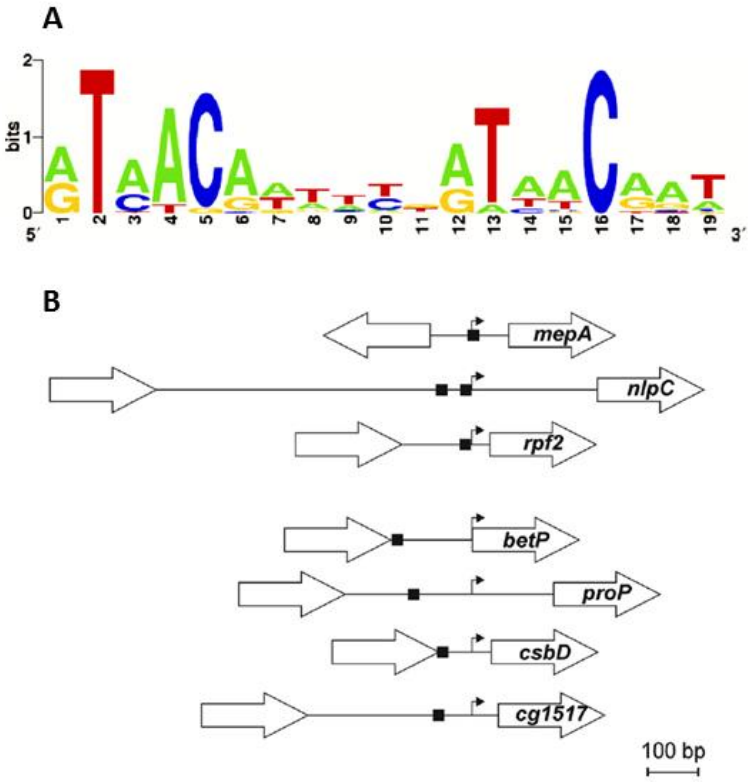

Figure 1.7 A 19 bp consensus motif of MtrA in *C. glutamicum* derived from the verified binding sites. **B** Gene repression (top) or activation (bottom) of MtrA in *C. glutamicum*. Reprinted (adapted) with permission from (Brocker et al. 2011). Copyright (2017) Journal of Bacteriology.

The phosphorylated form of MtrA is the active form *in vivo* in *C. glutamicum* as shown by the mutation of the phosphorylation site D53N (Brocker et al. 2011). Furthermore the authors demonstrated by size exclusion chromatography that MtrA can be phosphorylated *in vitro* by phosphoramidate but not by acetyl phosphate (Brocker et al. 2011). In comparison MtrA in *M. tuberculosis* can be phosphorylated by phosphoramidate but phosphorylation by acetyl phosphate has a much slower rate (Friedland et al. 2007).

To summarise, the work by Möker and colleagues shows in an elegant way how MtrB senses osmotic stress and transfers this signal to MtrA. However not only osmotic stress response genes are targets of MtrA but also genes for cell cycle progression. Thus, it might be possible that MtrB could sense additional signal(s) via the extracellular sensor domain which do not involve osmotic stress. It is worth mentioning that in *C. glutamicum dnaA* is not a target of MtrA in contrast to *M. tuberculosis* which could mean that the MtrAB-LpqB operon could have different function in different organisms even within these closely related genera. It is also possible however that the *C. glutamicum* MtrA regulon has not been completely defined and that the *C. glutamicum mtrA* and *mtrAB* mutants may have acquired suppressors (to prevent lethality) since the mutants were never complemented.

1.6.3 MtrAB-LpqB in *Streptomyces coelicolor*

When MtrA was first discovered in *M. tuberculosis* (Via et al. 1996) it was assumed that MtrA-MtrB could be a homolog of AfsQ1/2 in *Streptomyces* spp. due to the high protein similarity (Zahrt & Deretic 2000). However whole genome sequencing of *S. coelicolor* (Bentley et al. 2002) revealed the true orthologue in *S. coelicolor*, which is not AfsQ1/2. Relatively little is known about the role of MtrAB-LpqB in *Streptomyces* species but it is conserved in the genus *Streptomyces* and the above described model organisms and other Actinobacteria, (see section 1.7). Thus, it can be speculated that there might be a functional overlap of MtrAB-LpqB in the distantly related genera *Streptomyces*, *Mycobacterium* and *Corynebacterium*.

In the *M. avium mtrB* mutant the *mce* (mammalian cell entry) genes are downregulated (Cangelosi et al. 2006). *Mycobacteria* possess several *mce* operons and *mce* mutants were shown to be reduced in viability in mouse infection models (Gioffré et

al. 2005) but hyper virulent in macrophages in *M. tuberculosis* (Shimono et al. 2003). In comparison, the disruption of the *mce* cluster in the non-virulent *S. coelicolor* resulted in virulence towards amoebae and reduced colonization of plant models (Clark et al. 2013). Furthermore, it was shown that MtrA is required for expression of the *mce* genes in *S. coelicolor* because semi-quantitative RT-PCR showed that the *mce* locus was not transcribed in an *mtrA* mutant (Clark et al. 2013). This indicates that the *mce* cluster is a direct target of MtrA in *S. coelicolor*.

Knowles (2014) investigated the MtrAB-LpqB TCS in *S. coelicolor*. Deletion mutants of each single gene in the TCS operon were made and these mutants displayed a cell division defect and overproduced antibiotics. After many attempts to delete *mtrA* it was possible to obtain only a single colony of an *mtrA* mutant (Ryan Seipke, personal communication). The mutant colony is smaller than wild-type and is delayed in sporulation and overproduces actinorhodin and undecylprodigiosin. This mutant could not be complemented with a complementation construct containing *mtrA* under the control of the promoter upstream of *SCO3014*, the first gene in the *mtrAB-lpqB* operon. A second attempt to complement the Δ*mtrA* mutant involved a vancomycin inducible promoter, *vanJp*. The Δ*mtrA* mutant only complemented partially (Knowles 2014), see **Figure 1.8**. The difficulty in obtaining a double crossover Δ*mtrA* mutant and the fact that the Δ*mtrA* mutant that was isolated could only be partially complemented suggests that MtrA might be essential in in *S. coelicolor* and that the removal of *mtrA* from the *S. coelicolor* might be only possible because of one or more suppressor mutations. In comparison, the *mtrB* and *lpqB* mutants could be fully complemented using constructs driven by the *SCO3014* promoter and are therefore not essential in *S. coelicolor*. The Δ*mtrB* mutant shows a drastic cell division phenotype, see **Figure 1.9**. The colonies are smaller and the *mtrB* mutant forms irregular septa. In contrast, the *lpqB* mutant displays normal colony morphology but this mutant also forms irregular septa (**Figure 1.9**). Both mutants overproduce coloured pigments when grown in liquid supplemented minimal medium (SMM) medium. Initially a yellow pigment is visible in the liquid culture and as the culture continues to grow the culture supernatant turns purple. It was hypothesised that the yellow pigment is most likely the actinorhodin precursor (s)-DNPA and not the yellow γCPK (Gottelt et al. 2010). The purple colour can be explained by the overproduction of undecylprodigiosin.

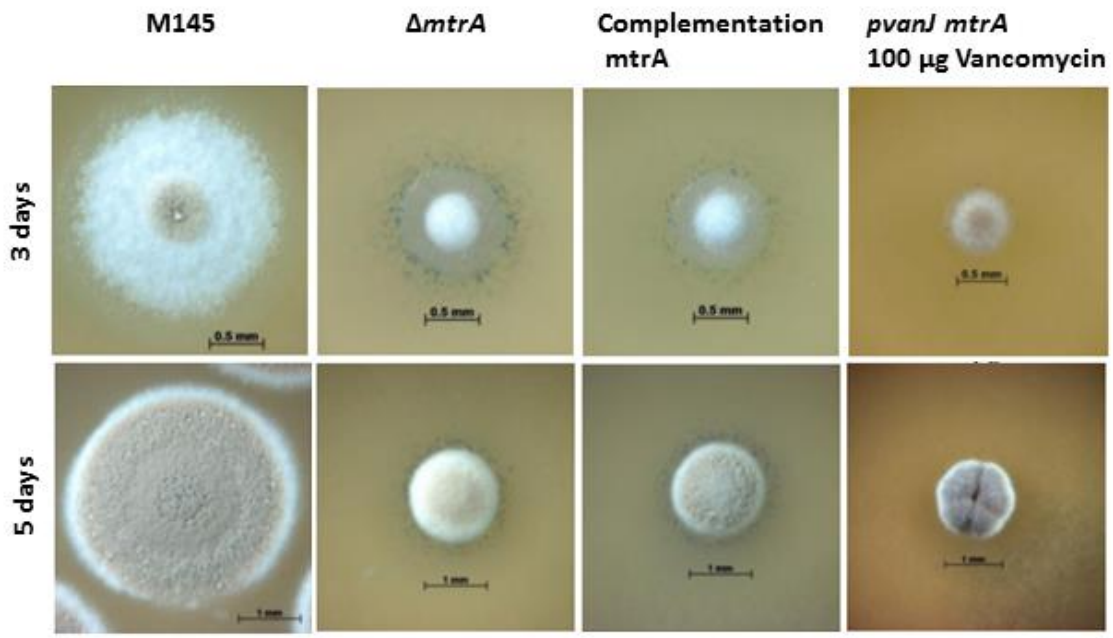

Figure 1.8 Deletion and complementation of Δ*mtrA* mutant in *S. coelicolor*. For the complementation of Δ*mtrA* the plasmid pA11912 containing *mtrA* under the promoter upstream of *SCO3014* was integrated in the ΦC31 integrative site. *mtrA* was cloned in pIJ6883 (Hong et al. 2004) under the control of the vancomycin inducible promoter *vanJp*. Both complementation constructs show only partial complementation. Images modified from (Knowles 2014).

Due to the cell division defect, it was hypothesised that MtrAB-LpqB in *S. coelicolor* might be involved in cell division and that *ftsZ* could be a target of MtrA. Therefore, the *ftsZ* expression in the Δ*mtrA* mutant was determined by qRT-PCR (Knowles 2014). The transcription of *ftsZ* in the Δ*mtrA* mutant is significantly lower than in the wild-type suggesting that MtrA might activate FtsZ in *S. coelicolor*. In summary, previous work indicates the involvement of MtrAB-LpqB in cell division in *S. coelicolor* which is coherent with the work in *M. tuberculosis*. Additionally, the deletion of single components of the TCS leads to overproduction of the antibiotic undecylprodigiosin but underlying mechanisms remain elusive. Finally, the data indicates that *mtrA* is likely to be essential in *S. coelicolor* as it is in *M. tuberculosis*.

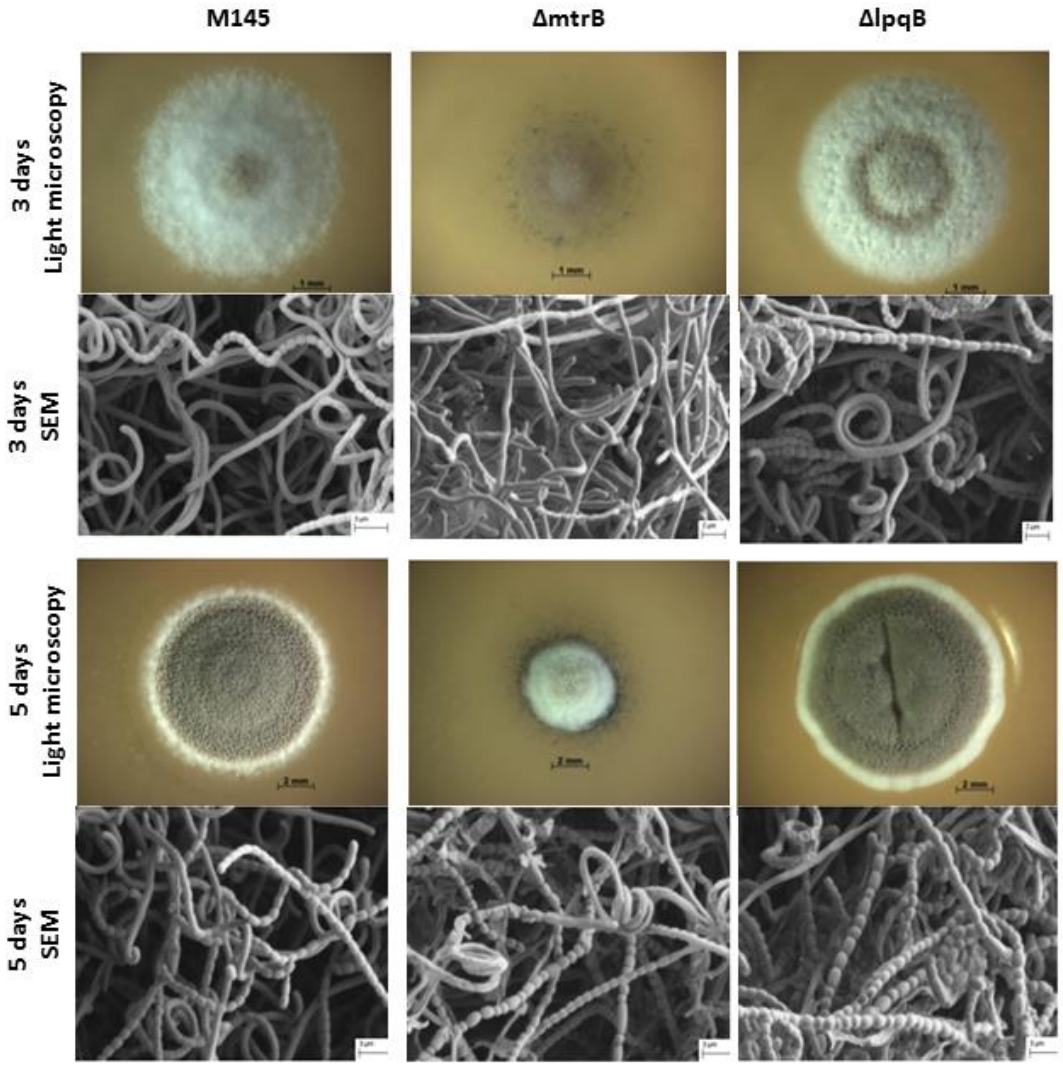

Figure 1.9 Growth defect phenotypes in *S. coelicolor* Δ*mtrB* and Δ*lpqB* mutant. The *S. coelicolor* M145 wild-type and mutants were cultivated on SFM medium for three and five days. Pictures were taken by light microscopy, top panel, and scanning electron microscopy (SEM), bottom panel. The ability to sporulate is reduced in the Δ*mtrB* mutant and it forms irregular spore septa at five days of cultivation. Additionally, the Δ*mtrB* mutant produces a blue pigment after five days of growth. The Δ*lpqB* mutant form irregular spore septa. Images modified from (Knowles 2014).

1.6.4 Transcription of MtrAB-LpqB in *S. venezuelae*

During this work, transcriptomic data for the *mtrAB-lpqB* operon became available. Differential RNA sequencing (dRNA-seq) was performed by John Munnoch and revealed that the *mtrAB-lpqB* operon is transcribed from two transcriptional start sites while microarray data shows the expression of MtrAB-LpqB during the developmental time course (**Figure 1.11**).

The dRNA-seq data is freely available: accession number GSE81104. The data suggest that two promoters drive expression of the *mtrAB-lpqB* operon (**Figure 1.10**).

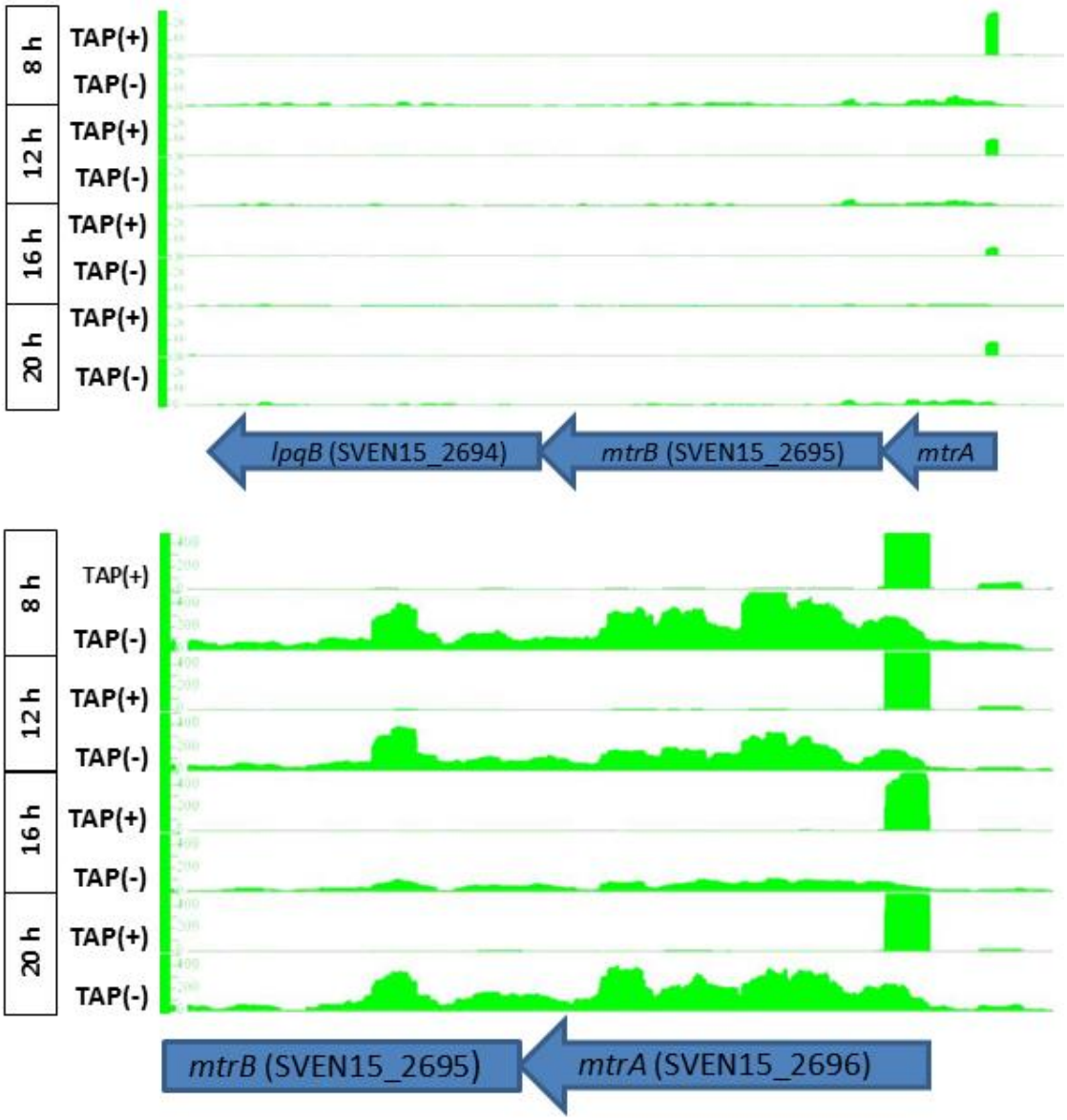

Figure 1.10 Transcriptional start sites of the *mtrAB-lpqB* operon. dRNA-seq in *S. venezuelae* conducted by John Munnoch, accession number: GSE81104. The top and bottom image show both the *mtrAB-lpqB* operon but the bottom image is zoomed in to present the second transcriptional start site (TSS). TAB(+) shows the TSS with enriched 5'PPP RNA and the TAB(-) show the whole transcript with 5'P RNA.

The P1 promoter generates a leaderless transcript starting at +1 and P2 generates a leadered transcript starting at -79. It is typical for TCS that a weak promoter constitutively expresses the RR and HK at a low level to be able to respond to the external stimulus (Groisman 2016). The microarray data shows that *mtrAB-lpqB* operon is constitutivly expressed during the life cycle with the highest level during vegetative growth at 10 till 12 hours and a subsequent drop in expression at 14 hour (**Figure 1.11**).

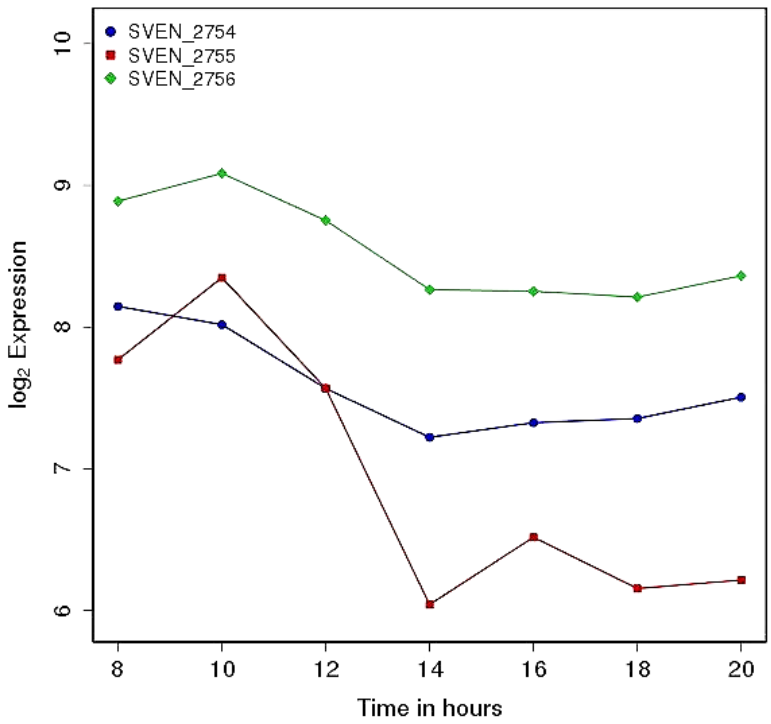

Figure 1.11 Microarray data of the *mtrAB-lpqB* operon in *S. venezuelae.* Kindly provided by Mark Buttner at the John Innes Centre.

1.7 An update on the conservation of MtrAB in Actinobacteria

In 2006 it was proposed that MtrAB-LpqB is highly conserved in the phylum Actinobacteria (Hoskisson & Hutchings 2006). At this time only 17 completely sequenced genomes of Actinobacteria were available (Gao et al. 2006) which belong to 10 genera within the order of Actinomycetales and one species belongs to Bifidobacteriales. These genomes span a wide range of Actinobacteria but no sequenced genomes of the classes Nitriliruptoria, Acidimicrobiia, Coriobacteriia, Thermoleophilia and Rubrobacteria were available at this time. MtrAB-LpqB was conserved in all sequenced Actinobacteria in 2006 with the exception of *T. whipplei* an intracellular pathogen with a reduced genome (Hoskisson & Hutchings 2006). Now in 2016 due to the rapid development of sequencing technology ~7781 Actinobacterial genomes are available (www.patricbrc.org). The following section explores if MtrAB-LpqB is still conserved in Actinobacteria.

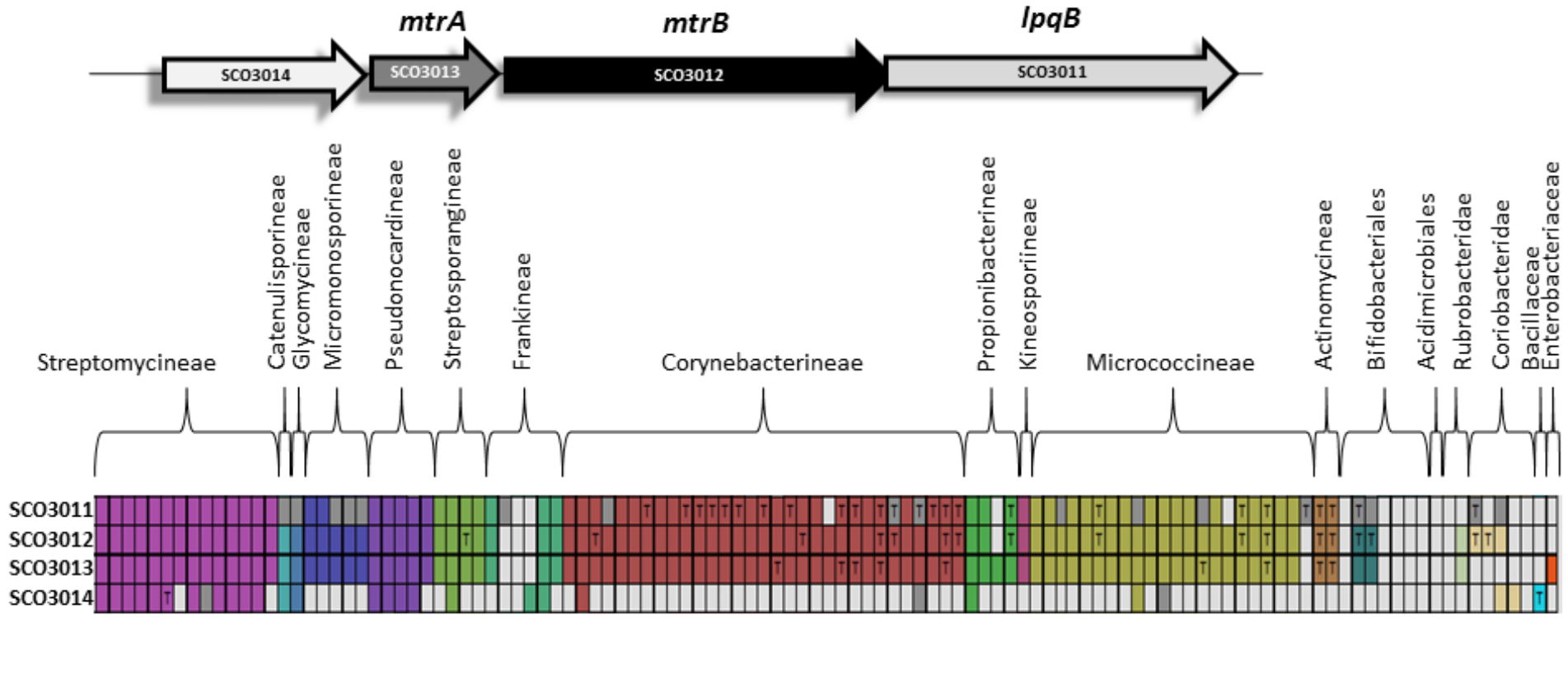
mtrA
mtrB
lpqB
SCO3014
SCO3013
SCO3012
SCO3011
Streptomycineae
Catenulisporineae
Glycomycineae
Micromonosporineae
Pseudonocardineae
Streptosporangineae
Frankineae
Corynebacterineae
Propionibacterineae
Kineosporiineae
Micrococcineae
Actinomycineae
Bifidobacteriales
Acidimicrobiales
Rubrobacteridae
Coriobacteridae
Bacillaceae
Enterobacteriaceae
SCO3011
SCO3012
SCO3013
SCO3014

Figure 1.12 Top: Genomic organisation of *SCO3014-mtrAB-lpqB* in *Streptomyces coelicolor*. **Bottom:** Distribution of probable orthologues of MtrA, MtrB and LpqB proteins of *S. coelicolor* encoded in more than 100 Actinobacterial genomes, as detected by reciprocal BLASTP best hits from **http://streptomyces.org.uk/actinoblast/**. Each column represents one genome, and the genomes are grouped and coloured to indicate subgroup relationships (e.g. *Corynebacterineae* columns, including *Mycobacterium*, *Nocardia* and *Corynebacterium*, were coloured Indian red). Grey boxes indicate reciprocal hits falling below the minimal criteria adopted for orthology. White boxes indicate the absence of a reciprocal hit. The presence of the rare TTA codon is highlighted by a T in the coloured box. The sources of genomes are listed in Table 1 of Gao & Gupta (2012). Organisms were as follows (in order across the tabulation). Magenta: *Streptomycineae, S. lividans* TK24, *S. viridochromogenes* DSM 40736, S. *scabiei* 87.22, *S. sviceus* ATCC 29083, *S. avermitilis* MA-4680, *S. griseoflavus* Tu4000, *S. venezuelae* ATCC 10712, *S. griseus* subsp. *griseus* NBRC 13350, *S. hygroscopicus* ATCC 53653, *S. pristinaespiralis* ATCC 25486, *S. roseosporus* NRRL15998, *S. albus* G J1074, *S. clavuligerus* ATCC 27064, *Kitasatospora setae* KM-6054. Turquoise: *Catenulispora acidiphila* DSM 44928. Light blue: Stackebrandtia *nassauensis* DSM 44728. Dark blue: *Salinispora*, *S. tropica* CNB-440, S. arenicola CNS-205; Micromonospora, M. sp. L5, M. sp. ATCC39149, M. *aurantiaca* ATCC 27029. Purple: *Saccharomonospora viridis* DSM 43017; *Saccharopolyspora erythraea* NRRL 2338; *Amycolatopsis mediterranei* U32; *Actinosynnema mirum* DSM 43827; *Thermobispora bispora* DSM 43833. Yellow green: *Streptosporangium roseum* DSM 43021; *Thermomonospora curvata* DSM 43183; *Thermobifida fusca* YX; *Nocardiopsis dassonvillei* subsp. *dassonvillei* DSM 43111. Blue green: *Acidothermus cellulolyticus* 11B; *Frankia*, *F*. sp. EAN1pec, *F*. sp. CcI3, *F. alni* ACN14a; *Geodermatophilus obscurus* DSM 43160; *Nakamurella multipartita* DSM 44233. Rust red: *Gordonia bronchialis* DSM 43247; *Nocardia farcinica* IFM 10152; *Segniliparus rotundus* DSM 44985; *Tsukamurella paurometabola* DSM 20162; *Rhodococcus, R. opacus* B4, *R. jostii* RHA1, *R. erythropolis* PR4, *R. equi* 103S; *Mycobacterium, M. vanbaalenii* PYR-1, *M. ulcerans* Agy99, *M.* sp. Spyr1*, M*. sp. MCS, *M*. sp. KMS, *M*. sp. JLS, *M. smegmatis* str. MC2 155, *M. marinum*, *M. leprae* Br4923, *M. gilvum* PYR-GCK, *M. abscessus* ATCC 19977, *M. avium* subsp. *paratuberculosis* K-10, *M. avium* 104, *M. tuberculosis* H37Rv, *M. bovis* AF2122/97; *Corynebacterium, C. urealyticum* DSM 7109, *C. pseudotuberculosis* FRC41, *C. kroppenstedtii* DSM 44385, *C. jeikeium* K411, *C. glutamicum* ATCC 13032 2, *C. efficiens* YS-314, *C. diphtheriae* NCTC 13129, *C. aurimucosum* ATCC 700975. Bright green: *Nocardioides* sp. JS614; *Kribbella flavida* DSM 17836; *Propionibacterium, P. freudenreichii* subsp. *shermanii* CIRM-BIA1, *P. acnes* KPA171202. Plum: *Kineococcus radiotolerans* SRS30216. Olive yellow: *Beutenbergia cavernae* DSM 12333; *Cellulomonas flavigena* DSM 20109; *Brachybacterium faecium* DSM 4810; *Kytococcus sedentarius* DSM 20547; *Intrasporangium calvum* DSM 43043; *Jonesia denitrificans* DSM 20603; *Clavibacter michiganensis* subsp. *michiganensis* NCPPB 382; *Leifsonia xyli* subsp. *xyli* str. CTCB07; *Microbacterium testaceum* StLB037; *Arthrobacter*, *A*. sp. FB24, *A. phenanthrenivorans* Sphe3, *A. chlorophenolicus* A6, *A. aurescens* TC1, *A. arilaitensis* Re117; *Kocuria rhizophila* DC2201; *Micrococcus luteus* NCTC 2665; *Renibacterium salmoninarum* ATCC 33209; *Rothias, R. mucilaginosa* DY-18, R. *dentocariosa* ATCC 17931; *Xylanimonas cellulosilytica* DSM 15894; *Sanguibacter keddieii* DSM 10542; *Tropheryma whipplei* str. Twist. Brown: *Mobiluncus curtisii* ATCC 43063; *Arcanobacterium haemolyticum* DSM 20595. Cyan: *Gardnerella vaginalis* ATCC 14019; *Bifidobacterium, B. longum* NCC2705, *B. longum* DJO10A, *B. dentium* Bd1, *B. bifidum* PRL2010, *B. animalis* subsp. *lactis* Bl-04, *B. adolescentis* ATCC 15703. Pink: *Acidimicrobium ferrooxidans* DSM10331. Pale grey green: *Conexibacter woesii* DSM14684; *Rubrobacter xylanophilus* DSM9941. Beige: *Atopobium parvulum* DSM 20469; *Cryptobacterium curtum* DSM 15641; *Eggerthella lenta* DSM 2243; *Olsenella uli* DSM 7084; *Slackia heliotrinireducens* DSM 20476. (Chandra & Chater 2014)

1.7.1 The conservation of MtrAB in 100 Actinobacteria

Chandra and Chater published an extensive web tool which compares the protein identities of each gene in *S. coelicolor* with 100 Actinobacterial genomes (Chandra & Chater 2014). This analysis shows that MtrAB-LpqB is conserved in Actinobacteria and the order of the *mtrAB-lpqB* operon is also conserved. However, in some *Streptomyces* species, including *S. coelicolor*, a putative eukaryotic translation initiation factor lies upstream of *mtrA* (**Figure 1.12**). The *S. venezuelae* operon does not contain this *Streptomyces* specific putative translational initiation factor. Also, members of the closely related genus *Kitasatospora* miss this putative translation initiation factor. The deletion mutant of the putative translation initiation factor in *S. coelicolor* (SCO3014) seems to precociously sporulate on agar plates (Knowles 2014) but the function remains elusive and the sporulation phenotype may be due to downstream effects on the (over) expression of *mtrAB-lpqB*. Since *S. venezuelae* does not contain the putative translation initiation factor this gene is not further investigated in this study.

Throughout Actinobacteria the upstream and downstream genes relative to *mtrAB-lpqB* are not conserved. However in Corynebacterineae there are two conserved upstream and downstream genes: *sahH* (S-adenosylhomocysteine hydrolase), *tmk* (thymidylate kinase), hypothetical cytosolic protein belonging to the amidophosphoribosyl transferase family and hypothetical cytosolic protein belonging to σ^{54} modulation protein family and S30AE family of ribosomal proteins (Möker et al. 2004). If these genes are co-transcribed or influence the MtrAB-LpqB operon remains to be seen.

The MtrAB-LpqB operon can be found in the majority of the investigated Actinobacteria but MtrAB-LpqB is less conserved in the more distantly related families Bifidobacteriales, Acidimicrobiales, Rubrobacteridae and Coriobacteridae and absent in Frankia which form N_2 fixing root nodules and intracellular pathogens as well as commensals (**Table 1.1**). It seems that many intracellular pathogens and commensals have lost the MtrAB-LpqB TCS. However, the family of *Corynebacterineae* contains pathogens like *M. tuberculosis* and *M. leprae*, which possess MtrAB-LpqB. The latter strain is especially interesting because this pathogen has a greatly reduced genome and does not contain most of the sigma factors and TCS which can be found in other Mycobacteria (Madan Babu 2003). Additionally it was shown that MtrA is actively

expressed by detection of MtrA in 2D gel electrophoresis of soluble cell extracts from *M. leprae* (Marques et al. 1998).

In summary, the work of Chandra and Charter revealed that MtrAB-LpqB is conserved in most Actinobacteria but distant related families to *Streptomyces* lost the MtrAB-LpqB operon. One exception is the genus Frankia. Further studies need to be conducted to see if MtrAB-LpqB has the same physiological role in the different genera to address the question why Frankia, human pathogens and commensals lost this TCS.

Table 1.1 Species from **Figure 1.12** in which MtrAB-LpqB is absent.

Family	Species	Environment	Reference
Frankineae	*F.* sp. EAN1pec *F.* sp. CcI3, *F. alni* ACN14a;	Form N2 fixing root nodules	(Chaia et al. 2010)
Micrococcineae	*Tropheryma whipplei* str. Twist.	Intracellular pathogen	(Raoult et al. 2003)
Bifidobacteriales	*Gardnerella vaginalis* ATCC 14019	Commensal of the human vaginal microbiome but also linked to bacterial vaginosis	(Yeoman et al. 2010)
	B. dentium Bd1	opportunistic cariogenic pathogen of the human oral cavity	(Ventura et al. 2009)
	B. bifidum PRL2010	Dominant in the infant intestinal microbiome	(Turroni et al. 2010)
	B. animalis subsp. **lactis** Bl-04,	intestinal microbiome	(Barrangou et al. 2009)
	B. adolescentis ATCC 15703.	intestinal microbiome	(Duranti et al. 2013)
Acidimicrobiales	*Acidimicrobium ferrooxidans* DSM10331	isolated from hot acidic springs	(Clum et al. 2009)
Rubrobacteriadae	*Conexibacter woesii* DSM14684	non sporulating motile soil bacterium	(Abt et al. 2010)
Coriobacteridae	*Olsenella uli* DSM 7084	frequently isolated from dental plaque in periodontitis patients and can cause primary endodontic infection	(Göker et al. 2010)
	Slackia heliotrinireducens DSM 20476.	originally isolated from the ruminal flora of a sheep	(Pukall et al. 2009)

1.8 Aims and objectives

MtrAB is conserved in Actinobacteria and previous studies demonstrated that MtrAB takes part in cell division in *Mycobacteria* and osmoprotection in *C. glutamicum*. The RR MtrA is essential in *M. tuberculosis* but dispensable in *C. glutamicum*. The reason for the essentiality in *M. tuberculosis* is most likely the involvement of MtrAB in cell division. Previous work in *S. coelicolor* indicated that MtrA might be involved in the regulation of cell division. Therefore, I aimed to investigate the regulatory role of MtrAB-LpqB in the two *Streptomyces* strains *S. venezuelae* and *S. coelicolor* in this study. To achieve this aim I had the following experimental objectives:

- To generate deletion mutants of *mtrA*, *mtrB* and *lpqB* in *S. venezuelae* and compare the phenotype with *S. coelicolor* Δ*mtrA*, Δ*mtrB* and Δ*lpqB* mutants (Chapter 3).
- To produce MtrA gain of function proteins in *S. venezuelae* (Chapter 3).
- To determine MtrA target genes in *S. venezuelae* and *S. coelicolor* by Chromatin Immunoprecipitation and sequencing (ChIP-seq), (Chapter 4).
- To investigate the phosphotransfer from the histidine kinase MtrB to the response regulator MtrA in *S. venezuelae* (Chapter 5).

2 Material and Methods

2.1 Chemicals and Reagents

Chemicals and reagents used are laboratory standard grade or above, purchased from Sigma Aldrich (UK) or Thermo Fisher Scientific (UK) unless otherwise stated. All media and solutions were made using dH_2O except SFM and MYM which contain 50% tap water.

2.2 Bacterial strains and plasmids

The bacterial strains, and plasmids used or constructed in this study are listed in **Table 2.1** and **Table 2.2**. Growth media used are listed in **Table 2.3**. Liquid cultures of *E. coli* were routinely grown shaking at 220 rpm, in LB broth at 37°C unless stated otherwise. Liquid cultures of *S. coelicolor* or *S. venezuelae* were grown at 30°C, shaking at 220 rpm. Typically, 35 ml of liquid culture was grown in 250 ml flasks containing springs or glass beads. Cultures grown on solid media were grown at the same temperatures listed above, unless stated otherwise.

Table 2.1 Bacterial strains used throughout this work. Abbreviation of resistances can be found in **Table 2.4.**

Strain	Description	Plasmid	Resistance	Reference
E. coli				
Top10	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G			Invitrogen™
BW25113	λ⁻, Δ(*araD-araB*)*567*, Δ*lacZ4787*(*::rrnB-4*), *lacIp-4000*(*lacIQ*), *rpoS369*(*Am*), *rph-1*, Δ(*rhaD-rhaB*)*568, hsdR514*	pIJ790	Cml^R	(Datsenko & Wanner 2000)
ET12567	*dam⁻ dcm⁻ hsdS⁻*	pUZ8002	Cml^R/Tet^R	(MacNeil et al. 1992)
BL21	*fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdSλ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*			(Studier & Moffatt 1986)
S. coelicolor				
M145	SCP1⁻ SCP2⁻ Pgl+			(Hopwood et al. 1986)
ΔmtrB	M145 *mtrB::scar*			(Knowles 2014)
ΔlpqB	M145 *lpqB::scar*			(Knowles 2014)
NS0171	M145 *mtrA::scar* ΦBT1 *mtrAp mtrA*-3xFlag	pNS109		This work
S. venezuelae				
S. venezuelae ATCC 10712	Wild-type			(Pullan et al. 2011)
NS003-005	ΦBT1 *mtrAp mtrA*-3xFlag	pNS109	Hyg^R	This work
NS012, NS021 and NS022	*mtrB*::*aac(3)IV oriT*		Apr^R	This work
NS013-15	ΦBT1 *mtrAp mtrA*(TB)	pNS103	Hyg^R	This work
NS016-18	ΦBT1 *mtrAp mtrA*$(TB)_{Y102C}$	pNS104	Hyg^R	This work

NS029	ΦBT1 pMS82	pMS82	Hyg^R	This work
NS033-35	ΦBT1 *ermE* mtrA*	pNS102	Hyg^R	This work
NS036-38	ΦBT1 pIJ10257	pIJ10257	Hyg^R	This work
NS039-41	ΦBT1 *mtrAp mtrA*	pNS108	Hyg^R	This work
NS042-44	*mtrA*::*aac(3)IV oriT* ΦBT1 *mtrAp mtrA*-3xFlag	pNS109	Apr^R / Hyg^R	This work
NS052	*mtrA*:: *aac(3)IV oriT* ΦBT1 *mtrAp mtrA*	pNS108	Apr^R / Hyg^R	This work
NS093-95	*mtrB*:: *aac(3)IV oriT* ΦBT1 *mtrAp mtrB*	pNS107	Apr^R / Hyg^R	This work
NS099-101	ΦBT1 *mtrAp* $mtrA_{Y99C}$	pNS105	Hyg^R	This work

Table 2.2 Plasmids and cosmids used throughout this work. Abbreviation of resistances can be found in **Table 2.4.**

Plasmids	**Genotype/description**	**Resistance**	**Reference**
pIJ773	*aac(3)IV oriT bla*	Apr^R	(Gust et al. 2004)
pIJ790	*araC-Parab,Y, β, exo, cat, repA1001ts, oriR101*	Cml^R	(Gust et al. 2004)
pUZ8002	RK2 derivative with a mutation in *oriT*	Kan^R	(Kieser et al. 2000)
pMS82	*ori*, pUC18, *hyg*, *oriT*, RK2, int ΦBT1	Hyg^R	(Gregory & Smith 2003)
pIJ10770	*ori*, pUC18, *hyg*, *oriT*, RK2, int ΦBT1 Δ *aac(3)IVp*	Hyg^R	Susanne Schlimpert, unpublished
pIJ10257	*oriT*, ΦBT1 *attB-int*, Hygr, *ermEp**, pMS81 backbone	Hyg^R	(Hong et al. 2005)
pNS074	pETduet-1 *mtrA*(Sv)	Amp^R	Mahmoud Al-Bassam, unpublished
pET-28a(+)	f1 origin, T7 *lac*, *IacI*, *ori*	Kan^R	Novagen™
pETduet-1	f1 origin, T7 *lac*, *IacI*, *ori* with two multiple cloning sites	Amp^R	Novagen™
pNS100	pET28a *mtrB* His-Cterm	Kan^R	This work
pNS101	pET28a *mtrB* His-Nterm	Kan^R	This work
pNS102	pIJ10257 *mtrA*	Hyg^R	This work
pNS103	pIJ10770 *mtrAp mtrA*(TB)	Hyg^R	Manufactored by GenScript™
pNS104	pIJ10770 *mtrAp mtrA*$(TB)_{Y102C}$	Hyg^R	Manufactored by GenScript™
pNS105	pIJ10770 *mtrAp mtrA*$(Sv)_{Y99C}$	Hyg^R	Manufactored by GenScript™
pNS106	pMS82 *mtrAp lpqB*	Hyg^R	This work
pNS107	pIJ10770 *mtrAp mtrB*	Hyg^R	This work
pNS108	pMS82 *mtrAp mtrA*	Hyg^R	This work
pNS109	pMS82 *mtrAp mtrA*-3xFlag	Hyg^R	This work
Cosmids			
SV-6-A04	Supercos-1 Cosmid with a 40.2kbp chromosomal fragment with *mtrAB-lpqB*		(Pullan et al. 2011)
pNS069	SV-6-A04 *lpqB*:: *aac(3)IV oriT*		This work
pNS070	SV-6-A04 *mtrB*:: *aac(3)IV oriT*		This work
pNS071	SV-6-A04 *mtrA*:: *aac(3)IV oriT*		This work

Table 2.3 Bacterial growth media. All media were made in 1000ml dH_2O except for SMM which is made up in 100ml.

Medium	Composition	Weight, %v/v, %w/v
Lennox Broth (LB)	Tryptone	10 g
	Yeast extract	5 g
	NaCl	5 g
For solid medium	Agar	15 g
For soft LB	Agar	7.5 g
2xYT	Tryptone	20 g
	Yeast extract	10 g
DNB	Difco Bacto tryptone	10 g
	Difco yeast extract	5 g
	Glucose	1 g
Tryptone Soya Broth (TSB)	TSB powder (Oxoid™)	30 g
MYM-tap	Maltose	4 g
	Yeast extract	4 g
	Malt extract	10 g
	Tap water	500 ml
	[1]R2 Trace elements	
For solid medium	Agar	20 g
[1]R2 Trace elements filter sterilised and added after autoclaving	$ZnCl_2$	40 mg
	$FeCl_3 \cdot H_20$	200 mg
	$CuCl_2 \cdot 2H_20$	10 mg
	$MnCl_2 \cdot 4H_20$	10 mg
	$Na_2B_40_7 \cdot 10H_20$	10 mg
	$(NH_4)6Mo_7O_{24} \cdot 4H_20$	10 mg
SFM (or MS)	Mannitol	20 g
	Soya flour (added in aliquots to flasks)	20 g
For solid medium	Agar	20 g
SMM Each solution is autoclaved separately, then mixed in the order given; SMM without PEG was made with dH_20 instead of PEG	PEG 6000 (BDH 6.1% w/v in dH_20) or replaced with dH_20	81.9 ml
	$MgSO_4 \cdot 7\ H_20$ (24 g l^{-1})	2.5 ml
	TES buffer (0.25M, pH 7.2)	10 ml
	$NaH_2PO_4 + K_2HPO_4$ (50mM each)	1 ml
	Glucose (50% w/v)	2 ml
	Antifoam 289 (Sigma A 5551; 1% w/v)	0.1 ml
	[2]Trace element solution	1 ml
	Difco Casaminoacids (20% w/v)	1 ml
	Glycerine (20% solution)	2.5 ml
[2]Trace elements for SMM (filter sterilised)	$ZnSO_4 \cdot 7H_2O$	0.1 g
	$FeSO_4 \cdot 7H_2O$	0.1 g
	$MnCl_2 \cdot 4H_2O$	0.1 g
	$CaCl_2 \cdot 6H_2O$	0.1 g
	NaCl	0.1 g

2.3 Media

Media compositions are shown in **Table 2.3**. When required, media were supplemented with the appropriate antibiotic (**Table 2.4**). In general media for *Streptomyces* mutants were not supplemented with antibiotics because *Streptomyces* mutants are genetically stable and added antibiotics can affect the morphology or growth behaviour. This is especially important to consider for liquid growth cultures.

Table 2.4 Concentrations of antibiotics used during this thesis. All antibiotics were filter sterilised with 0.22µm filter.

