Svoluji k zapůjčení své diplomové práce ke studijním účelům a prosím, aby byla vedena přesná evidence vypůjčovatelů. Převzaté údaje je vypůjčovatel povinen řádně ocitovat.

Charles University in Prague

Faculty of Science

Department of Genetics and Microbiology

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Bc. Milada Kambová

SigN from *Bacillus subtilis*: Functional characterization.

SigN z *Bacillus subtilis*: Funkční charakterizace.

Diploma Thesis

Supervisor: doc. Mgr. Libor Krásný, Ph.D.

Prague, 2021

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

Declaration:

I hereby declare that I have written this Diploma Thesis independently and that I have cited all sources and literature. This Thesis has not been submitted for any other degree or purposes.

V Praze, in Prague Signature:

Acknowledgments:

I want to thank all members of the Krásný lab for a warm, friendly and always helpful environment. I want to especially thank my supervisor doc. Mgr. Libor Krásný, Ph.D. for his guidance and Mgr. Hana Šanderová, Ph.D. for her counsel and help with experimental work. I also want to thank Ing. Marek Schwarz for his bioinformatical prediction of σ^N-dependent promoters in chromosomal DNA. Last but not least, I would like to thank Mgr. Petra Sudzinová, Ph.D. for her invaluable advice.

Abstract

Bacillus subtilis strain 3610 is an ancestral undomesticated strain. It differs from the laboratory strain 168 in many aspects. One difference in strain 3610 is the presence of plasmid pBS32 encoding the sigma factor N (σ^N). This σ factor is activated when DNA damage occurs and induces the bacteria's cell death. The aim of the Thesis was a systematic characterisation of σ^N-dependent transcription. First, I showed that plasmid-borne but not chromosome-borne predicted σ^N-dependent promoters were active in transcription *in vitro*. Next, the affinities of RNAP with σ^N for DNA, initiating NTP (iNTP) were determined for both relaxed and supercoiled DNA templates. Surprisingly, the activity of RNAP on relaxed σ^N-dependent promoters was higher than on their supercoiled versions, an opposite trend than displayed by RNAP associated with other σ factors. This property of σ^N-dependent promoters was not encoded by the core promoter sequence. In summary, this Thesis contributed to our understanding of the bacterial transcription apparatus.

Key words: SigN, *Bacillus subtilis*, plasmid pBS32, transcription, RNA polymerase

Abstrakt

Bacillus subtilis kmen 3610 je ancestrální nedomestikovaný kmen. Liší se od laboratorního kmene 168 v mnoha ohledech. Jedna z odlišností v kmeni 3610 je přítomnost plasmidu pBS32 kódujícího sigma factor N (σ^N). Tento σ faktor je aktivovaný po poškození DNA, a jeho exprese indukuje buněčnou smrt. Cíle této práce byla systematická charakterizace transkripce tohoto nového σ faktoru. Za prvé jsem ukázala, že pouze predikované promotory na plasmidu, ne na chromosomu, byly aktivní v *in vitro* transkripcích. Dále jsem určila afinitu RNAP se σ^N k DNA a iniciační NTP (iNTP) jak na relaxované, tak na DNA s nadšroubovicovým vinutím. Aktivita RNAP na relaxovaných σ^N-dependentních promotorech byla překvapivě vyšší než na promotorech v DNA s nadšroubovicovým vinutím, což je protikladem k RNAP asociovaných s jinými σ faktory. Tato vlastnost σ^N-dependentních promotorů nebyla kódovaná v promotorovém jádře. Shrnutě, tato práce přispěla k našemu pochopení bakteriální transkripce.

Klíčová slova: SigN, *Bacillus subtilis*, plazmid pBS32, transkripce, RNA polymeráza

Contents

1 Abbreviations

- BSA – Bovine serum albumin
- BSB1 – BaSysSio = *B. subtilis* 168 trp +
- ECF – extracytoplasmic function
- gDNA – genomic = chromosomal DNA
- iH_2O – aqua pro injection
- [iNTP] – concentration of iNTP
- IPTG – Isopropyl β-D-1-thiogalactopyranoside
- LIN DNA – linearised = relaxed DNA
- ME – 2-mercaptoethanol
- nt – nucleotide
- OD – optical density
- ONPG – ortho-Nitrophenyl-β-galactoside
- PAGE – Polyacrylamide Gel Electrophoresis
- (p)ppGpp – guanosine 3',5' bisphosphate for ppGpp and guanosine 3',5' trisphosphate for pppGpp
- RNAP – RNA polymerase
- RP_O and RP_C – open and closed promoter complex
- *rut* site – rho utilisation site
- SC DNA – supercoiled DNA
- UP element – upstream element