Antibiotic	Abbreviation	Stock concentration (mg/ml)	Working Concentration (µg/ml) for media	Working Concentration (mg/ml) for overlay conjugation plates
Ampicillin	Amp^R	100	100	
Apramycin	Apr^R	50	50	1.25
Chloramphenicol	Cml^R	25	25	
Hygromycin	Hyg^R	50	50	1.25
Kanamycin	Kan^R	50	50	
Nalidixic acid	Nal^R	25 in 0.3 M NaOH	25	0.5

2.4 *S. venezuelae* growth curves

To determine the developmental growth in liquid culture *S. venezuelae* and mutant strains were grown in 35 ml MYM in 250 ml conical flasks containing springs at 30°C at 220 rpm. A spore inoculum sufficient to reach an OD(600) of 0.35 after 8 hours of growth was added to 35 ml of MYM tap media in 250 ml flasks containing springs. The culture was measured at OD(600). When the cultures reach an OD(600) > 0.5 the samples were diluted with MYM to reach an OD(600) under 0.5. The growth rate was calculated as described previously (Widdel 2007) with the formulas:

$$N = N_0 \, e^{\mu t}$$

N is the number of cells, N_0 is any number of cells, e is the Euler number (e = 2.71828…), µ is the growth rate and t is time. The doubling time td is dependent on the growth rate:

$$\mu = \frac{ln2}{td}$$

Microsoft excel was used to calculate the growth rate during the exponential phase. The growth curve was converted to a logarithmic y-axis and then data point which

form an exponential trend were chosen and the excel software predicted the exponential curve containing the data points of the growth curve. The resulting formula of the exponential curve contains the growth rate in the first formula described above.

2.5 Microscopy

2.5.1 Light microscopy

Liquid cultures were examined with the GXML3000B from GX optical. Pictures were taken with the Dino-eye Eyepiece camera. Pictures of solid growth colonies were taken with the Zeiss SVII stereo microscope.

2.5.2 Scanning electron microscopy (SEM)

S. venezuelae and *S. venezuelae* Δ*mtrB* spores were diluted to obtain single colonies. The dilution was plated on MYM agar plates and incubated for 10 days at 30°C and imaged by the bioimaging facility at the John Innes Centre by using cryo-SEM technique. First, the sample is cryo-fixed, generally by plunging it into sub-cooled nitrogen (nitrogen slush) close to the freezing point of nitrogen at -210^{o}C, then the sample is transferred *in vacuo* to the cold-stage of the SEM cryo-preparation chamber, where fracturing can be performed if necessary. After sputter coating with metal (usually gold or platinum), the sample is transferred into the SEM chamber, where it remains frozen during imaging on another cold-stage, cooled by nitrogen.

2.6 General techniques

2.6.1 Polymerised Chain Reaction (PCR)

Two different DNA polymerases were used depending on the application. PCRBIO® Taq DNA Polymerase (from PCR Biosystems) was used to test if a DNA fragment was integrated in a plasmid or genomic DNA. Q5® High-Fidelity DNA polymerase was used to amplify DNA fragments for subsequent cloning because this polymerase has a lower error rate (> 100-fold lower than that of Taq DNA Polymerase). Template DNA was purified chromosomal DNA, PCR product, plasmid DNA and synthesised constructs. Calculated specific primer melting temperatures (T_m) and adjusting values accordingly for each enzyme used, determined the annealing temperature (T_a). PCR programmes (**Table 2.6** and **Table 2.8**) were conducted using a DNA engine

PTC 300 (BIORAD®) PCR machine. Primers used for the generation of cloning products are shown in **Table 2.9**. All primers used throughout this work were synthesised by Integrated DNA Technologies (IDT).

2.6.1.1. Taq polymerase

Table 2.5 Master mix composition for Taq polymerase in 20µl reaction volume.

Reagent	20µl reaction	Final concentration	Notes
2x PCRBIO Taq Mix	10 µl	1x	
DMSO	1 µl	5%	Reduces T_a
Forward primer (5µM)	0.5 µl	125nM	
Reverse primer (5µM)	0.5 µl	125nM	
Template DNA	0.5 µl	variable	<10 ng plasmid or gDNA
dH_2O	7.5 µl		

Table 2.6 Cycling conditions for Taq polymerase

Cycles	Temperature	Time	Notes
1	95°C	1 min	Initial denaturation
25	95°C	10 sec	Denaturation
	55°C to 72°C	10 sec	Anneal (Ta was calculated using NEB calculator (http://tmcalculator.neb.com/#!/))
	72°C	15 sec per kb	Extension
1	72°C	2 min	Final extension

2.6.1.2. Q5 polymerase

Table 2.7 Master mix for Q5 polymerase in 50µl reaction volume.

Reagent	50 µl reaction	Final concentration	Notes
5X Q5 Reaction Buffer	10 µl	1x	
5X Q5 High GC Enhancer	10 µl	0.5%	Reduces T_m
10 mM dNTPs	1 µl	200 µM	
Forward primer (10 µM)	0.5 µl	100 nM	
Reverse primer (10 µM)	0.5 µl	100 nM	
Template DNA	0.5 µl	variable	<10ng plasmid or gDNA
Q5 High-Fidelity DNA Polymerase	0.5 µl		
dH_2O	27 µl		

Table 2.8 Cycle condition for Q5 polymerase.

Cycles	Temperature	Time	Notes
1	98°C	30 sec	Initial denaturation
35	98°C	10 sec	Denaturation
	55°C to 72°C	10 sec	Anneal (T_m was calculated using NEB calculator)(http://tmcalculator.neb.com/#!/)
	72°C	20 sec per kb	Extension
1	72°C	2 min	Final extension

2.6.2 Primers

Table 2.9 Primers used throughout this work. JM0012-JM0016 were provided by John Munnoch (Munnoch 2015).The universal M13 primers are shown in bold.

Primer	Description	Sequence
NS-040	*mtrA* (SVEN15_2696) forward disruption primer (Redirect)	GCCTTGAGACTGATACGGAAATGGGATGATGTCGATATGATTCCGG GGATCCGTCGACC
NS-041	*mtrA* (SVEN15_2696) reverse disruption primer (Redirect)	TCCCCGGGCTTCGGAGCAGCACTGCCTGTACTCATGTCATGTAGGC TGGAGCTGCTTC
NS-044	*mtrB* (SVEN15_2695) forward disruption primer (Redirect)	TCCGTGGTGTCGGGTACAAGGCGGGACCGAGCTGACATGATTCCG GGGATCCGTCGACC
NS-080	*mtrB* (SVEN15_2695) reverse disruption primer (Redirect)	* ctcccgcacccaccgtcccgctcgggcgTCAgcgccCACtgtaggctggagctgcttc
NS-056	*lpqB* (SVEN15_2694) forward disruption primer (Redirect)	CACGACGCCGGCATGCCGCGCCGCGCCGGGGCCGCTATGTAGG CTGGAGCTGCTTC
NS-079	*lpqB* (SVEN15_2694) forward disruption primer (Redirect)	* aacgcggagcgggaggacaggacacGTGggcgcTGAcgcattccggggatccgtcgacc
NS-131	*mtrA* (SVEN15_2696) forward test primer -141	TGA CAT CCA TGT CTG GCA TCA ACA CCC A
NS-132	*mtrA* (SVEN15_2696) reverse test primer -120	CGA CGA TCC TCA GCT GGA TGT TCC GT
NS-070	*mtrB* (SVEN15_2695) forward test primer -100	CAC GTC CAG CGG CTG CGC TCG AAG G
NS-071	*mtrB* (SVEN15_2695) reverse test primer -100	GTC GGG CAT GGT CGC GCA CCC GGT G
NS-072	*lpqB* (SVEN15_2694) forward test primer -100	CGG ACG CCG GCG GAC GGC GAC GGG A
NS-073	*lpqB* (SVEN15_2694) reverse test primer -100	GCC AAC CCC TGT GGA AAA CCT CCG G
NS-238	*lpqB* (SVEN15_2694) forward test primer	AGTACGAGCTCTACTACCTTTT
NS-239	*lpqB* (SVEN15_2694) forward test primer	ACCTCCTGGAGATCAGCCGCTTCGA
NS-240	*lpqB* (SVEN15_2694) reverse test primer	TCATCAGGTCGTCCACGAGGACCAC
P1	*aac(3)IV* resistance cassette test primer	ATT CCG GGG ATC CGT CGA CC
P2	*aac(3)IV* resistance cassette test primer	TGT AGG CTG GAG CTG CTT C

NS-064	pMS82 forward test primer	GCAACAGTGCCGTTGATCGTGCTATG
NS-065	pMS82 reverse test primer	GCC AGT GGT ATT TAT GTC AAC ACC GCC
NS-115	*mtrA* (SVEN15_2696) NdeI forward	$ gcgCATATGATGAAGGGACGCGTT
NS-116	*mtrA* (SVEN15_2696) HindIII reverse	$ gcgAAGCTTTCAGCTCGGTCCCGC
NS-121	pIJ10257 forward test primer	AGGTACCAGCCCGACCCG
NS-122	pIJ10257 reverse test primer	ATCAGCGAGCTGAAGAAA
NS-123	*mtrA* (SVEN15_2696) HindIII forward 1	$ gcgAAGCTTCCG TGG GCC GGT CCC GCC TC
NS-124	*mtrA* (SVEN15_2696) KpnI reverse	$ gcgGGTACCTCAGCTCGGTCCCGCCTTGT
NS-125	*mtrB* (SVEN15_2695) reverse 2	CGGAGCAGCACTGCCTGTACTCATATCGACATCATCCCATTt
NS-126	*mtrB* (SVEN15_2695) forward 3	ATG AGT ACA GGC AGT GCT GCT CCg AAG CCC
NS-127	*mtrB* (SVEN15_2695) NsiI reverse 4	$ gcgATGCATTCAGCGCCCACGTGTCCTGT
NS-128	Rev 2 *lpqB* (SVEN15_2694) reverse 2	TCCCGCTCGGGCGTCAGCGCCCACATCGACATCATCCCATTT
NS-129	For 3 *lpqB* (SVEN15_2694) forward 3	GTGGGCGCTGACGCCCGAGCGGGACGGTGG
NS-130	*lpqB* (SVEN15_2694) KpnI reverse 4	$ gcgGGTACCCTAGCCCGGGTAGACGGGCG
NS-145	*mtrA* (SVEN15_2696) forward BamHI (pETduet)	GGATCCTAAAGGCCGCGTTCTGGTCGTC
NS-146	*mtrA* (SVEN15_2696) reverse HindIII (pETduet)	AAGCTT TCA GCTCGGTCCCGCCTTGTACC
NS-160	*mtrB* (SVEN15_2695) forward internal test primers	TTCAACGTGGTCGCGCTCTCCCTCG
NS-161	*mtrB* (SVEN15_2695) reverse internal test primers	GCCAGCTTCACCACGACGTCCTTGC
NS-162	*mtrB* (SVEN15_2695) forward HIS N-term NdeI (pET28a)	$ gcgCATATGATGCAGCGGCGGTTCGTCTCGGAC
NS-163	*mtrB* (SVEN15_2695) reverse HIS N-term HindIII (pET28a)	$ gcgAAGCTTTCAGCGCCCACGTGTCCTGTCCTC
NS-164	*mtrB* (SVEN15_2695) forward HIS C-term NcoI (pET28a)	$ gcgCCATGGGACAGCGGCGGTTCGTCTCGGACGTC
NS-165	*mtrB* (SVEN15_2695) forward HIS C-term HindIII (pET28a)	$ gcgAAGCTTGCGCCCACGTGTCCTGTCCTCCCG
NS-166	pET28a(+) forward test primers	CTCGATCCCGCGAAATTAATACGAC

NS-167	pET28a(+) reverse test primers	GGGCTTTGTTAGCAGCCGGATC
NS-283	pSS170 forward test primer	CGGGGTCTGACGCTCAGTGGAACGAAA
Universal M13-6FAM Labelled Primers	Forward	CTAAAACGACGGCCAGT
	Reverse	CAGGAAACAGCTATGAC
NS-176	M13FAM nested SVEN15_ 0205p (*ectA*)forward	CTA AAA CGA CGG CCA GTCACCGAACGGAGCCGGGC
NS-177	M13FAM nested SVEN15_ 0205p (*ectA*)reverse	CAG GAA ACA GCT ATG ACGGGCGCCGAAGGTACCGA
NS-286	M13FAM nested SVEN15_3571p (*dnaA*) forward	CTA AAA CGA CGG CCA GTC GGT TCG CAA GGA TGG CTC GGC
NS-287	M13FAM nested SVEN15_3571p (*dnaA*) reverse	CAG GAA ACA GCT ATG ACT GCG GCA AGA TCA GCA GGA ACG TCA
NS-290	M13FAM nested SVEN15_2524p (*adpA*) forward	CTA AAA CGA CGG CCA GTG CGT ACG GGG GCG TTC GCC
NS-291	M13FAM nested SVEN15_2524p (*adpA*) reverse	CAG GAA ACA GCT ATG ACG ACG CTA AGC CCC CCT CGG TGT
NS-294	M13FAM nested SVEN15_0880p (*cmlR2*) forward	CTA AAA CGA CGG CCA GTG AAA AAG CTC CAA CTA CAT CGC AGA
NS-295	M13FAM nested SVEN15_0880p (*cmlR2*) reverse	CAG GAA ACA GCT ATG ACC CAT CAT GAT CAT GGC TGT CTG GTG
JM0012	6FAM labelled forward primers for hmpA2 (SCO7094)	ACCCGGTCTCCGGCTTACC
JM0013	6FAM labelled reverse primers for hmpA2 (SCO7094)	ACGGGACGCTCCTCGAACA
JM0015	6FAM labelled forward primers for hmpA1 (SCO7428)	ACACTCGACCCACTGACC
JM0016	6FAM labelled reverse primers for hmpA1 (SCO7428)	TGGGCGTCGAAGAGCTTG

*Start and stop codon of *mtrB* and *lpqB* are in capital letters

[$] Most restriction enzymes require additional base pairs to their recognition site to restrict the DNA. Here gcg were added which are not binding to the DNA template indicated by lower case letters

2.6.3 PCR Purification

PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN) as per the manufacturer's instructions. Products were eluted from purification columns in 50 µl of nuclease-free dH_2O and stored at -20°C. Alternatively PCR products were purified using agarose gel electrophoresis to clean up the PCR product from any unspecific products (2.5.4).

2.6.4 Agarose Gel Electrophoresis

DNA fragment size was determined using agarose gels. Gels were made with 0.7-2% agarose in TBE buffer (90 mM Tris HCl, 90 mM Boric Acid, 2 mM EDTA) depending on the application. Large DNA fragments (plasmids) were analysed on 0.7% agarose gels. DNA fragments 1000 - 2000 bp were analysed on 1% gels fragments and if the DNA fragment of interest was <1000 bp a 2% agarose gel was used. Before pouring 2 µg/ml ethidium bromide was added to the melted agarose. DNA loading buffer (5x) (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene-cyanol blue, 40% (w/v) sucrose in water) was added to DNA samples amplified with Q5 or restriction digests. The 1 kb plus DNA ladder was loaded on the gel for size determination. Electrophoresis occurred at 120 V (Sub-Cell GT electrophoresis system, BIOLINE) for 30-60 minutes (depending on size: larger fragments were run longer for clearer separation). DNA was visualised by UV-light, using a Molecular Imager Gel Doc System (BIO-RAD).

2.6.5 Restriction Digestion

Both Roche and NEB restriction enzymes were used in digests plasmid DNA in 50 µl total volumes in accordance with manufacturer's guidelines. Digests were carried out with optimal buffer, which was outlined by Roche or NEB. Digestion of 1 µg of DNA was typically performed at 37°C for 1 hour adding 1 unit of the respective restriction enzyme. Then the restriction enzymes were heat inactivated at respective temperatures and the Shrimp alkaline phosphatase was added to dephosphorylate the digested plasmid DNA. Digests were then analysed by gel electrophoresis; desirable bands were excised and gel extracted.

2.6.6 Gel Extraction

Gel fragments containing DNA bands of interest were excised using a scalpel and extracted using a Qiaquick Gel Extraction Kit (QIAGEN), according to the manufacturer's instructions. DNA was eluted in 50 µl autoclaved dH_2O.

2.6.7 Ligation

Ligations where carried out following instructions of manufacturers for T4 DNA ligase. A standard ratio for a plasmid / insert ligation was 1:3. Ligation ratios were calculated using an online calculation tool (http://www.insilico.uni-duesseldorf.de/Lig_Input.html). A vector only ligation (re-ligation control) was included as a negative control.

2.6.8 Colony PCR

20 µl of PCRBIO Taq mix were inoculated with a transformed *E. coli* culture to test for correct insertion of amplified DNA in the plasmid of interest. The initial denaturing stage of the PCR programme chosen was extended to 5 min at 98°C to allow cell lysis and DNA release. The remaining stages of the chosen programme were completed as standard, see section 2.6.1.1.

2.6.9 Plasmid Preparation

Plasmid DNA was prepared using Qiaprep Spin Miniprep kits (QIAGEN) from 5 – 10 ml overnight cultures as per manufacturer's instructions. Plasmids where eluted from the column routinely using 50 µl autoclave distilled water (dH_2O).

2.6.10 Cosmid Preparation

Cosmid DNA was prepared by Wizard® Plus SV Miniprep DNA Purification System as per the manufacturer's instructions. Cosmids where eluted from the column routinely using 50 µl autoclave distilled water (dH_2O).

2.6.11 Preparing and transforming electrocompetent *E. coli* cells

E. coli ET12567 and Top10 cells were grown as 10 ml overnight cultures in LB (containing appropriate antibiotics) to an OD(600) of 0.4 - 0.6 and pelleted by

centrifugation in a 15 ml falcon tube at 4000 rpm for 5 minutes at 4°C. Cells were resuspended and pelleted in 1 ml ice cold 10% v/v glycerol six times and either flash frozen using liquid nitrogen and stored at -80°C for future use or used immediately for transformation. DNA (cosmid 2 µg, plasmid 100 ng) was added to ~50 µl of cells immediately before electroporation. Cells and DNA were transferred to an ice-cold electroporation cuvette and electroporated using the BioRad® Electroporator set to: 200 Ω, 25 µF and 2.5 kV. The electroporated cells were diluted in LB and transferred from the cuvette to a micro-centrifuge tube, incubated for 1 hour shaking (220 rpm) at 37°C before plating onto LB containing appropriate antibiotic selection. Plasmids containing the hygromycin resistance were transformed in *E. coli* cells and the transformation mix was plated on DNB plates and incubated for ~24 hours.

2.6.12 Preparing and transforming $CaCl_2$ competent *E. coli* Top 10 cells

A modified version of the $CaCl_2$ protocol by (Cohen et al. 1972), as described below, was used. All centrifugation steps were at 4000 rpm, 4°C for 10 minutes to pellet cells. An over-night culture (~16 hours) of *E. coli* Top10 strain was grown in 10 ml of sterile LB broth from frozen cells stored at -80°C, grown at 37°C, 250 rpm. Aliquots (100 µl) of these were then added to 500 ml of sterile LB broth. The cells were then grown at 37°C, 250 rpm, until an optical density OD(600) of 0.3 - 0.4 was reached (1 cm path length). At all steps the reagents and equipment were maintained at ~4°C beyond this point. The samples were centrifuged; the bacterial pellet was resuspended and washed gently in 125 ml of ice cold sterile 100 mM $MgCl_2$. The suspension was centrifuged and the pellet resuspended in 25 ml of ice cold sterile 100 mM $CaCl_2$ then a further 225 ml was added. The suspension was left on ice for ~25 minutes. Finally, the suspensions were centrifuged and resuspended in 10 ml ice cold sterile 100 mM $CaCl_2$, 20% v/v glycerol, dispensed into 100 µl aliquots and stored at -80°C.

Transformation of *E. coli* Top 10 was carried out by first gently thawing a 100 µl aliquot of pre-prepared competent cells on ice. Aliquots (1 µl) of plasmid DNA were added to the sample reactions. Both were left on ice (4°C) for at least 30 minutes then heat shocked for 90 seconds at 42°C then immediately cooled at 4°C for 2 minutes. Aliquots (1 ml) of LB was added to each reaction and incubated at 37°C for 1 hour at 220 rpm then plated on LB plates (containing appropriate antibiotics when applicable) at

37°C for ~16 hours unless otherwise stated. Plasmids containing the hygromycin resistance were transformed and plated on DNB plates and incubated for ~24 hours.

2.6.13 Storing *E. coli* using glycerol stocks

E. coli was stores in glycerol stocks by taking 10 ml of *E. coli* overnight culture, pelleting by centrifugation cells and re-suspending it in 500 µl of sterile, 20% glycerol. Glycerol stocks were stored at -20°C.

2.6.14 Phenol / Chloroform extraction of genomic DNA

Streptomyces cultures were grown overnight in a mixture of 0.8 ml 50% TSB / 50% YEME media at 30°C shaking at 220 rpm. Cells were isolated using a desktop centrifuge set to 13000 rpm for 5 minutes. The supernatant was discarded and the cells resuspended in 200 µl SET buffer (75 mM NaCl, 25 mM EDTA pH 8, 20 mM Tris-HCl pH 7.5. 10 µl of a mixture of lysozyme (100 mg/ml) and achromopeptidase (10 mg/ml) was added and incubated 30 minutes at 37°C to lyse the cells. 200 µl (one volume) of 1:1 phenol-chloroform was added and samples were mixed thoroughly by vortexing for 1 minute and then centrifuged at 13,000 rpm for 20 minutes. The upper aqueous phase (containing the DNA) was removed and transferred to a fresh microfuge tube. The phenol-chloroform step was repeated until the upper phase was clear (i.e. protein free). The clear aqueous layer was transferred to a fresh microfuge tube. Then 2.5 ml of 100% ethanol was added to the tube and was mixed by inversion and incubated at -20°C followed by centrifugation for 10 minutes at 13000 rpm. Ethanol was removed and the DNA pellet was washed in 200 µl 70% ethanol and centrifuged for a further 5 minutes at 13000 rpm. The DNA pellet was dried at room temperature ensuring all ethanol was removed and the pellet was finally resuspended in 50 µl sterile dH_2O and stored at 4°C.

2.6.15 Sequencing

Cloned DNA constructs were confirmed by Sanger sequencing using the Mix2Seq service from Eurofins Genomics. Plasmids DNA was diluted according to the manufactures instruction and test primers (**Table 2.9)** were added directly to the mix.

2.7 Constructing gene knockouts via lambda λ RED method (Redirect methodology)

Deletion mutants were produced by replacing genes of interest with an antibiotic cassette (Datsenko & Wanner 2000). This λ RED methodology was adapted for *Streptomyces* species by (Gust et al. 2003). A linear PCR product (FRT-flanked resistance gene construct) was produced (2.6.2) and purified (2.6.3) before conjugated (2.7.5) into an appropriate host strain. After replica plating potential mutant strains were verified by PCR (2.6.14), and long-term spore stocks were produced for each mutant strain (2.7.6).

2.7.1 Generation of FRT-flanked resistance gene construction

The apramycin antibiotic resistance cassette (APR) from pIJ773 containing *aac(3)IV* gene and an origin of transfer (*oriT*), were amplified by PCR using primers specific for the disruption of the gene of interest (see **Table 2.9**). Details of plasmids used and how to design appropriate primers to leave in frame gene knockouts (KO) were reported by (Gust et al. 2003). The forward primers consisted of 39 nucleotides (nt) upstream of the gene of interested ending in ATG, the translation start codon, with the 20 nt P1 sequence corresponding to the 5' end of the antibiotic resistance cassette. The reverse primer had 39 nt of antisense sequence ending TGA, the translational stop codon of each gene plus a 19 nt sequence of P2 corresponding to the end of the antibiotic resistance cassette. *mtrB* and *lpqB* overlap 11 bp in *S. venezuelae*. It is for this reason that the reverse primer for the deletion of *mtrB* ends with GTGggcgcTGA to leave the start codon for *lpqB* intact.

The PCR cycling conditions consisted of:

1. Denaturation:	94°C, 2min
2. Denaturation:	94°C, 45sec
3. Primer annealing:	**50°C**, 45sec
4. Extension:	72°C, 90sec; repeat 10x from step 2
5. Denaturation:	94°C, 45sec
6. Primer annealing:	**55°C**, 45sec
7. Extension: 72°C,	90sec; repeat 15x from step 5
8. Final extension:	72°C, 5min

PCR reactions typically were carried out using Q5. PCR products were checked by agarose gel electrophoresis, gel extracted then stored at -20°C until use.

2.7.2 Preparing of *E. coli* containing λ RED plasmid and *S. venezuelae* cosmid

The cosmid SV-6-A04 containing the wild-type *mtrAB-lpqB* genes to be targeted was kindly provided by Mark Buttner, John Innes Centre. An aliquot (~50 µl) of *E. coli* BW25113 / pIJ790 electrocompetent cells were transformed with ~2 µg of cosmid DNA. The cells were r own in 1 ml of LB for 1 hour and plated onto LB agar plates containing ampicillin and kanamycin to select for the incoming cosmid and chloramphenicol to select for the λRED recombinant plasmid pIJ790. The plates were incubated at 30°C overnight. Cosmid identity was assessed using BamHI digests (1 µl BamHI, 5 µl Roche Buffer B, 17 µl cosmid DNA, 27 µl dH_2O) incubated at 37°C for 1 hour and separated on a 0.7% agarose gel compared to in silico results obtained using ApE- A plasmid Editor v2.0.46 and sequence data from http://strepdb.streptomyces.org.uk. Additionally, SV-6-A04 was confirmed by positive amplification of the gene of interest. *E. coli* BW25113 / pIJ790 (50 µl aliquot) was transformed by electroporation as previously described (2.6.11) using 2 µg of isolated wildtype cosmid DNA grown for 1 hour at 30°C shaking 220 rpm and plated as described. A single colony was selected, picked and transferred to a 10 ml vial of LB containing antibiotic selection and grown at 30°C overnight and stored as a glycerol stock as previously described (2.6.13).

2.7.3 PCR-Targeting of *Streptomyces* cosmid SV-6-A04

From an overnight culture of *E. coli* BW25113 / pIJ790 containing SV-6-A04, 100 µl was inoculated and grown in 10ml LB broth containing the appropriate antibiotic selection and 100 µl 1 M L-arabinose, at 30°C for 3-4 hours to an OD(600) of ~0.4. The arabinose is essential as it induces the λRED genes on pIJ790 facilitating transformation with linear DNA. These cells were then made electrocompetent by washing in ice cold glycerol and a 50 µl aliquot was electroporated with 2 µl of the KO PCR product with flanking regions homologous to the gene of interest to cause an in-frame deletion of the chosen gene. These cells were then incubated for 1 hour shaking at 37°C and ultimately plated on to LB plates containing kanamycin (selection for the cosmid) and apramycin (selection for the gene deletion). Each plate was incubated overnight at 37°C to promote

loss of the temperature sensitive plasmid, pIJ790. Single colonies where then picked and grown for 16 hours at 37°C.

2.7.4 PCR conformation of mutagenized cosmid

The PCR targeted cosmids were isolated from overnight cultures as described and checked by PCR for the gene disruption. Specific primers for the flanking wild-type region as well as primers specific for the disruption cassette (P1 and P2) were used in combination, to check the disruption was successful, (primers see **Table 2.9**).

2.7.5 Transfer of the mutant cosmids into *Streptomyces*

S. coelicolor contains a methylation-sensing restriction system and as such it is essential to passage disruption cosmids through a non-methylating (*dam- dcm*) *E. coli* strain ET12567 before introduction into *S. coelicolor*. This is not required for *S. venezuelae* but the ET12567 strain containing the driver plasmid pUZ8002 is still used for all conjugations. ET12567 / pUZ8002 (**Table 2.1** and **Table 2.2**) was transformed by electroporation with 2 μg of cosmid DNA and subsequently plated onto LB agar containing chloramphenicol (to maintain *dam* mutation) and apramycin (to select for the incoming cosmid). Plates were incubated overnight at 37°C and single colonies were selected and grown in 10 ml LB broth at 37°C overnight in the presence of the previous antibiotics in addition to kanamycin and ampicillin. A sample inoculum from the overnight cultures (500 μl) was diluted in 10 ml of fresh LB broth containing antibiotics for selection was grown shaking at 37°C until cells reached an OD(600) of 0.4 - 0.6. Cultures were centrifuged at 13,000 rpm for 5 minutes and the resulting pellet washed in 10 ml fresh LB twice, to remove the selection antibiotics (potentially harmful to non-resistant wild-type *Streptomyces* species). Cell pellets were resuspended in 1 ml of LB broth. Washed *E. coli* cells (500 μl) were mixed with 500 μl of LB broth containing heat shocked (50°C for 10 minutes) *Streptomyces* (>1 x 10^7) spores. The mixture was centrifuged briefly as before and the supernatant removed, with the resulting pellet resuspended in ~300 μl of LB and plated on three SFM plates and incubated overnight for 16 - 20 hours at room temperature. SFM is not the standard medium for *S. venezuelae* but conjugations of *S. venezuelae* seem to work better on SFM than MYM. Following this incubation period, each conjugation plate was overlaid with 1 ml of sterile dH_2O containing 0.5 g nalidixic acid, selective bactericidal antibiotic for *E. coli* and 1.25 mg of

apramycin to select for insertion of the incoming cosmid by recombination. The overlay solution was distributed over the surface and incubated at 30°C for four days or until colonies appeared. Double crossover events were selected using apramycin resistance and kanamycin sensitivity as the marked antibiotic cassette has now replaced the target gene in the chromosomal DNA and the cosmid has been lost. Kanamycin sensitive and apramycin resistant colonies were re-streaked for at least three times to insure genetic stability of the gene knock out. Spore stocks were prepared (2.7.6.) for these double cross-over exconjugants and stored at -20°C.

2.7.6. *Streptomyces* spore stocks

S. venezuelae wild-type and mutant strains were re-streak with a cotton bud evenly on MYM plates and incubated for at least 3 days at 30°C. To harvest the spores 1 ml of 20% v/v glycerol was added to the plate and then the spores were removed from the vegetative mycelium by rubbing the cotton bud gently on the surface. The spores in 20% v/v glycerol were removed by pipetting through the cotton bud to separate the spores from any mycelium. The spores were then centrifuged at 10 minutes at 4000 rpm, 4°C and re-suspended in fresh 20% v/v glycerol and stored at -20°C.

2.7.7 Integration of plasmids *S. venezuelae*

Plasmids can be integrated in the genomic DNA of *S. venezuelae* by conjugation described in 2.7.5. *S. venezuelae* is a new model organism and only one integrative site (ΦBT1) is available in this strain. In this work plasmid DNA was integrated in the ΦC31 site but these mutants all displayed a developmental phenotype (data not shown).

2.7.8 Genetic complementation of knock out strains

Complementation constructs were designed to verify the Δ*mtrB* mutant and to show that *mtrA* or *lpqB* is essential. Therefore, the promoter of the *mtrAB-lpqB* operon was amplified with primer 1 and 2 and the respective genes with primer 3 and 4, see **Figure 2.1**. The promoter fragment and the gene fragment overlap and can be fused together in a second amplification step. The fused promoter and gene were then subsequently cloned into pMS82 (*mtrA* and *lpqB*) or pIJ10770 (*mtrB*) and conjugated in the mutant strains.

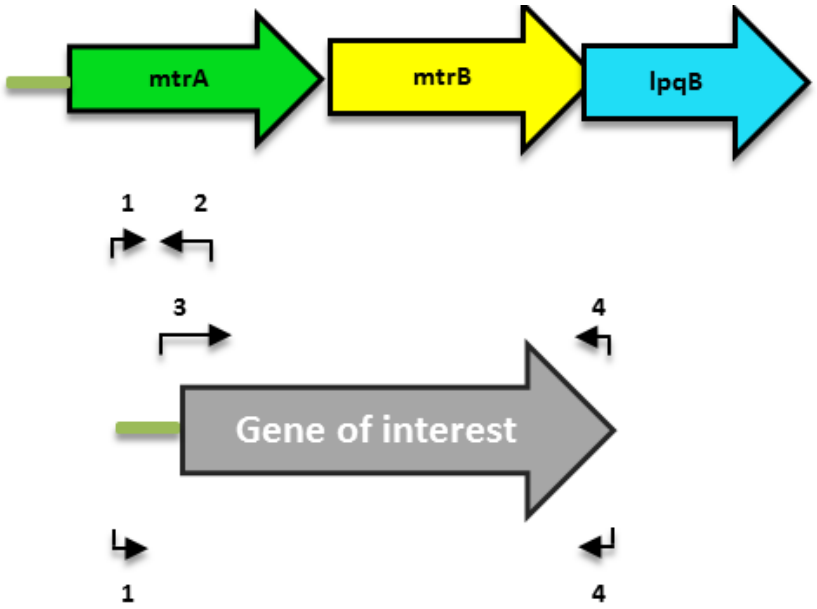

Figure 2.1 Construction of complementation constructs for gene knock outs in the MtrAB-LpqB operon. **Top:** Organisation of the MtrAB-LpqB operon. The native promoter is shown in green. **Bottom:** Fragments which are amplified by PCR to fuse the promoter with the gene of interest.

2.8 Protein Methods

2.8.1 Protein purification

Proteins were tagged with a 6xHis C-terminal (MtrB) or 6xHis N-terminal epitope (MtrA and MtrB) and purified from whole cell lysate, post overexpression, using Ni-NTA Agarose (QIAGEN) by batch purification according to the manufacturer's instructions. Genes were cloned into overexpression vectors under the control of IPTG inducible promoters (**Table 2.2).**

2.8.1.1 Test expression assay

Small-scale test expression assays were conducted to ascertain stable and high yielding conditions for recombinant protein overexpression. Overnight cultures, supplemented with ampicillin (100 µg/ml), of *E. coli* BL21 harbouring the overexpression construct were used to inoculate 10 ml LB broth (1% v/v) containing ampicillin (50 µg/ml). Cultures were incubated (37°C, 220 rpm) for three hours or until 0.5 - 1 OD(600). 'Zero-time point' samples were taken by collecting 1 ml samples from each culture; cells were harvested by centrifugation (2 minutes, 14,800 x g) and the supernatant removed prior to storing pellets at -20°C. 1 mM IPTG in sterile dH_2O was added to the cultures aseptically to induce protein expression. Samples (0.5 ml) were taken after two, three and

four hours incubation at 37°C, 220 rpm and harvested as per 'zero-time point' samples. Cell pellets were snap frozen and stored at -20°C.

2.8.1.2 Expression sample analysis by SDS-PAGE

To confirm overexpression and stability of recombinant proteins prior to large-scale purification, IPTG induced samples were analysed by SDS-PAGE (**Table 2.11**). Sample cell pellets were defrosted on ice (or analysed immediately post collection) and resuspended in 50 µl SDS loading buffer (950 µl Bio-Rad® laemmli buffer, 50 µl β-mercaptoethanol) before boiling (95°C, 3 minutes). Cell debris was pelleted by centrifugation (5 minutes, 14,800 x g) and 20 µl of sample loaded onto SDS-PAGE gels (15% v/v acrylamide) with 3 µl PageRule Prestained Protein Ladder® (Thermo Scientific) as a marker (details SDS-PAGE see 0.).

2.8.1.3 Large-scale cell harvest

Once protein overexpression conditions had been established through test expression assays (2.8.2), the appropriate conditions were repeated with a larger volume of culture to maximise protein yield. Overnight cultures, supplemented with ampicillin (pETduet) or kanamycin (pET28a(+)), were produced in 50 mL LB and used to inoculate (1% v/v) 1 l LB broth containing ampicillin or kanamycin in 2 l conical flasks. Cultures were incubated (37°C, 220 rpm), and 1 mL samples harvested after three hours growth, as described in 2.8.2. Overexpression was induced upon the addition of 1 mM IPTG. Cultures were incubated for a further three hours (37°C, 220 rpm) before 1 ml samples were collected and analysed by SDS-PAGE, as described previously (2.8.1.1 and 2.8.1.2), to confirm overexpression and stability of recombinant proteins at these larger volumes. The remaining culture was transferred to 95 x 191 mm, 1 l volume polycarbonate centrifugation bottles (Beckman Coulter) and cells harvested by centrifugation (20 minutes, 6000 x *g*, 4°C) in a Beckman Coulter Avanti® J-20 high performance centrifuge using a JLA-8.1000 rotor (Beckman Coulter). Cell pellets were transferred to 50 mL Falcon conical centrifugation tubes, snap frozen and stored at -20°C.

2.8.1.4 Purification of proteins with Ni-NTA beads in batch

Cell pellets (2.8.1.3) were defrosted on ice and resuspended in 25 ml lysis buffer (**Table 2.10**) and incubated for 30 minutes at room temperature. The cell lysate was then sonicated two times for 40 seconds at 50 Hz with 1 minute in between sonication steps. The cell debris was removed by centrifugation at 18 000 rpm for 20 minutes at 4°C in Beckman Coulter Avanti® J-20 high performance centrifuge using a JLA-25-50 rotor (Beckman Coulter). The supernatant was transferred in a fresh 50 ml falcon tube and 350 µl of Ni-NTA agarose beads (Qiagen) were added and incubated under gentle agitation for 1 hour at 4°C. The Ni-NTA agarose beads were spun down gently (maximum of 1200 rpm, 4°C) and the supernatant was discarded. The Ni-NTA beads were resuspended in 2 ml wash buffer (**Table 2.10**) and transferred in polypropylene columns (1 ml, Qiagen). The beads in the column were washed with 20 ml wash buffer (**Table 2.10**). The protein was eluted from the beads with 2.5 ml elution buffer (**Table 2.10**).

Table 2.10 Buffer for purification of MtrA and MtrB

	MtrA	MtrB
Lysis buffer	75 mM NaCl, 20 mM Tris-HCl pH 8 0.1% TritonX 100x 50 µl Lysozyme of 10mg/ml 3 x Pierce Protease Inhibitor Mini Tablets, EDTA free (Thermo Scientific)	50 mM NaH_2PO_4 pH 8 300 mM NaCl 10 mM imidazol 0.1% TritonX 100 x 50 µl Lysozyme of 10 mg/ml 3 x Pierce Protease Inhibitor Mini Tablets, EDTA free (Thermo Scientific)
Wash buffer	80 mM Tris-HCl pH 8 200 mM NaCl 10% Glycerol (v/v) 10 mM MgCl2 0.1 mM 1,4-Dithiothreitol (DTT) 20 mM β-mercaptoethanol	50 mM NaH_2PO_4 pH 8 300 mM NaCl 20 mM imidazol 10% Glycerol (v/v)
Elution buffer	80 mM Tris-HCl pH 8 200 mM NaCl 10% Glycerol (v/v) 10 mM MgCl2 0.1 mM 1,4-Dithiothreitol (DTT) 20 mM β-mercaptoethanol 350 mM imidazol	50 mM NaH_2PO_4 pH 8 300 mM NaCl 250 mM imidazol 10% Glycerol (v/v)
Storage buffer	20 mM HEPES pH 7.4 100 mM KCl 2 mM $MgCl_2$ 0.1 mM EDTA 0.1 mM DTT	50 mM Tris-HCl pH 8 150 nM NaCl 20% Glycerol (v/v) 0.1 mM EDTA

2.8.1.5 Desalting of purified protein

For long term storage, it was necessary to exchange the elution buffer which is high in NaCl with a storage buffer (**Table 2.10**). Sephadex G-25 in PD-10 Desalting Columns (GE Healthcare Life Sciences) were used as per manufacturer's instructions. Aliquots of proteins were stored at 4°C, -20°C and -80°C.

2.8.1.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE allows denatured proteins to be separated according to size along an acrylamide gel matrix. A standard resolving gel of 12% (w/v) ProtoGel™, Acrylamide/methylene Bis-Acrylamide solution (37.5:1 ratio) (National Diagnostics) and a stacking gel (5% w/v), was used throughout this research (**Table 2.11**), cast using Mini-PROTEAN® Tetra handcast systems (BIO-RAD) (0.75 mm and 1 mm combs and integrated spacer plates) and left to polymerise at room temperature, 30 minutes minimum. Samples were electrophoresed (120 V, 90 minutes) in 1 x TGS running buffer (0.025 M Tris-HCL, 0.192 M glycine, 1% SDS w/v) using Mini-PROTEAN® Tetra Cell systems (BIO-RAD), and stained with InstantBlue Protein Stain (Expedeon) with gentle agitation for a minimum of 15 minutes. Gels were de-stained in dH_2O for at least one hour. Gels were imaged using white light on a Molecular Imager® Gel Doc™ System (BIO-RAD).

Table 2.11 Composition of acrylamide gels for SDS-PAGE. The mixture is for 10 ml resolving gel and 5 ml stacking gel which is sufficient for 2 gels of 12%.

Compound	Stock solution	Volume	Final concentration
Resolving gel			
Acrylamide/Bis-Acrylamide	30% (w/v)	4 ml	12%
Tris-HCl, pH8.8	1.5 M	2.5 ml	375 mM
SDS	10% (w/v)	0.1 ml	0.05%
dH_2O		3.4 ml	
Tetramethylethylenediamine (TEMED)	>99%	5 µl	0.005%
ammonium persulfate (APS)	10% (w/v)	50 µl	0.1%
Stacking gel			
Acrylamide/Bis-Acrylamide	30% (w/v)	0.65 ml	5%
Tris-HCl, pH6.8	1M	1.25 ml	63 mM
SDS	10% (w/v)	50 µl	
dH_2O		3.05 ml	
TEMED	>99%	5 µl	
APS	10% (w/v)	25 µl	0.1%

2.8.2 Western Blot

2.8.2.1 Bradford assay

The Bradford dye-binding method (Bradford, 1976), provides a simple technique for the quantification of protein at low concentrations by comparing protein samples of unknown concentration to a standard curve produced from BSA samples of known concentration. Bovine serum albumin (BSA) standards (from 1 mg/ml stock in dH_2O) and unknown samples were diluted in Bradford Dye Reagent solution (BIO-RAD) and dH_2O in 1.6 mL cuvettes (semi-micro disposable polystyrene 10 mm path length; Fisherbrand) and mixed by inverting. The standards were set up in triplicate and the unknown proteins samples in duplicate. Absorbance was measured at A595 and a standard curve produced by plotting BSA standards A595 values against their known protein concentration (mg/ml). Using this, the protein concentration of unknown samples was deduced from their A595 values.

Table 2.12 Composition of Bradford assay reaction mix, BSA standards for production of a standard curve and unknown samples to be tested.

Sample	BSA (µl)	dH_2O (ml)	Bradford Reagent (ml)
†*Standards*			
1	0	0.8	0.2
2	1	0.8	0.2
3	5	0.795	0.2
4	10	0.790	0.2
5	20	0.780	0.2
6	40	0.760	0.2
	*Unknown Sample**		
As many as required	*2*	0.78	0.2

*Unknown sample volume can be increased as required to produce a sufficient colorimetric change if low protein concentration is present. †Increase concentration of standards as required.

2.8.2.2 Membrane transfer and membrane blocking

Protein samples were subjected to SDS-PAGE (2.8.1.6) prior to nitrocellulose Biodyne A membrane (Pall Corporation) transfer in a Trans-Blot® SD Semi-Dry Transfer Cell (BIO-RAD). Three layers of blotting paper, equal size to the gel, were soaked in 1 x transfer buffer (25 mM Tris, 192 mM Glycine, 0.1 % SDS) and placed on the transfer cell anode plate. Nitrocellulose membrane was soaked in 100% methanol (1 minute), followed by washing in transfer buffer (5 minutes), and placed on top of the blotting paper. The SDS polyacrylamide gel of proteins to be transferred was placed on top of the

membrane, followed by three more layers of soaked blotting paper before transfer took place (10 V, 1 hour). A blocking solution of 5% (w/v) fat-free skimmed milk powder in 1 x TBST (50 mM Tris Cl pH 7.5, 150 mM NaCl, 1% Tween) was poured over the membrane and incubated either at room temperature for 1 hour or overnight at 4°C with gentle agitation.

2.8.2.3 Antibody staining and blot imaging

Anti-His antibodies were used to check the correct size of purifies proteins. Anti-Flag antibody was used to check strains containing MtrA-3xFlag and anti-MtrA was used as a control. Anti-His antibody (QIAGEN) and anti-Flag (SIGMA), conjugated to horseradish peroxidase (HRP), were diluted 1: 20,000 in 1 x TBST. Anti-MtrA was diluted 1:5000. The membrane was incubated in 20ml of the respective antibody suspension at room temperature for 1 hour or 4°C overnight. Membranes incubated with anti-MtrA were washed 3 x for 1 minute and 3 x for 5 minutes in TBST and the HRP conjugated antibody Anti-Rabbit IgG (SIGMA) was diluted 1:12,000 in TBST and added to the membrane and incubated as above. After the incubation with the antibody(s) the membrane was washed 3 x for 1 minute and 3 x for 5 minutes in TBST. Antibody-tagged proteins were detected using a luminol-based chemiluminescent detection system (QIAGEN).

2.9 Phosphotransfer Assay

2.9.1 Autophosphorylation of MtrB

The autophosphorylation and phosphotransfer was carried out as previously described (Hutchings et al. 2006; Molle & Buttner 2000) with the following modifications. For the autophosphorylation reaction, MtrB (40 pmol) was incubated with 10 μCi (0.37MBq) [^{32}P]ATP at room temperature for 1 hour in 50 mM HEPES, pH 7.2, 5 mM $MgCl_2$ (50 μl total volume) and samples were taken at 1, 5, 15, 30 and 60 minutes and quenched immediately by addition of an equal volume of SDS-PAGE loading dye. The samples were loaded on a 12% SDS-PAGE gel and run for 1.5 hours at 120 V. Gels were transferred from glass plates to Whatman paper and dried for 30 minutes using a model 583 gel dryer/HydroTech vacuum pump (BioRad). Labelled protein was visualised using a phosphoimager plate exposed for 24 hours and scanned at 635 nm using the purple IP filter on a Typhoon FLA 9500 (GE Healthcare Life Sciences).

2.10 Electrophoretic mobility shift assay (EMSA)

2.10.1 EMSA 6-FAM™-fluorescein Labelled Probe Production

Fluorescently labelled DNA probes were generated by PCR amplification of target gene promoters (~250-500 bp in length, upstream of the start codon) using sequence specific oligonucleotides (**Table 2.9**). Negative control probes were produced by amplifying the promoter region of *hmpA2* (*SCO7094*) and *hmpA2* (*SCO7094*). Following purification (2.6.6), PCR products were diluted 1:50 to minimise carry-over of unlabelled DNA. Universal 6-FAM™-fluorescein labelled primers (**Table 2.9** Primers) were used to amplify 1:50 template DNA during a second round of PCR, producing 6-FAM™ labelled probes. Labelled probes were purified as previously described (2.6.6) before use in EMSA reactions.

2.10.2 EMSA reaction and gel imaging

EMSA's are carried out using non-denaturing PAGE gels. Four gels (5% acrylamide) were poured from a 20 ml total volume. Gels were produced using the using a Mini Protean III system (BioRad). Before loading the acrylamide, gels were pre-run at 30 mA for 1 hour at 4°C prior to use.

Table 2.13 Composition of native acrylamide gels for PAGE. The mixture is for 20 ml which is sufficient for 4 gels of 5%.

Compound	Stock solution	Volume	Final concentration
Acrylamide/Bis-Acrylamide	30% (w/v)	3.34 ml	5%
TBE	10 x	2 ml	1 x
TEMED	>99%	20 µl	0.1%
dH_2O		13.72 ml	
DTT (1,4-Dithiothreitol)	100 mM	20 µl	0.1 mM
APS	10% (w/v)	300 µl	0.15%

Unless otherwise stated, each binding reaction was prepared with ~10 ng 6'FAM labelled DNA probe (1 µl), 11 µl buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA pH 8.0, 5 mM DTT, and 5% glycerol), X µl MtrA and Y µl of dH_2O (up to a final volume of 20 µl). When acetyl phosphate (50 mM) was added to the buffer MtrA was added and incubated for 15 minutes to allow phosphorylation of MtrA. After adding the DNA samples were incubated for 15 minutes at room temperature. Poly(dIdC) is a polymer which is commonly used and provides an excess if non-specific binding to

out-compete any low-affinity binding. This is important when whole cell extract is used. Here poly(dIdC) was only used for the *ectAp* probe to demonstrate that it does not make a difference if poly(dIdC) is added to the EMSA reaction. The samples were loaded on the pre-run native gel and run for 3 hours at 30 mA at 4°C. Gels were visualised using a Typhoon FLA 9500 laser scanner (GE Healthcare) with LBP/BPB1 emission filter, Exmax 495 nm Emmax 576 nm, at 50 µM resolution. A Typhoon FLA 9500 laser system and filters provided results comparable in resolution and sensitivity to those obtained for radiolabelled probes.

2.11 Bioassay of *S. venezuelae* Δ*mtrB* mutant

S. venezuelae Δ*mtrB* was tested against Gram positive *Bacillus subtilis* and the Gram negative *Escherichia coli* to test the bioactivity of this strain.

2.11.1 Spot Assays

2 µl of spores of *S. venezuelae* wild-type and *S. venezuelae* Δ*mtrB* (~10^7 CFU per 1 µl) was spotted onto LB or MYM agar and incubated for 24 hours. Then overnight cultures (2 µl) of the test strains *B. subtilis* and *E. coli* were added and incubated for another 24 hours.

2.11.2 Disc Assays with Methanol Extracts

S. venezuelae wild-type and *S. venezuelae* Δ*mtrB* were cultivated in 35 ml as described in 2.4. 750 µl of the culture at 24 hours of growth were either snap frozen or immediately used for methanol extraction. An equal volume of 100% methanol was added to the aliquot of the culture and vortexed for 30 minutes. The methanol culture mix was then centrifuged for 20 minutes at 13,000 rpm. The upper phase was transferred in a fresh tube and stored at 4°C or used directly in the disc assay. 50 µl of the methanol extracts were added to discs (Grade AA, 6mm, GE Healthcare Life Science) in a maximum of 20 µl aliquots to allow the methanol extract to dry. An overnight culture of *B. subtilis* (5 ml) was used to inoculate 200 ml LB soft agar (**Table 2.3**). The LB soft agar containing *B. subtilis* was poured in petri dishes and the methanol extract soaked disks were placed on the LB soft agar and incubated for 24 hours at 30°C.

2.12 Liquid Chromatography–Mass Spectrometry (LCMS)

All LCMS experiments presented in this work were carried out by Dr Daniel Heine at the John Innes Centre.

Analytical HPLC was carried out on an HPLC 1100 system (Agilent Technologies) using a Gemini® 3 µm NX-C18 110Å, 150×4.6 mm column (Phenomenex). All solvents for analytical and semi-preparative HPLC measurements were obtained commercially in HPLC grade. To avoid microbial growth, 0.1% formic acid was added to the water.

2.13 Chromatin Immunoprecipitation Sequencing (ChIP-Seq)

S. venezuelae and NS042 (*S. venezuelae* Δ*mtrA* ΦBT1 *mtrAp mtrA-3xFlag*) as well as *S. coelicolor* and NS171 (*S. coelicolor* Δ*mtrA* ΦBT1 *mtrAp mtrA-3xFlag*) were grown in MYM in the same way as described before (2.4). Following growth to the chosen time point, the entire content of the flask was transferred to a 50 ml falcon tube for crosslinking, which was carried out by incubation at 30°C for 30 minutes with 1% final concentration of formaldehyde (v/v). Crosslinking was quenched by incubation at room temperature with glycine (final concentration of 125 mM). Mycelium was harvested by centrifugation 4000 rpm at 4°C for 10 minutes and washed twice with ice cold PBS (Phosphate Buffer Saline Tablets, Oxoid) before transfer to a 2 ml centrifuge tube. Pellets were resuspended in 0.75 ml lysis buffer (10 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mg/ml lysozyme, 1 x Pierce Protease Inhibitor Mini Tablets) and incubate at 37°C for 30 minutes. Then 0.75 ml 1 x IP buffer (100 mM Tris-HCl pH 8.0, 250 mM NaCl, 0.5% Triton X-100, 0.1% SDS, 1 x Pierce Protease Inhibitor Mini Tablets) were added and samples mixed by pipetting up and down. Samples were sonicated 20 x at 50Hz, 10 seconds/cycle with a 1 minute incubation on ice after each cycle. DNA fragmentation was checked by agarose gel electrophoresis (2.6.4) following phenol extraction (2.6.14) of 25 µl of the crude lysate mixed with 75 µl of TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA) with 100-200 µl of phenol / chloroform. Contaminating RNA was removed with 2 µl RNase (1mg/ml) added to extracted DNA followed by incubation for 30 minutes at 37°C. A smear of DNA from 200 to 1000 bp with the majority of DNA around 500 bp should be visible. Crude lysate was centrifuged at 13,000 rpm for 15 minutes at 4°C to clear the sample of cell debris. M2 affinity beads (Sigma-Aldrich #A2220) were prepared by washing in ½IP buffer following manufacturer's instructions. The cleared lysate was

incubated with 40 µl of washed M2 beads and incubated for 16 hours at 4°C in a vertical rotor. The lysate was removed and the beads pooled into one 1.5 microfuge tube and washed in ½IP buffer by incubating on a vertical rotor for at least 10 minutes. The beads were transferred to a fresh microfuge tube and washed a further three times removing as much buffer as possible without disturbing the beads. The DNA protein complex was eluted from the beads with 100µl elution buffer (50 mM Tris-HCl pH 7.6, 10 mM EDTA, 1% SDS) by incubating at 65°C overnight. Removing the ~100 µl elution buffer, an extra 50 µl of elution buffer was added and further incubated at 65°C for 5 minutes. To extract the DNA 2 µl proteinase K (10 mg/ml) were added to 150 µl eluate and incubated 1.5 hours at 55°C. To the reaction 150 µl phenol / chloroform were added. Samples were vortexed and centrifuged at full speed for 10 minutes. The aqueous layer was purified using the Qiaquick column from Qiagen with a final elution using 50 µl EB buffer (Qiagen). The concentration of samples was determined by nanodrop measurement.

2.13.1 Data analysis of ChIP-seq data

The ChIP-seq samples were sequenced via Illumina HiSeq2500 with 100 bp single-end reads by the Earlham Institute. Sequencing reads were processed by Dr Govind Chandra. Every 25 nt an enrichment was calculated as follow: An area of 4000 nt was divided by the reads of the central 50 nt. These enrichment values were analysed by negative binominal distribution. All enrichment values $p > 0.05$ were not included in further analysis unless otherwise stated. A $p = 0.05$ correlates with an enrichment value of seven. Thus, every peak shows the enrichment of the sequencing reads in correlation to the surrounding area.

2.14 MtrA pull down

2.14.1 Immunoprecipitation of MtrA-flag

The immunoprecipitation was performed in the same way as for ChIP-seq (section 2.13). But the immunoprecipitated beads containing the DNA-MtrA-3xflag were eluted by adding 20 µl 2 x SDS sample buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 20% Glycerol and 0.004% bromphenol blue and boiled for 3 minutes. Then the beads were centrifuged down at 13000 rpm for 1 minute. The supernatant was transferred in a clean tube and loaded on a 10% SDS gel without stacking gel. The samples were separated by electrophoresis a few millimetres into the gel. After the run the gel was rinsed with tap

water to remove any residual detergent. The protein band was cut with a clean scalpel and stored at -20°C.

2.14.2 Purification of immunoprecipitated samples for trypsin digest

The samples were prepared as follows for a trypsin digest and MALDI-TOF. The gel slices were de-stained with 30% ethanol for 30 minutes at 65°C in low binding tubes. The ethanol was replaced until the gel was clear. Each washing step was for 15 minutes. The de-stained gel was washed with 50 mM TEAB (Sigma Aldrich) in 50% Acetonetril. Then the gel slices were incubated in 10 mM DTT in TEAB for 30 minutes at 55°C. The DTT was removed and 30 mM Iodoacetamide (IAA) in 50 mM TEAB was added and incubated for 30 minutes in the dark while vortexing. IAA was removed and the gel slices were washed with 50 mM TEAB in 50% acetonitrile. The protein bands were cut and transferred in fresh low bind tubes and washed with 50 mM TEAB in 50% acetonitrile. Then the gel pieces were washed in 100% acetonitrile until they shrunk and became hard and white. The gels were dried in a speed vac for 30 minutes and stored at -20°C.

2.14.3 Trypsin digest and MALDI-TOF

The MtrA-Flag pull down samples were analysed in the proteomics suit at the John Innes Centre. The samples were trypsin digested and further analysed by LCMS on nanoLC Orbitrap Fusion mass spectrometer. The identified proteins were investigated using the Scaffold 4 software version 4.5.3.

2.15 RNA-seq

RNA isolation and purification was carried out by John Munnoch as described in (Munnoch et al. 2016) and analysis of RNA sequencing reads was performed by Dr Govind Chandra.

Mycelium was harvested at experimentally appropriate time points and immediately transferred to 2 ml round bottom tubes, flash frozen in liquid N_2, stored at -80°C or used immediately. All apparatus used was treated with RNaseZAP (Sigma) to remove RNases for a minimum of 1 hour before use. RNaseZAP treated mortar and pestles were used, the pestle being placed and cooled on a mixture of dry ice and liquid N_2 with liquid N_2 being poured into the bowl and over the mortar. Once the bowl had

cooled the mycelium samples were added directly to the liquid N_2 and thoroughly crushed using the mortar leaving a fine powder of mycelium. Grindings were transferred to a pre-cooled 50 ml Falcon tube and stored on dry ice. Directly to the tube, 2 ml of TRI reagent (Sigma) was added to the grindings and mixed. Samples are then thawed while vortexing intermittently at room temperature for 5 - 10 minutes until the solution cleared. To 1 ml of TRI reagent resuspension, 200 µl of chloroform was added and vortexed for 15 seconds at room temperature then centrifuged for 10 minutes at 13,000 rpm. The upper, aqueous phase (clear colourless layer) was removed into a new 2 ml tube. The remainder of the isolation protocol follows the RNeazy Mini Kit (Qiagen) instructions carrying out both on and off column DNase treatments. On column treatments were carried out following the first RW1 column wash. DNaseI (Qiagen) was added (10 µl enzyme, 70 µl RDD buffer) to the column and stored at RT for 1 hour. The column was washed again with RW1 then treated as described in the manufacturer's instructions. Once eluted from the column, samples were treated using TURBO DNA-free Kit (Ambion) following manufacturer's instructions to remove residual DNA contamination.

2.16 *In silico* analysis of MtrAB-LpqB

2.16.1 Protein or gene similarity

In order to compare two proteins or genes BLAST online tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used. Protein or DNA sequences were obtained from http://strepdb.streptomyces.org.uk/. The protein similarity of MtrA, MtrB and LpqB was compared using http://streptomyces.org.uk/actinoblast/ (Chandra & Chater 2014).

2.16.2 Modelling of MtrA structure

The crystal structure of un-phosphorylated MtrA(TB) was solved (Friedland et al. 2007). Two different modelling software were used to compare MtrA(Sv) with the solved crystal structure of MtrA(TB) (https://toolkit.tuebingen.mpg.de/hhrep and http://www.sbg.bio.ic.ac.uk/phyre2).

3 Genetic Manipulation of MtrAB-LpqB in *S. venezuelae*

Previous studies investigated the MtrAB-LpqB TCS in Mycobacteria, *C. glutamicum* and *S. coelicolor*. In the following chapter I investigate the function of MtrAB-LpqB in the new model organism *S. venezuelae*. To investigate the function of MtrAB-LpqB I attempted to generate deletion mutants of the single genes of the TCS. This is a first step in understanding the role of MtrAB-LpqB by examining the arising phenotypes of the deletion mutants. Additionally, I described the design of a MtrA gain of function protein and the resulting phenotype in the wild-type background. I used Redirect PCR targeting method to create in frame deletions of the three genes in the TCS. The phenotypes were examined on solid medium and in liquid culture.

3.1 Genetic Manipulation of mtrA in *S. venezuelae*

3.1.1 *mtrA* is essential in *S. venezuelae*

The response regulator MtrA is essential in *M. tuberculosis*, but dispensable in *C. glutamicum*. To test if *mtrA* is essential in *S. venezuelae* a cosmid carrying the genomic region including *mtrA* was disrupted via PCR targeting to delete the *mtrA* gene and replace it with an apramycin resistance cassette (Gust et al. 2004), see section 2.7. The *mtrA* deletion cosmid was then introduced into *S. venezuelae* by conjugation. Several attempts were made to delete the *mtrA* gene in *S. venezuelae* but they were all unsuccessful. To investigate if *mtrA* is essential, a second copy of *mtrA* under the control of its native promoter was introduced *in trans* into the ΦBT1 integrative site of wild-type *S. venezuelae* using pMS82 (Gregory & Smith 2003). Additionally, *mtrA* with a C-terminal linker (GGGGSGGGGSGGGGS) and 3xFlag-tag was introduced in the ΦBT1 integrative site of the wild-type strain for further analysis, including immunoblotting, immunoprecipitation and ChIP-seq. It was possible to delete *mtrA* in its native locus in both strains and the correct integration of the apramycin resistance cassette (APR) was tested by PCR (**Figure 3.1**). This suggests *mtrA* is essential and, importantly, that the C-terminal 3xFlag-tagged MtrA is functional. Furthermore, the *mtrA* strains Δ*mtrA* ΦBT1 *mtrAp-mtrA* (NS052) and Δ*mtrA* ΦBT1 *mtrAp-mtrA*-flag (NS042-044) grew normally on solid MYM (**Figure 3.2**) and in liquid MYM (**Figure 3.3**) which indicates that the 3xFlag-tag does not affect the function of MtrA.

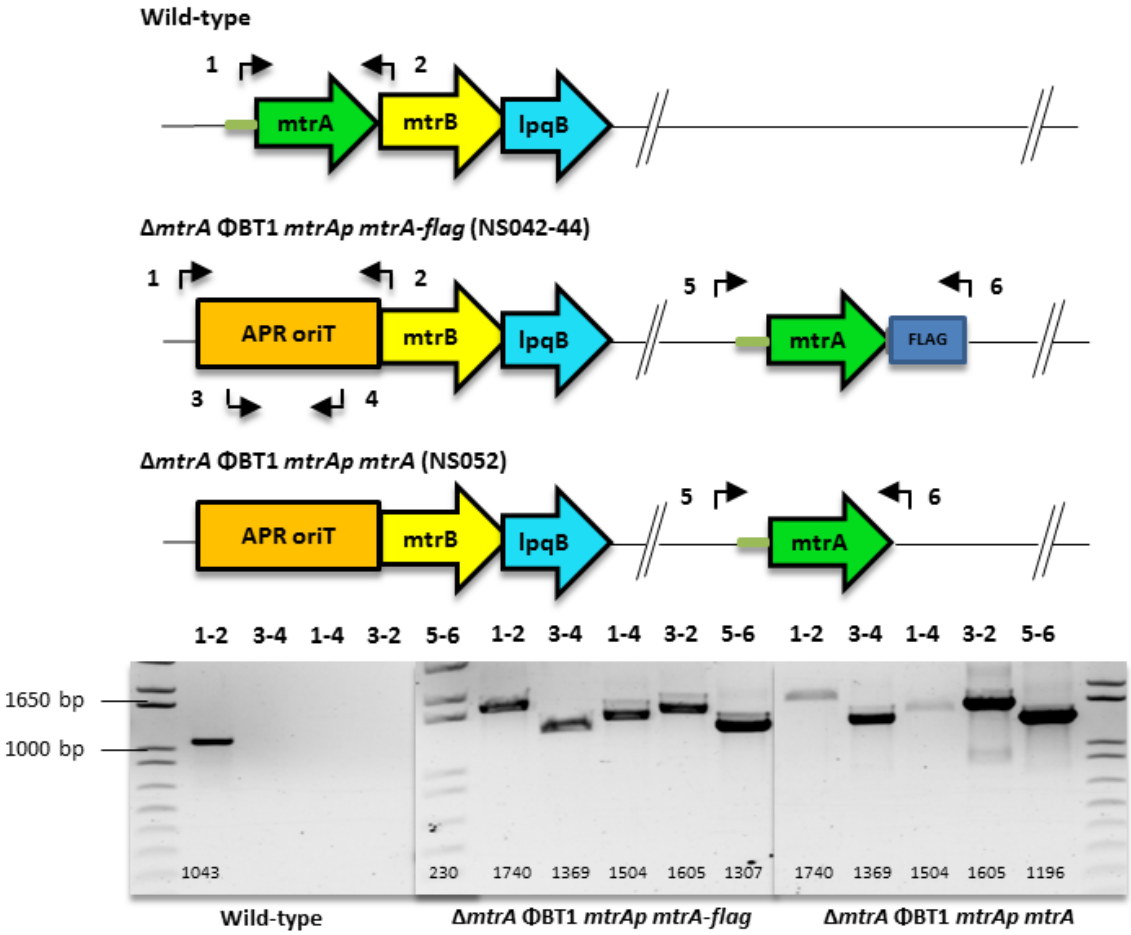

Figure 3.1 Top: Schematic overview of *mtrA* mutants. **Bottom**: PCR confirmation of the *mtrA* mutants with Primers P131-132 for 1-2; P1-P2 for 3-4; P131-P2 for 1-4; P1-P132 for 3-2 and P64-P65 for 5-6. The expected sizes of the PCR products are at the bottom in base pairs (bp).

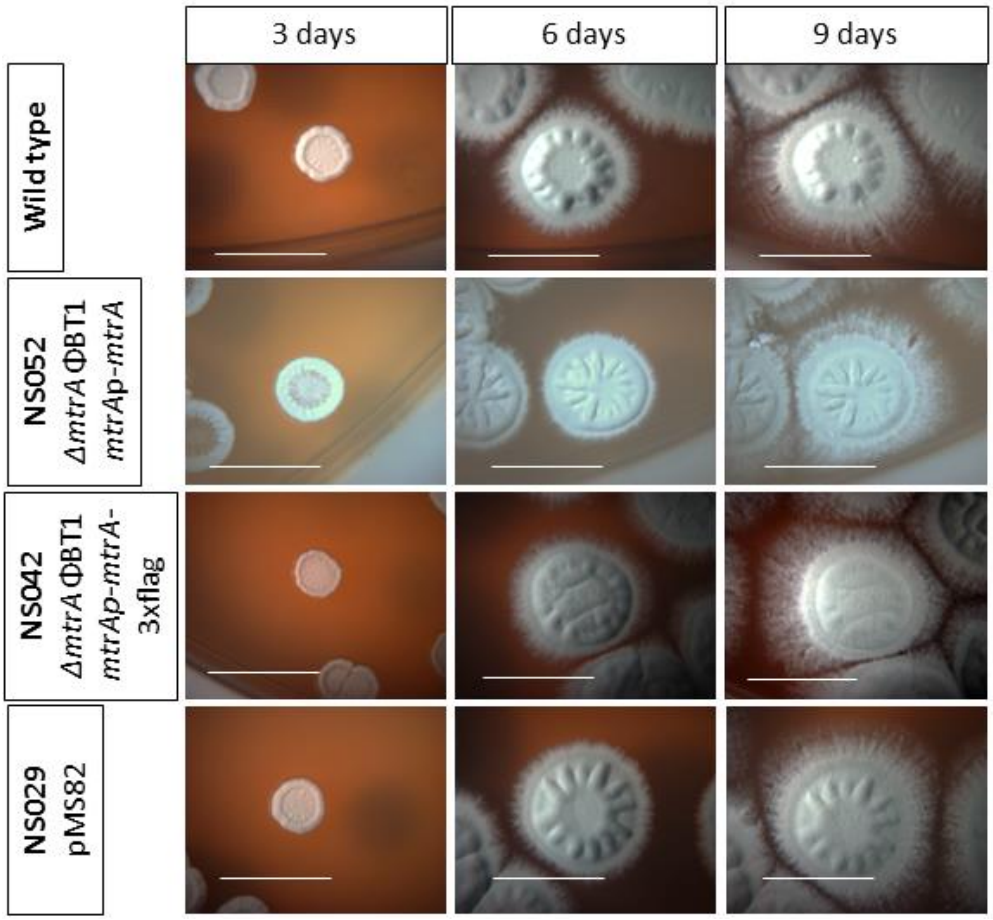

Figure 3.2 Phenotype of *S. venezuelae*, NS052 (Δ*mtrA* ΦBT1 *mtrAp-mtrA*), NS042 (Δ*mtrA* ΦBT1 *mtrAp-mtrA*-3xflag) and NS029 (*S. venezuelae* pMS82) on MYM plates after 3, 6 and 9 days. Scale bar: 1 cm. Additional colony pictures of the strains and additional identical genetic clones are shown is **S1-S6**. The images of clone NS052 are brighter due to overexpression during microspcopy.

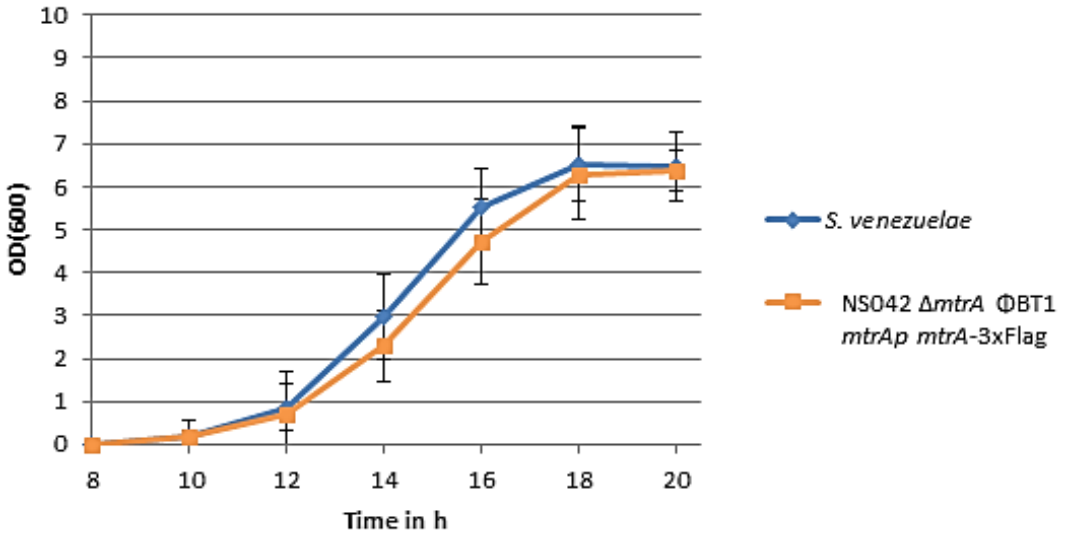

Figure 3.3 Growth curve of *S. venezuelae* wild-type and NS042 (*mtrA*::APR ΦBT1 *mtrAp-mtrA-3xflag*) strains in MYM. The average of six biological replicates is shown. The growth rate μ (calculation see methods 2.4) during exponential growth is 0.61 ± 0.005 and 0.56 ± 0.002 for *S. venezuelae* and NS042 respectively. During exponential growth, the growth rates are statistically similar with p(T<=t) two-tail = 0.21.

3.1.2 Overexpression of *mtrA* using *ermEp** does not induce a phenotype in wild-type *S. venezuelae.*

Since *mtrA* cannot be deleted in *S. venezuelae* the effect of constitutive expression of MtrA in the wild-type background was investigated. *mtrA* was cloned in pIJ10257 (Hong et al. 2005) downstream of the *ermEp** promoter which is constitutively active. The resulting overexpression of MtrA does not induce a phenotype on MYM solid or liquid medium (**Figure 3.4**. and **Figure 3.5**)

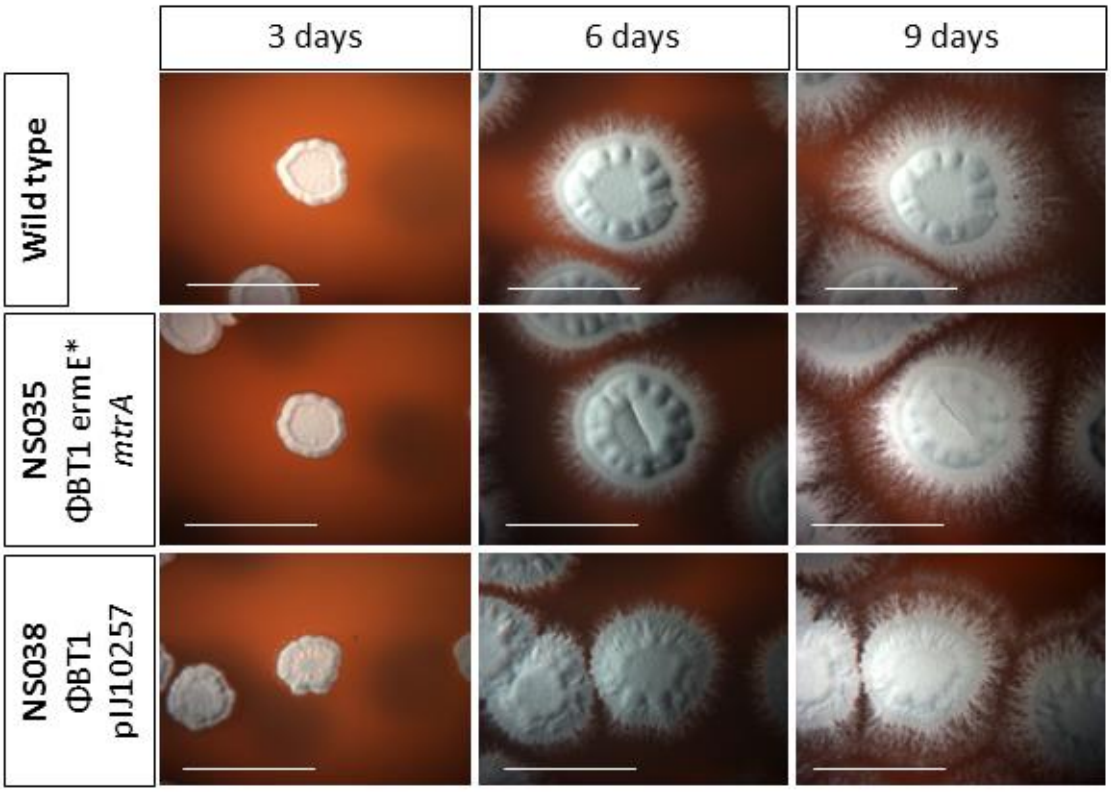

Figure 3.4 Phenotype of *S. venezuelae* wild-type, NS035 (*S. venezuelae* ΦBT1 *ermEp*-mtrA*) and NS038 (*S. venezuelae* ΦBT1 pIJ10257) strains on MYM after 3, 6 and 9 days. Scale bar: 1 cm. Additional colonies of the strains and additional genetic identical clones are shown is **S7-S12**.

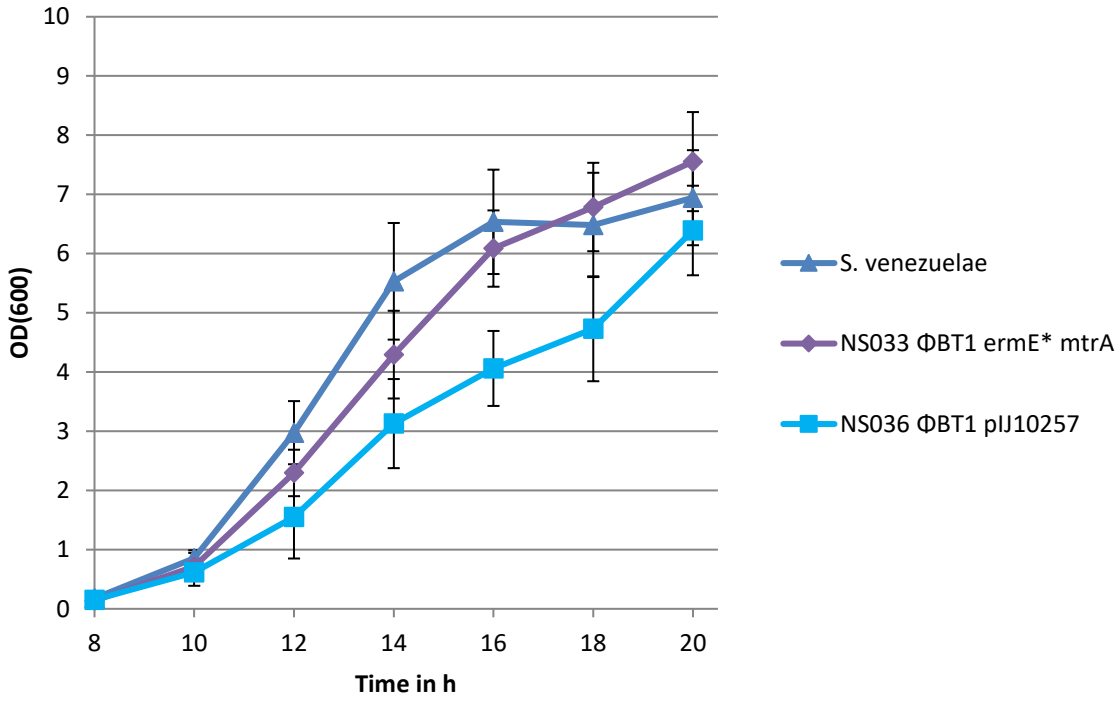

Figure 3.5 Growth curve of strain NS033 (ΦBT1 *ermEp*-mtrA*) and the empty vector control strain NS036 (ΦBT1 pIJ1257) in liquid MYM medium. The average of six biological replicates is shown. The growth rate μ is 0.56 ± 0.04, 0.58 ± 0.07 and 0.5 ± 0.04 for *S. venezuelae* wild-type, NS033 and NS036 strains, respectively. The growth rate of *S. venezuelae* wild-type was not significantly different from either of NS033 (p(T<=t) two-tail = 0.63) or NS036 (p(T<=t) two-tail = 0.06) which was tested by t-test.

3.2 Deletion of *mtrB* in *S. venezuelae*

3.2.1. Construction of an in-frame deletion of *mtrB* in *S. venezuelae*

MtrB is not essential in *M. tuberculosis* or *C. glutamicum.* To investigate the function of MtrB in *S. venezuelae* a cosmid carrying the genomic region including *mtrB* was deleted via PCR targeting to delete the *mtrB* gene and replace it with an apramycin resistance cassette (Gust et al. 2004), see section 2.7. Strains which were apramycin resistant and kanamycin sensitive were tested by PCR for integration of the apramycin resistance cassette. Three individual *mtrB* mutants (named NS012, NS021 and NS022) were confirmed and spores were harvested and stored for further analysis, see **Figure 3.6**.

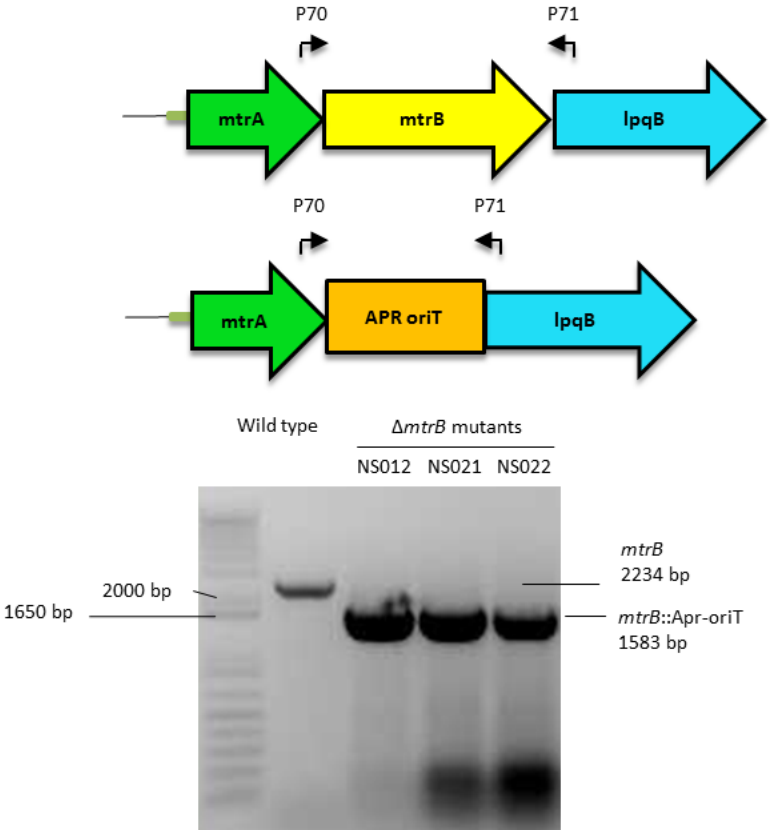

Figure 3.6 Top: Schematic overview of the apramycin resistance cassette integration in the *mtrB* mutants. **Bottom:** PCR confirmation of three individual clones with the primers P70 and P71. The presence of the apramycin resistance cassette was also tested by PCR using internal primers in the apramycin cassette, data not shown. All three clones contain the apramycin resistance cassette

3.2.2 The *S. venezuelae* Δ*mtrB* mutant is slightly delayed in aerial hyphae formation on MYM agar plates

The *S. venezuelae* Δ*mtrB* mutant is viable and is slightly delayed early in the developmental cycle on solid MYM medium. At three days of growth the colonies of the *S. venezuelae* Δ*mtrB* mutant are slightly smaller than the wild-type and in the middle of the colony the aerial hyphae seem to be delayed in growth due to the lack of the typical fuzzy white appearance (**Figure 3.7**). After six and nine days of growth the *S. venezuelae* Δ*mtrB* mutant colonies are slightly larger than the wild-type colonies and not as raised up as the wild-type. To confirm these changes in colony morphology are due to the deletion of *mtrB* a copy of *mtrB* under its native promoter was introduced in *trans*. The complementation strain seems to form aerial hyphae at three days and at six and nine days the colony size and height are more comparable to the wild-type than the Δ*mtrB* mutant. However, the colony morphology of the complementation strain does not seem to fully complement the deletion of *mtrB*.

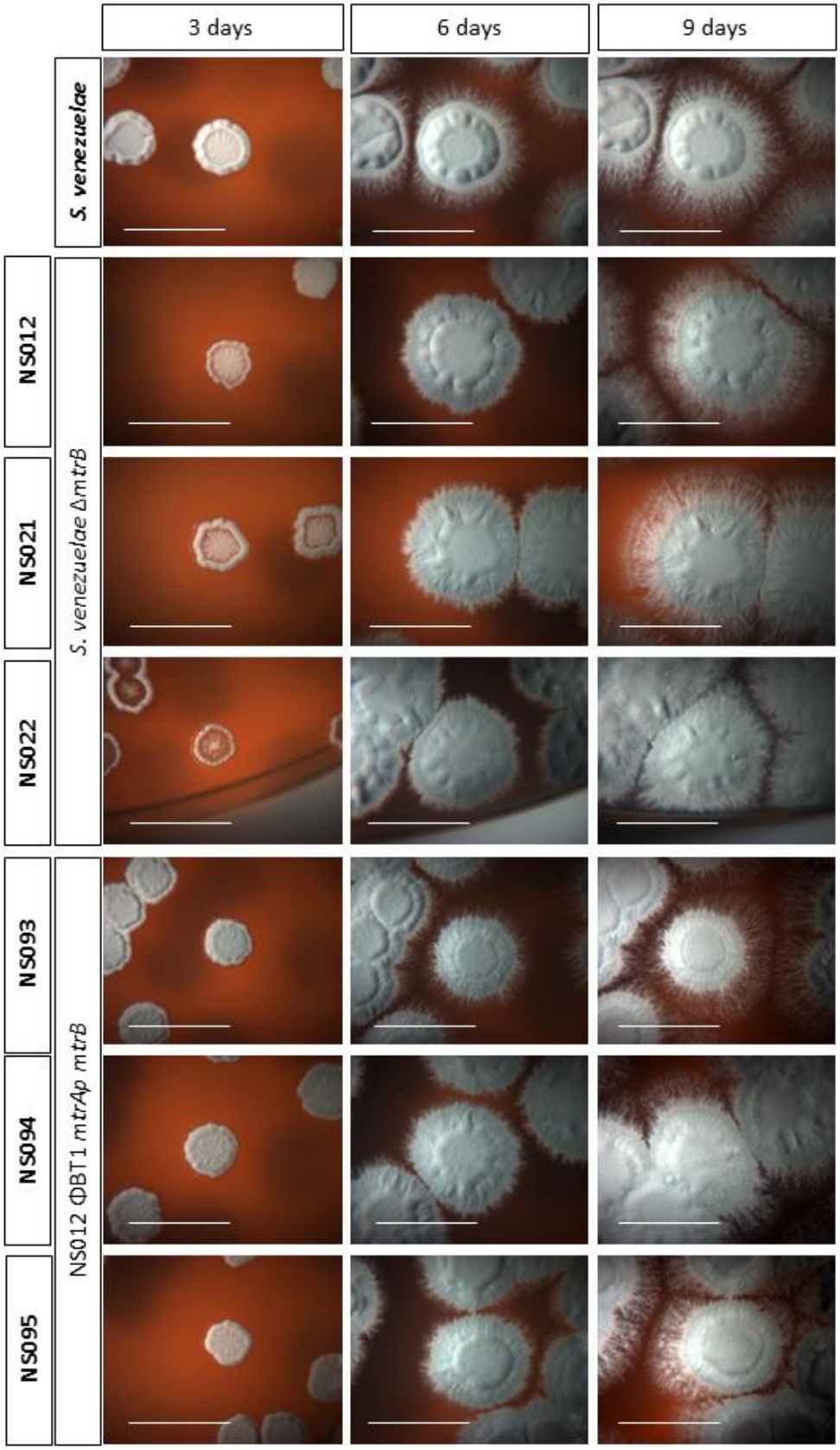

Figure 3.7 Phenotype of *S. venezuelae* wild-type, three independent clones of *S. venezuelae* Δ*mtrB* NS012, NS021, NS22, and three independent clones of the complemented NS012 Δ*mtrB* strain NS093-95 (NS012 ΦBT1 *mtrAp mtrB*) on MYM plates after 3, 6 and 9 days. Scale bar: 1 cm. Additional colonies of the strains are shown in **S13-S18**.

3.2.3 The *S. venezuelae* Δ*mtrB* mutant grows normally in liquid MYM medium

To test if the *S. venezuelae* Δ*mtrB* mutant displays a growth or developmental phenotype in liquid culture, three replicates were grown in liquid MYM. At the top of **Figure 3.8** the growth of three replicates of *S. venezuelae*, *S. venezuelae* Δ*mtrB* and the complemented Δ*mtrB* strain is shown. The mean growth rate for the wild-type strain (0.42 ± 0.04) was compared, by t-test, to *S venezuelae* Δ*mtrB* (0.36 ± 0.002) and the complemented strain, *S. venezuelae* Δ*mtrB* ΦBT1 *mtrAp-mtrB* (0.40 ± 0.02), in turn. No significant difference was observed between the wild-type and *S. venezuelae* Δ*mtrB* strain (p(T<=t) two-tail = 0.09) or the wild-type compared to *S. venezuelae* Δ*mtrB* ΦBT1 *mtrAp-mtrB* (p(T<=t) two-tail = 0.49).

Since no defect in the growth rate in the *S. venezuelae* Δ*mtrB* mutant was observed it was of interest to see if the *S. venezuelae* Δ*mtrB* mutant sporulates at the same time as the wild-type strain. Therefore, the strains were investigated using microscopy at individual time points and this revealed that all three strains start to sporulate at 16 hours in liquid MYM (**Figure 3.9**). However, the quantity of sporulation remains elusive in this experiment.

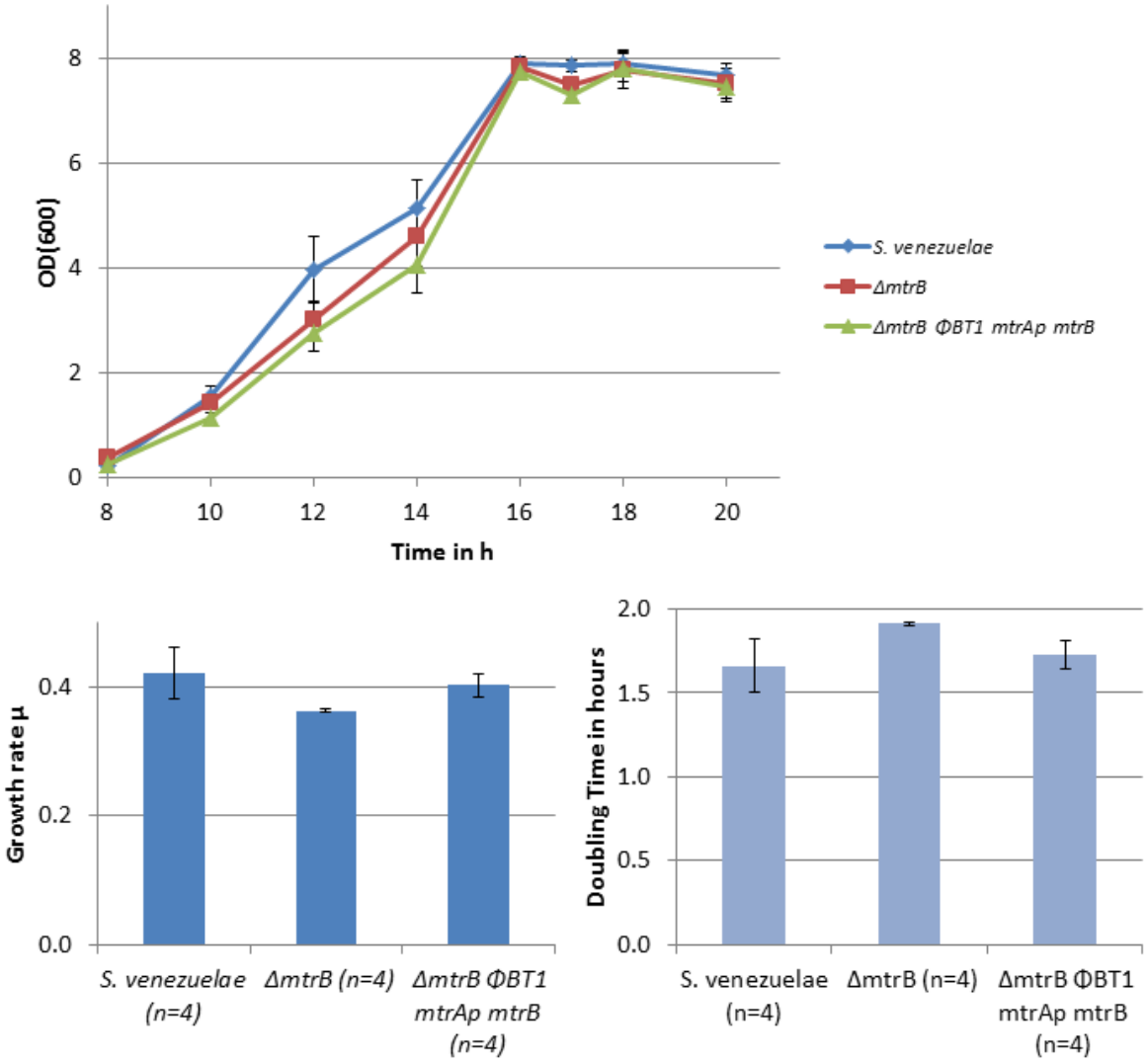

Figure 3.8 Top: Growth curve of four individual cultures of *S. venezuelae*, *S. venezuleae* Δ*mtrB* (NS012) and *S. venezuelae* Δ*mtrB* ΦBT1 *mtrAp mtrB* (NS093) in liquid MYM. **Bottom:** Growth rate and doubling time of liquid cultures during exponential growth. A t-test revealed no significant difference between *S. venezuelae* and either of *S. venezuelae* Δ*mtrB* or the complemented Δ*mtrB* strain (*S. venezuelae* growth rate compaired with *S. venezuelae* Δ*mtrB* P(T<=t) two-tail = 0.09 and *S. venezuelae* growth rate compaired with *S. venezuelae* Δ*mtrB* ΦBT1 *mtrAp mtrB* P(T<=t) two-tail = 0.49).

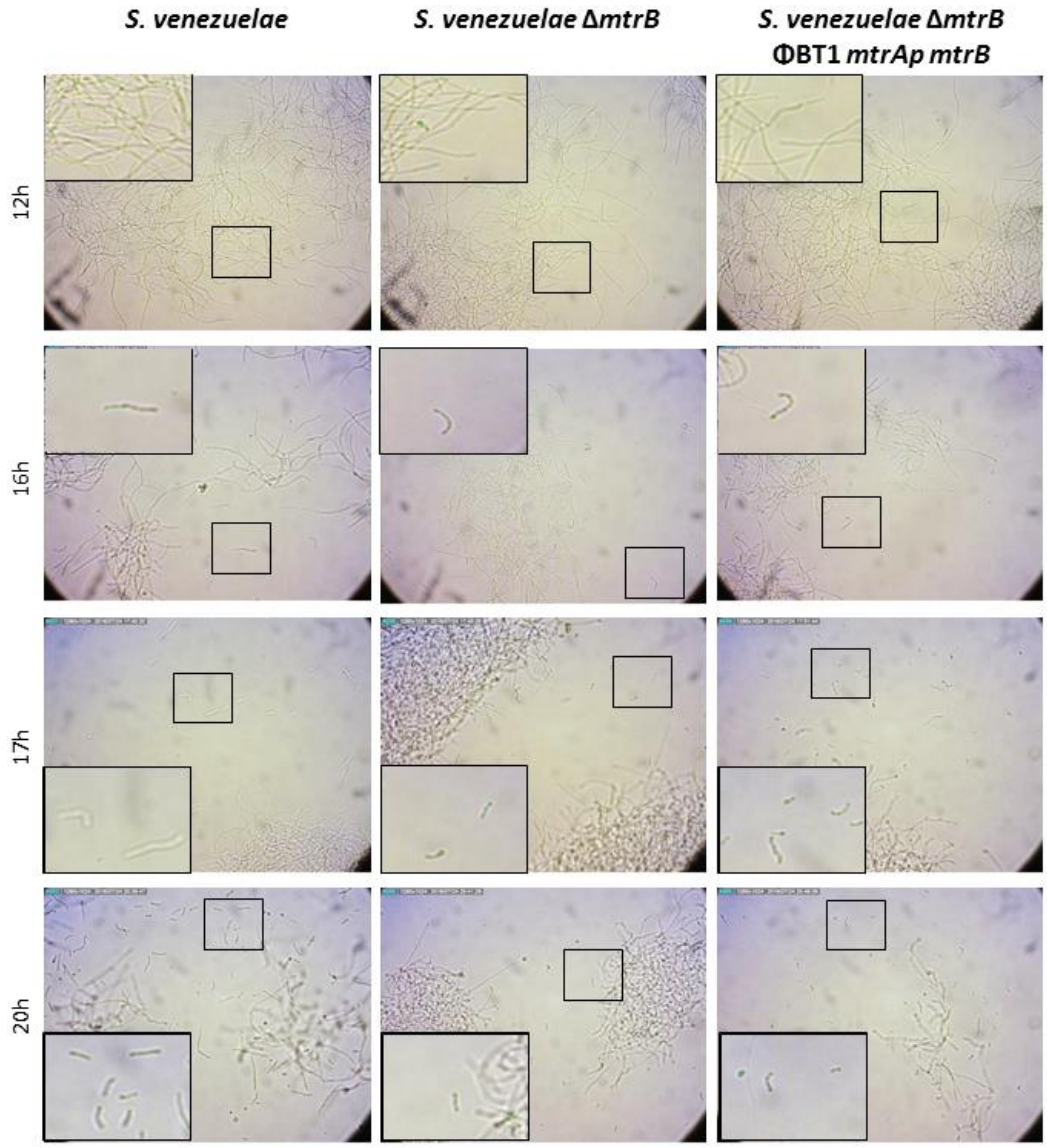

Figure 3.9 Microscopy of cultures shown in **Figure 3.8.** All three strains start to sporulate at 16 hours. The small box is magnified and displayed in the corner.

3.2.4 The *S. venezuelae* Δ*mtrB* colonies have an unusual growth defect in the middle of the colony

To further investigate the phenotype of the *S venezuelae* Δ*mtrB* mutant, Scanning Electron Microscopy (SEM) was performed. Since the *S. venezuelae* Δ*mtrB* mutant appears to have a delay in vegetative growth in the middle of the colony images were taken from the outside and inside of the Δ*mtrB* colonies (**Figure 3.10**). The results show that the *S. venezuelae* Δ*mtrB* mutant sporulates normally on the outside of the colony but shows a drastic growth defect in the middle of the colony. At the time when the SEM images were taken the *mtrB* complementation strain was not available. It is necessary to investigate the *mtrB* complementation strain via SEM microscopy to determine if this strain fully complements the growth defect in the middle of the colony.