2 Introduction

Bacillus subtilis is a model organism for gram-positive bacteria. it is often used as a model for pathogenic bacteria (as a close relative), sporulating bacteria and bacteria important for biotechnological production. For laboratory experiments, the "wild type" strain is strain 168. However, this strain was isolated after UV and radioactive irradiation, selected for its fast growth on a relatively nutritionally poor media (Burkholder & Giles, 1947).

B. subtilis strain NCBI 3610, also known as *B. subtilis* 3610, is the ancestral undomesticated strain. It differs from strain 168 in a number of aspects. One difference is the presence of plasmid pBS32 in strain 3610, which was likely lost in irradiation experiments leading to strain 168 (Earl et al., 2007; Burkholder & Giles, 1947). This plasmid encodes sigma N factor (σ^N) (Burton et al., 2019), also formerly known as ZpdN (Myagmarjav et al., 2016). σ^N is activated when DNA damage occurs and subsequently facilitates the bacterial programmed suicide.

In a previous study (Burton et al., 2019) σ^N-dependent promoter sequences were predicted. This Thesis experimentally confirmed and characterised selected σ^N-dependent promoters, defining their affinities for RNAP σ^N and the concentration of the initiating substrate (iNTP), performing the studies with supercoiled as well as relaxed DNA templates. The aim of this Thesis was to provide novel insights into the bacterial transcription machinery and its role in the programmed cell death.

3 Aims of the Thesis

The aim of this Thesis was to characterise transcription from σ^N-dependent promoters. To goals were to:

- Isolate σ^N and *B. subtilis* RNAP.
- Prepare DNA constructs for transcriptions.
- Experimentally test a selected number of previously predicted σ^N-dependent promoters.
- Determine the concentration of regulating iNTP for σ^N-dependent promoters.
- Investigate the *in vitro* effect of DNA topology on σ^N-dependent promoters.
- Investigate the *in vivo* effect of DNA topology on σ^N-dependent promoters.

4 Literature review

4.1 Bacterial transcription

Transcription is a process that produces RNA, a complementary copy of DNA. RNA has many divergent functions, the most notable are roles in protein production (mRNA, rRNA and tRNA), regulation of various processes (e.g. small RNAs) and toxin-antitoxin interactions. Bacterial transcription is mediated by one type of DNA dependent RNA polymerase (RNAP) in contrast to eukaryotic transcription that has five types of RNAP (Hurwitz, 2005).

4.1.1 Promoter

Promoters (Fig. 1) are specific regions in DNA lying upstream of a gene sequence (Burgess et al., 1969). Promoter sequences are recognised by σ subunits of RNAP and their nucleotide sequences are highly conserved for specific σ factors. The most important parts are -35 and -10 elements (ca 35 or 10 nucleotides upstream of transcription start site +1, respectively) recognised by σ factors (with the exception of σ^{54}) (Hawley & McClure, 1983).