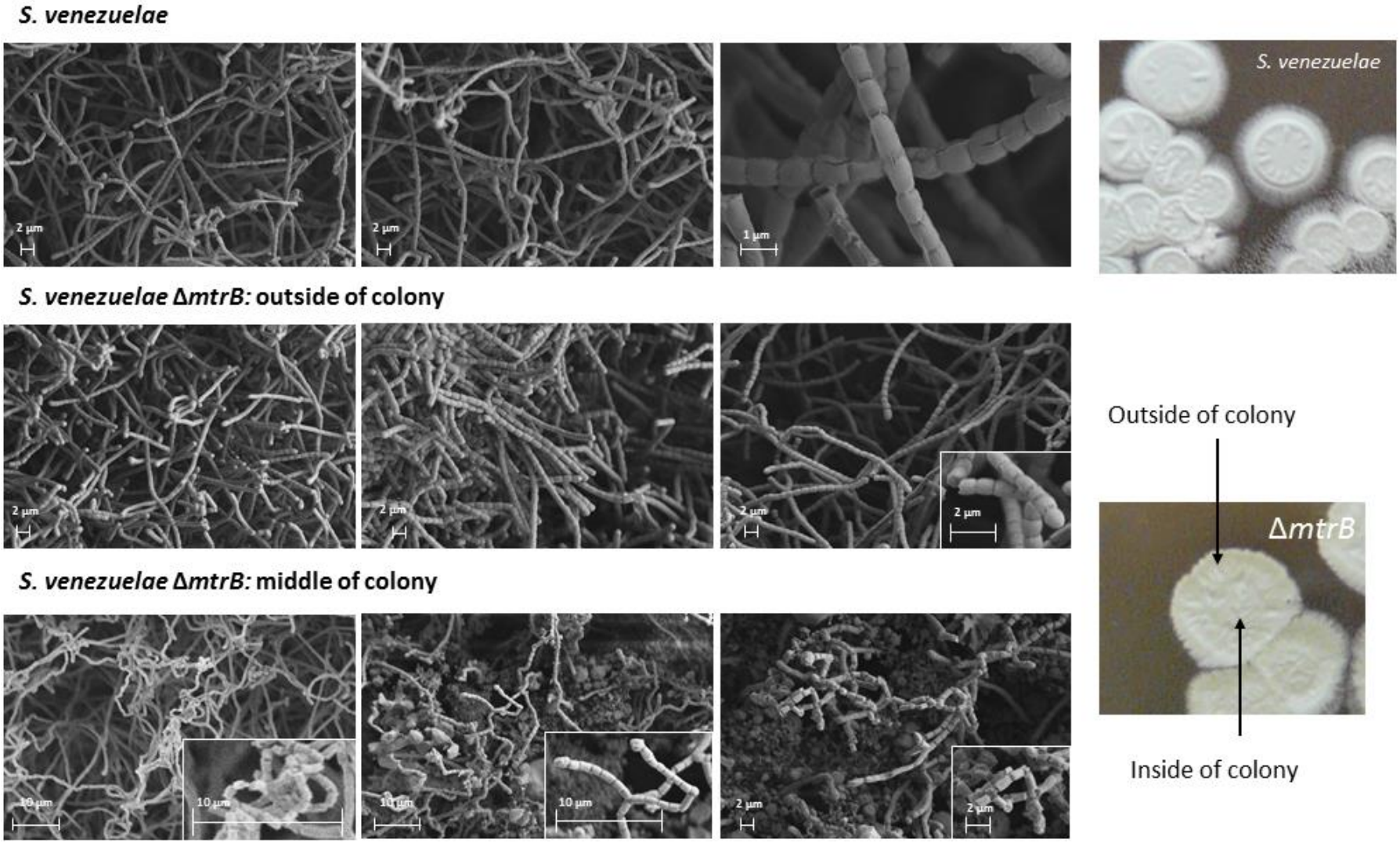

Figure 3.10 Scanning Electron Microscopy (SEM) images of *S. venezuelae* and the *S. venezuelae* Δ*mtrB* mutant. Cells were grown on MYM for 10 days at 30°C. It was not possible to take high magnification images of the *S. venezuelae mtrB* mutant because the spores were destroyed by the electron beam at high magnifications (Elaine Barclay, personal communication). Therefore, sections of the *S. venezuelae* Δ*mtrB* images are shown magnified.

3.2.5 The Δ*mtrB* mutant is sensitive to salt stress (osmotic stress phenotype)

C. glutamicum MtrAB is involved in cell wall metabolism and osmoprotection. It was shown that the histidine kinase MtrB can be activated by various solutes, sugars, amino acids and polyethylene glycol (Möker, Kramer, et al. 2007). To test if MtrAB is involved in osmoprotection in *Streptomyces* species, *S. venezuelae* and *S. coelicolor* Δ*mtrB* mutants were grown on agar plates containing different NaCl concentration to induce osmotic stress. Note, the *S. coelicolor* Δ*mtrB* mutant was constructed previously by Felicity Knowles (Knowles 2014). In both *Streptomyces* species, the Δ*mtrB* mutant is more sensitive to salt stress than the wild-type (**Figure 3.11** and **Figure 3.12**). *S. venezuelae* is delayed in aerial hyphae formation at three days but fully sporulates at six and nine days on MYM agar medium containing 0.3 M or 0.5 M NaCl. On MYM agar plates containing 0.7 M NaCl the wild-type colonies are extremely small at three days but then grow larger than the wild-type colonies without NaCl at nine days. After nine days of growth on NaCl the wild-type strain forms aerial hyphae but does not sporulate. In comparison, the *S. venezuelae* Δ*mtrB* mutant shows a drastic phenotype under salt stress. At three days of growth the *S. venezuelae* Δ*mtrB* mutant colonies are smaller than the wild-type under salt stress. After six days on MYM agar plates containing 0.3M NaCl the *S. venezuelae* Δ*mtrB* mutant produces the green spore pigment but it does not produce spore pigment on MYM agar plates containing 0.5 M NaCl. Also, the *S. venezuelae* Δ*mtrB* colonies are completely flat compared to the *S. venezuelae* Δ*mtrB* mutant growing in the absence of NaCl. The lack of the erection of the colonies in the *S. venezuelae* Δ*mtrB* mutant under NaCl could be visualised by cross section the colonies. Furthermore the *S. venezuelae* Δ*mtrB* mutant under NaCl seems to form aerial hyphae due to the white colour but this could be investigated with higher magnification microscopy. It seems that NaCl stress exacerbates the phenotype of the *S. venezuelae* Δ*mtrB* mutant. The *mtrB* complementation strains seems to be more like the Δ*mtrB* mutant than the wild-type under salt stress. The Δ*mtrB* strain containing a copy of *mtrB* in *trans* only partially complements under salt stress.

In contrast, the *S. venezuelae* Δ*mtrB* mutant grows similarly to the wild-type in liquid medium (**Figure 3.13**). When 0.5 M NaCl is added to *S. venezuelae* and *S. venezuelae* Δ*mtrB* cultures at 12 hours both cultures do not grow exponentially which would be the case in growth without NaCl. However, the strains do grow but more slowly

and they enter stationary phase after 30 hours. Interestingly, no spores could be observed after 32 hours of growth of the wild-type strain or the Δ*mtrB* mutant.

Similar to *S. venezuelae*, the growth of the *S. coelicolor* wild-type strain is reduced with increasing salt stress. *S. coelicolor* was grown on the standard medium SFM instead of MYM to see if there are any developmental defects. In addition to the reduction in growth of the *S. coelicolor* wild-type it also produces blue and red secondary metabolites under salt stress (**Figure 3.12**) which are most likely actinorhodin and undecylprodigiosin, respectively. All *S. coelicolor* strains produce a red secondary metabolite at three days under salt stress. The *S. coelicolor* Δ*mtrB* mutant however does not seem to produce coloured secondary metabolites on SFM plates. It is noteworthy that the *S. coelicolor* Δ*mtrB* mutant seems to be genetically unstable and produces colonies with several different phenotypes under salt stress (see supplementary data **S31-34**).

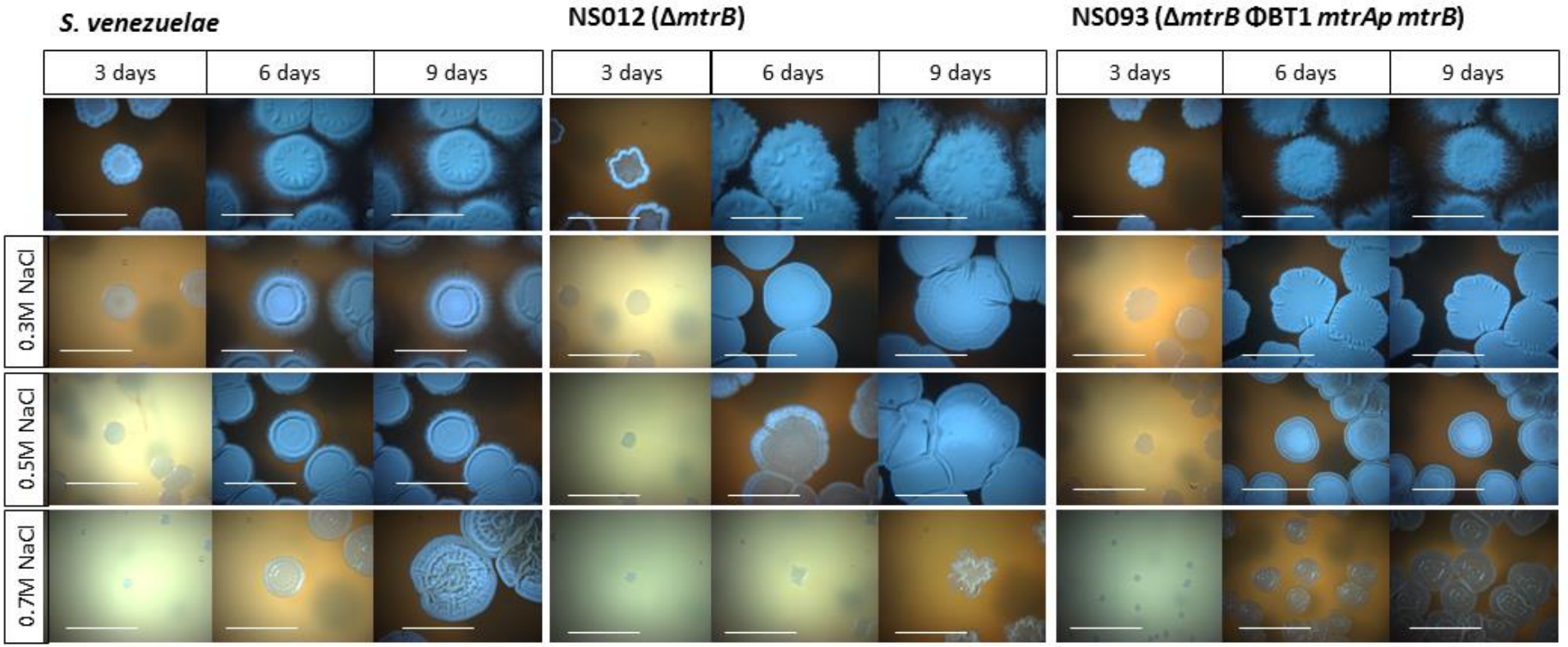

Figure 3.11 Phenotype of *S. venezuelae*, *S. venezuelae* Δ*mtrB* (NS012) and *S. venezuelae* Δ*mtrB* ΦBT1 *mtrAp mtrB* (NS093) on solid MYM medium with different salt concentrations. Scale bar 1 cm.

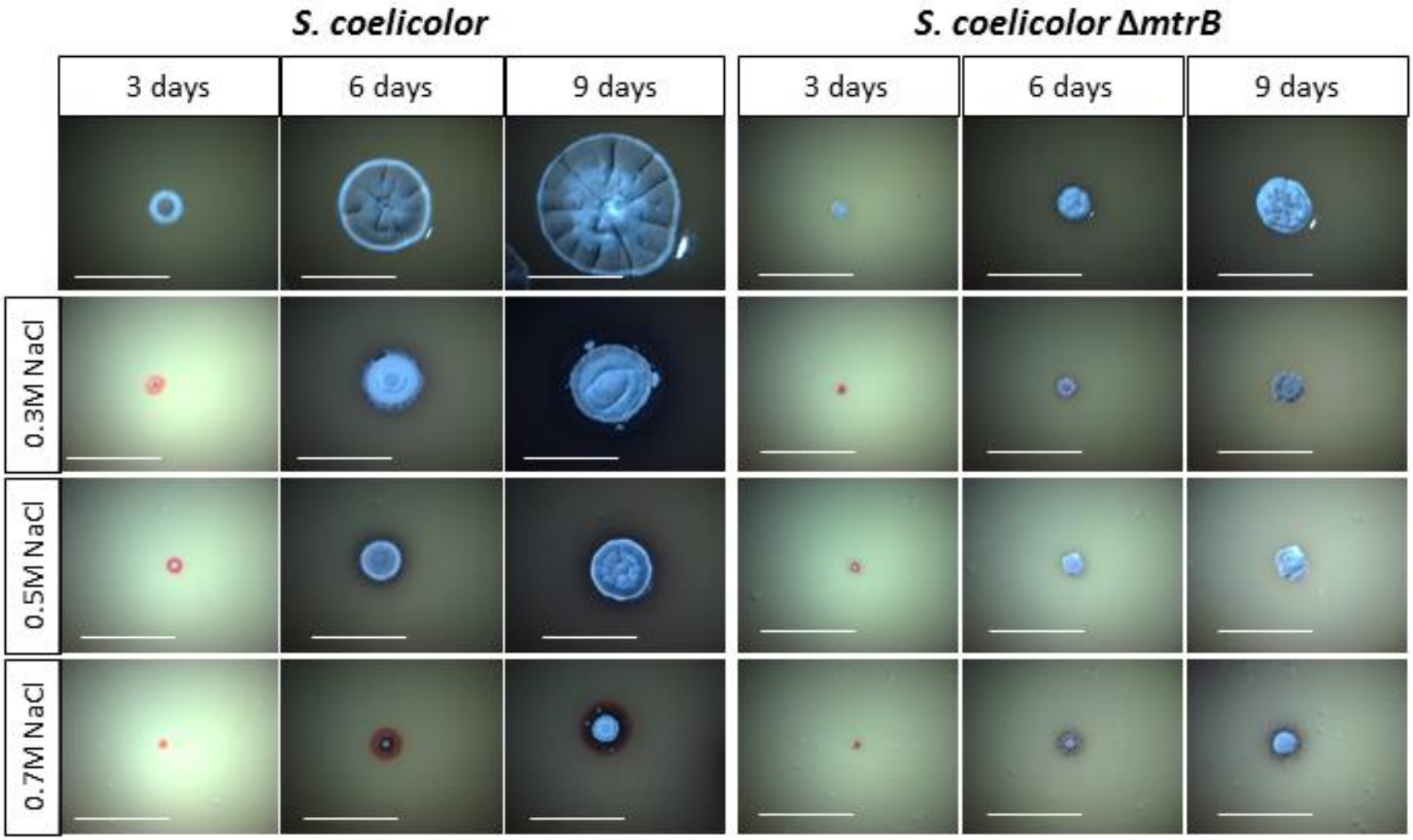

Figure 3.12 Phenotype of *S. coelicolor* and *S. coelicolor* Δ*mtrB* on solid SFM medium with different salt concentration. Scale bar 1 cm.

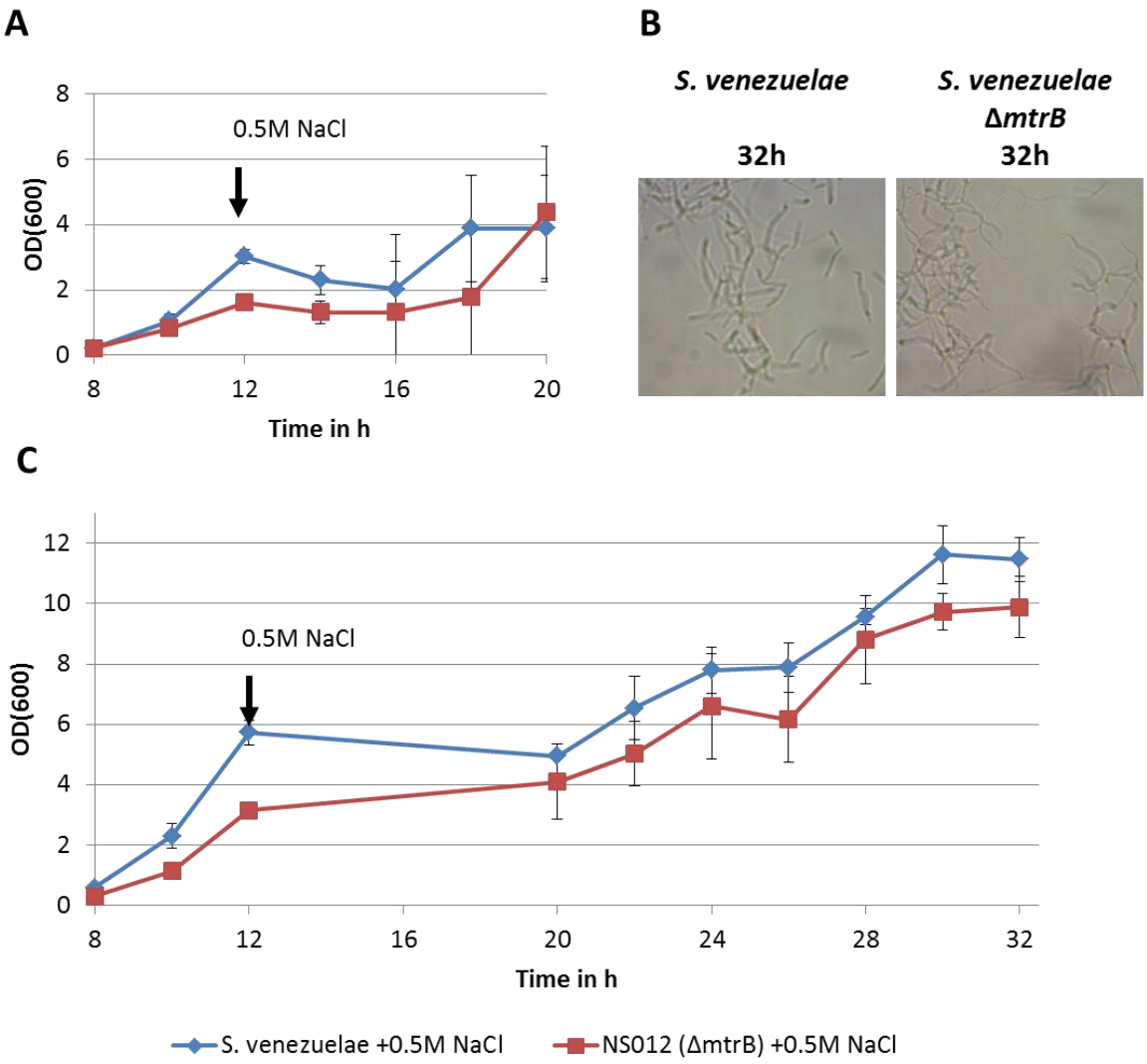

Figure 3.13 Growth curve of *S. venezuelae* wild-type and *S. venezuelae* Δ*mtrB* under salt stress. (**A)** Three individual cultures grown till 20 hours after NaCl stress. (**B)** Microscopic image of *S. venezuelae* and *S. venezuelae* Δ*mtrB* under NaCl stress at 32 hours. Spores could not be observed in both cultures. (**C)** Two individual cultures grown up to 32 hours after NaCl stress. Replicates of cultures were grown normally until 12 hours when 0.5 M NaCl was added. The cultures were inhibited in growth and did not grow exponentially. Thus, the growth rate cannot be calculated.

3.2.6 In the *S. venezuelae* Δ*mtrB* mutant secondary metabolites are increased in production

It was reported by Knowles (2014) that a *S. coelicolor ΔmtrB* mutant overproduces the antibiotic undecylprodigiosin. *S. venezuelae* does not produce any pigmented antibiotics, but it does encode gene clusters for the biosynthesis of the antibacterial agents chloramphenicol and jadomycin so we screened the Δ*mtrB* mutant for bioactivity against the Gram positive bacterium *Bacillus subtilis* and the Gram negative bacterium *Escherichia coli.*

3.2.7 *S. venezuelae* Δ*mtrB* inhibits the growth of *B. subtilis* and *E. coli*

Initially, wild-type *S. venezuelae* and *S. venezuelae* Δ*mtrB* were tested in a spot assay. Spores (2 µl) of each *Streptomyces* strain were spotted onto LB or MYM agar and incubated for 24 hours. Then cultures (2 µl) of the test strains were added and incubated for another 24 hours. No inhibition was observed on MYM medium but the growth of *B. subtilis* and *E. coli* was inhibited by both *Streptomyces* strains on LB medium. However, it seems that the *S. venezuelae* Δ*mtrB* mutant (NS012) inhibits the growth of the bacterial test strains more than the wild-type (**Figure 3.14**). To verify this result *S. venezuelae* and NS012 (Δ*mtrB*) were grown in liquid MYM and then the culture was methanol extracted. Methanol is an amphiphilic compound and can extract polar as well as non-polar compounds. The methanol extract was added to discs which were placed on top of *B. subtilis* in soft LB agar and incubated for 24 hours. The wild-type *S. venezuelae* methanol extract does not show any inhibition of *B. subtilis* whereas the methanol extract of NS012 (Δ*mtrB*) shows a clear zone of inhibition which shows that the *S. venezuelae* Δ*mtrB* mutant produces one or more antibiotics which inhibit the growth of *B. subtilis* (**Figure 3.14**).

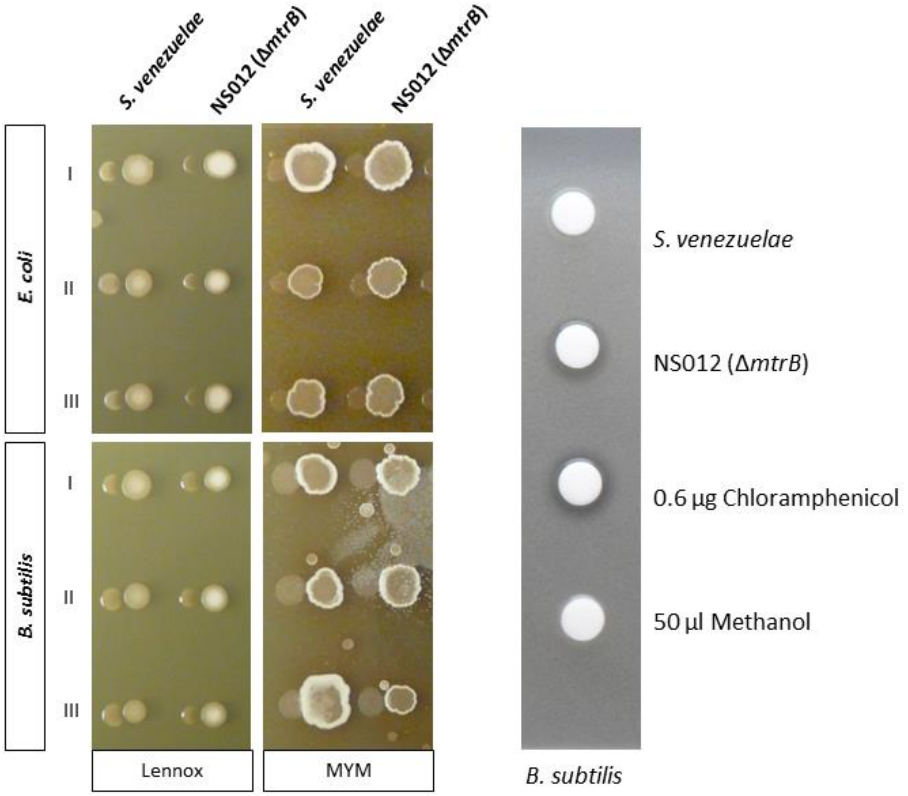

Figure 3.14 Left: Bioassay of *S. venezuelae* Δ*mtrB* against *E. coli* and *B. subtilis* on LB and MYM. Shown are three (I-III) technical replicates. **Right:** Methanol extraction of *S. venezuelae* wild-type and *S. venezuelae* Δ*mtrB* (NS012) cultures. Cells were grown for 24 hours and then methanol extracted. 50 µl of the extract was added to discs and then spotted on *B. subtilis* in soft LB agar and incubated for 24 hours.

3.2.8 Chloramphenicol production is increased in *S. venezuelae* Δ*mtrB* (NS012)

The methanol extracts of *S. venezuelae* and *S. venezuelae* Δ*mtrB* were analysed by LCMS by Dr. Daniel Heine (John Innes Centre). Three replicates were grown in liquid MYM for 12, 16, 24 and 36 hours. The results show that *S. venezuelae* Δ*mtrB* cultures produce more chloramphenicol than the wild-type (**Figure 3.15**). The Δ*mtrB* complemented strain was not available at the time of the experiment. It is necessary to repeat the chloramphenicol measurement with the complemented Δ*mtrB* mutant to verify that loss of *mtrB* is responsible for the increased production of chloramphenicol.

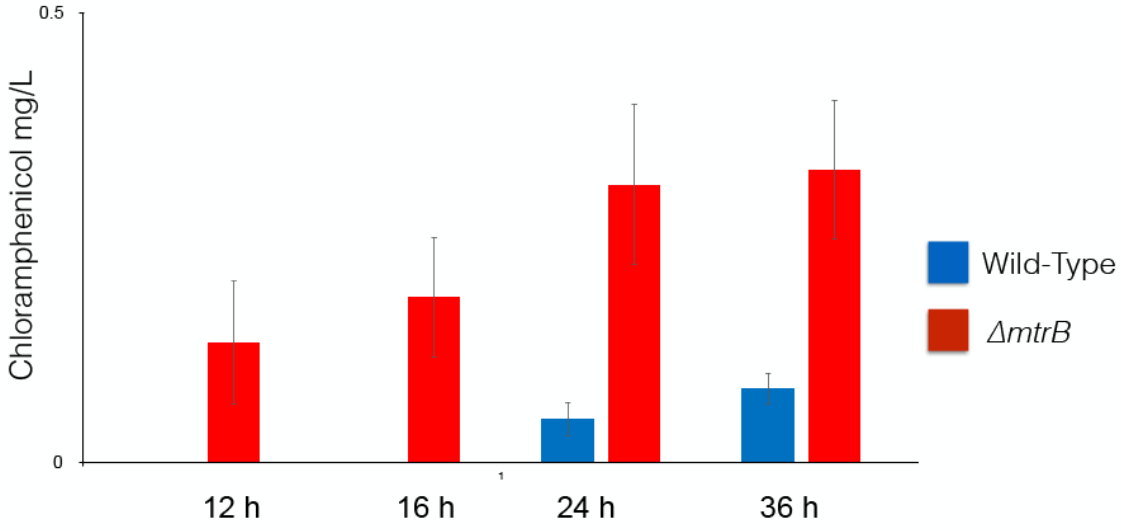

Figure 3.15 Concentration of chloramphenicol in *S. venezuelae* and *S. venezuelae* Δ*mtrB* culture at different time points, (Dr Daniel Heine, unpublished). The Δ*mtrB* complemented strain was not available at the time of the experiment.

3.2.9 *S. coelicolor* Δ*mtrB*: Increase in production of secondary metabolites

As mentioned above, Knowles (2014) reported that the *S. coelicolor* Δ*mtrB* mutant overproduces the red antibiotic undecylprodigiosin in liquid SMM medium. SMM medium contains ~82% of polyethylene glycol 6000 (PEG) which disturbs the analysis by LCMS. Therefore the *S. coelicolor* Δ*mtrB* mutant was grown in SMM without PEG, several different rich media including MYM and in minimal medium (MM). Since actinorhodin and undecylprodigiosin are coloured secondary metabolites the increase in production of these antibiotics can be easily spotted. The *S. coelicolor* Δ*mtrB* mutant produced only small amount of coloured pigments in SMM without PEG and MM whereas *S. coelicolor* Δ*mtrB* cultures were dark purple in liquid MYM after 2 days of

growth (**Figure 3.16**). This was the justification for choosing MYM to test for the production of secondary metabolites in *S. coelicolor*.

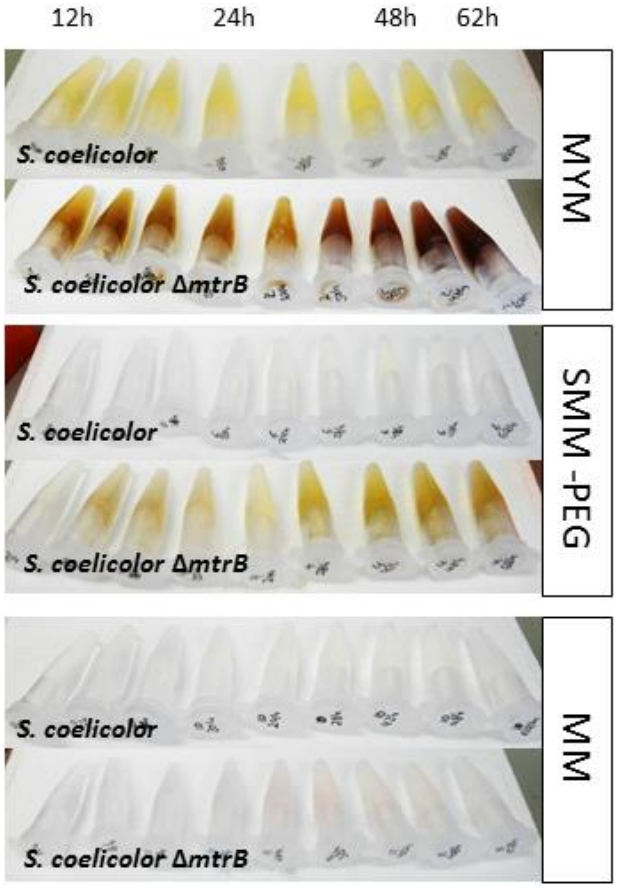

Figure 3.16 Culture supernatants of *S. coelicolor* and *S. coelicolor* Δ*mtrB* grown in different media.

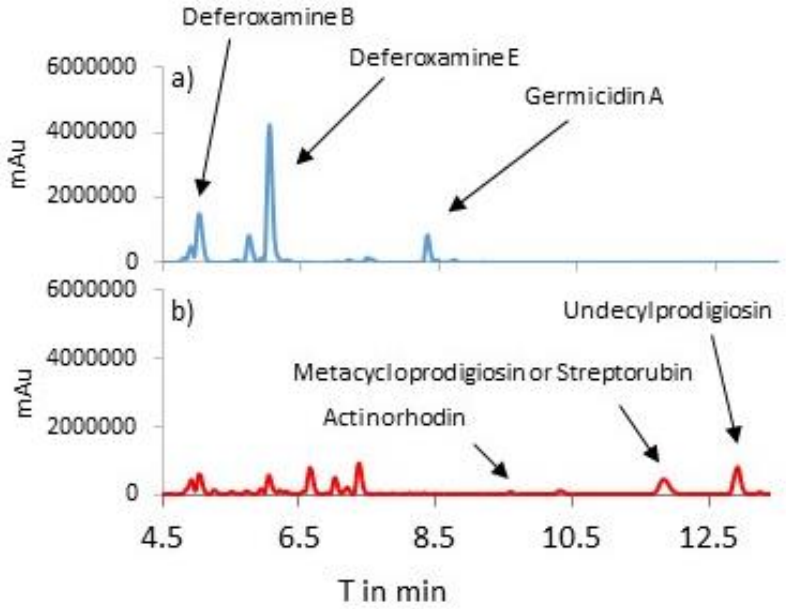

Figure 3.17 HPLC-MS analysis of extracts from a) *S. coelicolor* wild type and b) *S. coelicolor* Δ*mtrB* mutant. The Y-axis has been normalized for both profiles and shows the TIC (Total Ion

Chromatogram). The siderophores desferrioxamine B and E and germicidin A are reduced in production upon loss of MtrB while production of the antibiotics undecylprodigiosin and actinorhodin are upregulated. Metacycloprodigiosin and streptorubin has the same mass. To identify the compound, it has to be isolated and tested by NMR, (Dr Daniel Heine, unpublished).

The *S. coelicolor* Δ*mtrB* mutant over-produces the red antibiotic undecylprodigiosin and the blue antibiotic actinorhodin and we detected significant amounts of a compound which could be either metacycloprodigiosin, a potent anticancer compound made by the same pathway as undecylprodigiosin or streptorubin. Both compounds have the same mass and to identify which compound is increased in production it has to be isolated and analysed by NMR. Other secondary metabolites are clearly reduced in yield in the Δ*mtrB* mutant, including the desferrioxamine B and E siderophores and germicidin A, see **Figure 3.17**. This preliminary data needs further validation by analysis of more biological replicates. Furthermore, the *S. coelicolor* Δ*mtrB* complementation strain has to be analysed to confirm that the increase in antibiotic production is caused by the loss of *mtrB.*

3.3 Gain of function MtrA

MtrA(TB) and MtrA(Sv) are highly similar with a protein identity of 74%. The crystal structure of unphosphorylated MtrA(TB) has been solved (Friedland et al. 2007) and based on this structure an $MtrA(TB)_{Y102C}$ protein was active independent of MtrB. In fact $MtrA(TB)_{Y102C}$ binds to its target genes independently of phosphorylation leading to a permanently active, gain-of-function MtrA protein (Plocinska et al. 2012; Satsangi et al. 2013).

The following section explores if the same MtrA gain of function mutation is functional in *S. venezuelae*. To compare MtrA in the distantly related *M. tuberculosis* and *S. venezuelae,* two different protein modelling tools were used with the results showing that the tertiary structure of MtrA in both organisms is likely to be highly similar (**Figure 3.18**). The phosphorylation domains (in yellow / orange) of MtrA(TB) and MtrA(Sv) contain a conserved tyrosine residue Y102 and Y99, respectively, which forms a hydrogen bond with the aspartic acid residue D190 or D187, respectively, in the DNA binding domain (green / olive green). If this tyrosine is changed to a cysteine MtrA(TB) becomes permanently active, most likely because the hydrogen bond between the two amino acids is broken.

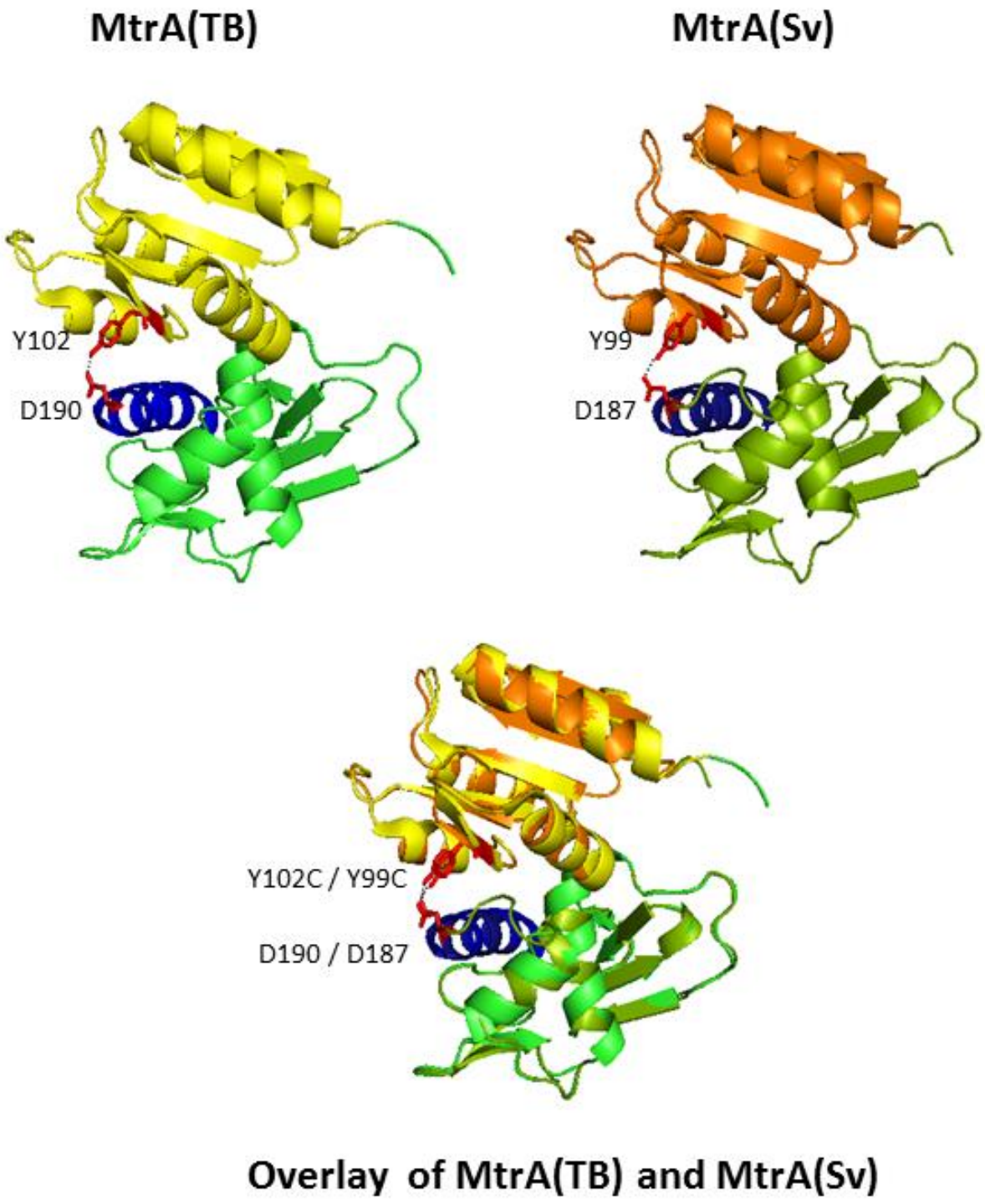

Figure 3.18 Left: Crystal structure of MtrA(TB), (Friedland et al. 2007). **Right**: The modelled structure of MtrA(Sv). **Bottom**: Overlay of MtrA(TB) and MtrA(Sv). The MtrA(TB) structure was obtained from the protein data bank (http://www.rcsb.org). Accesion number for MtrA(TB) is 2GWR. The structure of MtrA(Sv) was modelled with https://toolkit.tuebingen.mpg.de/hhpred and http://www.sbg.bio.ic.ac.uk/~phyre2. Both models were similar and for simplicity only the phyre2 model is shown.

To test if this gain-of-function mutation has an effect in *S. venezuelae*, two different gain of function constructs were designed with MtrA(TB)$_{Y102C}$ and MtrA(Sv)$_{Y99C}$ under the control of the *S. venezuelae mtrA* promoter (*mtrA*(Sv)*p*). Additionally, the wild-type MtrA(TB) protein was also expressed using *mtrA*(Sv)*p* to investigate if MtrA(TB) is functional in *S. venezuelae*. All three constructs were introduced in the ΦBT1 site of *S. venezuelae*.

The *S. venezuelae* Δ*mtrB* mutant produced more chloramphenicol than the wild-type after 24 hours of growth in liquid MYM medium. Therefore, the gain of function mutants were tested for chloramphenicol production at 24 hours of growth by LCMS by Dr Daniel Heine. Three individual clones of the *S. venezuelae* Δ*mtrB* and the MtrA mutant proteins [MtrA(TB), MtrA(TB)$_{Y102C}$ and MtrA(Sv)$_{Y99C}$] were grown and measured in technical replicates by LCMS. The LCMS traces of each biological and technical replicate are shown in the supplementary data, **Figure S39-45**. **Figure 3.19** shows the average chloramphenicol concentration of *S. venezuelae*, Δ*mtrB,* and the MtrA(TB), MtrA(TB)$_{Y102C}$ and MtrA(Sv)$_{Y99C}$ mutant strains. The *S. venezuelae* wild-type produced 0.05 ± 0.02 µg/ml. The Δ*mtrB* mutant produced 0.4 ± 0.03 µg/ml which is 8-times more chloramphenicol than the wild-type. The MtrA(TB) and MtrA(TB)$_{Y102C}$ mutant strains produced 0.64 ± 0.28 and 0.7 ± 0.18 µg/ml chloramphenicol, respectively. The increase in chloramphenicol production in the MtrA(TB) and MtrA(TB)$_{Y102C}$ mutant strains is statistical significant with $p = 5.8 * 10^{-7}$ for both strains compared to the wild-type chloramphenicol concentration. Additionally, in both strains containing the MtrA(TB) protein the chloramphenicol production is significantly higher than in the Δ*mtrB* mutant ($p = 0.033$). The average chloramphenicol concentration in the MtrA(Sv)$_{Y99C}$ mutant was 0.38 ± 0.48 µg/ml and no statistical significance between this mutant and the wild-type could be observed. Thus, the individual clones of the MtrA(Sv)$_{Y99C}$ were investigated individually. The first clone, NS099, produced nearly 4-times more chloramphenicol than the wild-type and this increase in production is statistical significant ($p = 0.015$). The second clone, NS100, produced similar concentrations of chloramphenicol than the wild-type. The third clone, NS101, produced 1.21 ± 0.51 µg/ml which is 24-times more than the wild-type. However, the increase of chloramphenicol production is not statistically significant due to the high sample variation. Although some variation between the biological and technical replicates can be observed, the increased production of chloramphenicol is most likely due to the introduction of the gain of function MtrA

proteins because introduction of a second copy of MtrA(Sv) under its native promoter in the integrative site does not result in an increased production in chloramphenicol (**Figure S44**). Due to the high sample variation, especially in the MtrA(Sv)$_{Y99C}$ mutant it is necessary to repeat the experiment in future work to verify that the increased production is based on the introduction of the gain of function MtrA proteins.

To see if the gain of function proteins and MtrA(TB) are functional attempts were made to delete the native MtrA but all attempts were unsuccessful, probably because they are constitutively active and therefore toxic.

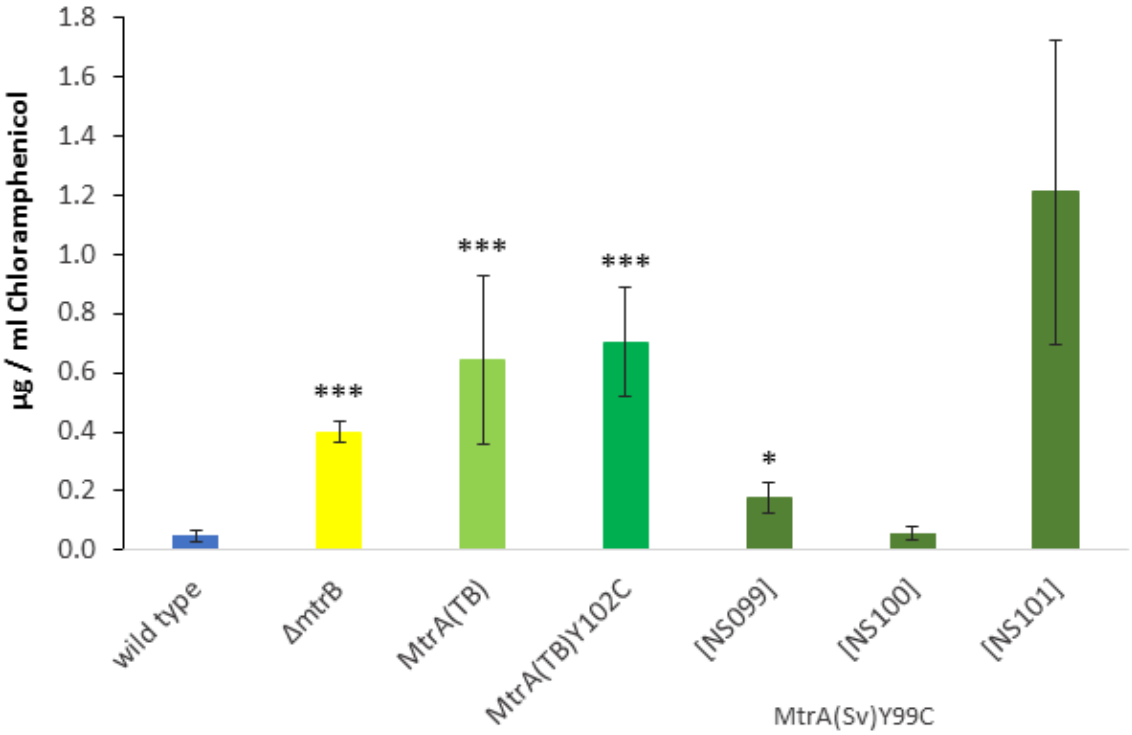

Figure 3.19 Average chloramphenicol concentration of methanol extracted cultures containing different MtrA mutant proteins. For each mutant three individual isolated clones were measured in three technical replicates. The average of three technical replicates of three individual clones of the MtrA(Sv)$_{Y99C}$ are shown to demonstrate the high sample variation in this strain. LCMS chromatograms of samples are shown in **S39-45**. Statistical significance was determined by t-test, *** $p \leq 0.005$, * $p \leq 0.05$

3.4 Discussion

3.4.1 MtrA is essential in *S. venezuelae*

It was only possible to delete *mtrA* in *S. venezuelae* in the presence of a second copy which was integrated *in trans* in the genome. That suggests that *S. venezuelae* cells need MtrA in order to survive. This is consistent with published reports on *M. tuberculosis* in which *mtrA* is also essential and can only be deleted if a second copy is present *in trans* (Zahrt & Deretic 2000). However, MtrA is not essential in all Actinobacteria since *mtrA* can be deleted in *C. glutamicum* (Möker et al. 2004; Brocker & Bott 2006). Why is MtrA essential in *M. tuberculosis* but not in *C. glutamicum*? The answer could be found in the targets bound and controlled by MtrA. Confirmed targets of MtrA(TB) include *dnaA*, which encodes the chromosome replication initiation protein DnaA and the origin of DNA replication, *oriC*. MtrA(TB) also interacts directly with the DnaA protein and controls a number of genes required for cell division, including *sepF* (Purushotham et al. 2015). This suggests that MtrA(TB) is involved in coordinating DNA replication and cell division, both of which are essential processes in all living organisms. In *C. glutamicum*, MtrAB are known to be involved in sensing and responding to osmotic stress but they do not regulate DNA replication or any essential cell division genes, which could explain why MtrA is dispensable in this strain. However, it is also possible that the regulon is not completely defined since they used ChIP-chip and not ChIP-seq which is a lot more sensitive. They also defined the regulon under a single growth condition. Furthermore, neither the Δ*mtrA* or Δ*mtrAB* mutants were complemented in *C. glutamicum* so it is feasible they have acquired compensatory secondary mutations.

Since MtrA is essential in *S. venezuelae* it is difficult to manipulate the *mtrA* gene in order to study its function. We attempted to make MtrA(Sv) more tractable by adding a 3xFlag-tag to MtrA. Because the *mtrA-3xflag* gene was integrated *in trans* and then the native *mtrA* gene was deleted it was important to analyse if there are any polar effects on MtrB and LpqB but this is unlikely to be the case because the *S. venezuelae mtrA*::APR ΦBT1 *mtrAp-mtrA* (NS042-44) strains do not show any abnormalities in growth or development under the tested conditions (**Figure 3.2** and **3.3**). A similar observation was made when MtrA was constitutively expressed under the *ermE** promoter and this is consistent with the findings in *M. tuberculosis*. When MtrA(TB) was overexpressed the growth of *M. tuberculosis* was inhibited in macrophages, mice lungs and spleens but the growth in broth was not affected (Fol et al. 2006). Furthermore, the decreased replication

in macrophages could be restored when MtrA(TB) was overexpressed with its cognate HK MtrB. The authors conclude that it is not the level of MtrA(TB) which regulates its targets but rather the phosphorylation state of MtrA(TB) (Fol et al. 2006). Since the overexpression of MtrA in *S. venezuelae* does not show a phenotype similarly to *M. tuberculosis* growth in broth it is likely that the MtrAB TCS in *M. tuberculosis* and *S. venezuelae* function in a similar way which could indicate that the function of MtrA(Sv) is determined by its phosphorylation state rather than the level of expression.

S. venezuelae grows exponentially in MYM medium after an 8-hour lag phase and starts to sporulate at approximately 16 hours. It is noteworthy that *S. venezuelae*, as a filamentous organism, is difficult to grow consistently. Therefore, four replicates were used to calculate the growth rate. Although great care was taken to grow *S. venezuelae* strains consistently, the growth can vary due to the filamentous growth. The strains containing pIJ102357 seems to grow slower than wild-type and the MtrA overexpression strain. However, this was not investigated further in this work because the difference is not statistically significant (P(T<=t) two-tail = 0.06 and *S. venezuelae* and *S. venezuelae ermEp* mtrA* grow at the same growth rate which shows that increased level of MtrA(Sv) do not influence the growth in liquid culture.

We conclude from these experiments that MtrA is essential in *S. venezuelae* which might indicate an involvement in essential processes such as DNA replication and / or cell division. We further conclude that the cellular function and activity of MtrA(Sv) is controlled by altering the ratio of phosphorylated to unphosphorylated MtrA during the *S. venezuelae* life cycle.

3.4.2 Deletion of *mtrB*

To study the function of the histidine kinase MtrB the encoding gene was deleted in *S. venezuelae* using the λ RED based PCR targeting system (Gust et al. 2003). The *mtrB* deletion mutants in *M. tuberculosis*, *C. glutamicum* and *S. coelicolor* show a severe cell division defect. In *M. tuberculosis* (Plocinska et al. 2012) and *C. glutamicum* (Möker et al. 2004) Δ*mtrB* mutant cells are elongated which are similar to temperature sensitive depletion mutants of FtsZ in *E. coli* (Addinall et al. 1996). The *S. coelicolor* Δ*mtrB* mutant cells also show irregular septum formation (Knowles 2014). Surprisingly, *S. venezuelae* Δ*mtrB* has a divalent phenotype with normal growing vegetative and aerial hyphae at the outside and growth defect at the inside of the colony. The *S. venezuelae* Δ*mtrB* mutant

seems to have a mild growth defect on solid MYM medium and, although the growth rate is lower for *S. venezuelae* Δ*mtrB* compared to the wild-type in liquid culture, (0.36 ± 0.002 compared to 0.42 ± 0.041, respectively) this was not statistically significant (**Figure 3.8**).

Interestingly the *S. venezuelae* Δ*mtrB* mutant although being delayed in aerial hyphae formation sporulates in a similar manner to the wild-type at 16 hours in liquid MYM medium and sporulates like the wild-type at six days on solid MYM medium. With the time points presented here for solid medium it is difficult to say if there is a delay in sporulation on solid medium. However, it is possible that there is no delay in sporulation on agar plates in the *S. venezuelae* Δ*mtrB* mutant because this mutant starts to sporulate at the same time as the wild-type in liquid culture. The sporulation in liquid culture of *S. venezuelae* wild-type and the *S. venezuelae* Δ*mtrB* mutant were investigated visually but not quantitatively. It is possible that the *S. venezuelae* Δ*mtrB* mutant produces less spores at 16-hour time point. This could be measured by separation of the spores from the vegetative mycelium by filtration and subsequent plating of serial dilutions to determine to spore count in the mutant compared to the wild-type.

Additionally, the SEM images reveal that the spores and the septa of the *S. venezuelae* Δ*mtrB* mutant look like wild-type spores except for the drastic growth defect in the middle of the colony. The *S. venezuelae* Δ*mtrB* spore chains on the outside of the colony have regular septa. This was unexpected because the *S. coelicolor* Δ*mtrB* mutant has irregular septa (Knowles 2014). In *M. tuberculosis*, MtrB interacts with components of the divisome, Wag31 and FtsI (homologs of DivIVA and PBPB in *Streptomyces*, respectively) (Plocinska et al. 2014). Since the deletion of *mtrB* leads to the formation of irregular septa in *S. coelicolor* and given the localisation of MtrB(TB) at the cell division site it is likely that MtrB is also involved in regulating the divisome in *S. coelicolor* as well. Contradictorily, this does not seem to be the case in *S. venezuelae* at least in that deleting *mtrB* has little effect on cell division but we do not yet know anything about the localisation of MtrB(Sv) during the *Streptomyces* life cycle.

Deletion of *mtrB* in the distantly related species *S. coelicolor* and *S. venezuelae* results in quite different colony morphological phenotypes. *S. coelicolor* Δ*mtrB* colonies are small and produce few spores of irregular size that can be complemented by reintroduction of *mtrB* in *trans*. In contrast the *S. venezuelae* Δ*mtrB* colonies are larger than the wild-type and form normal spores, at least at the outside of the colony, but there

is a serious defect in the spore chains formed at the centres of the Δ*mtrB* colonies. It is not known if the *S. coelicolor* Δ*mtrB* mutant displays a different phenotype in the middle of the colony. Thus, it is possible that MtrB has different roles in these two organisms or perhaps just that loss of MtrB has a more drastic effect in *S. coelicolor* whereas *S. venezuelae* somehow compensates better for this loss. It is noteworthy that *S. venezuelae* Δ*mtrB* and *S. coelicolor* Δ*mtrB* were grown on MYM and SFM respectively. These media are standard for phenotypic analysis. It is possible that the different phenotypes of the deletion of *mtrB* in *S. venezuelae* and *S. coelicolor* could be a result of the different media compositions which could be tested by cultivating *S. venezuelae* Δ*mtrB* on SFM plates since *S. coelicolor* does not develop on MYM. However, both Δ*mtrB* mutant strains over-produce antibiotics and both are more sensitive to salt stress which indicates that MtrB could sense salt and/or osmotic stress. It remains to be determined if MtrB(Sv) is involved in the formation of the divisome.

The complementation strain of the *mtrB* deletion in *S. venezuelae* seemed to recover the loss of *mtrB* because the complementation strains forms aerial hyphae at three days similar to the wild-type whereas the Δ*mtrB* mutant is delayed in aerial hyphae formation. Despite the recovery of the early aerial hyphae formation phenotype the colony morphology of the complemented *mtrB* mutant resembles more of the Δ*mtrB* mutant than the wild-type. This indicates that the complementation of *mtrB* is only partially. This is even more obvious when the strains were cultivated on MYM plates containing increasing concentration of NaCl. The lack of complementation in the *mtrB* mutant could be due to a polar effect on *lpqB*, see section 3.4.3.

Why is there a drastic growth defect in the middle of the *S. venezuelae* Δ*mtrB* mutant colonies? To answer the question, it is important to look at the morphology of a *Streptomyces* colony. H. Wildermuth studied the structure of individual *S. coelicolor* colonies by microscopy of thin section (Wildermuth 1969). In the middle of the colony aerial hyphae grow on top of substrate mycelium and other aerial hyphae. The aerial hyphae at the bottom either sporulate or undergo cell lysis. The amount of lysed aerial hyphae increased towards the middle of the colony. When cells lyse they release the content of the cells into the surrounding environment which increases the osmotic potential. If MtrB(Sv) senses osmotic stress, the *S. venezuelae* Δ*mtrB* mutant would not be able to sense this osmotic stress caused by cell lysis in the middle of the colony and therefore could not induce the osmotic stress response. This possibly explains the growth

defect observed in *S. venezuelae* Δ*mtrB* grown on solid MYM agar medium and could be a possible reason why the *S. venezuelae* Δ*mtrB* mutant grows normally in liquid culture.

3.4.3 The role of LpqB

We know relatively little about the LpqB lipoprotein which is co-encoded with MtrAB in Actinobacteria, but it is one of the 233 conserved signature proteins in Actinobacteria (Gao et al. 2006). It was reported that in *M. smegmatis*, LpqB interacts with the sensor domain of MtrB on the outside of the cell and that this interaction modulates phosphotransfer from MtrB to MtrA (Nguyen et al. 2010). Unfortunately, all attempts to delete *lpqB* in *S. venezuelae* were unsuccessful, or at least the *lpqB* mutants that were generated could not be confirmed using PCR. It was possible to delete *lpqB* in *S. coelicolor* (Knowles 2014) but it remains to be determined if *lpqB* is essential or dispensable in *S. venezuelae*.

Deletion of *S. venezuelae mtrB* has a polar effect on *lpqB.* The *mtrB* and *lpqB* coding sequences overlap by 11 bp but the start codon of *lpqB* was left intact when the deletion of *mtrB* was designed. However, in the Δ*mtrB* mutant, the ribosomal binding site of *lpqB* is removed thus it is possible that *lpqB* is not transcribed in the Δ*mtrB* strain. This could be a possible explanation why the Δ*mtrB* mutant containing a copy of *mtrB* under its native promoter only complements partially under normal growth conditions and under salt stress. In contrast the *S. coelicolor* Δ*mtrB* mutant fully complements under normal growth conditions (Knowles 2014) and it remains to be subject of future work to determine if the *S. coelicolor* Δ*mtrB* complementation strains restores the wild-type phenotype under NaCl stress conditions. In the deletion of *mtrB* in *S. coelicolor* four base pairs were left in front of the start codon of *lpqB* which seems to be sufficient to ensure transcription of *lpqB.* The *S. coelicolor* Δ*lpqB* mutant sporulates normally but with irregular septa formation and the Δ*lpqB* mutant overproduces undecylprodigiosin. It remains elusive if the deletion of *lpqB* in *S. venezuelae* has a similar effect on development and secondary metabolite production.

3.4.4 Increased production of secondary metabolites in *Streptomyces* Δ*mtrB* mutants

Although the morphological phenotypes are different in *S. coelicolor* and *S. venezuelae* Δ*mtrB* mutants both strains show an increased production of antibiotics.

Chloramphenicol production is increased in the *S. venezuelae* Δ*mtrB* mutant on solid agar and in liquid culture and actinorhodin and undecylprodigiosin production is increased in the *S. coelicolor* Δ*mtrB* mutant in liquid MYM. It is possible that this is also linked to the osmotic stress response.

Osmotic stress is disastrous for a bacterial cell. During osmotic stress the turgor pressure rises which can be fatal if the bacterial cell does not counteract this stress. Therefore bacteria have many sensors to detect this fatal stress like sensor kinases, transporters, P-type ATPases and channels (Wood 1999). *Streptomyces* have at least two sensing mechanism: OsaAB (Bishop et al. 2004) which is modulated by the osmoregulator OsaC (Fernández Martánez et al. 2009) and the *kdpDEFABC* operon which contains the TCS KdpDF and a p-type ATPase (Hutchings et al. 2004). This means that MtrB, if indeed it is an osmosensor, is not the only system to sense osmotic stress and this could explain why the Δ*mtrB* mutant cells can still grow under osmotic stress conditions.

An *S. coelicolor* Δ*osaB* mutant overproduces actinorhodin and undecylprodigiosin when osmotic stress was induced (Bishop et al. 2004). The *S. coelicolor* Δ*osaB* mutant is not able to adapt to osmotic stress conditions and it was hypothesised that the overproduction of antibiotics could be due to the interference of the complex response to osmotic stress (Bishop et al. 2004). If MtrB senses osmotic stress in *Streptomyces* then loss of MtrB would mean that *Streptomyces* cells are not able to sense osmotic stress anymore with this system and the *Streptomyces* Δ*mtrB* mutants might overproduce antibiotics under non-stressful conditions, i.e. it is possible that the deletion of *mtrB* mimics osmotic stress because MtrA is permanently active.

MtrA(TB) can be phosphorylated by small phosphate donors (Friedland et al. 2007) and MtrB(Cg) can act as a phosphatase as well as a kinase (Möker et al. 2007). That implies that MtrA can be phosphorylated in the cell independently from MtrB and that MtrB regulates the phosphorylation status of MtrA by acting as an MtrA specific kinase or phosphatase under inducing and non-inducing conditions. That suggests that in an Δ*mtrB* mutant the MtrA protein is phosphorylated by cross talk and / or small phosphor donors and cannot be dephosphorylated by MtrB which leads to a permanent active MtrA protein which in turn could lead to the over production of antibiotics. To distinguish between these possibilities, I decided to investigate the regulon of MtrAB in *S. venezuelae* using ChIP-seq and this is described in Chapter 4.

3.4.5 Gain of function MtrA proteins

Expression of the gain of function MtrA proteins and wild-type MtrA(TB) resulted in the increased production of chloramphenicol in wild-type *S. venezuelae*. The wild-type strains expressing MtrA(TB) and MtrA(TB)$_{Y102}$ produce significantly more chloramphenicol than the Δ*mtrB* mutant and the wild-type whereas the production of chloramphenicol production in the MtrA(Sv)$_{Y99C}$ strain is not significant due to the high sample variation. In order to determine if the increased production of chloramphenicol is consistent more replicates need to be tested since the biological and technical replicates gain of function strains show a high sample variation (see **Figure 3.19** and **S39 - 45**).

All attempts to delete the native *mtrA* in the presence of the gain of function proteins were unsuccessful which indicates that the gain of function proteins are either not functional or toxic to the cell. MtrA should be expressed in a mixture of native MtrA and gain of function protein. It is expected that MtrA and gain of function *mtrA* proteins should be expressed in a ratio of 50:50 because both copies are expressed by the native *mtrA* promoter. However, we know very little about the autoregulation of *mtrA* and the introduction of the gain of function proteins in the integrative site could lead to a different expression than the wild-type protein due to the different location in the chromosome. The possible difference in expression could lead to different ratios of native to gain of function MtrA. This in turn could result in different production of chloramphenicol in two genetically similar gain of function strains. Also, it is possible that the gain of function proteins are not regulating the production of chloramphenicol directly. The possible toxicity of the gain of function MtrA could lead to an overall stress response in the cell which can lead to increased antibiotic production (Vohradsky et al. 2000). It has to be determined in future experiments if the gain of function MtrA mutant proteins are not functional or toxic to the cell.

For unknown reasons the chloramphenicol BGC is silent in wild-type *S. venezuelae* (Fernández-Martínez et al. 2014). It is unlikely that the overproduction of chloramphenicol is due to a second copy of MtrA which could lead to elevated levels of MtrA since a strain containing the native MtrA and a second copy of MtrA under its native promoter integrated in the ΦBT1 site does not overproduce chloramphenicol (see **S44**).

Intriguingly, *S. venezuelae* containing MtrA(TB) overproduces chloramphenicol. It is likely that MtrA(TB) binds to the same binding site as MtrA(Sv) to activate

chloramphenicol production because MtrA(TB) and MtrA(Sv) are very similar in their DNA binding domain. Therefore MtrA(TB) must be more active which means a greater proportion of MtrA(TB) is phosphorylated. MtrA is very similar in protein identity but MtrB only shares 55% protein identity in *S. venezuelae* and *M. tuberculosis*. This could mean that the specificity of the protein-protein interaction of MtrB(Sv) with MtrA(TB) is reduced and thus MtrB(Sv) dephosphorylates MtrA(TB) with lower efficiency which leads to higher levels of active MtrA(TB) in the cell. This could also be the reason why the production of chloramphenicol in *S. venezuelae* MtrA(TB)$_{Y102C}$ increased compared to the wild-type and Δ*mtrB* mutant. However, the phosphorylation state of the gain of function protein and MtrA(TB) have to be determined experimentally.

Additionally, the presence of the gain of function mutation causes the overproduction of chloramphenicol. The amino acid change from Y102C/Y99C removes the hydrogen bond between Y102/Y99 and D190/D187 in the inactive form of MtrA(TB) (Friedland et al. 2007) which may lead to a change in the tertiary structure of MtrA. This change could in turn lead to an increased ability of MtrA to form dimers which promotes DNA binding. It is interesting that the strain containing MtrA(TB)$_{Y102C}$ produces more chloramphenicol than MtrA(Sv)$_{Y99C}$. It is likely that the DNA binding affinity of MtrA(TB)$_{Y102C}$ is higher than native MtrA and thus MtrA(TB)$_{Y102C}$ could outcompete the native MtrA.

Further experiments are needed to demonstrate if MtrA gain of function mutant proteins induce overproduction of silent gene clusters. If it can be proven, then MtrA gain of function proteins could be a new tool to activate silent gene clusters in *Streptomyces* since MtrA is highly conserved.

3.5 Conclusion and future work

The genetic manipulation of the MtrAB-LpqB system in *S. venezuelae* outlined in this chapter implicates that *mtrA* is essential and that this TCS is involved in the regulation of secondary metabolite production and osmotic stress response in *S. venezuelae*. Furthermore, the overexpression of *mtrA* in the wild-type background does not cause a visible phenotype, which indicates that it is the phosphorylation state of MtrA and not the proteins level which activates this RR. In contrast, it was possible to delete *mtrB*, which implies that MtrA is phosphorylated by small phosphor donors or other HKs. Otherwise the deletion of *mtrB* would be lethal.

The *S. venezuelae* Δ*mtrB* mutant has a divalent phenotype. The Δ*mtrB* colonies are delayed in aerial hyphae formation in the middle of the colony, whereas the outside of the colony seems to develop similar to the wild-type. A possible reason for this phenotype could be osmotic stress which occurs in the middle of *Streptomyces* colonies due to their unique growth. This hypothesis is supported by the cultivation of the *S. venezuelae* Δ*mtrB* mutant on agar plates contain increasing concentrations of NaCl. Under NaCl stress the Δ*mtrB* mutant is reduced in growth and development compared to the wild-type. The cultivation on NaCl plates also supported the partial complementation of the Δ*mtrB* mutant. Under normal growth conditions and under NaCl stress the Δ*mtrB* mutant partially complements. Therefore, it is necessary to investigate the complementation strain by SEM. Also, it is not clear with the data presented if the increased production of secondary metabolites is due to the loss of *mtrB* or if this is caused by the polar effect on *lpqB*. Thus, the LCMS analysis of the secondary metabolites has to be repeated with the Δ*mtrB* complementation strain in *S. venezuelae* and *S. coelicolor*. So far it remains elusive if LpqB is essential in *S. venezuelae* and the deletion of *lpqB* has to be confirmed either by PCR or southern blot to investigate if LpqB feeds additional signals in the TCS. Also, to avoid a polar effect *mtrB* should be deleted with the ribosomal binding site of *lpqB* remaining intact in the genome. Additionally, we investigated the chloramphenicol production in the MtrA gain of function strains. And the preliminary data indicates that the MtrA gain of function mutants produce chloramphenicol which is not produced under lab conditions. However, the gain of function strains need to be investigated further. The MtrA(TB) gain of function protein complements the Δ*mtrB* mutant in *M. smegmatis* which can be addressed in *S. venezuelae* by introducing the gain of function constructs in the Δ*mtrB* mutant.

The data presented in this chapter indicate that MtrA is involved in essential processes in *S. venezuelae* and that alteration of the ratio of phosphorylation of MtrA either by removing the cognate HK or introducing gain of function proteins leads to increased production of secondary metabolites and activation of silent gene clusters.

4 Targets and regulon of MtrA in *S. venezuelae* and *S. coelicolor*

The genetic manipulation of the MtrAB TCS outlined in chapter 3 showed that the RR MtrA is essential in *S. venezuelae* and that manipulating the MtrAB TCS by either removing the HK MtrB or introducing a MtrA gain of function protein leads to increased production of secondary metabolites in *S. venezuelae* and *S. coelicolor*. It is possible that the essentiality of MtrA arises due to the transcriptional regulation of essential target genes. MtrA(TB) regulates expression of the DNA replication initiation gene *dnaA* (Li et al. 2010) and also interacts on protein level with DnaA (Purushotham et al. 2015) which might be the reason for the essentiality of MtrA in *M. tuberculosis*. Furthermore, it is not clear if MtrA regulates secondary metabolite production by an indirect global response to stress or if secondary metabolites are directly regulated. To investigate the function of MtrAB in more depth it is necessary to identify target genes of MtrA. In this chapter I will identify target genes by Chromatin Immunoprecipitation and Sequencing (ChIP-seq) in *S. venezuelae* and *S. coelicolor*.

4.1 Chromatin Immunoprecipitation and Sequencing (ChIP-seq) of MtrA in *S. venezuelae* over the developmental time course

ChIP-seq is a technique which can reveal the genome wide binding of a protein to DNA. This is particularly of interest for transcription factors such as the RR MtrA. In recent years ChIP-seq was used in *S. venezuelae* to identify transcription factor involved in control of development (Bush et al. 2013; Tschowri et al. 2014; Al-Bassam et al. 2014; Bush et al. 2016). In ChIP-seq DNA binding proteins are crosslink with DNA with formaldehyde *in vivo* and then the chromosomal DNA is sheared by sonication to break the DNA in small fragments. Then the DNA-protein complex is immunoprecipitated with an antibody specific to the protein of interest. This can either be a polyclonal antibody or an antibody binding to a protein tag. In the last step, the crosslinking is reversed to release the DNA which can be determined by next generation sequencing.

MtrA target genes in Mycobacteria and *C. glutamicum* were identified by ChIP and subsequent QRT-PCR and ChIP-to-ChIP, respectively (Fol et al. 2006; Li et al. 2010; Brocker & Bott 2006). However the complete regulon in Mycobacteria is not published but MtrA regulates among others the DNA replication initiation protein DnaA (Fol et al. 2006). In *C. glutamicum* genes involved in the osmotic stress response were identified (Brocker & Bott 2006). Also, we know that MtrA targets *mce* in *S. coelicolor* (Clark et

al. 2013) and that RR autoregulate their own transcription (Groisman 2016). Thus, I would expect MtrA target genes involved in DNA replication and / or osmotic stress response and the promoter region of *mtrA* and *mce* should be bound by MtrA. In previous ChIP-seq experiments in *S. venezuelae* the deletion strain of the transcription factor was used as a negative control to identify unspecific binding and noise in the data (Bush et al. 2013; Tschowri et al. 2014; Al-Bassam et al. 2014; Bush et al. 2016). Since MtrA is essential in *S. venezuelae* this was not possible.

In a first attempt to perform ChIP-seq I introduced a MtrA-3xFlag under the control of *mtrAp* in the ΦBT1 integrative site in the wild-type background and performed ChIP-seq after 18 hours of growth. I used two independent cultures of the same clone (NS003) and no technical replicates because the variation between two technical replicates in ChIP-seq is low (Ho et al. 2011). Only five targets were enriched: These were the regions upstream of the *ectABCD* operon (SVEN15_0205-8), a TetR family transcriptional regulator (SVEN15_2165), a putative amino permease (SVEN15_2362), a probable nucleotide pyrophosphatase (SVEN15_2560) and a putative secreted oxidoreductase (SVEN15_2566), **Figure 4.1**. We speculated that MtrA-3xFlag binding was outcompeted by the wild-type MtrA and that the five targets do not reflect the complete regulon of MtrA because it does not include the known MtrA target *mce* in *S. coelicolor* (Clark et al. 2013) or *mtrA* since most RR bind to their own promoter (Groisman 2016).

In a second attempt a polyclonal α-MtrA antibody was used against the *S. venezuelae* wild-type strain. This antibody was raised against MtrA(SCO) and a previous attempt to perform ChIP-seq in *S. coelicolor* on solid agar was unsuccessful due to the non-specific binding of α-MtrA (Knowles 2014). However, in the ChIP-seq with the polyclonal antibody in *S. venezuelae*, I used cultures grown in liquid culture and speculated that the non-specific binding in *S. venezuelae* might be reduced. It was feasible to use the polyclonal α-MtrA antibody raised against MtrA(SCO) because MtrA in *S. coelicolor* and *S. venezuelae* share 99% protein homology. Only two amino acids differ in MtrA between the two distant related species: $A_{Sv}45T_{SCO}$ and $T_{Sv}129A_{SCO}$ which are not in the DNA binding helix. Two independent cultures were grown for 18 hours, then pooled and processes for ChIP-seq. Only six distinct peaks in promoter regions could be observed: *ectA* (SVEN15_0205), a putative glycosyl hydrolase (SVEN15_0947), a probable low-affinity inorganic phosphate transporter (SVEN15_1451), the chromosome

(plasmid) partitioning protein ParA or Sporulation initiation inhibitor protein Soj (SVEN15_3576), Phosphate regulon sensor protein PhoR (SphS) (SVEN15_3873) and a putative lipoprotein (SVEN15_4130), see **Figure 4.1**. Again, these targets might not reflect the complete MtrA regulon because the promoters of *mce* or *mtrA* are not enriched. Interestingly the promoter of the first gene in the ectoine BGC *ectA* is enriched in both ChIP-seq attempts. Additionally, it was contradictory that only the *ectA* promoter was enriched in both ChIP-seq experiments thus it is not clear if the targets enriched in ChIP-seq using α-MtrA in the wild-type are true MtrA targets. The flag antibody is very specific to the tagged protein whereas the polyclonal antibody might bind other OmpR RR in *S. venezuelae*. This is consistent with the attempts of ChIP-seq with the α-MtrA in *S. coelicolor* (Knowles 2014), which showed that the polyclonal antibody was not specific enough to perform ChIP-seq.

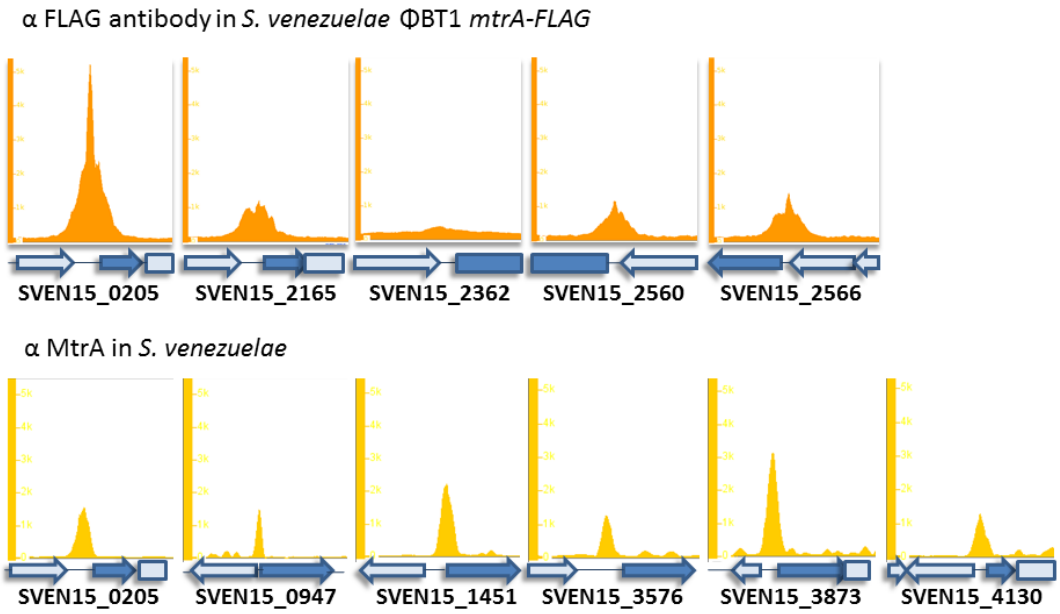

Figure 4.1 ChIP-seq using **Top**: α-FLAG antibody in *S. venezuelae* ΦBT1 *mtrAp mtrA-3xFlag* at 18 hours. Sequences reads were aligned to the old *S. venezuelae* genome and the SVEN15 numbers for the targets are: *ectA* SVEN15_0205, TetR family transcriptional regulator SVEN15_2165, putative amino permease (SVEN15_2362) probable nucleotide pyrophosphatase SVEN15_2560 and putative secreted oxidoreductase SVEN15_2566. **Bottom:** α-MtrA antibody in *S. venezuelae*. Sequences reads were aligned to the old *S. venezuelae* genome and the SVEN15 numbers for the targets are: *ectA* SVEN15_0205, putative glycosyl hydrolase SVEN15_0947, Probable low-affinity inorganic phosphate transporter SVEN15_1451, chromosome (plasmid) partitioning protein ParA or Sporulation initiation inhibitor protein Soj SVEN15_3576, Phosphate regulon sensor protein PhoR (SphS) SVEN15_3873 and putative lipoprotein SVEN15_4130.

In a third attempt the native *mtrA* was deleted in the strain containing *mtrAp mtrA*-3xFlag in the ΦBT1 site (NS042). This strain does not show a mutant phenotype which means that the MtrA-3xFlag protein is functional (see section 3.1.1). NS042 was cultivated in two independent culture and harvested at 18 hours of growth. In this attempt, we used ChIP-exo which is a methodology that can produce near base pair resolution for binding sites. ChIP-seq samples are normally processed but in this experiment the protein was not removed from the immunoprecipitated sample before it was sent to Peconic Genomics. The ChIP-exo technique uses a lambda exonuclease to remove unspecific DNA. The DNA-protein complex is treated with the lambda exonuclease which digests double DNA strands from the 5' end until the digestion is stopped by the protein bound to DNA. Additionally, contaminating DNA is degraded by the addition of a second single-strand specific exonuclease. Then, the crosslinking is reversed and the DNA can be identified by sequencing.

In the ChIP-exo experiment, 1138 targets were enriched but only around 12% were exo peaks with a clear distinct binding site (**Figure 4.2**). This indicates that the exonuclease step was not very efficient but the peaks still reflect the binding of MtrA to target promoters. A list of target genes is provided in the supplementary data (**Table S2**). Compared to the previous attempts of ChIP-seq the enriched genes are potentially part of the MtrA regulon because the promoter region of *mtrA* and *mce* are enriched. Additionally, many promoters of genes involved in development and cell cycle progression including *dnaA, dnaN, smc, whiD, whiB, whiH, ftsZ, divIVA, filP, scy, adpA* and several *bld* genes were enriched. Additionally, the *ectA* promoter was enriched again with extremely high reads (over 300 000) which is much higher than the rest of the peaks. Other interesting targets include cold shock proteins and other transcription factors. This data suggested that MtrA is involved in regulating development and we decided to perform a ChIP-seq during the developmental cycle of *S. venezuelae* to verify the target genes of MtrA and to investigate in which part of the developmental stage MtrA is most active because the ChIP-exo experiment was performed at 18 hours in which the culture is in early sporulation.

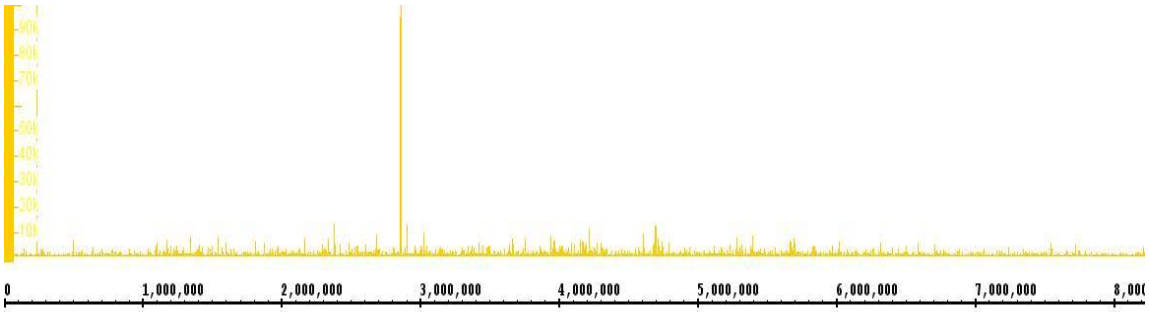

Figure 4.2 Sequencing reads enriched in ChIP-exo with strain NS042 (*mtrA*::*aac(3)IV mtrAp mtrA*-3xFlag in the ΦBT1 site) at 18 hours. The sequencing reads were aligned to the SVEN genome.

4.1.1 MtrA is a global regulator

The ChIP-exo experiment indicated a role for MtrA in regulating development so we decided to perform ChIP-seq throughout the *S. venezuelae* developmental life cycle which completes in 20 hours in liquid MYM. Samples were thus taken at 8, 10, 12, 14, 16, 18 And 20 hours (**Figure 4.3** and **Figure 4.4**).

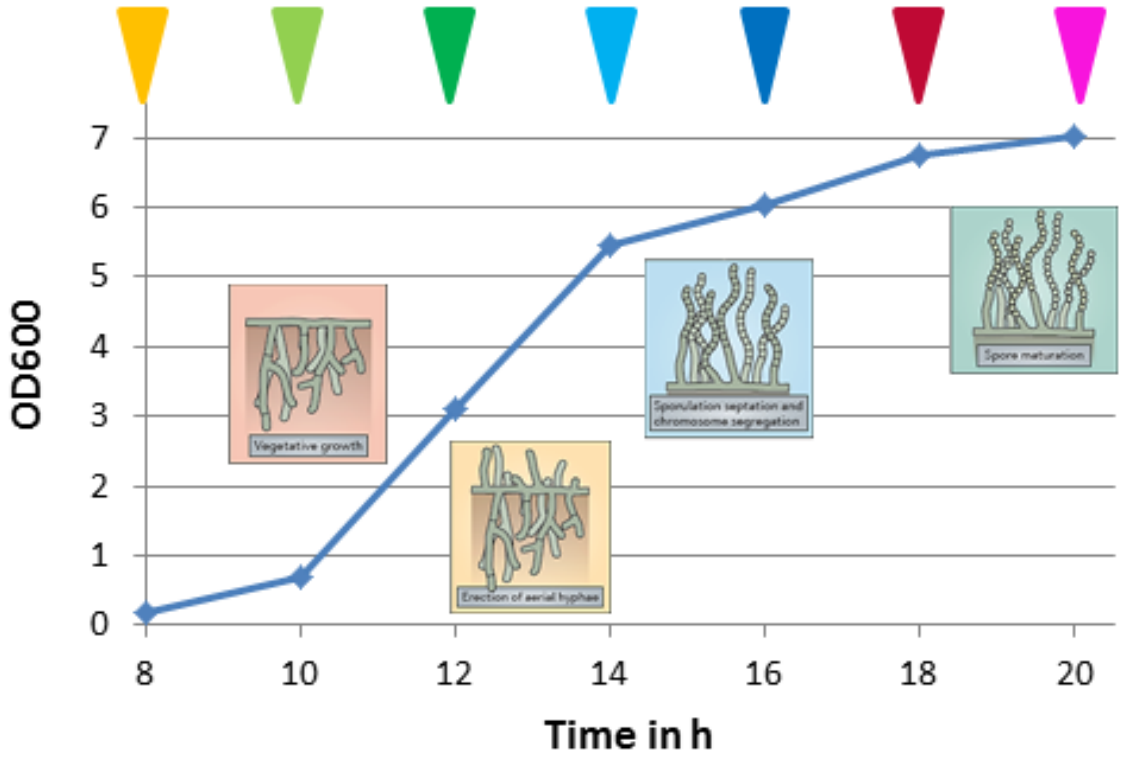

Figure 4.3 OD(600) of samples used for ChIP-seq analysis of *S. venezuelae mtrA*::APR ΦBT1 *mtrAp mtrA-3xFlag.* Images of life cycle adapted with permission from (Bush et al. 2015). Copyright (2017) Nature Reviews Microbiology.

S. venezuelae enters the exponential phase after approximately 10 hours growth in liquid MYM medium and enters stationary phase between 16 and 18 hours. In the exponential growth phase the culture grows as vegetative mycelium until 14 hours. From 14 till 16 hours the culture grows as hyphae and begins to sporulate at 16 hours and at 20 hours most of the hyphae have undergone cell division to form prespores or mature spores. However, the transition of the culture from vegetative growth to aerial hyphae is not visible in liquid culture and is only reflected by molecular changes in the cell like chromosome segregation and division septum formation.

Sequencing reads from the ChIP-seq time course experiment were processed by Dr Govind Chandra at the John Innes Centre. The number of peaks differed between the different time points (**Figure 4.4** and **Figure 4.5**). Only one promoter region, *ectA*, was enriched at the 8-hour time point and at all other time points. All other peaks were under the cut off of $p > 0.05$. At the 8-hour time point the cultures reached an OD = ~ 0.35 which equates a low biomass thus the yield of the DNA was very low although three 35 ml cultures were combined and used to perform ChIP-seq. This generated a low number of sequencing reads and it is possible that some targets might not be enriched due to the low number of sequencing reads.

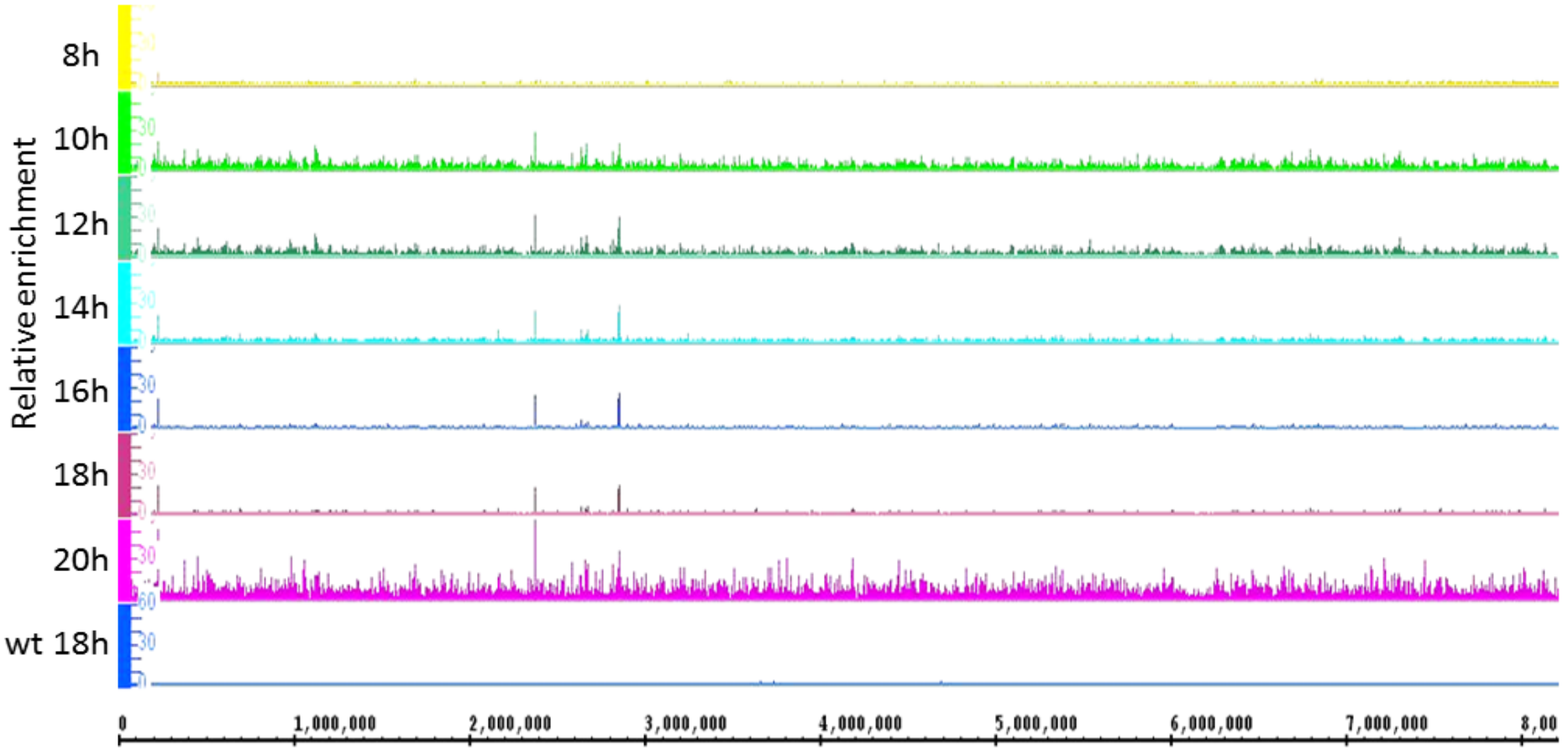

Figure 4.4 ChIP-seq peaks at different time points of *S. venezuelae mtrA*::APR ΦBT1 *mtrAp mtrA-3xFlag*. The wild-type control (wt) was subtracted from the reads in the different time points to eliminate background noise. The peaks are a relative enrichment of the sequencing reads. A 50 bp region was compared to the surrounding 4000 bp.

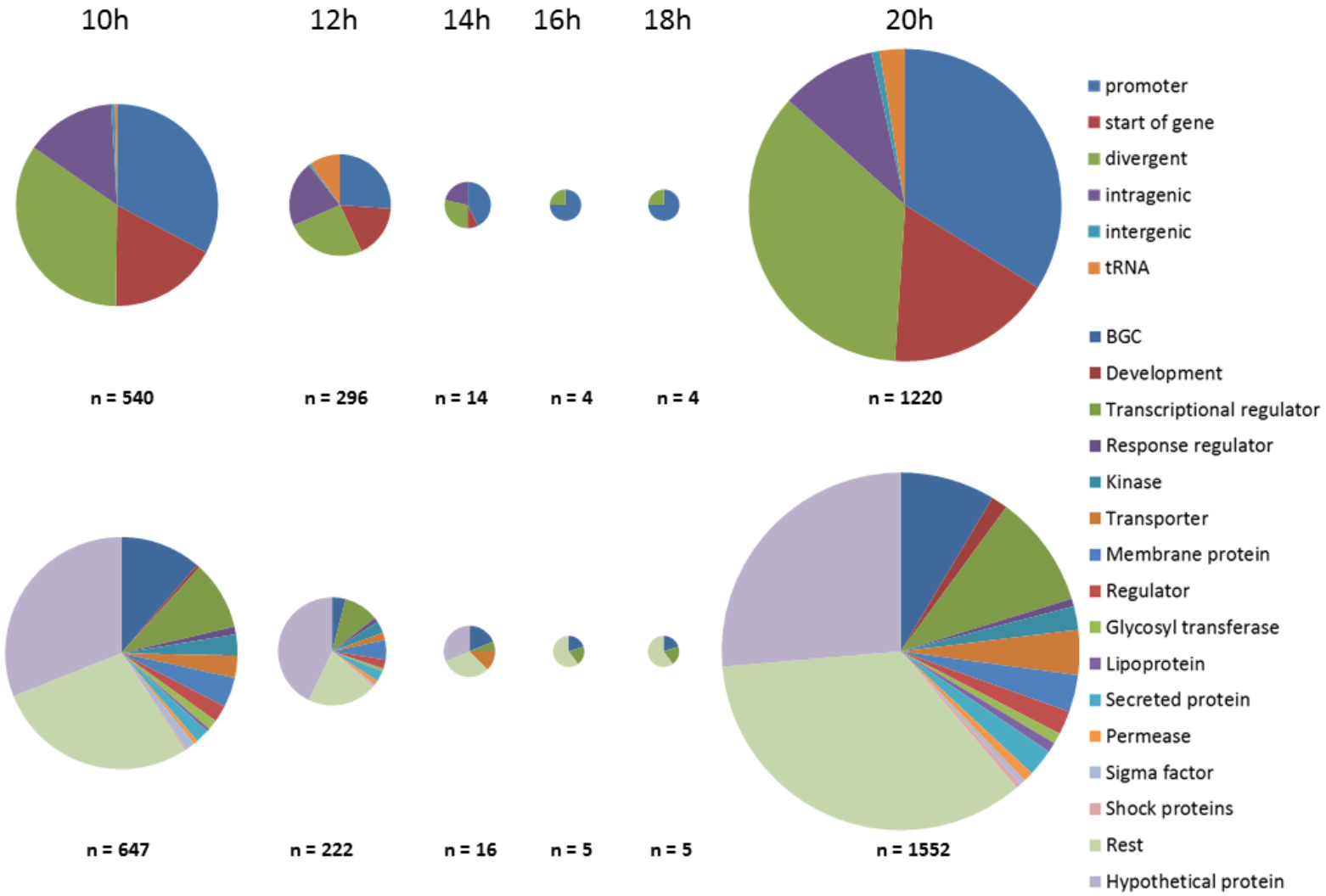

Figure 4.5 Representation of enrichment peaks in the ChIP-seq data during the time course. The size of the cycles represents the amount of data points (not to scale). **Top**: Location of enrichment peak in relation to the transcriptional start site (TSS). Enrichment peaks are count as promoter if they are 500 bp within the TSS, start of the gene from -20 till +500 bp of the TSS, intragenic >500 bp away from the TSS, intergenic means an enrichment peak between genes at the 3` end, tRNA peaks in promoter regions or in tRNAs. **Bottom**: General function of genes downstream of promoter, start of gene or divergent peaks.

The number of enrichment peaks were 1, 540, 296, 14, 4, 4 and 1220 for 8, 10, 12, 14, 16, 18 and 20 hours, respectively, see **Figure 4.5**. The enrichment peak location was investigated in relation to the transcriptional start site (TSS) of the adjacent gene. During the developmental time course, most of the enrichment peaks were in the promoter region, divergent or in the start of a coding sequence.

Around 17% of the enrichment peaks were inside a gene (intragenic) in 10, 12 and 20 hours and less than 1% of the enrichment peaks were between the stop codon of two different genes (intergenic). Enrichment peaks were classed as intragenic when they were inside the coding sequence and more than 500 bp away from the TSS. The biological relevance of protein binding within a gene is not clear. Thus, intragenic and intergenic enrichment peaks were not investigated further. A small proportion of enrichment peaks were tRNAs, 0.4, 10 and 3% for 10, 12 and 20 hours, respectively. Most of the enrichment peaks covered tRNA genes and it is known that the Flag antibody can pull out tRNAs non-specifically (Matt Bush, personal communication).

To investigate the role of MtrA during the developmental time course the genes adjacent to enrichment peaks located in promoter regions, between divergent genes or in the start of the gene were sorted by annotation (**Figure 4.5)**. MtrA was most active at 10, 12 (vegetative growth) and 20 hours (sporulation). Three target genes at 10 hours and 23 target genes at 20 hours are involved in cell division were enriched which is also described in detail in section 4.1.2. MtrA also binds to two promoter regions of target genes involved in osmotic stress which is described is section 4.1.3. Also, MtrA targets most of the BGC in *S. venezuelae* and *S. coelicolor* described in section 4.1.4 and 4.2, respectively. Around 10% of the enriched promoters at 10, 12 and 20 hours were transcriptional regulators. Additionally, RR and HK, regulators and sigma factor genes are targets of MtrA which indicates that MtrA sits on top of a regulatory cascade.

The data from the ChIP-seq time course cannot be directly compared to the ChIP-exo data because the two methods were different in sequencing and sample treatment. However, 39% of the ChIP-exo target genes can be found in the ChIP-seq time course data mainly at 20 hours but also at other time points. Nine target genes of the ChIP-exo data can be found only at 10 hours and one target only at 14 hours. Despite the differences in sample treatment it seems that the sample at 18 hours in the ChIP-exo data (1138 target genes) represents an intermediate time point of the ChIP-seq time course of 18 (five target genes) and 20 hours (1552 target genes), see **Table S1**. This difference of the state of the

colony at the same time courses is most likely due to the filamentous growth of *S. venezuelae* in liquid culture.

To identify the expression of MtrA targets John Munnoch carried out RNA-seq in the *S. venezuelae* Δ*mtrB* mutant at 14 and 20 hours and Dr Govind Chandra analysed the RNA-seq sequencing reads. The RNA-seq data is added to the ChIP-seq data (**Table 4.1**)

4.1.2 MtrA targets involved in development

Many target genes involved in development are enriched during the developmental time course in the ChIP-seq data, including *oriC* and sites upstream of genes required for growth, DNA replication and cell division. Many of these target gene promoters are also enriched in MtrA ChIP-seq data for *S. coelicolor* (

Table 4.1).

4.1.3 BldD-c-di-GMP

There is no enrichment peak over the statistical cut off in the promoter region of the master regulator *bldD* and no significant fold change in the expression of *bldD* could be observed in the *S. venezuelae* Δ*mtrB* strain, see

Table 4.1. However, MtrA likely has an indirect effect on BldD because it binds to several promoters of GGDEF / EAL domain proteins which are responsible for the synthesis or degradation of c-di-GMP. The genome of *S. venezuelae* has ten GGDEF / EAL domain genes. MtrA binds to promoter regions of three of them (*SVEN15_4502, cdgB SVEN15_3942* and *rmdB SVEN15_5058*). The promoter region of SVEN15_4502 is bound by MtrA at 10 and 20 hours and is 4-fold downregulated at 14 hours. The promoter regions of *cdgB* and *rmdB* are bound at 20 hours but the expression is not up or downregulated in the Δ*mtrB* mutant. Two of the ten GGDEF / EAL domain genes are not targeted by MtrA but upregulated in the *S. venezuelae* Δ*mtrB* mutant (SVEN15_0422 and SVEN15_5080) at 14 and 20 hours and only 14 hours, respectively which could be a pleiotropic effect (

Table 4.1).

4.1.4 bld gene targets

MtrA binds to the promoter regions of seven *bld* genes. The *bldG* promoter region is enriched at 20 hours but the expression of the gene is not significantly changed at 14 or 20 hours. There is no binding of MtrA over the statistical cut off (**Figure 4.6**) but the expression of *adpA* is nearly four-fold downregulated in the Δ*mtrB* mutant at 20 hours. MtrA binds to the promoter of *bldM* and *bldN* at 20 hours and the expression of *bldM* is nearly two-fold upregulated at 14 hours in the Δ*mtrB* mutant.