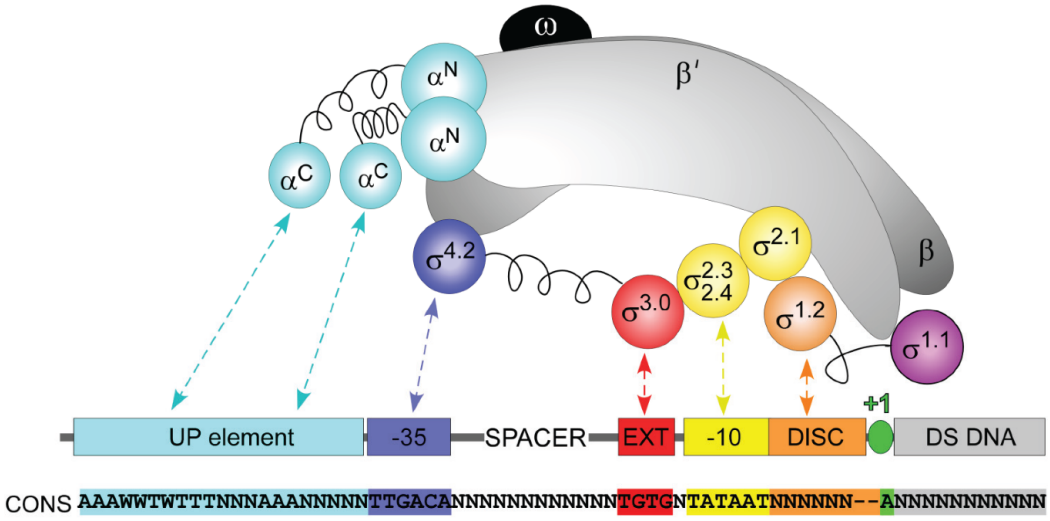

Figure 1: Interactions between DNA promoter and sigma regions of the RNAP holoenzyme. The consensus sequence of σ^{70} family promoters (CONS) is shown below the figure (Ruff et al., 2015).

Upstream of the promoter sequence sometimes lies the Upstream (UP) element. It is an AT-rich sequence approximately between positions -60 and -40 and is recognised by α-CTD domains of the RNAP α subunits (Blatter et al., 1994; Estrem et al., 1999). UP elements typically precede rRNA promoters and are involved in their regulation

(Condon et al., 1995).

The -35 element is highly conserved and is identical in both *E. coli* and *B. subtilis* ($^{-35}$TTGACA^{-30}) (Hawley & McClure, 1983) and is recognised by a helix-turn-helix motif in $\sigma_{4.2}$ (σ^{70} family) (Kenney & Moran, 1991).

The -35 and -10 elements are separated by the spacer. The typical spacer is 17 bp long and even one nucleotide deviation may result in large changes in transcription (Sztiller-Sikorska et al., 2011). Although its nucleotide sequence is not conserved, some nucleotide changes in the spacer can influence transcription (Liu et al., 2004).

The consensus sequence ($^{-12}$TATAAT^{-7}) of the -10 element (or alternatively called Pribnow box) is even more conserved than the consensus sequence of the -35 element (Hook-Barnard & Hinton, 2007). It is recognised by domains $\sigma_{2.3}$ and $\sigma_{2.4}$ in *E. coli*. The most important interaction between σ and the -10 element occurs at bases A_{-11} and T_{-7} (Feklistov & Darst, 2011). It is no surprise that the most conserved base of the -10 element is A_{-11} (Shultzaberger et al., 2007).

Upstream of the -10 element is the extended -10 motif ($^{-15}$TGn^{-13}) that can be found in 20 % of *E. coli* promoters (Burr et al., 2000). The extended -10 motif is recognised by $\sigma_{3.0}$ (Koo et al., 2009). Promoters in *E. coli* can be divided to promoters with -10 and -35 elements that are highly similiar to canonical -10 and -35 elements (but do not require extended -10 motif), and promoters with the extended -10 motif with the canonical -10 element but without the canonical -35 element (Hook-Barnard et al., 2006). Thus, the extended -10 motif is important for promoters with weak -35 elements and also for promoters with long spacers (Mitchell et al., 2003).

Downstream of the -10 element is the discriminator. It is a GC rich region with optimal sequence $^{-6}$GGG^{-4}; it is usually 7 nt long but the length can differ (Haugen et al., 2008). It is recognised by the unstructured $\sigma_{1.2}$ domain and is involved in the transition between initial transcription phases RP_O and RP_C (more about them below) (Haugen et al., 2008, 2006). It is present in rRNA promoters and thus the promoters' open complex is destabilised (when the transcription bubble is formed) and more prone to regulation by ppGpp (Haugen et al., 2006).

4.1.2 Bacterial RNAP

Bacterial RNAP is a ca 400 kDa large protein complex. Two α subunits, β, β' and ω form the RNAP core that is highly conserved in all three life domains (Murakami & Darst, 2003).

The assembly of the RNAP core (Fig. 2) is as follows: two copies of α form a homodimer. Then, β attaches to the homodimer. β' with bound ω binds to the complex and thus completes the formation of the core (Ghosh et al., 2001; Ganguly & Chatterji, 2011). This RNAP core by itself cannot initiate transcription. For transcription initiation RNAP needs to bind an appropriate σ factor that can recognise the promoter sequence (Feklístov et al., 2014). *B. subtilis*, the model organism used in this Thesis, contains also accessory subunits, termed δ and ε (Papoušková et al., 2013; Keller et al., 2014).