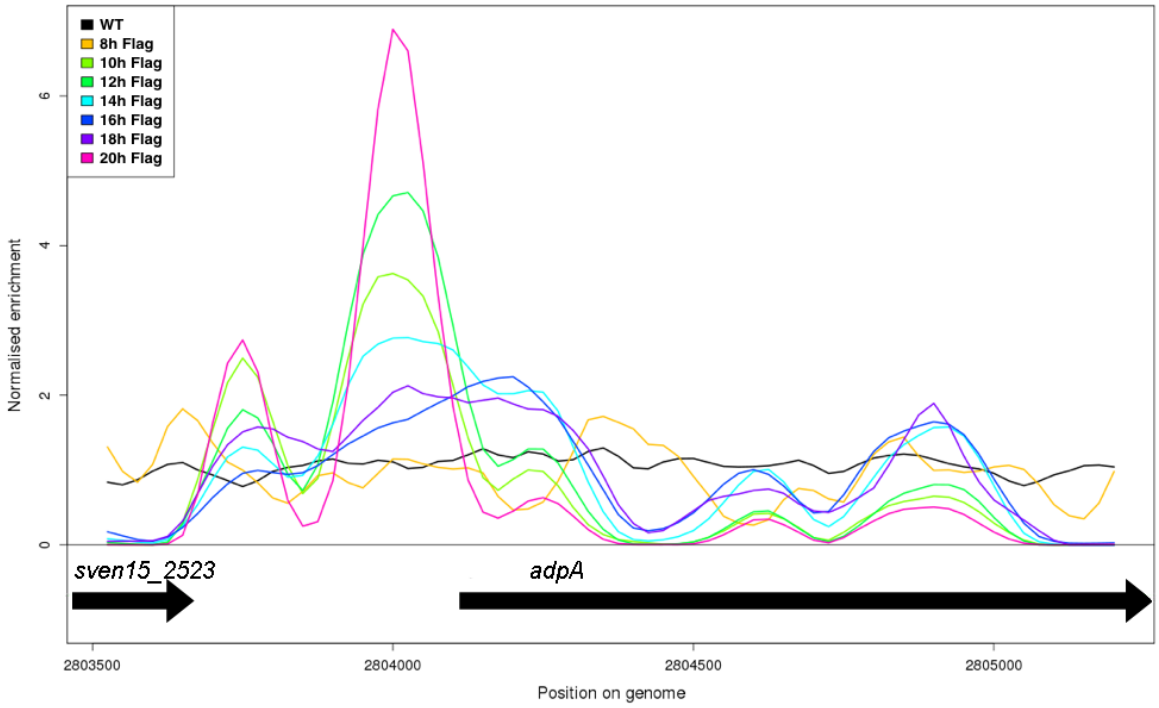

Figure 4.6 Relative enrichment of sequencing reads of the promoter region of *adpA* (SVEN15_2524) at different time points indicated by different colours. Black represents the wild-type control.

4.1.5 whi gene targets

MtrA binds to the promoter regions of 11 genes required for sporulation. *wblE*, *whiB*, *whiG*, *whiH*, *whiI*, *ssgA* (**Figure 4.7**), *B* (**Figure 4.8**), *D*, *E* and *G* promoter regions are most enriched at 20 hours and the expression of all these targets are upregulated at 14 hours. *whiD* is the only *whi* gene bound by MtrA which is not upregulated in the Δ*mtrB* mutant. Interestingly MtrA does not directly bind to the promoter region of *wblM* and *sspA* but *wblM* is upregulated three-fold at 14 hours in the Δ*mtrB* mutant and *sspA* is two-fold upregulated in the Δ*mtrB* mutant.

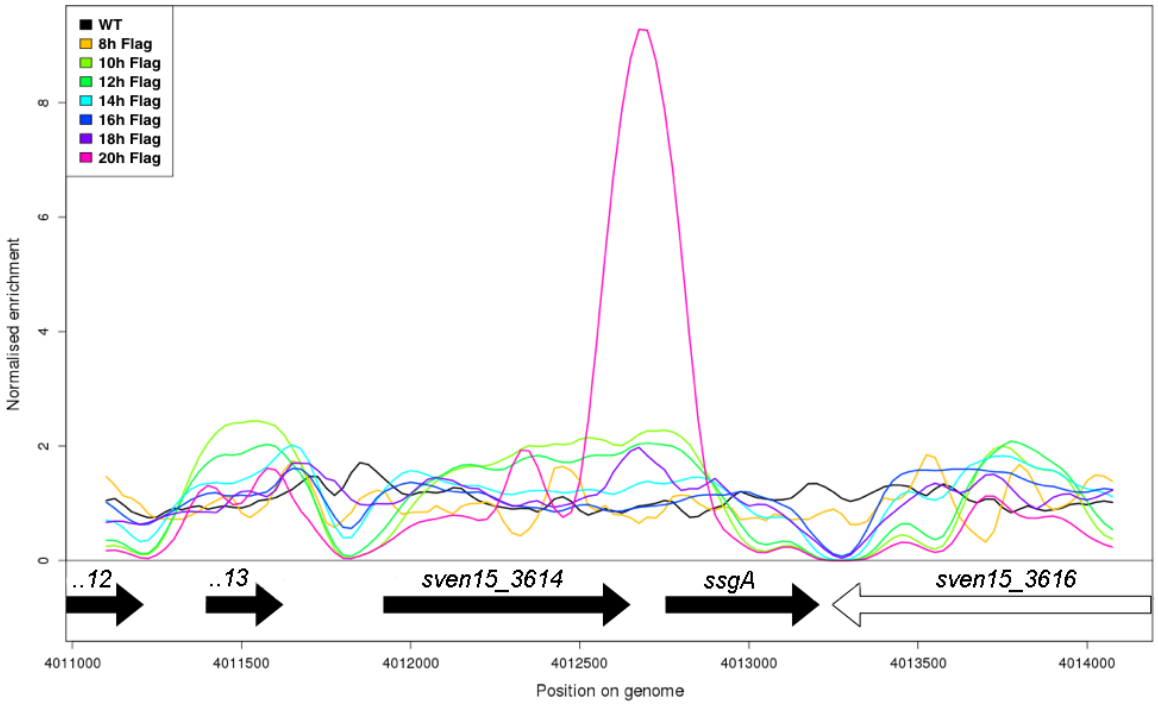

Figure 4.7 Relative enrichment of sequencing reads of the promoter region of *ssgA* (SVEN15_3615) at different time points indicated by different colours. Black represents the wild-type control.

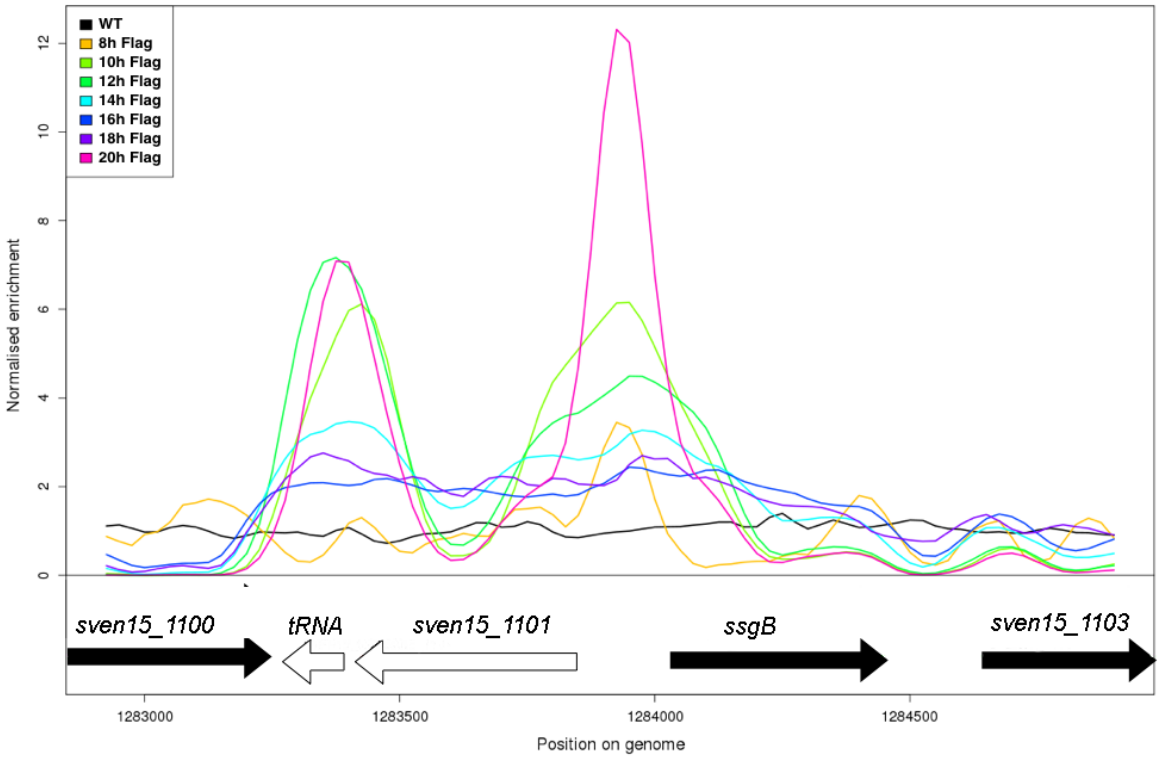

Figure 4.8 Relative enrichment of sequencing reads of the promoter region of *ssgB* (SVEN15_1102) at different time points indicated by different colours. Black represents the wild-type control.

4.1.6 Hydrophobic sheath targets

Ten genes involved in the formation of the hydrophobic sheath are regulated by MtrA. The chaplins *chpC, H, E, G, F, D, sapB,* and three rodlins are upregulated at 14 hours in the *S. venezuelae* Δ*mtrB* mutant. The promoter regions of the chaplins *chpH, chpF* and *chpD* as well as *sapB* are bound by MtrA at 20 hours (**Table 4.1**).

4.1.7 Cell division targets

MtrA binds to nine promoter regions which are involved in cell division. There are two enrichment peaks in the promoter region of *dnaN* which is also part of the origin of replication (oriC) and the promoter region of *dnaA* (**Figure 4.9**). However, *dnaN* and *dnaA* expression is not significant different in the Δ*mtrB* mutant compared to the wild-type. A similar situation can be observed for the promoter region of *ftsZ* and *divIVA* (

Table 4.1 and **Figure 4.10**). MtrA does not bind to the promoter regions of *ftsI* and *sffA* however the expression of *ftsI* is three-fold more in the Δ*mtrB* mutant at 14 hours and the expression of *sffA* is three-fold more in the Δ*mtrB* at 20-hour time point. The expression of *filP* is the only one downregulated at 20 hours and *smeA* is three-fold upregulated at 20 hours.

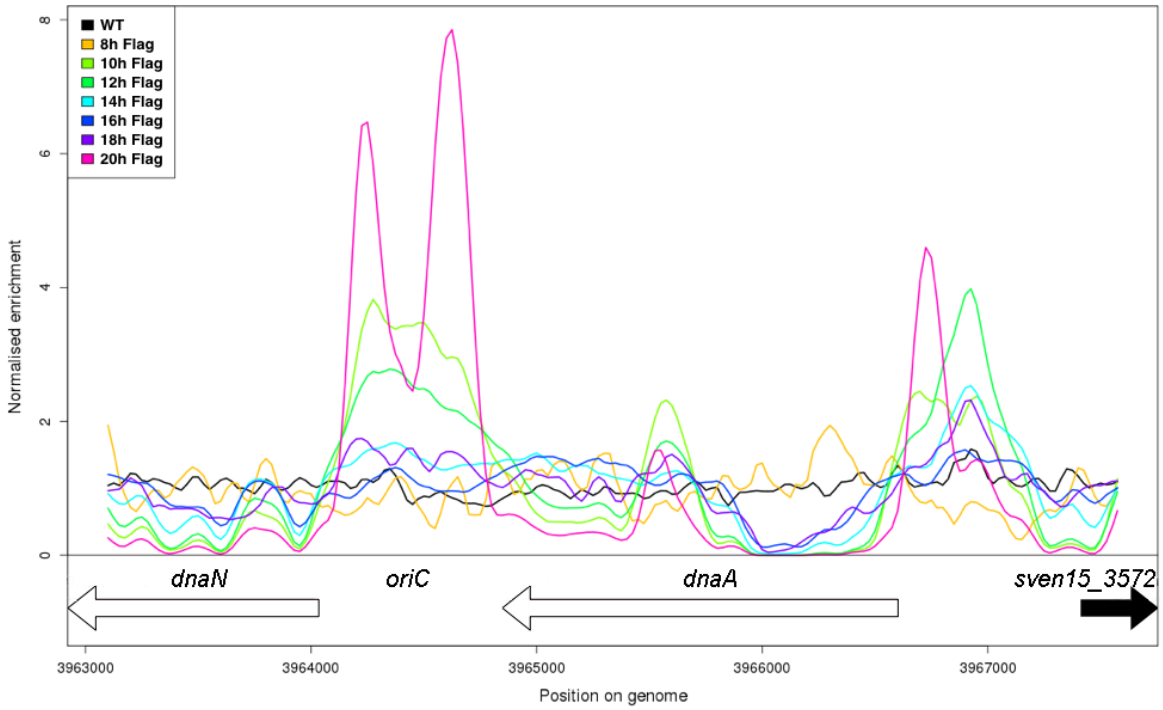

Figure 4.9 Relative enrichment of sequencing reads of the promoter region of *dnaA* (SVEN15_3570) and *dnaN* (SVEN15_3571) at different time points indicated by different colours. Black represents the wild-type control.

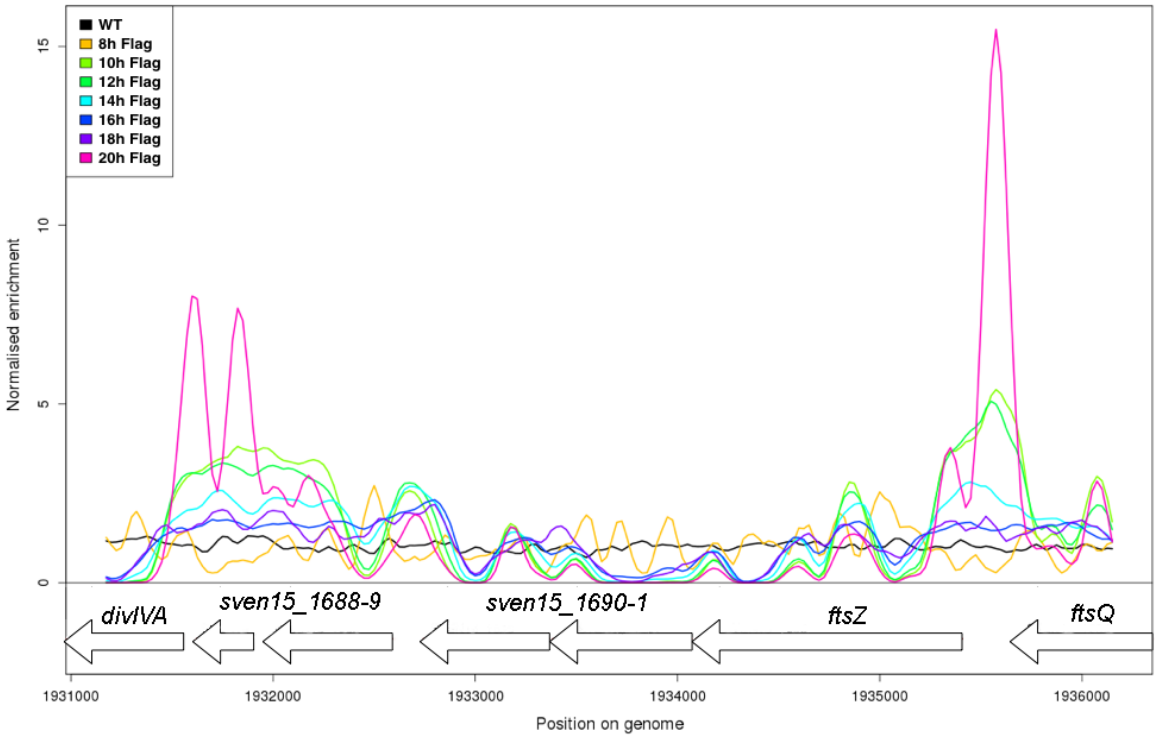

Figure 4.10 Relative enrichment of sequencing reads of the promoter region of *divIVA* (SVEN15_87, left) and ftsZ (SVEN15_1692, right) at different time points indicated by different colours. Black represents the wild-type control.

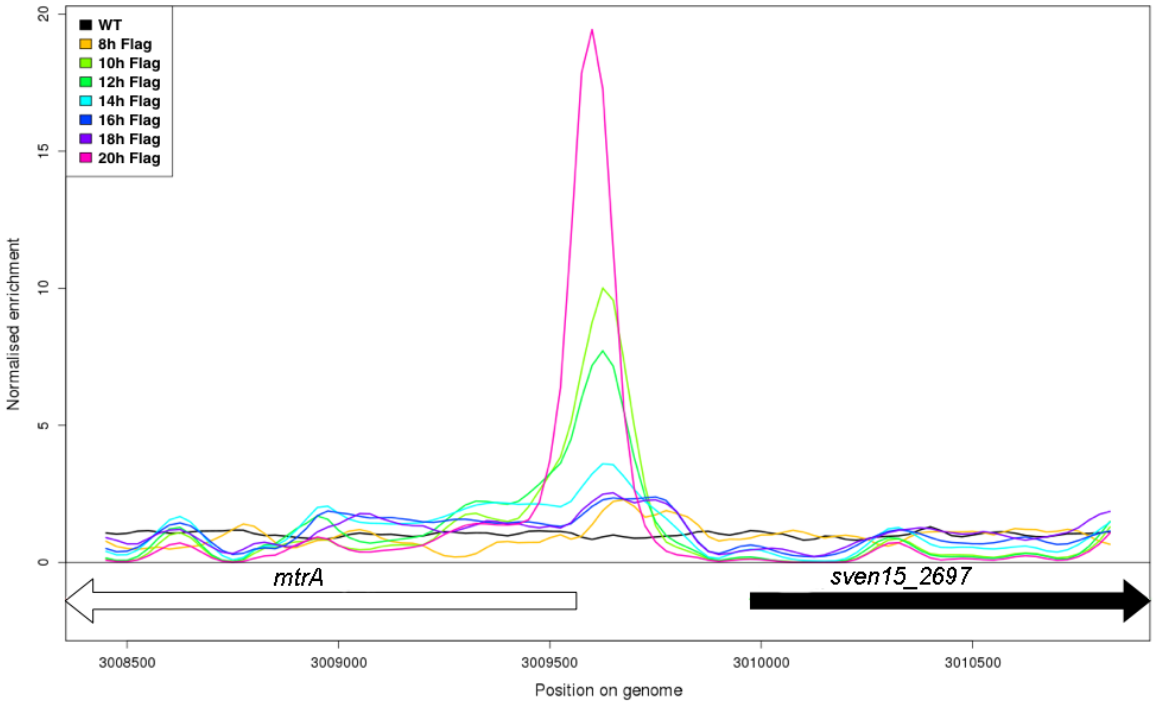

Figure 4.11 Relative enrichment of sequencing reads of the promoter region of *mtrA* (SVEN15_2696) at different time points indicated by different colours. Black represents the wild-type control

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4.1.8 MtrA binds to its own promoter

MtrA, like most other RRs, autoregulates its own expression. It binds to its own promoter (**Figure 4.11**) and in the Δ*mtrB* mutant the expression of *mtrA* is three-fold upregulated at 14 and 20 hours (

Table 4.1).

4.1.9 MtrA binds to target genes involved in osmotic stress response

The response to osmotic stress involves many genes. However, MtrA targets a few of them under normal conditions. MtrA binds to the promoter region of the *ectABCD* operon (*SVEN15_0205-0208*) which encodes the biosynthetic genes of ectoine and 5-hydroxyectoine. Additionally, MtrA binds to the promoter region of two sigma factors involved in osmotic and global stress response *hrdD* (*SVEN15_2993*) and σ^N (*SVEN15_3694*). The expression of the ectoine operon or *hrdD* does not change in the Δ*mtrB* mutant. The expression of σ^N is nearly four-fold higher at 14 hours in the Δ*mtrB* mutant (

Table 4.1).

Table 4.1 ChIP-seq and RNA-seq data for target genes involved in development. An enrichment value in the ChIP-seq data below seven is not statistically significant. A fold change less than two is not statistically significant in the RNA-seq data. The ChIP-exo data from section 4.1 is added.

				ChIP-seq										RNA-seq			
											Exo *1000			wt	ΔmtrB	14h	20h
							SVEN				SCO	SVEN	strand	distance to TSS	14h compared to 20h		wt versus ΔmtrB	
Genes required for formation of aerial hyphae			**Product**	**10**	**12**	**14**	**16**	**18**	**20**	**16**	**18**						
bldD	*SCO1489*	*SVEN15_1052*	DNA binding transcription factor	1.7	1.5	0.5	0.1	0.3	1.7	6.0	1.6			-0.6	0.5	-0.5	0.6
c-di-GMP																	
	SCO4931	*SVEN15_4502*	GGDEF domain protein	8	4.8	1.6	0.3	0.8	20	8.8		1	-42	**-4.8**	**0.5**	-4.4	0.5
cdgB	*SCO4281*	*SVEN15_3942*	GGDEF domain protein	4.8	3.5	1.5	0.4	0.4	10.7	8.3	1.4	1	-58	0.0	0.4	0.0	0.2
		SVEN15_0422	GGDEF / EAL domain protein	2.2	3	2.2	1.2	1.2	3.8	/		-1	-447	5.4	6.2	2.1	2.9
	SCO5495	*SVEN15_5058*	GGDEF / EAL domain protein	5.6	2.9	0.6	0	0.5	18.9	8.3		1	-465	-0.1	0.0	-0.3	-0.2
	SCO5511	*SVEN15_5080*	GGDEF / EAL domain protein	3.8	2.8	1.7	0.8	0.8	4.3	7.5		1	-160	**3.0**	**-0.1**	2.9	**-0.2**
Bald phenotype																	
bldC	*SCO4091*	*SVEN15_3754*	putative MerR-like DNA binding protein	2.5	1.5	0.5	0.3	0.2	6.9	/	3	1	-268	0.1	0.4	**0.1**	**0.4**

bldG	*SCO3549*	*SVEN15_3247*	putative anti-anti sigma factor	4.3	3.8	1.5			9.6	/	2.4	-1	79	-1.9	-1.0	-1.6	-0.7
adpA	*SCO2792*	*SVEN15_2524*	DNA binding transcription factor	2.4	3.5	1.6	0.5	0.9	5.8	/	1.8	1	-112	**-0.7**	**-3.6**	-0.6	**-3.6**
bldM	*SCO4768*	*SVEN15_4355*	Two-component RR	3.6	2.7	1.3	0.4	0.9	9.1	9.5	5.3	1	-257	**0.7**	**-2.4**	1.8	-1.3
bldN	*SCO3323*	*SVEN15_3116*	ECF sigma factor	2.9	2.1	0.6	0.1	1.1	12.3	7.3	3.4	1	-141	0.5	-0.9	1.2	-0.1
citA	*SCO2736*	*SVEN15_2480*	Citrate synthase	1.8	1.3				4.5	/	1.7	-1	24	**-3.1**	**-0.3**	-2.6	0.2
clpP1	*SCO2619*	*SVEN15_2352*	Clp protease proteolytic subunit	0.8	0.5				1.1	/	1.7	-1	68	**-2.4**	**-0.8**	-2.1	-0.3
dasR	*SCO5231*	*SVEN15_4793*	GntR-like transcription factor	3.4	3.9	2.2	1.5	2.2	15	/	3	-1	153	0.8	-0.3	0.1	-0.4
amfT	*SCO6681*	*SVEN15_6340*	lantibiotic biosynthetic protein	2.6	2.7	1.2	0.8	0.3	2.7	6.5		-1	-260	**2.1**	**-1.4**	**0.9**	**-5.1**
				3.9	3.2	1.7	0.7	0.6	3.4	/		-1	16				
Genes required for sporulation																	
wblC	*SCO5190*	*SVEN15_4738*		2.7	2.8	1.5	0.3	0.2	0.9	/	2.7	1	-168	**-1.8**	**-0.4**	-1.1	0.3
whiD	*SCO4767*	*SVEN15_4354*	Putative transcription factor	3.8	2.7	1.3	0.4	0.9	10	9.5		-1	176	**8.3**	**10.6**	**-0.8**	**1.6**
wblE	*SCO5240*	*SVEN15_4802*		2.9	3.7	1.4	0.3	1.7	9.2	8.8	7.8	-1	33	**3.4**	**-0.1**	**2.1**	**-1.4**
wblM	*SCO6922*	*SVEN15_5661*												**3.0**	**-0.1**	**2.7**	**-0.4**
whiB	*SCO3034*	*SVEN15_2717*	Putative transcription factor	3.2	2.5	1	0.3	0.9	10.6	11.3	4	-1	135	**2.9**	**0.5**	**2.5**	**0.2**
sspA	*SCO5321*	*SVEN15_6651*												6.5	7.4	1.2	2.2
whiG	*SCO5621*	*SVEN15_5191*	Sigma factor	5.2	3.4	1.6	0.9	1.1	5	/		1	-253	**2.0**	**0.1**	**2.6**	**0.8**

whiH	*SCO5819*	*SVEN15_5388*	GntR-like transcription factor	3.3	2.9	1.3	0.2	1	12	7.6	2.2	1	41	**2.0**	**0.1**	**2.6**	**0.8**
whiI	*SCO6029*	*SVEN15_5706*	Two-component RR	7.3	5.6	2.7	0.8	1.3	20.6	17.6	1.5	-1	137	**5.7**	**2.2**	**4.0**	**0.4**
ssgA	*SCO3926*	*SVEN15_3615*	putative regulator	1.3	1	0.4		0.9	8.3	7.6	2.9	1	-79	**0.3**	**-2.1**	**2.0**	**-0.4**
ssgB	*SCO1541*	*SVEN15_1102*	putative regulator	5.1	3.3	1.9	1.2	1.2	11.3	/	2.4	1	-111	**7.2**	**4.2**	**4.3**	**1.3**
ssgD	*SCO6722*	*SVEN15_6261*	putative regulator	2.6	2.8	1.3	0.6	0.4	2.8	/		-1	27	**0.5**	**-3.9**	**2.5**	**-1.9**
ssgE	*SCO3158*	*SVEN15_2937*	putative regulator	4.9	3.5	1.2	0.3	0.5	15.3	8.2		1	-70	1.5	0.8	1.9	1.3
ssgG	*SCO2924*	*SVEN15_2622*	putative regulator	8.7	4.7	1.7	0.5	0.9	6.4		1.2	1	-182	**4.2**	**-0.9**	**5.1**	**0.1**
Hydrohpobic sheath																	
chpC	*SCO1674*	*SVEN15_1231*												2.1	-2.4	**3.9**	**-0.6**
chpH	*SCO1675*	*SVEN15_1232*	Hydrophobic cell wall-associated proteins	3.4	1.6	0.5		0.6	8.6	/		-1	57	**3.1**	**-0.9**	**3.4**	**-0.6**
chpE	*SCO1800*	*SVEN15_1396*		0.4	0.8	0.6		0.3	1.4	/		-1	49	-1.0	-4.9	2.8	-1.0
chpG	*SCO2699*	*SVEN15_4550*		2.3	2.8	1	1.8	1.2	5.6	/		1	55	**3.8**	**1.7**	**3.0**	**0.9**
chpF	*SCO2705*	*SVEN15_4546*		2.8	2.3	2	1	1.6	6.8	14.1	1.9	1	-183	**7.8**	**4.1**	**5.2**	**1.5**
chpD	*SCO2717*	*SVEN15_4533*		3.2	2.7	1	0.5	1.1	7.5	/		1	-40	2.9	1.8	2.8	1.7
sapB	*SCO6682*	*SVEN15_6339*	Lantibiotic-like peptide surfactant	6.8	5.2	2.3	1	0.7	8.1	14.7		-1	-12	**3.3**	**-2.2**	**2.5**	**-3.0**
rdlA	*SCO2719*	*SVEN15_4528*	Secreted cell wall-associated protein	0.2	0.6	0.6	0.3	0.5	1	/		-1	60	**5.8**	**1.3**	**3.0**	**-1.5**
rdlB		*SVEN15_4531*	Secreted cell wall-associated protein	1.8	1.2	0.9	0.7	0.4	1.5	/		-1	46	**4.5**	**1.2**	**3.2**	**0.0**

rdlA		*SVEN15_4532*	Secreted cell wall-associated protein	1.4	1.4	0.7	0.4	0.7	2.9	/	1.4	1	-21	**3.8**	**1.2**	**2.9**	**0.3**
Osmotic stress response																
ectA	*SCO1864*	*SVEN15_0205*	L-2,4-diaminobutyric acid acetyltransferase	29	**34**	**31**	**30**	**30**	**54**	**10.9**	305	**1.0**	-161	0.7	1.3	0.8	1.5
hrdD	*SCO3202*	*SVEN15_2993*	RNA polymerase principal sigma factor HrdD	8.0	7.7	3.3	1.9	3.5	23.1	23.1	4	-1	67	0.0	-1.1	1.0	-0.1
σ^N	*SCO4034*	*SVEN15_3694*	putative RNA polymerase sigma factor	4.1	3.7	2.1	1.4	2.1	7.5	7.5	2.8	-1	159	1.2	-3.4	3.8	-0.7
Cell division initiation																
dnaN / oriC	*SCO3878*	*SVEN15_3570*	DNA polymerase III / origin of replication	3.1	1.5	0.4		0.6	6.3	6.7	5.7	-1	235	0.0	-0.8	0.2	-0.6
				1.2	2.3		0.1	0.7	8	/		-1	611				
dnaA	*SCO3879*	*SVEN15_3571*	Chromosome replication initiation protein	0.7	0.9				4.5	/	6.4	-1	125	0.6	0.6	0.4	0.4
Cell division																
ftsZ	*SCO2082*	*SVEN15_1692*	Tubulin-like cell division protein	4.8	4	1.5	0.5	0.6	15.5	10.2	1.8	-1	165	0.7	0.0	1.2	0.5
ftsI	*SCO2090*	*SVEN15_1700*	Penicillin binding protein											**2.5**	**1.2**	**2.6**	**1.3**
divIVA	*SCO2077*	*SVEN15_1687*	Tip-associated protein	1	2	1	0.5	0.5	8	/	3	-1	47	**1.1**	**0.2**	**0.0**	**-1.0**
filP	*SCO5396*	*SVEN15_4942*		3.2	2.4	1	0.4	0.5	9.8	11.5	1.7	-1	57	**-0.2**	**-1.2**	**-1.3**	**-2.3**

smeA	*SCO1415*	*SVEN15_0971*		5.8	4.9	1.6	0.6	1.7	21.5	15.6	1.6	1	23	7.4	8.5	1.5	2.6
sffA	*SCO1416*	*SVEN15_0972*												6.3	7.8	1.8	3.3
smc	*SCO5577*	*SVEN15_5163*	Chromosome-associated ATPase	3.2	2.5	1.1	0.9	0.9	2.4	8.4	7			3.8	4.5	0.0	0.7
mtrAB-lpqB																	
mtrA	*SCO3013*	*SVEN15_2696*	Two component RR	10	7.7	2.4	1.2	1.4	19.4	6.0	3.1	-1	42	-0.2	0.1	2.9	3.2

4.1.10 MtrA targets in biosynthetic gene cluster (BGC)

S. venezuelae produces chloramphenicol and jadomycin. The BGCs encoding these antibiotics have been defined, see **Figure 1.4** (Fernández-Martínez et al. 2014; Wang & Vining 2003). However, *S. venezuelae* contains many BGCs which can be predicted with the online tool antiSMASH (http://antismash.secondarymetabolites.org/). This software can predict undescribed BGC however the edges of the clusters need to be determined experimentally. The software predicted 31 BGCs and MtrA binds to sites spanning 28 of genes that may form part of BGCs (**Figure 4.2**). The only BGCs not bound by MtrA are those encoding biosynthesis of the desferrioxamine siderophores, the WhiE polyketide spore pigment and a putative insecticidal complex. Of these three clusters, the WhiE BGC is upregulated in the Δ*mtrB* mutant suggesting indirect regulation by MtrA, possibly via BldM (

Table 4.1). The desferrioxamine siderophores and the putative insecticidal complex BGC are not regulated by MtrA because there is no change in expression of these two clusters in the *S. venezuelae* Δ*mtrB* mutant. Of the 28 predicted BGCs that are bound by MtrA, nine have genes that are positively regulated by MtrA, ten have genes that are negatively regulated by MtrA and three have genes that are subject to both positive and negative regulation by MtrA. The remaining six BGCs contain promoter regions which are bound by MtrA but their expression profiles are not altered significantly in the *S. venezuelae* Δ*mtrB* mutant under the tested conditions. Therefore, MtrA directly affects the expression of genes in at least 22 predicted BGCs in *S. venezuelae* and indirectly activates the WhiE gene cluster.

Table 4.2 Biosynthetic gene clusters (BGCs) predicted by antiSMASH analysis of the *S. venezuelae* genome. MtrA regulation of each BGC is indicated. BGC coloured in amber are bound by MtrA and the regulation is not known or individual genes are positively and negatively regulated, green BGC are positively regulated, red BGC are negatively regulated and yellow BGC are not bound by MtrA. N/S: Not significant. BGCs marked with * are defined.

BGC	Product	SVEN15 gene nos.	ChIP target	RNA-seq LogFC WT/ΔmtrB	MtrA regulation	Reference
1	*Ectoine	*0205-8*	*0205*	N/S	Not known	(Shao et al. 2015)
2	PKS/NRPS	*0448-72*	*0450* *0451*	1.24 (14h) N/S	Positive Not known	
3	Thiazostatin	*0473-85*	*0473*	N/S	Not known	

			0475	-0.97 (14h)	Negative	
4	Lantibiotic	*0506-18*	*0512*	N/S	Not known	
			0513	N/S	Not known	
			0514	-3.18 (20h)	Negative	
			0515	-3.43 (20h)	Negative	
5	Lantibiotic	*0585-91*	*0585*	N/S	Not known	
			0591	-1.18 (14h)	Negative	
6	Arcyriaflavin	*0724-34*	*0725*	N/S	Not known	
			0731	N/S	Not known	
			0732	-1.07 (14h)	Negative	
7	*Chloramphenicol	*0877-94*	*0879*	N/S	Not known	(Fernández-Martínez et al. 2014)
			0878	-1.59 (14h)	Negative	
			0880	3.01 (20h)	Positive	
8	Other	*1792-832*	*1802*	-0.82 (14h)	Negative	
			1815	N/S	Not known	
			1820	N/S	Not known	
			1821	N/S	Not known	
			1828	-1.82 (14h)	Negative	
			1830	N/S	Not known	
9	Desferrioxamine	*2514-7*	No	N/S	None	
10	Lassopeptide	*3042-65*	*3043*	N/S	Not known	
11	Lankamycin-like	*3973-4016*	*4014*	N/S	Not known	
			4016	1.15 (20h)	Positive	
12	Butyrolactone	*4085-95*	*4093*	N/S	Not known	
			4094	-1.49 (14h)	Negative	
			4095	-1.79 (14h)	Negative	
13	Melanin	*4547-57*	*4548*	0.979 (14h)	Positive	
			4556	N/S	Not known	
			4557	0.991 (20h)	Positive	
14	Butyrolactone	*4983-99*	*4996*	-2.033	Negative	
			4998	N/S	Not known	
15	Thiopeptide	*5018-38*	*5028*	0.76	Positive	
			5029	N/S	Not known	
16	T3 PKS	*5241-73*	*5265*	N/S	Not known	
17	Siderophore	*5304-15*	*5307*	N/S	Not known	
			5308	1.35	Positive	
			5311	N/S	Not known	
18	Siderophore	*5361-72*	*5362*	N/S	Not known	
19	Bacteriocin	*5709-19*	*5709*	N/S	Not known	
			5712	N/S	Not known	
			5714	2.4 (14h)	Positive	
			5717	-2.1 (14h)	Negative	
20	*Jadomycin	*5847-73*	*5858*	N/S	Not known	(Wang & Vining 2003)
			5861	N/S	Not known	
			5863	N/S	Not known	
			5868	N/S	Not known	
21	Herbimycin-like	*5995-6029*	*5995*	N/S	Not known	
			6000	N/S	Not known	
			6008	N/S	Not known	
			6009	N/S	Not known	

			6010	-2.09 (14h)	Negative	
			6012	N/S	Not known	
22	NRPS	*6050-77*	*6052*	-1.72 (14h)	Negative	
			6053	-0.79 (14h)	Negative	
			6063	-0.92 (20h)	Negative	
23	NRPS-PKS	*6101-25*	*6101*	N/S	Not known	
			6108	-0.89	Negative	
			6109	N/S	Not known	
			6111	1	Positive	
			6120	-1.19	Negative	
			6124	0.88	Positive	
			6125	N/S	Not known	
24	Terpene	*6296-6320*	*6296*	N/S	Not known	
			6297	2.15 (14h)	Positive	
			6311	0.84 (20h)	Positive	
			6315	N/S	Not known	
			6316	N/S	Not known	
			6317	-3.17	Negative	
25	Bacteriocin	*6385-6393*	*6385*	1.31	Positive	
			6390	1.79	Positive	
26	*WhiE	*6643-6652*	No	All genes >1 in ΔmtrB except 6652 at 20h	Indirect, positive	(Kelemen et al. 1998)
27	Melanin	*6688-6695*	*6688*	-0.9	Negative	
			6689	-3.41	Negative	
28	NRPS	*6883-6907*	*6901*	N/S	Not known	
			6904	N/S	Not known	
29	Terpene	*6950-67*	*6951*	0.971 (20h)	Positive	
			6964	N/S	Not known	
			6966	2.65 (14h)	Positive	
			6968	N/S	Not known	
30	T3 PKS	*7070-103*	*7074*	1.27 (14h)	Positive	
			7080	N/S	Not known	
			7083	N/S	Not known	
			7092	0.86 (14h) / 1.40 (20h)	Positive	
			7103	N/S	Not known	
31	Insecticidal	*7258-63*	No	N/S	None	

To further examine the effects of deleting *mtrB* on the wider metabolome I cultivated the wild-type strain and three independently isolated Δ*mtrB* mutants in biological and technical triplicates and Dr Daniel Heine analysed the extracts by UPLC/HRMS using untargeted metabolomics. Runs were aligned to compensate for between-run variation and a peak-picking algorithm was applied to allow for the

immaculate matching of each feature (a discrete m/z value and its retention time) among all runs. Following normalisation, features could be compared quantitatively and their putative identity proposed based on their high-resolution MS-signal. Comparing the level of metabolite signals, it appeared that all Δ*mtrB* mutants showed an extensive alteration of global metabolite levels. To display multidimensional data, we used Principle Component Analysis (PCA; **Figure 4.2**). Each sphere in the 3D Plot represents one dataset obtained from a single UPLC-HRMS run. Data from the individual Δ*mtrB* mutant strains clearly group together, and are distinct from data obtained from the wild type, while variations within each group are comparably small. The 3D Plot therefore shows consistent and global changes in the metabolome upon loss of MtrB (**Figure 4.2**).

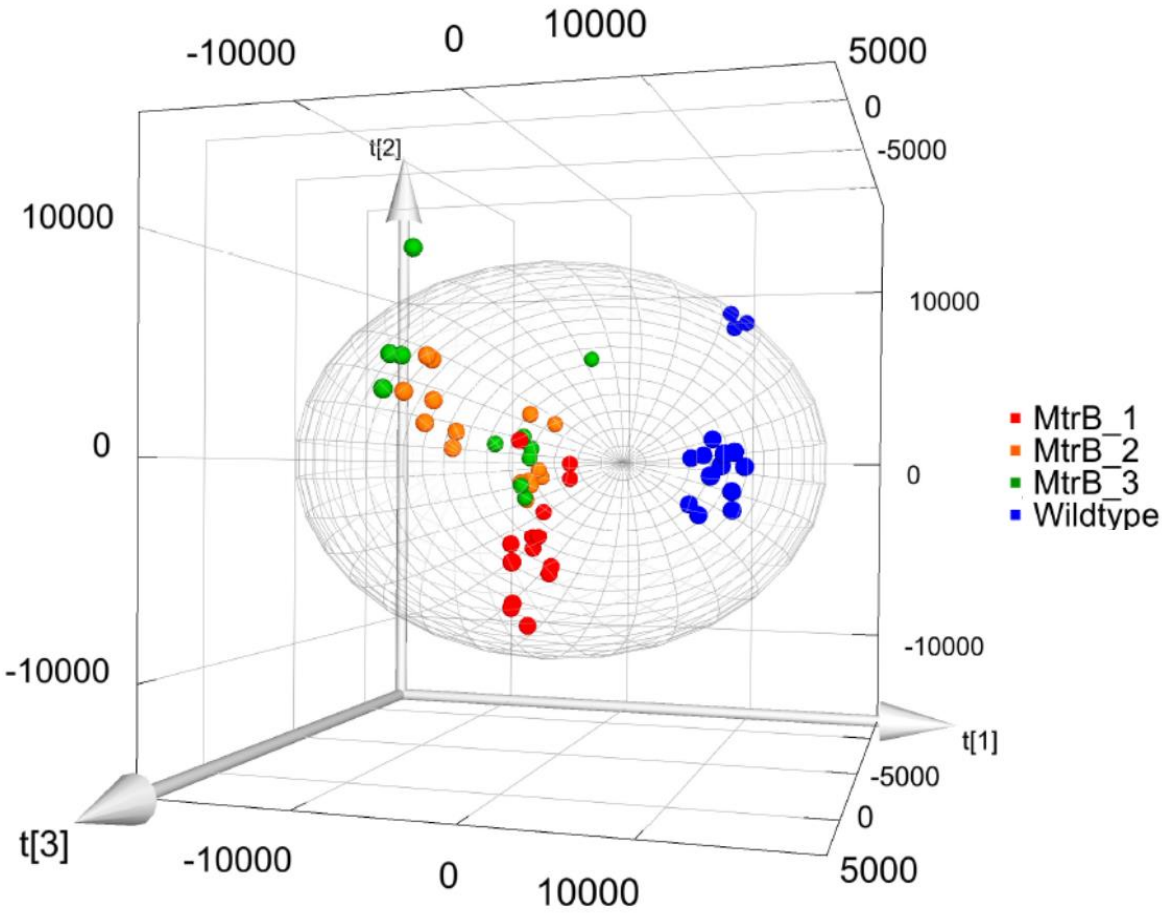

Figure 4.2 3D Plot of the principle component analysis (PCA) showing a global change in the metabolism following deletion of *mtrB*. Red, orange and green dots are triplicate Δ*mtrB* samples and blue dots are the wild-type (Dr Daniel Heine, unpublished).

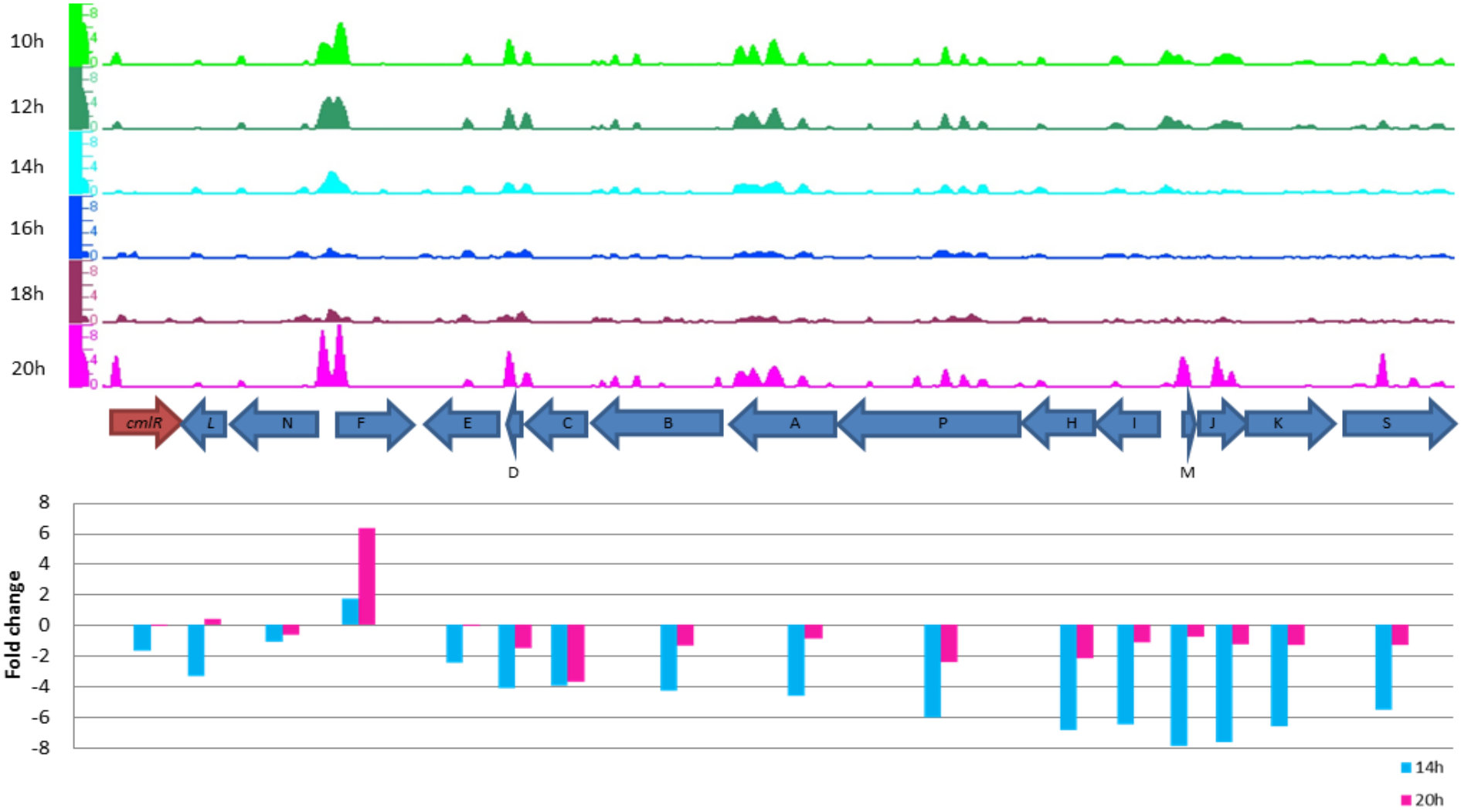

Figure 4.3 ChIP-seq and RNA-seq of the Chloramphenicol BGC of *S. venezuelae*. **Top:** ChIP-seq enrichment peaks over the developmental time course. Shown is the chloramphenicol cluster SVEN15_0877 (*cmlR*) – SVEN15_0892 (*cmlS*). Regulatory genes are shown in red. **Bottom:** Relative expression change of the respective genes in the *S. venezuelae* Δ*mtrB* mutant at 14 and 20 hours.

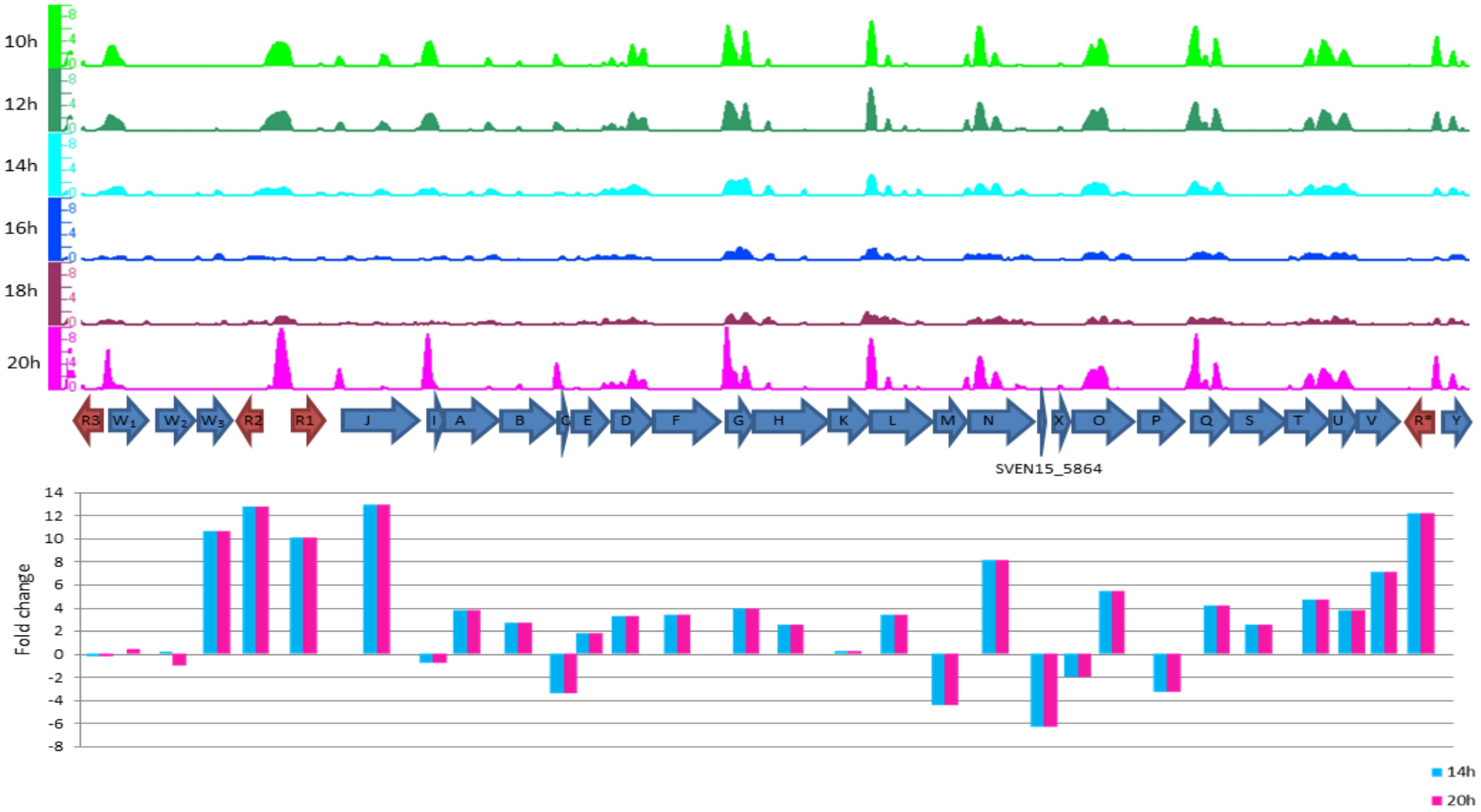

Figure 4.4 ChIP-seq and RNA-seq of the jadomycin BGC in *S. venezuelae*. **Top**: ChIP-seq enrichment peaks over the developmental time course. Shown is the jadomycin cluster *SVEN15_5844* (*R3*) – *SVEN15_5874* (*jadY*). Regulatory genes are shown in red. **Bottom**: Relative expression change of the respective genes in the *S. venezuelae* Δ*mtrB* mutant at 14 and 20 hours.

4.2 Chromatin Immunoprecipitation and Sequencing (ChIP-seq) of MtrA in *S. coelicolor*

MtrA is conserved throughout the genus *Streptomyces*. To investigate the function of MtrA in more detail I performed ChIP-seq in *S. coelicolor*, which is distantly related to *S. venezuelae*. The MtrA-3xFlag construct was introduced in the *S. coelicolor* Δ*mtrA* mutant. Take note that this mutant does not look like the wild-type upon complementation which means this mutant probably has one or more secondary mutations. ChIP-seq was performed at 12, 16 and 20 hours in liquid MYM medium in which *S. coelicolor* grows vegetatively. DNA obtained for the 12-hour time point was contaminated with *E. coli* DNA and was not investigated further.

The results showed that 1098 sites are enriched at 16 hours and 16 sites were enriched at 20 hours (**Figure 4.5**) and are above the cut off of $p > 0.05$ which equals an enrichment value of 7 (**Table S3**). All the sites which were enriched at 20 hours were enriched in the 16-hour sample. *S. coelicolor* does not complete the developmental cycle and does not sporulate in liquid culture. Thus, it is unlikely that the difference in enrichment sites at 16 and 20 hours was due to changes in development. Here 16 hours represent the vegetative growth of *S. coelicolor* however the cell physiology might be completely different from cells growing on solid medium. However, the following section is focused on the results from ChIP-seq at the 16-hour time point.

The distribution of the enrichment peaks was similar to MtrA binding in *S. venezuelae* (**Figure 4.6**). Most enrichment were in promoter, intragenic or divergent between two genes and 20% of the enrichment peaks are in the start of a gene. 12% of the enrichment peaks were intragenic which means that the enrichment was in a coding sequence and more than 500 bp away from the TSS. 1% of enrichment peaks were between the stop codons of two different genes (intergenic). tRNAs were not enriched in the *S. coelicolor* data. In *S. coelicolor* at 16 hours, 100 promoter regions of BGC genes were bound by MtrA which accounts for 9% of the total enrichment peaks. Around 6% of the total enrichment peaks were transcriptional regulator genes and 4% account for putative regulatory genes. At 16 hours, 25 genes involved in development were bound by DNA. This included the MtrA target genes identified in *S. venezuelae* and listed in

Table 4.1 although some *S. venezuelae* MtrA targets were not bound by MtrA in *S. coelicolor*, including *bldG, adpA, sspA, ssgB, ssgD, ssgG*, most of the chaplin genes, the rodlin genes, *dnaA* and *divIVA.*

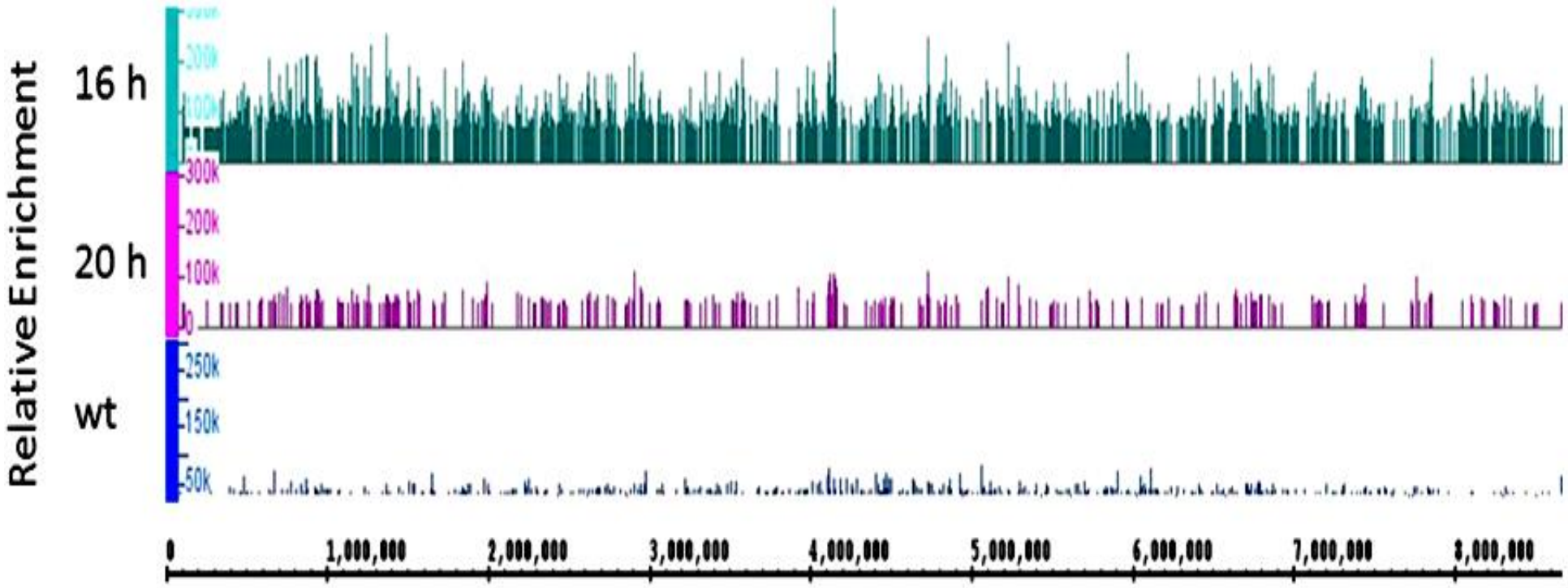

Figure 4.5 ChIP-seq peaks at 16 and 20 hours of *S. coelicolor* Δ*mtrA* ΦBT1 *mtrAp mtrA-3xFlag*. The wild-type control (wt) was subtracted from the reads in the different time points to eliminate background noise. The peaks are a relative enrichment of the sequencing reads. A 50 bp region was compared to the surrounding 4000 bp.

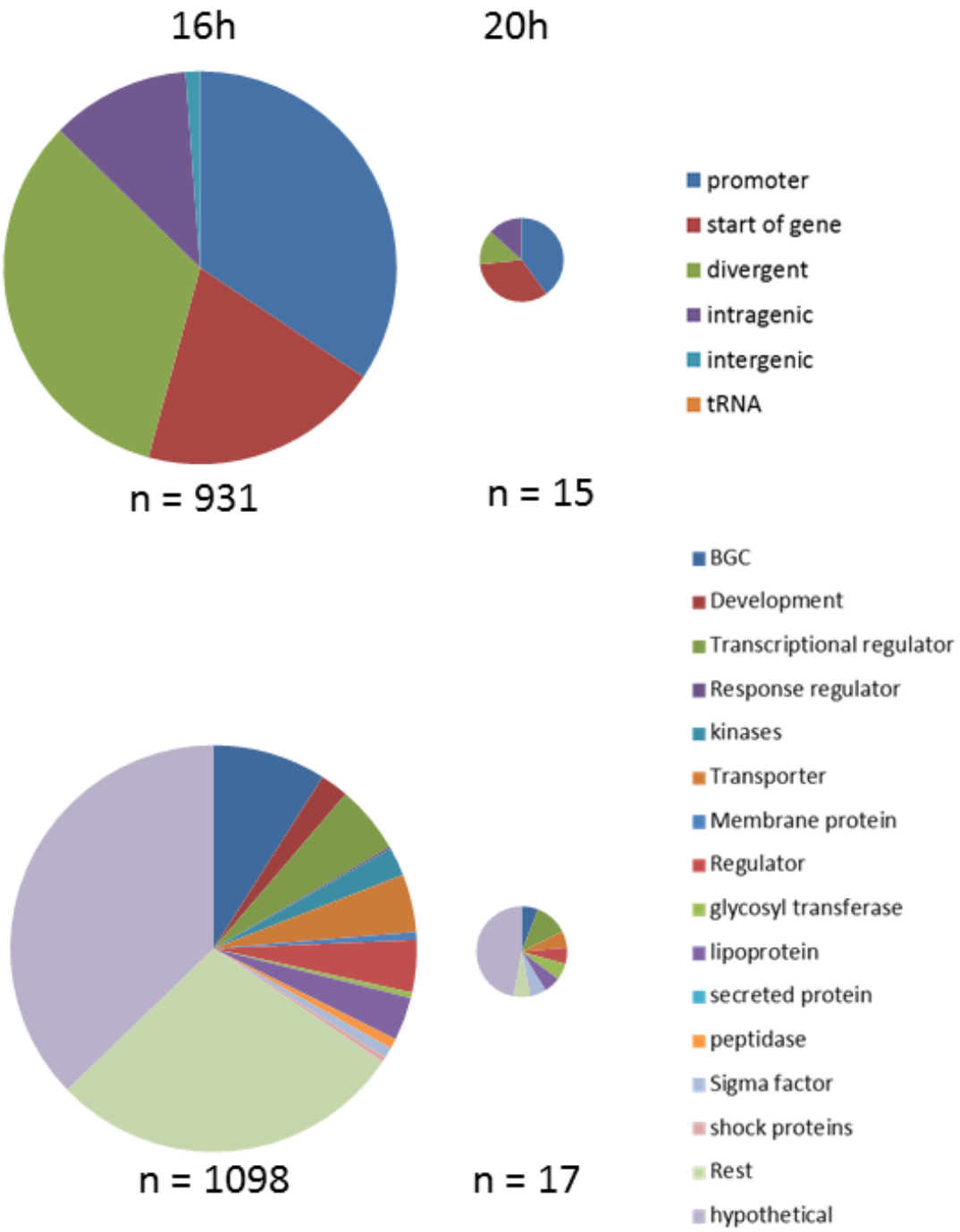

Figure 4.6 Representation of enrichment peaks in the ChIP-seq data at 16 and 20 hours in *S. coelicolor*. The size of the cycles represents the amount of data points (not to scale). **Top:** Location of enrichment peak in relation to the transcriptional start site (TSS). Enrichment peaks are count as promoter if they are 500 bp within the TSS, start of the gene from -20 till +500 bp of the TSS, intragenic >500 bp away from the TSS, intergenic means an enrichment peak between genes at the 3` end, tRNA peaks in promoter regions or in tRNAs. **Bottom:** General function of genes downstream of promoter, start of gene or divergent peaks.

As mentioned above 9% of the enrichment peaks were in BGCs and distributed across 24 out of 27 BGC predicted by antiSMASH. MtrA did not bind to cluster 5, 9 and 24 which encode biosynthesis of a bacteriocin, desferrioxamine and a lantipeptide,

respectively. I cannot predict if the BGCs bound by MtrA were up- or downregulated, however as outlined in section 3.2.6.3 *S. coelicolor* Δ*mtrB* overproduces actinorhodin, metacycloprodigiosin / streptorubin and undecylprodigiosin which indicates that MtrA positively regulates the actinorhodin and undecylprodigiosin BGCs. This is in conformation with the ChIP-seq data because MtrA binds to the promoter region of the CSR *actII-4* in the actinorhodin BGC and *redZ* the CSR in the undecylprodigiosin BGC.

Table 4.3 BGCs predicted by antiSMASH in *S. coelicolor* and genes within the predicted cluster bound by MtrA. BGCs marked with * are defined.

BGC	Product	SCO gene nos.	ChIP target SCO gene nos.	Reference
1	Other-Type 1 PKS	*0104-123 (other) 0124-48 (T1PKS)*	*0126, 0127, 0130*	
2	Isorenieratene	*0177-0202*	*0185, 0198, 0201*	
3	Lantipeptide	*0247-0279*	*0249, 0250, 0252, 0253, 0261, 0268, 0275*	
4	Coelichelin	*0473-0509*	*0475, 0484, 0485, 0486, 0487, 0488, 0489, 0490*	
5	Bacteriocin	*0750-756*	/	
6	Type 3 PKS	*1185-1226*	*1185, 1187, 1195, 1200, 1201, 1209, 1210*	
7	*Ectoine	*1864-67*	*1864*	(Shao et al. 2015)
8	Melanin	*2693-2707*	*2705, 2706*	
9	Desferrioxamine	*2780-90*	/	
10	*CDA	*3210-3249*	*3229, 3230, 3232, 3234, 3236, 3241*	(Hojati et al. 2002)
11	*Actinorhodin	*5067-5110*	*5068, 5069, 5082, 5083, 5085, 5096, 5100, 5104*	(Fernández-Moreno et al. 1991)
12	Albaflavenone	*5212-5231*	*5217*	
13	Spore pigment	5293-5336	*5299, 5308, 5309*	
14	Siderophore	5797-5804	*5801*	

15	*Undecylprodigiosin	5875-5900	*5881, 5883, 5886*	(Cerdeno et al. 2001)
16	Bacteriocin	6041-52	*6042, 6044, 6049, 6050*	
17	Terpene	6064-81	*6080*	
18	Siderophore	6221-32	*6222, 6223, 6226, 6227*	
19	*Coelimycin (CPK)	6265-88	*6265, 6266, 6273, 6280*	(Gomez-Escribano et al. 2012)
20	NRPS	6419-51	*6426, 6445, 6447, 6448*	
21	Lantipeptide	6668-87	*6671, 6675, 6676, 6683*	
22	Terpene	6750-74	*6751, 6752, 6772, 6773*	
23	Other-Type 1 PKS	6808-45	*6845*	
24	Lantipeptide	6921-43	/	
25	Other	7176-7200	*7176, 7177*	
26	Indole	7454-77	*7462, 7463, 7468*	
27	Coelibactin	7648-723	*7664, 7674, 7681, 7682, 7684,7687, 7695, 7696, 7708, 7709*	

4.3 Discussion

4.3.1 MtrA is a global regulator of development

The ChIP-seq data over the *S. venezuelae* developmental time course together with ChIP-seq in liquid cultures of *S. coelicolor* and the RNA-seq data all suggest that MtrA is a master regulator of development and secondary metabolite production in *Streptomyces* species. It is possible that MtrA was not discovered by classical screens for developmental regulatory genes because it is essential.

BldD is a major regulator for development and during vegetative growth it represses most sporulation genes. BldD regulates the expression of at least 167 genes, including 42 genes (~25% of the regulon) that encode regulatory proteins (Elliot et al. 2001; Hengst et al. 2010). Many BldD target genes are crucial for the regulation of development in *Streptomyces* including other *bld* regulators (e.g., *bldA, bldC, adpA/bldH, bldM,* and *bldN*), *whi* regulators required for the differentiation of aerial hyphae into spores (e.g., *whiG* and *whiB*) and central components of cell division and chromosome

segregation machineries such as FtsZ, SsgA, SsgB, and the DNA translocase SffA (Hengst et al. 2010; McCormick 2009).

MtrA bind to 1615 different target genes during the time course in *S. venezuelae* and is most active at 10, 12 and 20 hours. The RNA-seq data show that 11% of the total genes are downregulated and 17% are upregulated at 14 hours in liquid culture whereas only 5% of the total genes are up or downregulated at 20 hours in the *S. venezuelae* Δ*mtrB* mutant. This indicates that MtrA can activate or repress genes. This is in conformation with the regulation of target genes by MtrA in *C. glutamicum*. Depending on the location of the MtrA binding site in relation to the TSS MtrA can activate or repress gene transcription (Brocker et al. 2011). However, it is not clear yet which genes are directly regulated by MtrA and which genes are differently expressed due to downstream effects of MtrA which are likely to be many because 10% of the MtrA target genes are regulatory. However, many target genes of MtrA overlap with the BldD regulon, including the Bld regulators AdpA, BldN, BldM, white regulators like WhiB and WhiG and the cell division and chromosome segregation proteins FtsZ, SmeA, SffA, SsgA and SsgB. The overlap of target genes of BldD and MtrA could indicate that the two regulators have a similar function to regulate the complex development of *Streptomyces* (**Figure 4.7**).

However, MtrA seems to regulate additional genes which are not in the BldD regulon. MtrA binds to the promoter region of *divIVA* and *filP* which are both components of the polarisome (Flärdh 2003; Bagchi et al. 2008; Fuchino et al. 2013). *filP* is twofold downregulated in an *S. venezuelae* Δ*mtrB* mutant at 20 hours whereas the fold change of *divIVA* is not significant. The effect of MtrA on the polarisome might not be important during normal growth and MtrAB might regulate hyphal tip growth in response to a stressful condition.

Other target genes which are not included in the BldD regulon but targeted by MtrA are the *whiB* like (wbl) genes *whiD*, *wblE* and *wblM* and the WhiG direct targets *whiH* and *whiI* as well as several SsgA-like proteins (SALPs). All genes except *whiD* are at least twofold upregulated at 14 hours in *S. venezuelae* Δ*mtrB* whereas the expression of the genes does not significantly change at 20 hours. We assume that in the *S. venezuelae* Δ*mtrB* mutant MtrA is permanently phosphorylated and this could be the reason why the above *whi*, *wbl* and SALP genes are upregulated at 14 hours which is two hours before the first spores appear in liquid culture because the temporal dephosphorylation of MtrB does not occur in the Δ*mtrB* compared to wild-type. However,

the *S. venezuelae* Δ*mtrB* mutant seems to sporulate at the same time as the wild-type (see section 3.2.3) thus the MtrA induced upregulated expression of the *whi*, *wbl* and SALP MtrA targets might not be sufficient to alter the temporal spore formation. The downstream effect of MtrA on the *whi*, *wbl* and SALP genes remains elusive at this point and needs further investigation. However, it is likely that MtrA has a similar role to BldD, i.e. to repress sporulation genes during vegetative growth but also to keep them switched off in spores.

The genes encoding proteins responsible for the formation of the hydrophobic sheath are indirectly regulated by BldD via the regulators AdpA, BldA and BldN. Interestingly MtrA regulates the expression of some of the chaplins, rodlins and SapB. The chaplins and rodlins are at least twofold upregulated in the *S. venezuelae* Δ*mtrB* mutant at 14 hours whereas the expression is not significantly different at 20 hours in the Δ*mtrB* mutant. MtrA targets some of the chaplins at 20 hours (*chpC, chpF* and *chpD*) but none of the rodlins during the developmental time course. It is possible that the upregulation of the chaplins and rodlins at 14 hours is due to an indirect effect.

The chaplins and rodlins form the hydrophobic sheath which enables *Streptomyces* to break through the surface tension and form aerial hyphae. This gives the chaplins and rodlins an important role on solid growth medium. In liquid culture *S. venezuelae* does not have to break through the surface tension to erect aerial hyphae but nevertheless the chaplins and rodlins are expressed. *S. venezuelae* is a new model organism and the growth behaviour in liquid is not well described and the role of chaplins and rodlins and the possible regulation by MtrA remains elusive. However, it is possible that the artificial activation of MtrA by removal of MtrB could lead to the increased expression of proteins in the hydrophobic sheath in the *S. venezuelae* Δ*mtrB* although they might not be required in liquid growth.

Despite the similarity of the BldD and MtrA regulon and the regulation of MtrA of c-di-GMP synthesis genes it is not clear if MtrA directly regulates *bldD*. The enrichment peak of MtrA is under the statistical cut off of p = 0.05 (see

Table 4.1) and expression is not significantly different in the *S. venezuelae* Δ*mtrB* mutant. However the activity of BldD is c-di-GMP dependent (Tschowri et al. 2014). c-di-GMP is synthesized from two molecules of GTP by diguanylate cyclases (DGCs), which are characterized by active site GGDEF motifs (Paul et al. 2004; Chan et al. 2004)

and the cyclic dinucleotide is degraded by specific phosphodiesterases, which harbour EAL or HD-GYP domains (Schmidt et al. 2005; Christen et al. 2005; Ryan et al. 2006). *S. venezuelae* has 10 proteins involved in the synthesis of c-di-GMP: Three containing a GGDEF domain, two containing a HD-GYP domain and five containing a GGEDF and EAL domain (Tschowri et al. 2014). MtrA binds to the promoter regions of five of the 10 proteins predicted or known to be involved in c-di-GMP synthesis. The expression of the GGDEF domain protein CdgB (SVEN15_3942) and the GGDEF / EAL domain protein RmdB are not significantly changed in the *S. venezuelae* Δ*mtrB* mutant. *cdgB* deletion and overexpression leads to blocked development of aerial hyphae (Tran et al. 2011; Tschowri et al. 2014) and *cdgB* is a direct target of the developmental regulators WhiA and BldD (Bush et al. 2013). This indicates that CdgB has an important role during development. Deletion of the GGDEF / EAL protein RmdB results in decreased levels of spore-specific grey pigment and a delay in spore formation (Hull et al. 2012). The ortholog of the GGDEF / EAL domain protein SVEN15_5080 in *S. coelicolor* is directly regulated by BldD (Bush et al. 2013). The role of SVEN15_4502 and SVEN15_0422 has not been investigated yet. Since *cdgB* and *rmdB* are not changed in expression in *S. venezuelae* Δ*mtrB* it is possible that MtrA does not bind directly to the promoter region but instead to another DNA binding protein.

The GGDEF domain protein SVEN15_4502 is over four-fold downregulated at 14 hour in the *S. venezuelae* Δ*mtrB* mutant whereas the GGDEF / EAL proteins SVEN15_0422 and SVEN15_5080 are upregulated in *S. venezuelae* Δ*mtrB.* The upregulation of *SVEN15_0422* and *SVEN15_5080* is most likely an indirect effect because MtrA does not bind to the promoter regions of these two genes during the developmental time course. However, it is possible that MtrA is involved in the regulation of the GGDEF domain protein SVEN_4502 because it binds the promoter region at 10 and 20 hours and when MtrA does not bind *SVEN_4502* is downregulated. It is not clear yet how the genes of the c-di-GMP synthesis are exactly regulated but MtrA might play a crucial role because it binds to three of the ten genes involved in production and degradation of c-di-GMP. MtrA takes part in the regulation of the level of c-di-GMP in the cell and therefore regulates the activity of BldD which is intriguing because so far no protein has been identified to regulate the activity of BldD (Bush et al. 2015). Also, the overlap of the BldD regulon and additional target genes of MtrA indicate that MtrA is a

major regulator of development but feeds additional external signals in the regulatory system.

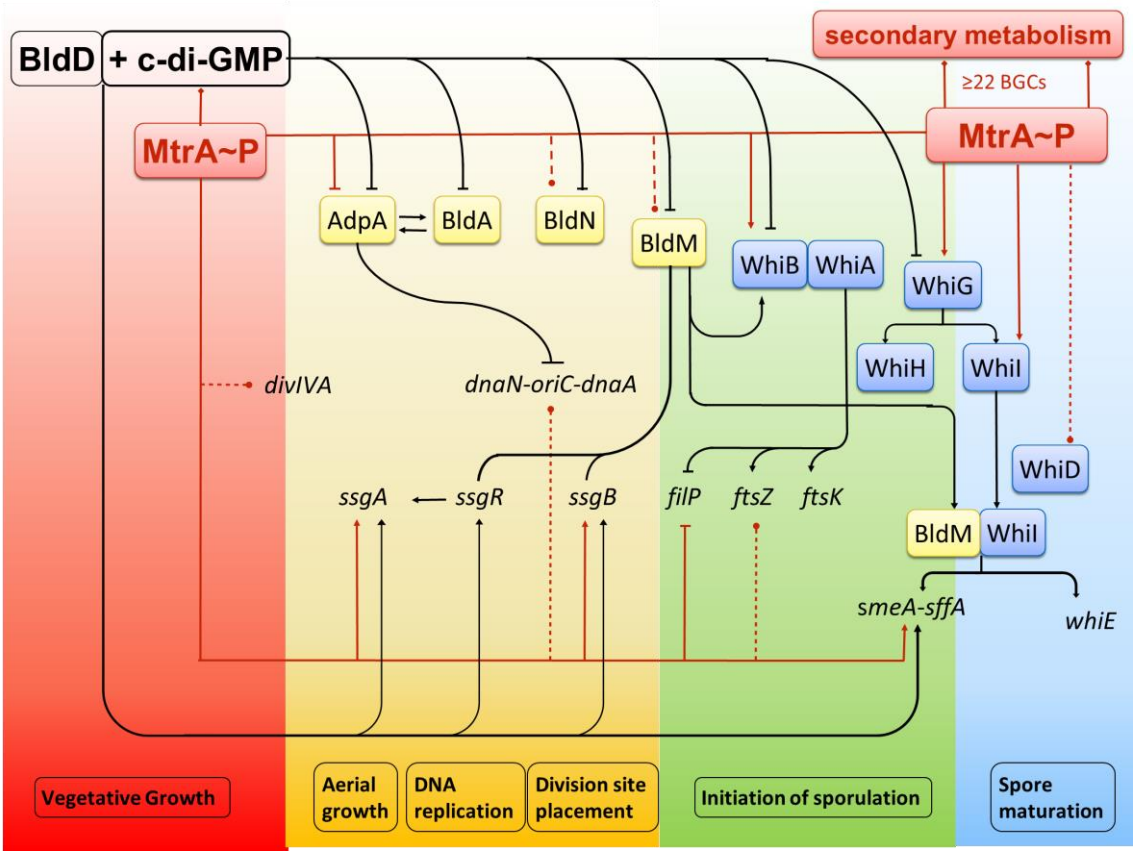

Figure 4.7 BldD regulon based on Bush et. al 2015 and the MtrA regulon. MtrA regulates the global regulators AdpA, BldN, BldM and WhiB. Additionally MtrA binds to promoter regions of genes involved in cell division and sporulation as well as to the origin of replication (oriC). Solid lines represents MtrA binding to taget genes and up or downregulation in the Δ*mtrB* mutant according to the RNA-seq data. Dotted lines are genes which are up or downregulated in the RNA-seq data but not directly bound by MtrA.

4.3.2 MtrA is a global regulator of secondary metabolite production

ChIP-seq and RNA-seq showed that MtrA directly affected the expression of genes near or in at least 22 out of 31 BGCs in *S. venezuelae* and indirectly activates the *whiE* gene cluster. Since most of the *S. venezuelae* BGCs are uncharacterised we do not know anything about their cluster specific regulation or their natural products but the ChIP- and RNA-seq data support the hypothesis that MtrA is a global regulator of secondary metabolism in *S. venezuelae*. However, some of the targets bound by MtrA are at the beginning or end of the predicted BGC and it is not clear if the MtrA target genes are within a true BGC. The defined BGCs which are regulated by MtrA are ectoine, chloramphenicol and jadomycin in *S. venezuelae* and ectoine, CDA, actinorhodin, undecylprodigiosin and CPK in *S. coelicolor*.

Principle Component Analysis (PCA) on *S. venezuelae* wild-type and Δ*mtrB* strains shows an extensive alteration of global metabolite levels following deletion of *mtrB* (**Figure 4.2**). ChIP-seq in *S. coelicolor* also shows that MtrA binding to sites spanning 24 out its 27 BGCs (**Table 4.3**). These data support the idea that MtrA is a global regulator of secondary metabolism in *Streptomyces* species and likely coordinates it with the developmental life cycle.

4.3.3 Chloramphenicol production in *S. venezuelae*

S. venezuelae has the chloramphenicol BGC but the wild-type does not produce chloramphenicol under laboratory conditions (Fernández-Martínez et al. 2014). The production of chloramphenicol is increased in *S. venezuelae* Δ*mtrB*. We assume that MtrA is permanently active in an *S. venezuelae* Δ*mtrB* mutant because otherwise the deletion of *mtrB* would be lethal. Presumably, in the absence of MtrB MtrA can be phosphorylated by small phosphor donors or cross talk from other HKs and it cannot be dephosphorylated. The dephosphorylation of MtrA by MtrB could not be determined in this work however the phosphatase activity was shown for the *C. glutamicum* MtrB (Möker, Reihlen, et al. 2007).

The chloramphenicol cluster contains one CSR *cmlR* which abolishes chloramphenicol production when deleted (Fernández-Martínez et al. 2014). However, the expression of *cmlR* does not change significantly in *S. venezuelae* Δ*mtrB*. The jadomycin CSR Jad1 activates jadomycin production and represses chloramphenicol production whereas the CSR Jad2 represses Jad1 and therefore activates chloramphenicol production. Furthermore, Jad1 is activated by Jad3 in response to γ-butyrolactone. The expression of *jad2* is upregulated in *S. venezuelae* Δ*mtrB* but the expression of *jad3* is not changed in *S. venezuela*e Δ*mtrB*. It is possible that Jad2 represses *jad1* which then stops inhibiting chloramphenicol production which is consistent with the overproduction of chloramphenicol and the lack of jadomycin production. However, the genetic mechanisms of initiation of chloramphenicol production are not known yet and MtrA binds to several promoter regions in the chloramphenicol cluster (**Figure 4.3**). MtrA binds directly to the divergent genes *cmlN* and *cmlF* and this was confirmed by EMSA, see chapter 5. The fold change in *cmlN* expression is not significant but *cmlF* expression is over six-fold higher in the *S. venezuelae* Δ*mtrB* at 20 hours. CmlN is a putative ion antiporter (Fernández-Martínez et al. 2014) and CmlF is an efflux permease that is

predicted to export chloramphenicol (He et al. 2001; Piraee et al. 2004). The remaining genes in the chloramphenicol cluster are downregulated. Antibiotics can autoregulate the expression of the respective cluster and chloramphenicol is probably accumulated in the cell which has to be exported outside of the cell by CmlF and the chloramphenicol excess inside the cell inhibits gene expression of structural chloramphenicol genes. However, it is still unclear if MtrA directly causes the overproduction of chloramphenicol or if the effect is indirect as a global regulator. Nevertheless, these data show clearly that MtrA is involved in the complex regulation of chloramphenicol production.

4.3.4 Actinorhodin and undecylprodigiosin production in *S. coelicolor*

The *S. coelicolor* Δ*mtrB* mutant overproduces undecylprodigiosin, actinorhodin and metacycloprodigiosin / streptorubin in liquid MYM whereas the desferrioxamine B and E siderophores and germicidin A are downregulated (see section 3.2.6.3). This is consistent with data presented in the PhD thesis of F. Knowles where *S. coelicolor* Δ*mtrB* overproduced coloured antibiotics (Knowles 2014).

MtrA binds to DNA that is near or in 24 of the 27 predicted BGCs in *S. coelicolor*. MtrA binds to the promoter region of the CSR *actII-4* and *redZ* which control the expression of actinorhodin and undecylprodigiosin, respectively. MtrA does not bind to the desferrioxamine BGC *(desABCD*) in *S. coelicolor* but it does bind upstream of *SCO4394* gene which encodes DesR, an iron dependent repressor of desferrioxamine biosynthesis (**Figure S47**). This is consistent with MtrA-mediated repression of siderophore biosynthesis, see **Table 4.3** (Flores et al. 2005).

The ActII-4 CSR controls the expression of all five transcriptional units in the actinorhodin cluster (Iqbal et al. 2012). The activation or repression of the actinorhodin cluster is channelled via ActII-4 in response to internal and external cellular processes. The expression of *actII-4* is activated by AdpA, AfsQ1, DraR and ROK7B7 whereas LexA and DasR repress expression of *actII-4*. It seems likely that MtrA~P in an *S. coelicolor* Δ*mtrB* counteracts the repression of *actII-4* which leads to the increased production of actinorhodin.

MtrA also binds to the promoter of undecylprodigiosin CSR RedZ. The production of undecylprodigiosin is regulated via the two CSRs RedZ and RedD. RedZ activates the expression of RedD which is the direct activator for the biosynthetic *red*

genes (White & Bibb 1997). Therefore, it is likely that MtrA activates RED production in a similar way as ACT by binding to the promoter region of the biosynthetic genes activating CSR.

The regulation of antibiotic production in *S. coelicolor* has been investigated by many studies and TCS appear to play a major role in regulating antibiotic production. Many TCS are known to activate or repress antibiotic production in *S. coelicolor* however only three TCS systems regulating antibiotic production have a known signal (Rodríguez et al. 2013). AfsQ1/Q2 and DraR/K sense nitrogen availability and PhoP/R senses phosphate availability. The TCS AfsQ activates RED, ACT, CDA and yCPK (Wang et al. 2013; Shu et al. 2009), DrAR/K represses RED and yCPK and activates ACT (Yu et al. 2012) and PhoP/R indirectly activates RED and ACT (Santos-Beneit et al. 2009). Therefore, it is not surprising that MtrA regulates antibiotic production in *Streptomyces* species. MtrAB reacts to an external stimulus and controls both development and antibiotic production.

4.3.5 Does MtrAB sense osmotic stress in *Streptomyces*?

MtrAB in *C. glutamicum* senses osmotic stress via its cytoplasmic domain and unknown signals via its extracellular sensor domain (Möker et al. 2007). The data presented in this work indicate that MtrAB in *Streptomyces* might have a similar role. As outlined in section 3.2.5 the Δ*mtrB* mutant in *S. venezuelae* and *S. coelicolor* is inhibited in growth by NaCl stress and deletion of *mtrB* possibly mimics osmotic stress due to the increased levels of MtrA~P. The ChIP-seq provides some evidence that MtrAB is involved in sensing and adapting to osmotic stress.

MtrA binds to the promoter region of the ectoine BGC in *S. venezuelae* (SVEN15_0205-08) and *S. coelicolor* (SCO1864-67) during the developmental time course (**Table S2** and **S3**) but no ectoine or 5-hyroxyectoine could be observed in cultures (Dr. Daniel Heine, personal communication). Thus, MtrA must negatively regulate the ectoine BGC. Ectoine and 5-hyroxyectoine (5HE) are compatible solutes which do not interfere with cellular function and can be accumulated in the bacterial cell to very high levels to counteract osmotic stress (Oren 2008). Ectoine and 5HE is synthesised in *S. coelicolor* upon osmotic and heat stress (Bursy et al. 2008) which connects MtrAB to the osmotic stress response. It makes sense that MtrA represses ectoine and 5HE production during normal growth but when osmotic stress occurs MtrA activates the

expression of the *ectABCD* operon. We did not verify if ectoine or 5HE is produced in the Δ*mtrB* mutant but lack of ectoine or 5HE would confirm that MtrAB derepresses ectoine or 5HE under osmotic stress.

MtrA binds to ten sigma factor gene promoters at 20 hours in *S. venezuelae*. Two of them are involved in global stress response. The sigma factor HrdD (SVEN15_2993) is speculated to coordinate "cross talk" from osmotic stress (σ^B), redox stress (σ^R) and cell envelope stress (σ^E) sensing system (Kang et al. 1997; Paget et al. 2001; Lee et al. 2005). The sigma factor σ^N (SVEN15_2993) is involved in stress response to heat, cold, acid, oxidation, salt and ethanol (Wang et al. 2010). Therefore, MtrA might be a major regulator of global stress response in *Streptomyces*.

Another connection of MtrAB to osmotic stress is the regulation of chaplins, rodlins and the surface active peptide SapB. Rodlins and SapB are essential for development in medium with high osmolality since *sapB* deletion mutants are not able to form aerial hyphae under high osmolality conditions because the cell turgor is not sufficient under osmotic stress to break the surface tension (Jong et al. 2012). Thus, it is possible that MtrAB could upregulate *sapB* and rodlins expression under osmotic stress.

A recent publication investigated the effect of osmotic stress on the polarisome in *S. coelicolor* (Fuchino et al. 2016). After osmotic upshift the polarisome is dismantled and re-growth of the hyphal tip occurs on a different site which includes the rearrangement of the polarisome. The authors tested if Scy, FilP, AfsK, OsaA, OsaB, SigB, SigH were responsible for the rearrangement of the divisome but none of the tested proteins caused this. One hypothesis is that MtrAB could be responsible for the rearrangement of the divisome after osmotic upshift. MtrB senses the osmotic shock and then activates MtrA which then regulates either indirectly or directly the expression of *divIVA* and other genes involved in this specific osmoadaptation. Also, MtrB(TB) interacts with the DivIVA homolog Wag31 in *M. tuberculosis* (Plocinska et al. 2014) which could indicate a possible interaction with DivIVA and MtrB in *S. venezuelae.* Taken together the MtrAB system might regulate the rearrangement of the divisome after and osmotic upshift.

4.3.6 Is the function of MtrAB conserved throughout Actinobacteria?

MtrAB is conserved in Actinobacteria and highly conserved in *Streptomyces*. MtrA binds to many developmental genes in *S. venezuelae* and *S. coelicolor* including c-di-GMP synthesising genes and *whiD, wblE, whiB, whiH, whiI, ftsZ* and *oriC*. MtrA binds to 235 orthologues in *S. venezuelae* and *S. coelicolor* which suggest that MtrA could have the same function in these two distant related *Streptomyces* strains. MtrA binds to the promoter of *ftsZ* in both species but not to *dnaAp*. It was speculated above that MtrA regulates the expression of *ftsZ* in a DnaA-dependent manner. However, we cannot be sure if that is the case in *S. coelicolor* because we do not have any expression data. The phenotypes of the Δ*mtrB* mutant in *S. venezuelae* and *S. coelicolor* are different. Deletion of *mtrB* causes irregular septa formation in *S. coelicolor* whereas the septa in *S. venezuelae* Δ*mtrB* mutant are not affected. Thus, MtrB might be involved in the formation of the divisome in *S. coelicolor* but not in *S. venezuelae*. More experiments need to be conducted to be certain if MtrA has the same role in *S. venezuelae* and *S. coelicolor*. It is not unusual that deletion mutants of genes involved in differentiation have distinctive phenotypes in different *Streptomyces* species. There are two known examples of genes displaying variable phenotypes upon deletion. The deletion mutant of the IclR family transcriptional regulator *samR* had a bold phenotype in *Streptomyces ansochromogenes* whereas the deletion mutant of the ortholog in *S. coelicolor* had a white phenotype with nonsporulating aerial hyphae (Tan et al. 2002). The second example is the membrane protein CrgA which is essential for sporulation in *Streptomyces avermitilis*. In contrast, the *crgA* mutant in *S. coelicolor* showed a precocious aerial mycelium growth and sporulation together with premature production of actinorhodin (Del Sol et al. 2003).

Here we present evidence that indicates that MtrA(Sv) interacts with DnaA(Sv) which is similar in *M. tuberculosis* (Purushotham et al. 2015). In *S. venezuelae* and *M. tuberculosis* MtrA is essential which is probably due to the regulation of *oriC* via interaction with DnaA(TB) (Purushotham et al. 2015). In contrast MtrA is not essential in *C. glutamicum* (Möker et al. 2004) because MtrAB is probably not involved in cell cycle progression and its main function is to sense osmotic stress (Brocker & Bott 2006). However, it can be speculated that MtrA acts as a major cell cycle regulator by regulating expression of *dnaA* and therefore when MtrA is essential it might have the same function in distantly related strains. But further work needs to be conducted to understand the role of MtrA in *Streptomyces* to see if MtrAB has the same role in *Streptomyces* and *Mycobacteria*.

The lipoprotein LpqB is conserved in Actinobacteria. It would be interesting to investigate the role of LpqB in *Streptomyces* and compare this with the role of LpqB in *M. tuberculosis* in which the lipoprotein modulates the activity of MtrAB (Nguyen et al. 2010). The translation initiation factor upstream of *mtrA* in *S. coelicolor* is conserved in the suborder Streptomycineae. Further investigations are necessary to identify the role of the translation initiation factor and why many *Streptomyces* species harbour this gene. Also, it would be of interest to repeat the ChIP-seq of *S. coelicolor* Δ*mtrA* ΦBT1 *mtrAp-mtrA* strain on solid culture to see if MtrA binds to different targets when *S. coelicolor* completes development.

Essential TCS are rare in bacteria and it seems that the role of MtrAB as a global regulator of cell cycle progression convergently evolved in other phyla. The RR CtrA in *C. crescentus* is essential and regulates cell cycle progression and DNA replication together with DnaA and GcrA. CtrA inhibits initiation of chromosome replication during differentiation in stalked and swarmer cells (Quon et al. 1996). MtrA could have the same role of inhibiting DNA replication during vegetative growth.

4.4 Conclusion and future work

The results outlined in this chapter show that MtrA binds to genes involved in development and genes in most of the BGCs, which implicates MtrA as a major regulator in both development and secondary metabolite production in *Streptomyces* species. The MtrA regulon overlaps with the BldD regulon and probably feeds external signal in the regulation of the developmental cycle and coordinated secondary metabolite production with developmental growth. So far, no protein was identified which regulates the activity of BldD. MtrA does not regulate BldD directly, however MtrA targets genes involved in the synthesis and degradation of c-di-GMP which activates BldD. Additionally, MtrA is involved in the regulation of the expression genes which form the hydrophobic sheath. This regulation becomes more important under osmotic stress on solid agar. *Streptomyces* use the hydrophobic sheath proteins to overcome the increased surface tension under osmotic stress. Also, MtrA targets the ectoine operon and two sigma factors involved in global and osmotic stress response. This indicates that the MtrAB TCS takes part in the osmotic stress response. Therefore, it would be interesting to perform ChIP-seq under osmotic stress conditions.

Furthermore, MtrA binds to the *oriC* and the promoter of *dnaA*. This is similar to the regulatory role of MtrA in *M. tuberculosis*. The ChIP-seq experiment indicates that MtrA is most active at 10, 12 (vegetative growth) and 20 hours (sporulation). This could be investigated further by analysing the phosphorylation state of MtrA during the developmental time course. Phosphorylated proteins can be identified on acrylamide gels by adding phos-tag which specifically binds to the phosphate group.

Also, the ChIP-seq showed that MtrA binds to promoter regions of genes of the divisome and MtrB(TB) localises at the mid-cell and the cell poles. To investigate the localisation of MtrB a fluorophore like GFP or mCherry could be added to MtrB for localisation studies. Additionally, the protein-protein interaction of MtrB could be investigated by BACTH. Attempts to screen a BACTH library with MtrB only demonstrated a self-interaction with MtrB but the library was most likely incomplete. Also, it would be interesting to perform ChIP-seq under osmotic stress to identify the response of MtrAB to osmotic stress and if MtrAB plays a role in the reassembly of the polarisome after osmotic stress.

5 Phosphorylation of MtrA

It was demonstrated that elevating the levels of MtrB along with overexpression of MtrA reverses the phenotype of MtrA overexpression in *M. tuberculosis* (Al Zayer et al. 2011). This indicates that MtrB regulates the phosphorylation status of MtrA and that the ratio of phosphorylated to non-phosphorylated MtrA is regulated during cell cycle progression in *M. tuberculosis*. Consistent with these findings the overexpression of *mtrA* in *S. venezuelae* does not result in a phenotype (section 3.1.2.) whereas the deletion of *mtrB* leads to different colony morphology and elevated production of antibiotics (section 3.2). The ChIP-seq data described in chapter 4 reveals the *in vivo* binding of MtrA to target promoters but it remains elusive what phosphorylation state of MtrA is the active form. In the following chapter I investigate the phosphorylation activity of MtrB by phosphotransferassay. Additionally, I use EMSAs to examine the *in vitro* binding of MtrA to target genes to verify the ChIP-seq data and use the *in vivo* and *in vitro* data to predict a putative MtrA binding site in *S. venezuelae*. Furthermore, I use MtrA immunoprecipitation to define the MtrA regulon.

5.1 Autophosphorylation of MtrA and MtrB

HKs typically autophosphorylate and transfer the phosphate to their cognate RR (Hoch 2000). MtrB is a membrane binding protein with two transmembrane domains, a HAMP linker and a Histidine Kinase A phosphoacceptor domain. In order to investigate MtrB a truncated version of MtrB(TB) (Al Zayer et al. 2011) and a full length MtrB(Cglu) integrated in phospholiposomes (Möker, et al. 2007) was investigated. Both studies demonstrated that MtrB transfers a phosphate group to MtrA. To test if this is the case for *S. venezuelae* MtrAB a truncated MtrB was purified and the transfer of phosphate was investigated.

The soluble domain of MtrB(Sv) is encoded by the N-terminus which contains the HAMP linker and a Histidine Kinase A phosphoacceptor domain. Thus, the N-terminal nucleotides 1045 – 2034 bp were cloned in pET-28a in two versions: One harbouring the His-tag at the C-terminus and the other at the N-terminus. Both plasmids (**see Table 2.2**) were introduced in BL21 (**see Table 2.1**) and individually expressed and purified. Both

purified proteins were incubated with [γ-^{32}P]ATP and separated on an SDS-polyacrylamide gel. None of the proteins accepted the phosphate group of [γ-^{32}P]ATP which might indicate that the truncated MtrB(Sv) is not functional.

MtrA was incubated with [^{32}P]-acetyl phosphate and N- and C-terminal His tagged MtrB were added to the phosphorylated MtrA to test for dephosphorylation activity (**Figure 5.1**). MtrA is phosphorylated by [^{32}P]-acetyl phosphate but the truncated MtrB did not show any dephosphorylation activity.

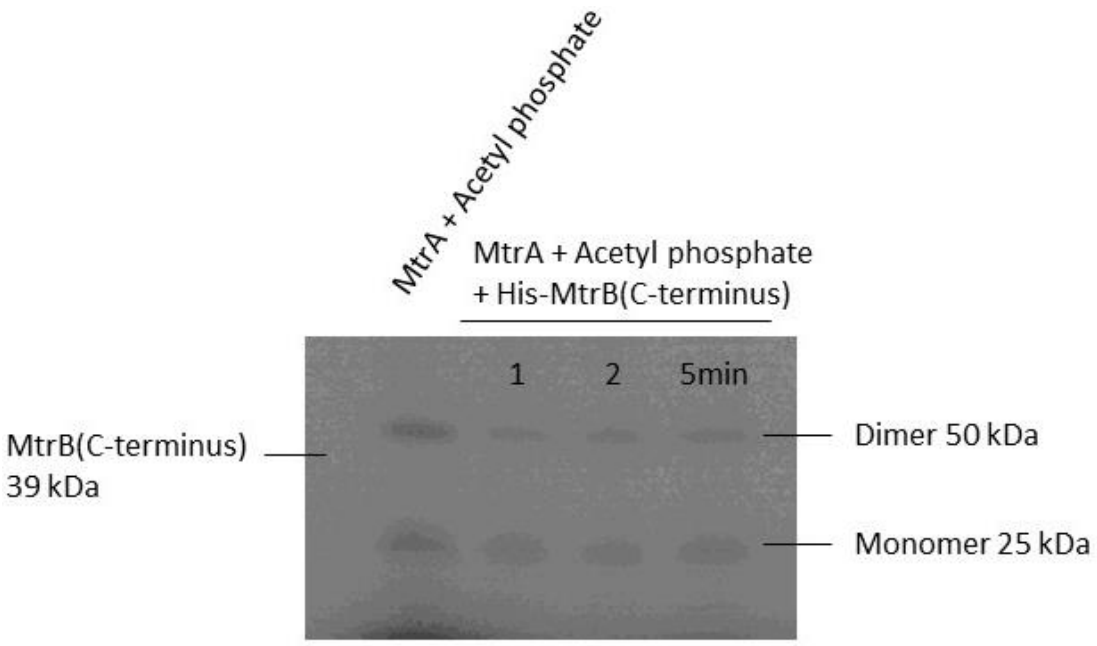

Figure 5.1 Autophosphorylation of MtrA by [^{32}P]-acetyl phosphate. A truncated version of MtrB was added to phosphorylated MtrA but the truncated protein does not seem to be functional.

5.2 Electrophoretic mobility shift assay (EMSA)

To confirm the MtrA *in vivo* binding obtained by the ChIP-seq experiment in chapter 4 it is possible to analyse the binding of a RR to DNA *in vitro*. ChIP-seq peaks are considered to be true binding sites over a p-value of 0.05 (equals an enrichment value of 7). However, the p-value reflects the probability that the ChIP-seq peaks are true binding sites. Thus, it is important to verify the MtrA binding to target promoter with additional experiments. One method to determine the binding of a RR to DNA is electrophoretic mobility shift assays (EMSA). EMSAs take advantage of the principle that protein-DNA complexes separate slower on a native polyacrylamide gel than unbound DNA. Thus, if the protein of choice interacts with the added DNA the visible band shifts up. I used universal 6-FAM-fluorescein primers to label the DNA of interest. First the promoter region is amplified with normal primers and then in a second PCR the

6-FAM label is added to the DNA. Therefore, I use EMSA to verify the MtrA binding to selected target promoters obtained in the ChIP-seq experiment outlined in section 4.1 in *S. venezuelae*.

The first gene of the ectoine cluster was identified as an MtrA target early in ChIP-seq experiments and was tested first. The purified MtrA was stored in the fridge several weeks prior to EMSAs and it was assumed that the purified protein was not phosphorylated. MtrA can be phosphorylated by acetyl phosphate as outlined in section 5.1 and by Friedland et al. (2007) for MtrA(TB). Thus, I used acetyl phosphate to autophosphorylate MtrA. When MtrA was phosphorylated by acetyl phosphate the binding affinity to the *ectA* promoter was higher compared to unphosphorylated MtrA (**Figure 5.2**). This is consistent with EMSA assays with MtrA(TB) shifting target DNA in *M. tuberculosis* (Li et al. 2010; Plocinska et al. 2012; Purushotham et al. 2015; Rajagopalan et al. 2010).

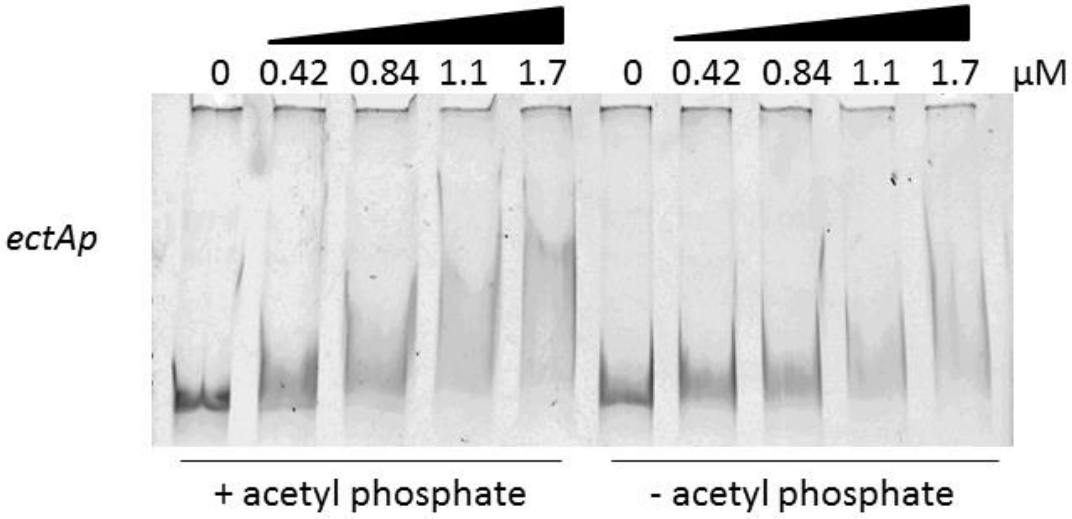

Figure 5.2 EMSA of *ectAp* with and without acetyl phosphate. MtrA used in this assay was stored in the fridge prior to the experiment. Here 8nM DNA were used.

Following the ChIP-seq time course experiment MtrA was again purified. It did not make any difference if acetyl phosphate was added to the newly purified MtrA in the EMSA (**Figure 5.3**). The *ectA* promoter was shifted the same way with and without acetyl phosphate. Thus, MtrA seems to be already phosphorylated which could be possible because MtrA can be phosphorylated by acetyl phosphate in *E. coli* BL21 which was used to overexpress MtrA. The intracellular concentration of acetyl phosphate is 3 mM in *E. coli* which is sufficient to phosphorylate RR (Klein et al. 2007). In contrast, MtrA stored in the fridge for several weeks might have lost the phosphate group due to auto-

dephosphorylation. Additionally, the effect of polydIdC on the EMSA reaction was tested. polydIdC removes non-specific binding which is important if whole cell lysate is used. However, in the case of MtrA and *ectAp* addition of polydIdC does not make a difference in the EMSA reaction. Therefore, in the following EMSAs acetyl phosphate or polydIdC was not added.

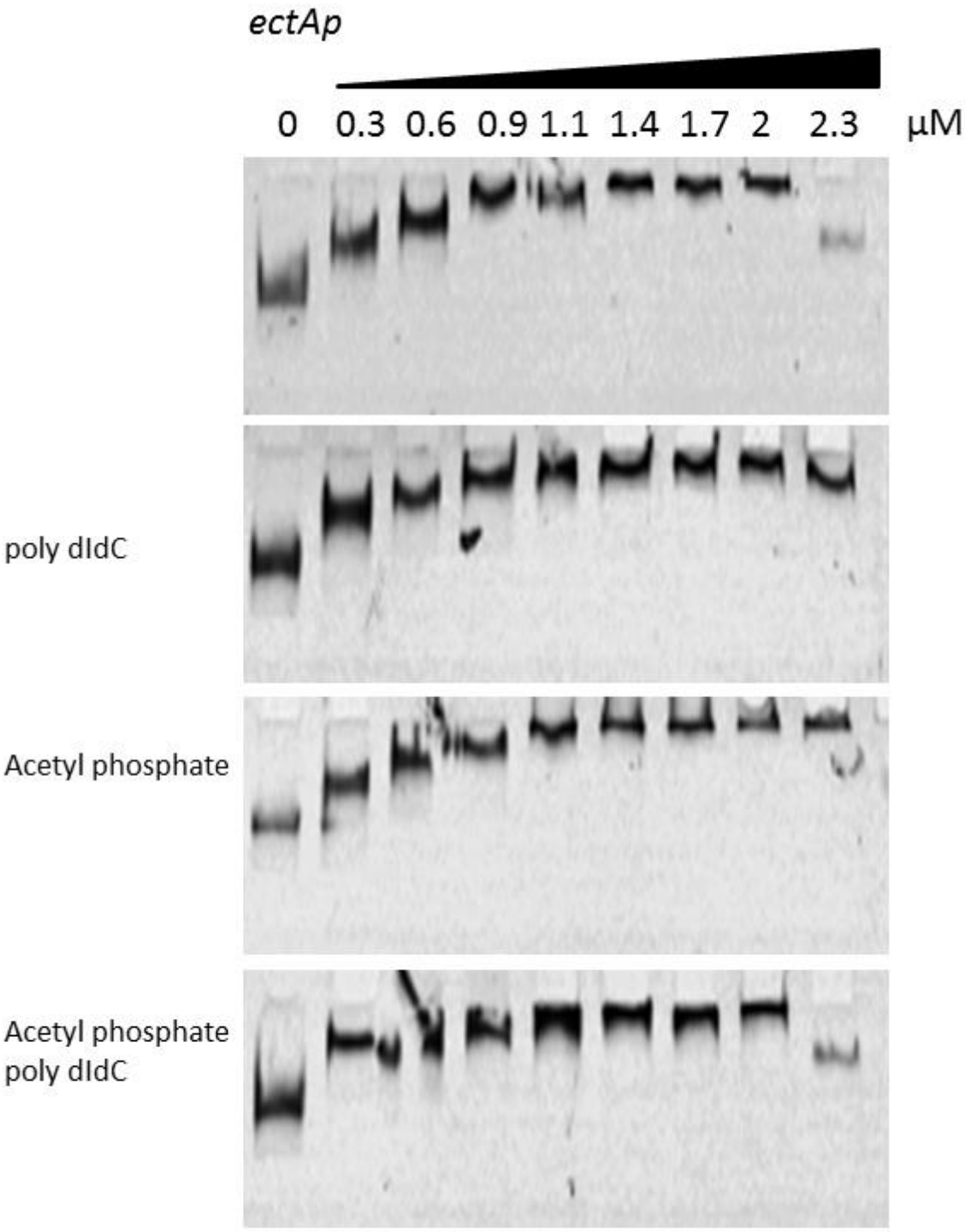

Figure 5.3 EMSA of *ectAp* without any additives, polydIdC (1 µg), acetyl phosphate (30 mM) and acetyl phosphate and polydIdC. 10 nM DNA was added to increasing concentrations of MtrA.

The two *S. coelicolor* promoters *hmpA2p* (*SCO7094*) and *hmpA1p* (*SCO7428*) were used as negative controls since there are no enrichment peaks in these promoters in the *S. coelicolor* ChIP-seq data at 16 and 20 hours. In the EMSA 10 nM of promoter DNA were added to increasing amount of MtrA. Non-specific binding could be observed when 0.9 µM of MtrA were added to the two negative controls. In contrast, in the EMSA with MtrA(TB) and the *ripA* promoter 0.1 – 1 µM of protein and 200 fM DNA resulted in a band shift and not unspecific binding (Li et al. 2010). In my experimental set up it was not possible to use less than 5 nM of DNA because otherwise the DNA bands were not detectable (data not shown). Thus, I had to use 10 nM of DNA causing unspecific shift of the DNA/protein complex due to the excess of DNA (Hellman & Fried 2007). Therefore, band shifts with 10 nM DNA and more than 0.9 µM of MtrA are non-specific binding under the assay conditions used in this work. However, the promoter of *dnaA*, *ectA* and *cmlF* (*SVEN15_0880*) were shifted by MtrA at much lower concentrations of protein indicating that MtrA binds to these promoter regions *in vitro* (**Figure 5.4**). The enrichment peak in *dnaAp* is with ≤ 5 under the enrichment value of 7 during the developmental time course. Despite that, the promoter of *dnaA* was shifted by purified MtrA. This means that the p-value of 0.05 (equals enrichment value of 7) cannot be seen as a strict cut off for MtrA binding to target promoters. In comparison, the enrichment value for *ectAp* is ≤ 54 and both DNA fragments containing *dnaAp* or *ectAp* shift at 0.25 µM MtrA. This equals a ratio of one DNA molecule to 25 MtrA dimers. This reflects the fact that ChIP-seq is not a quantitative method. The binding affinity of MtrA to different DNA fragments is not related to the enrichment of the peaks.

Therefore, I tested more promoter region of interesting target genes in *S. venezuelae*. The promoter region of *smc* is enriched with ≤ 3 during the developmental time course in. The promoter region of *ftsZ* is ≤ 5 from 10 – 18 hours and 16 at 20 hours. The enrichment of the promoter region of *adpA* is ≤ 6 during the developmental time course (**Table 4.1**).

According to the ChIP-seq data it was expected that the *ftsZp* would shift and *smcp* and *adpAp* should not shift. The promoter regions of *smc* and *adpA* did not shift and surprisingly the *ftsZp* did not shift as well with a protein concentration lower than 0.9 µM

(**Figure 5.5**). No unspecific binding could be observed for the *smcp*. A possible reason for this could be that the *smcp* fragment used in the EMSA was 158 bp which is at least 100 bp smaller than the other promoters tested in the assays. Due to the smaller size of the *smcp* DNA fragment it is possible that MtrA does not form an unspecific complex with *smcp* which does not result in an unspecific shift.

More promoter regions need to be tested to refine the statistical cut off in the ChIP-seq data. However, it is possible that MtrA does not bind directly to target promoters. The binding of MtrA could occur via other DNA binding proteins which leads to enrichment peaks in the ChIP-seq data but no direct binding of MtrA to target promoters.

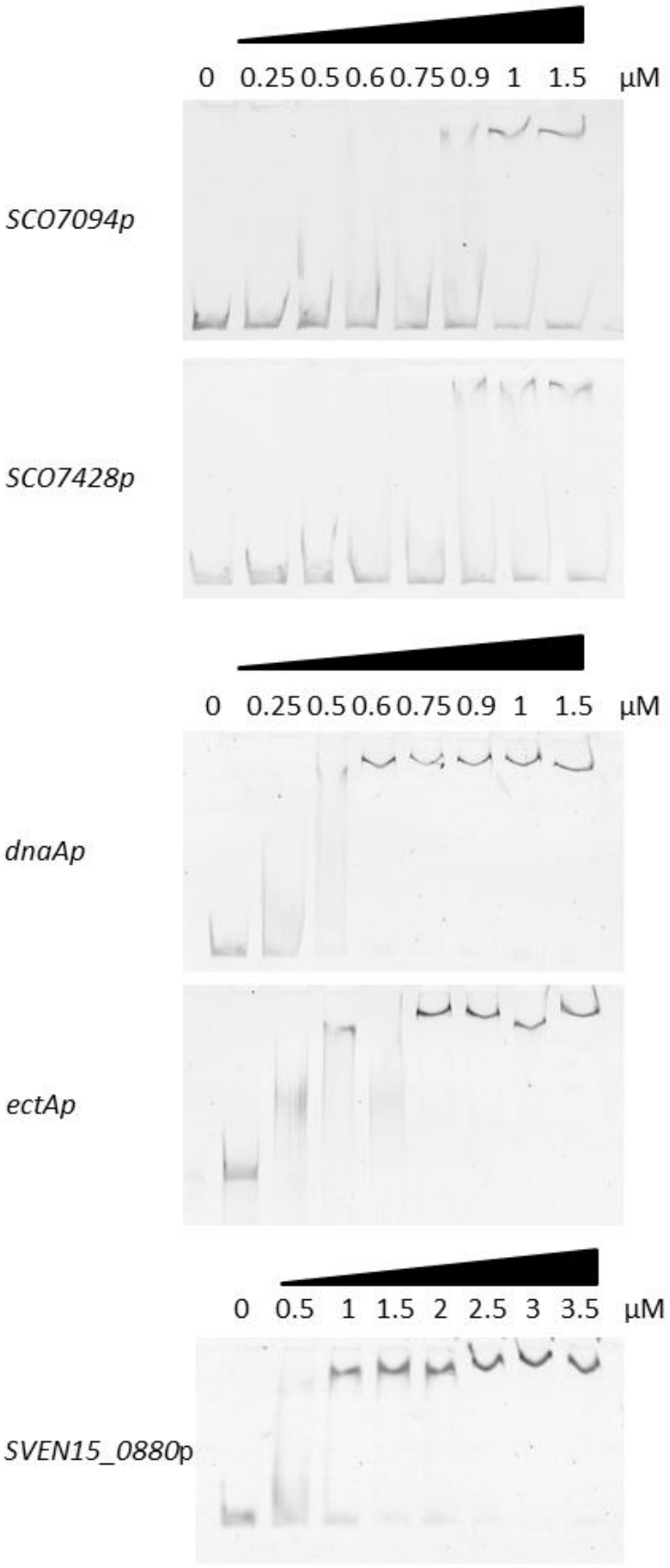

Figure 5.4 EMSA of two promoter *hmpA2p* (*SCO7094*) and *hmpA1p* (*SCO7428*) as negative control and shifts of *dnaAp* (*SVEN15_3571*), *ectAp* (*SVEN15_0205*) and *cmlF* (*SVEN15_0880*). 10 nM DNA was added to increasing concentrations of MtrA.

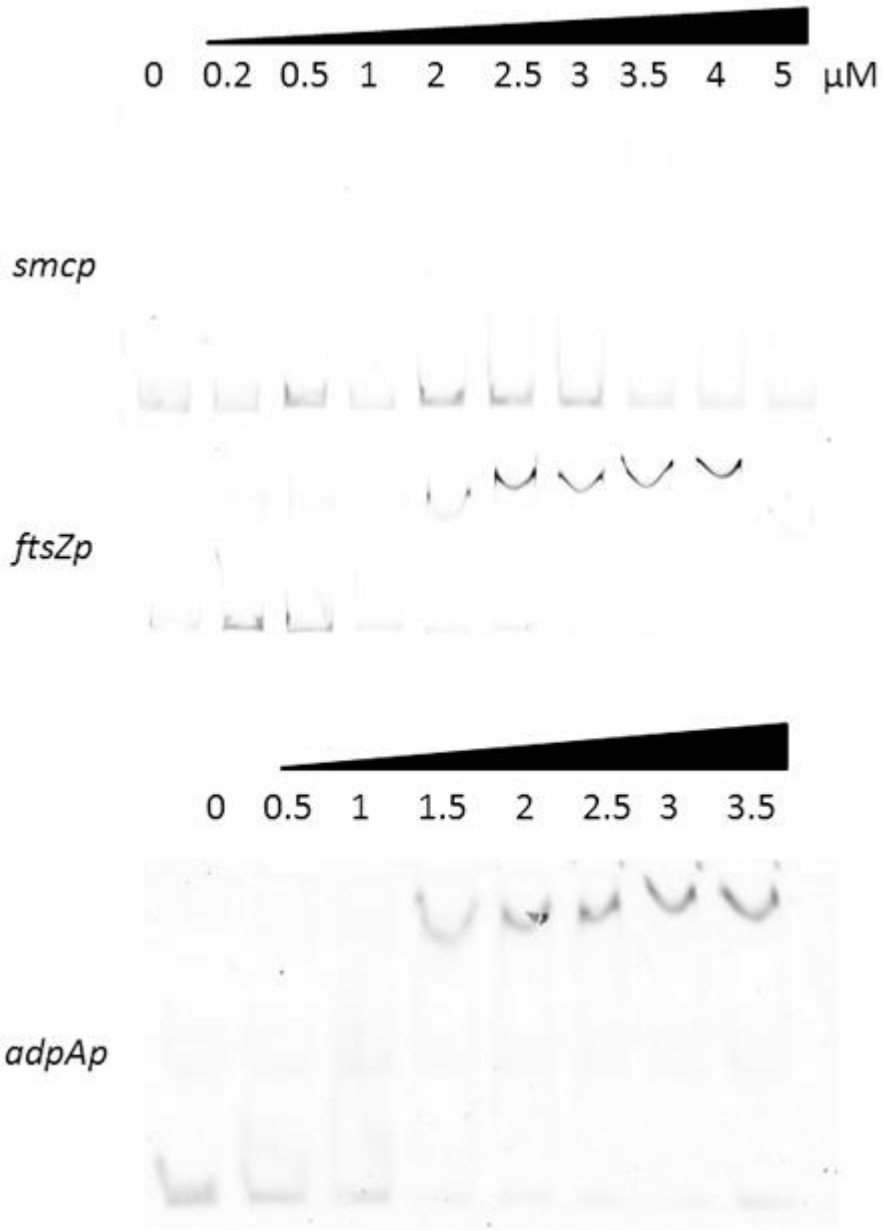

Figure 5.5 Promoter regions not shifted by purified MtrA. The shift of the *ftsZ* and *adpA* promoter is unspecific binding because the negative control start to shift at 0.9 µM of protein. The *smc* promoter is not shifted by increasing amount of MtrA which is most likely due to the smaller DNA fragment used for *smcp*. 10 nM DNA was added to increasing concentrations of MtrA.

5.3 *In silico* binding site of MtrA

The next step to identify the regulon of MtrA in more detail was to identify the binding site. Therefore, all enrichment peaks at the different time points were investigated via the motif predicting software MEME (http://meme-suite.org/). A 50 bp nucleotide sequence at the base of each enrichment peak was analysed by MEME. When the whole data set was analysed by MEME no significant binding site could be observed. Thus, the three promoter regions shifted by EMSA (see section 4.3.3), *ectAp* (SVEN15_0205), *dnaAp* (SVEN15_3571) and *cmlFp* (SVEN15_0880) were used as a starting point to find a conserved MtrA binding motif. All three promoters contain the binding site T (A/C) C G T T (C/A) T which is referred to here as motif [1], see **Figure 5.6**. When the promoters enriched at 10, 12 and 20 hours were analysed by MEME a direct repeat of [1] could be identified, referred to here as motif [2]. A similar direct repeat could also be observed when the individual time points were analysed (motifs [3] – [6]). However, only a small proportion of enrichment peaks contained the putative MtrA binding site. The enriched promoter regions of the different time points containing motifs [2] - [6] were then analysed by MEME to obtain a clear binding site, motif [7]. Binding motif [1] could not be observed in the 20-hour data set and thus the binding motif [7] was used to search in the 20-hour data set. The resulting binding motifs were added to the previous obtained binding sites and combined through MEME to identify motif [8]. This motif is a direct repeat and can be found in 43 enriched targets listed in **Table 5.1**. 28 of the target genes in **Table 5.1** are not significantly up or down regulated in the RNA-seq data, whereas 13 target genes are up or downregulated, see **Table S4**. Interestingly the list of targets containing the putative binding site does not include *dnaAp* and *cmlFp* which might indicate that these promoter regions contain only the half site (motif [1]) and not a full length direct repeat binding motif.

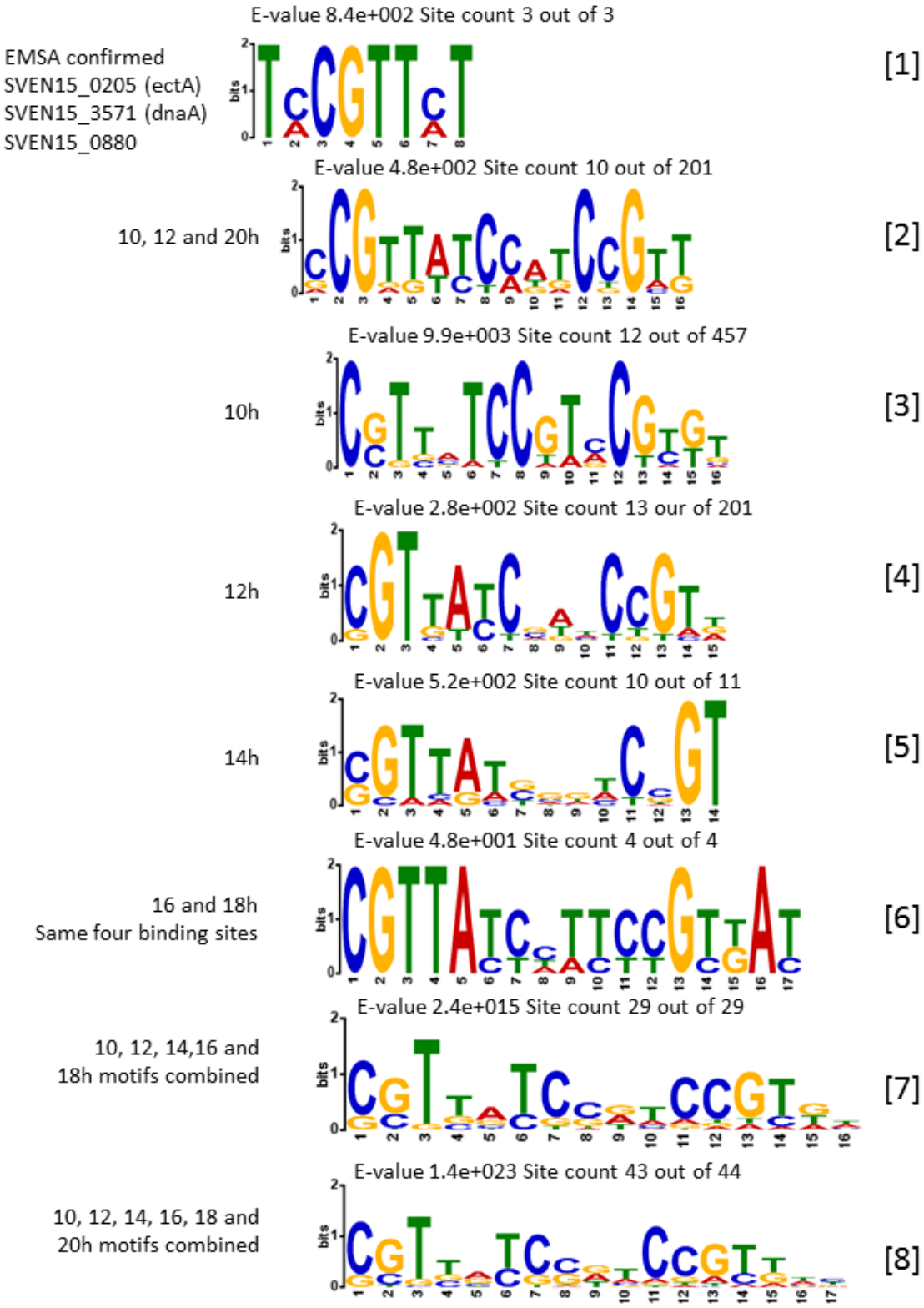

Figure 5.6 Putative MtrA binding site predicted by MEME software (Bailey et al. 2009). The motif [1] at the top was obtained with the three promoters shifted in the EMSA assay, see section 5.2. Data sets of the indicated time points were analysed with the MEME software and binding motifs similar to the motif at the top are shown.

Table 5.1 Putative MtrA binding sites in *S. venezuelae*. Bold genes are part of a BGC.

Gene	Strand	Distance from TSS	Annotation	Gene	Strand	Distance from TSS	Annotation	Time point of binding site
SVEN15_2560	-1	104	probable nucleotide pyrophosphatase					10h
SVEN15_4405	-1	-186	carboxymuconolactone decarboxylase domain or alkylhydroperoxidase AhpD family core domain protein					10h
SVEN15_7072	**1**	**209**	**hypothetical protein**					10h
SVEN15_3771	1	-208	putative peptidase					12h
SVEN15_6511	-1	46	Xylose isomerase	SVEN15_6512	1	-92	Xylose kinase	12h
SVEN15_6516	-1	83	Transcriptional regulator, HxlR family	SVEN15_6517	1	-66	Xylose repressor XylR	12h
SVEN15_1902	1	142	Ferrous iron transport periplasmic protein EfeO, contains peptidase-M75 domain and (frequently) cupredoxin domain					14h
SVEN15_2911	-1	169	Cell wall-binding protein					14h
SVEN15_6108	**-1**	**98**	**Anthranilate phosphoribosyl-transferase**	**SVEN15_6109**	**1**			14h
SVEN15_5419	-1	124	putative DNA-binding protein	SVEN15_5420	1			14h
SVEN15_0377	1	-45	Substrate-specific component CbiM of cobalt ECF transporter					20h
SVEN15_1274	1	-94	hypothetical protein					20h
SVEN15_1260	1	-31	hypothetical protein					20h
SVEN15_3174	-1	109	putative secreted protein					20h
SVEN15_0096	-1	7	hypothetical protein					20h
SVEN15_3317	-1	29	hypothetical protein	SVEN15_3318	1	-62	3-oxoacyl-[acyl-carrier protein] reductase	20h
SVEN15_7069	**-1**	**61**	**TetR-family transcriptional regulator**	**SVEN15_7070**	**1**	**-53**	**Long-chain-fatty-acid--CoA ligase**	20h
SVEN15_4448	-1	146	putative glycosyltransferase	SVEN15_4449	1	-74	hypothetical protein	20h
SVEN15_3473	-1	51	hypothetical protein	SVEN15_3474	1	-17	Methionyl-tRNA synthetase	20h
SVEN15_3850	-1	45	Transcriptional regulator, MarR family	SVEN15_3851	1	-41	hypothetical protein	20h
SVEN15_0505	1	37	hypothetical protein					20h
SVEN15_1266	-1	25	Butyryl-CoA dehydrogenase	SVEN15_1267	1	-38	Transcriptional regulator, TetR family	20h

SVEN15_3420	**1**	**41**	**S-adenosylmethionine synthetase**				20h
SVEN15_5187	1	83	Protein often found in Actinomycetes clustered with signal peptidase and or RNaseHII				20h
SVEN15_3517	-1	-386	Potassium channel protein				20h
SVEN15_0205	**1**	**-161**	**L-2,4-diaminobutyric acid acetyltransferase**				12, 14, 16 and 20h
SVEN15_2566	-1	80	putative secreted oxidoreductase				12, 14, 16 and 20h
SVEN15_6986	-1	119	ABC transporter solute-binding protein	SVEN15_6987	1	-89	D-amino-acid oxidase	10, 12, 20h
SVEN15_4857	1	-106	hypothetical protein					10, 12, 20h
SVEN15_0668	-1	67	hypothetical protein	SVEN15_0669	1	-209	putative integral membrane transport protein	10, 12, 20h
SVEN15_2757	-1	62	hypothetical protein					10, 12, 20h
SVEN15_1260	1	-31	hypothetical protein					10, 12, 20h
SVEN15_2164	-1	77	Beta-glucosidase	SVEN15_2165	1	-86	Transcriptional regulator, TetR family	10, 12, 20h
SVEN15_3771	1	-283	putative peptidase					10, 12, 20h
SVEN15_3825	-1	208	Transcriptional regulatory protein glnR	SVEN15_3826	1	-49	putative hydrolase	10, 12, 20h
SVEN15_2417	-1	151	hypothetical protein					12, 14, 20h
SVEN15_5675	1	14	hypothetical protein					10, 20h
SVEN15_0036	1	-203	hypothetical protein					10, 20h
SVEN15_0530	-1	76	Tyrosine protein kinase: Serine or threonine protein kinase: Sel1 repeat					10, 20h
SVEN15_1746	-1	69	hypothetical protein	SVEN15_1747	1	-44	Transcriptional regulator, PadR family	10, 20h
SVEN15_4327	1	-53	hypothetical protein					10, 20h
SVEN15_5674	1	21	hypothetical protein					12, 20h

5.4 MtrA immunoprecipitation

Since MtrA binds promoter regions of genes which do not differ in expression in *S. venezuelae* Δ*mtrB* mutant like *ftsZ* or *divIVA* it is possible that MtrA does not bind directly to the promoter region but instead binds to other DNA binding proteins. Additionally, it was very difficult to identify an MtrA binding motif which might also indicate the involvement of other DNA binding proteins. Also it was demonstrated that MtrA(TB) interacts with the DNA replication initiation protein DnaA (Purushotham et al. 2015). Thus, we performed immunoprecipitation in the strain *S. venezuelae* Δ*mtrA* ΦBT1 *mtrAp-mtrA-3xFlag* at 18 hours with crosslinked samples which are similar to the ChIP-seq samples and non-crosslinked samples and analysed the immunoprecipitated proteins by MALDI-TOF using the wild-type strain as a negative control.

In total 47 proteins were identified in both crosslinked and non-crosslinked experimental samples and were not present in the wild-type control experiments. One protein is part of BGCs and three pulled down proteins are involved in cell division and development, see **Table 5.2**. MtrA interacts with CmlS (SVEN15_0892) which is part of the chloramphenicol cluster and chlorinates an acetyl group of chloramphenicol (Latimer et al. 2009). Consistent with data in *M. tuberculosis* MtrA(Sv) interacts with DnaA(Sv). MtrA also interacts with two uncharacterised proteins SVEN15_1384 and SVEN15_3776 which are annotated as a SpoOJ, ParA or ParB or RepB family protein and a sporulation associated protein, respectively. MtrA binds to five regulatory proteins. The probable regulatory protein SVEN15_4644 contains a Serine/threonine phosphatase domain and a histidine kinase ATPase domain. SVEN15_2691, SVEN15_4036 and SVEN15_4568 are putative DNA binding proteins. MtrA also interact with three transporter proteins which are likely to be membrane bound. It is surprising to see membrane bound proteins interacting with MtrA in this experiment because the majority of membrane proteins were removed by centrifuging the cell lysate. This could be the reason why MtrB was not co-immunoprecipitated. It is likely that the interaction of DisA and the two ribosomal subunits could be just due to the proximity of these proteins to MtrA and DNA. The remaining 30 proteins are either involved in general metabolism or they are hypothetical genes.

Table 5.2 Proteins co-purified with MtrA by immunoprecipitation. Only proteins present in both crosslinked and non-crosslinked samples and not in the wild-type control are shown.

Function	SVEN15	kDa	Annotation	Comment	In ChIP-seq	
					Time point	Normalised enrichment
BGC	**SVEN15_0892**	**64 kDa**	**CmlS flavin-dependent halogenase**	**part of chloramphenicol gene cluster**	**no**	
Cell division	SVEN15_1384	47 kDa	SpoOJ or ParA or ParB or repB family protein	Chromosome segregation	no	
	SVEN15_3571	**66 kDa**	**Chromosomal replication initiator protein DnaA**		**20h**	**4.9**
	SVEN15_3776	51 kDa	sporulation associated protein		10, 12 and 20h	8, 8.1 and 9.5
Regulatory	SVEN15_2691	27 kDa	putative two-component system response regulator	RR of CitB family	no	
	SVEN15_4036	86 kDa	probable DNA-binding protein		no	
	SVEN15_4568	28 kDa	Transcriptional regulator	IclR family	no	
	SVEN15_4644	93 kDa	probable regulatory protein	Regulated by σ^U	10 and 20h	7 and 7.1
	SVEN15_6329	15 kDa	multi-component regulatory system-3, containing roadblock or LC7 domain		no	
Transporter	SVEN15_1516	33 kDa	ABC-type multidrug transport system, ATPase component		no	
	SVEN15_1941	36 kDa	putative iron-siderophore uptake system exported solute-binding component		20h	8.3
	SVEN15_4939	42 kDa	putative ABC transporter ATP-binding protein		no	
DNA repair	SVEN15_3142	38 kDa	DNA integrity scanning protein disA	DNA repair	no	
Ribosomal	SVEN15_1141	42 kDa	Ribosomal RNA small subunit methyltransferase C		no	
	SVEN15_2914	31 kDa	rRNA small subunit methyltransferase I		no	
Metabolism	SVEN15_0071	19 kDa	4-hydroxy-2-oxovalerate aldolase	metabolic pathways	20h	9.1
	SVEN15_1244	62 kDa	Fumarate or succinate or L-aspartate dehydrogenases	metabolic pathways	20h	17.7
	SVEN15_1457	48 kDa	Cobyrinic acid A, C-diamide synthase	metabolic pathways	10, 12 and 20h	8.4, 7.3 and 8.3
	SVEN15_1475	52 kDa	Ketoglutarate semialdehyde dehydrogenase	metabolic pathways	20h	7.9
	SVEN15_1718	42 kDa	Ferredoxin reductase	metabolic pathways	no	
	SVEN15_1807	29 kDa	Octanoate-[acyl-carrier-protein]-protein-N-octanoyl transferase (*lipB*)	Lipoic acid biosynthesis	no	
	SVEN15_2469	66 kDa	Epi-inositol hydrolase	metabolic pathways	no	
	SVEN15_2728	38 kDa	putative glycosyl transferase			
	SVEN15_4412	45 kDa	Serine hydroxymethyltransferase	metabolic pathways	no	

Metabolism	SVEN15_4420	45 kDa	putative D-alanyl-D-alanine carboxypeptidase	metabolic pathways	20h	12.3
	SVEN15_4667	78 kDa	putative peptidase	metabolic pathways	10h	8.1
	SVEN15_4676	36 kDa	putative acetyltransferase	metabolic pathways	20h	8.3
	SVEN15_5627	27 kDa	putative phospholipase protein	metabolic pathways	no	
	SVEN15_5681	81 kDa	putative bifunctional hydroxylase or oxidoreductase	metabolic pathways	no	
	SVEN15_5961	35 kDa	putative uricase	metabolic pathways	no	
	SVEN15_6151	38 kDa	Glycerate kinase	metabolic pathways	no	
	SVEN15_6256	38 kDa	Probable acyl-ACP desaturase, Stearoyl-ACP desaturase	fatty acid biosynthesis	20h	8.4
	SVEN15_6418	26 kDa	putative secreted protein		20h	10.3
	SVEN15_6432	35 kDa	putative periplasmic protein kinase ArgK and related GTPases of G3E family		no	
	SVEN15_6690	72 kDa	Succinate dehydrogenase flavoprotein subunit	Energy metabolism	no	
	SVEN15_7027	47 kDa	Glutamate-1-semialdehyde aminotransferase	metabolic pathways	no	
	SVEN15_7036	45 kDa	methyltransferase	metabolic pathways	no	
Hypothetical Proteins	SVEN15_1225	29 kDa	hypothetical protein		no	
	SVEN15_2323	42 kDa	hypothetical protein	RfaB multidomain: Glycosyltransferase involved in cell wall biosynthesis	no	
	SVEN15_2364	23 kDa	hypothetical protein		no	
	SVEN15_2396	47 kDa	hypothetical protein	Contains smc domain (Chromosome segregation ATPase)	no	
	SVEN15_3292	31 kDa	hypothetical protein		no	
	SVEN15_3538	52 kDa	hypothetical protein		no	
	SVEN15_4870	23 kDa	hypothetical protein	predicted aceyltranferase domain	10, 12 and 20h	10.5, 8.1 and 26
	SVEN15_5400	69 kDa	hypothetical protein	no		
	SVEN15_6683	30 kDa	hypothetical protein		20h	10.2
	SVEN15_6869	25 kDa	hypothetical protein		no	

5.5 Discussion

The MtrA regulon seems to be complex. EMSA showed that MtrA binds to target genes but also that promoter regions enriched in ChIP-seq are not directly bound by monomeric or dimeric purified MtrA. Promoter regions which are directly bound by MtrA are those of the ectoine BGC *ectA*, the chromosome replication initiation protein *dnaA* and *cmlF* which is part of the chloramphenicol cluster and encodes a chloramphenicol efflux pump (He et al. 2001). The enrichment peak in the promoter region of *dnaA* is not included in the ChIP-seq data because the enrichment is under the significant cut off (5 at 20 hours).

However, the promoter of *dnaA* is directly bound by MtrA which means that the statistical cut off of p = 0.05 might be too strict and that the binding of MtrA has to be confirmed with further experiments. The purified MtrA does not shift the promoter regions of *adpA* and *ftsZ*. This could implicate that MtrA binds to additional proteins which bind to the promoter DNA or that MtrA forms a heterodimer with another DNA binding protein which is discussed below.

MtrA directly binds to the promoter of *dnaA* but also to two sites between *dnaN* and *dnaA* which is where part of the origin of replication (*oriC*) can be found. It was not possible to amplify enough clean DNA for the *oriC* region to perform EMSA thus it remains unclear if MtrA binds directly to *oriC* or binds a protein which binds to *oriC*. However, immunoprecipitation suggested a protein-protein interaction of MtrA with DnaA. DnaA binds to a 9 bp consensus region (DnaA boxes) to unwind DNA to provide an entry site for the DnaB / DnaC helicase complex (Kornberg & Baker 1992). *Streptomyces* have 19 DnaA boxes in the *oriC* and two DnaA boxes upstream of *dnaA* which are conserved throughout *Streptomyces* species (Jakimowicz et al. 1998; Jakimowicz et al. 2000). Additionally AdpA competes for binding at *oriC* with DnaA in an ATP dependent manner to prevent initiation of DNA replication (Wolański et al. 2012). There are two possibilities which can explain the two enrichment peaks of MtrA in *oriC*. First MtrA could directly bind to *oriC* and second MtrA binds to DnaA which in turn binds *oriC*. If MtrA binds directly to *oriC* it would have a similar role as AdpA of preventing DnaA of binding. In this case binding of MtrA to *oriC* would be highest during vegetative growth and decrease at the onset of sporulation when chromosomes are actively replicated. However, this is not the case. MtrA does not bind to *oriC* in a high frequency represented by the low enrichment but the enrichment increases at 20 hours.

Thus, it is unlikely that MtrA has a similar role as AdpA. The binding of MtrA is more likely to be indirect via DnaA since the expression of *dnaN* is not significantly changed in *S. venezuelae* Δ*mtrB.* The data presented here do not elucidate the role of MtrA binding to *oriC* or *dnaAp* and needs further investigation. However, the interaction of MtrA with *oriC* and the protein interaction with DnaA clearly show that MtrA has an important role in the regulation of DNA replication in *Streptomyces*.

MtrA binds to the promoter region of *ftsZ*. The MtrA enrichment peak lies in the *ftsZ2p* which is controlled during development and specifically upregulated in sporulating aerial hyphae (Flärdh et al. 2000). A possible explanation why *ftsZ* expression is not altered in the Δ*mtrB* mutant could be that MtrA does not bind directly to the *ftsZ* promoter (**Figure 5.5**). It is possible that MtrA binds to an unknown DNA binding protein which in turn binds to the promoter region of *ftsZ*. In the *S. coelicolor* Δ*mtrA* mutant *ftsZ* expression is downregulated and the *S. coelicolor* Δ*mtrA* mutant forms irregular septa (Knowles 2014). The downregulation of *ftsZ* in *S. coelicolor* Δ*mtrA* together with the data obtained in *S. venezuelae* indicate that the effect of MtrA on *ftsZ* expression is indirect.

DnaA is not only the initiation protein of chromosome replication it also acts as a transcription factor which can activate or repress gene expression (Messer & Weigel 1997). The regulon of DnaA has been determined in *Caulobacter crescentus* and includes several components of the replisome, the global cell cycle regulator GcrA, the polar localization protein PodJ, nucleotide biosynthesis enzymes and FtsZ. The binding of DnaA to the promoter regions was shown by EMSAs (Hottes et al. 2005). The latter target of DnaA is of great interest because *ftsZ* is indirectly regulated by MtrA and it is possible that MtrA binds DnaA which then binds to the promoter region of *ftsZ* in *S. venezuelae* and *S. coelicolor*. However, it is not known so far if DnaA acts as a transcription factor regulating *ftsZ* in *Streptomyces* and this hypothesis needs experimental testing. One possible way to test this hypothesis could be ChIP-seq with DnaA in *S. venezuelae*.

It was demonstrated that DnaA and MtrA(TB) interact in *M. tuberculosis*. However, the nature of this protein-protein interaction was not investigated any further. RR form homodimers upon phosphorylation to bind to DNA. Heterodimer formation of two different RR is a common regulatory mechanism in eukaryotes (Reményi et al. 2004) but very rare in prokaryotes. In recent years, examples of RR heterodimer formation as regulatory tool in bacteria have been investigated. One example of heterodimer formation is the developmental genes *whiI* and *bldM* in *S. venezuelae*. BldM and WhiI belong both

to the NarL / FixJ subfamily of RR and are atypical RR regulator with an altered phosphorylation pocket. The homodimer of BldM regulates gene expression early in development whereas the BldM-WhiI regulates a different group of target genes later in the developmental cycle (Al-Bassam et al. 2014). Another example of heterodimer formation is the RR RcsB in *E. coli*. RcsB is phosphorylated by its cognate HK RcsD and forms a homodimer to activate target genes including *rprA*, *osmC*, *osmB* and *ftsZ* (Majdalani & Gottesman 2005; Majdalani & Gottesman 2007). RcsB forms a heterodimer with RcsA which has a typical LuxR- type C-terminal DNA-binding domain but the N-terminal region is not related to RR. The RcsB-RcsA heterodimer activates genes encoding for exopolysaccharide synthesis which are required for capsule formation. A third example of heterodimer formation is NarL-DevR in *M. tuberculosis*. NarL and DevR were shown to interact and both activate genes in nitrate / nitrite metabolism. Both RR belong to the LuxR family (Malhotra et al. 2015). It seems to be a common theme that RR which form heterodimers are from the same family. MtrA belongs to the OmpR family of RR. However, no OmpR family RRs could be observed in the immunoprecipitation (**Table 5.2**) but a RR of the CitB family and a transcriptional regulator of the IcIR family. The immunoprecipitation of MtrA is a preliminary result and the protein-protein interaction needs to be validated with further experiments like Bacterial Two Hybrid (BACTH) or protein pull down assay to investigate the protein-protein interaction of MtrA with other DNA binding proteins. However, it is likely that MtrA interacts with other DNA binding proteins because only 44 out of 1615 target genes contain the *in silico* direct repeat MtrA binding site (**Figure 5.6Figure**). The MtrA DNA binding site can be determined by DNase I footprinting in future experiments to verify that only a few target genes in the ChIP-seq time course data have a MtrA binding site.

Surprisingly the immunoprecipitation suggests that MtrA interacts with CmlS which chlorinates an acetyl group of chloramphenicol (**Table 5.2**). The chlorination of chloramphenicol is essential for its antibacterial activity (Suzuki et al. 1972; Podzelinska et al. 2010). This indicates that MtrA might not only regulate secondary metabolite production on transcriptional level but also could modify the synthesis of chloramphenicol by interacting with a protein involved in chloramphenicol synthesis which is not presented in the literature before.

5.6 Conclusion and future work

The data outlined in this chapter show that MtrA(Sv) can be phosphorylated by acetyl phosphate. However, the truncated version of MtrB was not functional and the phosphotransfer from MtrB to MtrA could not be tested. To investigate the phosphotransfer full length MtrB could be reconstituted in proteoliposomes in the same way MtrB(Cg) was investigated (Möker et al. 2007).

Furthermore, the *in vitro* binding of MtrA investigated by EMSAs indicates that the ChIP-seq data needs further validation. The enrichment peak of *dnaA* is under the statistical cut off but MtrA binds to the *dnaAp in vitro*. More promoter regions need to be tested either by EMSA or DNase I footprinting to verify the target genes list. However, the data outlined in this chapter suggests that MtrA binds to other DNA binding proteins which makes the MtrA regulon more complex.

6 Conclusion

This project identified the TCS MtrAB as a new master regulator of development and secondary metabolite production. However, this project just scratched the surface of the regulatory role of MtrAB and many questions remain unanswered and further investigation is needed.

The RR MtrA is essential in *S. venezuelae* most likely due to the involvement in the initiation of DNA replication. Removal of *mtrB* from the chromosome leads to permanent activation of MtrA most likely by small phosphor donors or cross talk from other HKs. The activation of MtrA is reflected by the increased production of secondary metabolites in *S. venezuelae* and *S. coelicolor*. Introducing a gain of function MtrA protein has a similar effect on secondary metabolite production as shown for chloramphenicol.

The MtrA regulon overlaps with the BldD regulon which is a master regulator of development. Furthermore, MtrA might regulate the synthesis and degradation of c-di-GMP which activates BldD. Other target genes of MtrA suggest that MtrB senses osmotic stress e. g. sigma factors, the ectoine operon and components of the polarisome. The complexity of the MtrA regulon is most likely due to the interaction of MtrA with other DNA binding proteins.

7 References

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8 Supplementary data provided on a CD at the end of the thesis