B. subtilis' α is a 34.64 kDa large protein. It consists of the N-terminal domain (α-NTD) and the C-terminal domain (α-CTD) connected by a flexible linker. α-NTD is responsible for α dimerisation (Blatter et al., 1994). α-CTD binds to AT-rich UP elements and could also interact with the C-terminal domain of σ (Blatter et al., 1994; Estrem et al., 1999).

α-CTD can bind various regulation factors, one of them is called Spx (Gaballa et al., 2013). It is a global regulator for oxidative and heat stresses in *B. subtilis*. Its binding to α-CTD blocks binding of other transcriptional regulators (Zhang et al., 2006), stimulates transcription from certain promoters (Rochat et al., 2012) and inhibits transcription of translation-related genes (Schäfer et al., 2019).

RNAP has a crab claw shape that consists of β (150 kDa) and β' (155 kDa). In this crab claw is the primary channel for DNA and DNA-RNA hybrids. At the back of the primary channel is the active centre with the catalytic Mg^{2+} ion. In RNAP also lies the secondary channel through which nucleotide triphosphates (NTPs) penetrate into the active site, it is separated from the primary channel by the bridge helix (Zhang et al., 1999).

The ω subunit (7.62 kDa in *B. subtilis*) is conserved among all domains of life (Minakhin et al., 2001). ω likely binds to β' before formation of the RNAP core (Gentry & Burgess, 1993) and helps β' to fold (Ghosh et al., 2003). In *E. coli* ω together with β' are important for the stringent response through binding of the ppGpp alarmone (Kåhrström, 2013). In *B. subtilis*, ppGpp does not bind to RNAP but instead lowers

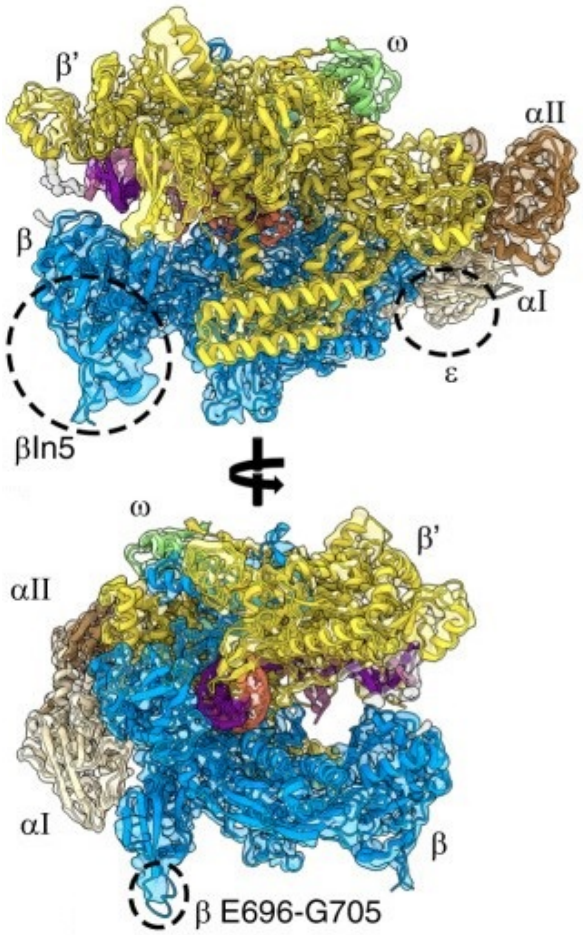

Figure 2: 3D structure from cryo-EM of *B. subtilis* RNAP in elongation complex. The colours of components are as follows: αI beige, αII brown, β azure, β' yellow, ω light green, template DNA purple, non-template DNA pink, and RNA orange. Dashed lines show the ε site, βln5 (β region that was not precisely calculated) and β E696-G705 (a protruding region that is unique to *B. subtilis*) (Newing et al., 2020).

GTP concentration, thereby decreasing transcription from GTP sensitive promoters (Krásný & Gourse, 2004; Kriel et al., 2012). ω' expression is stable during exponential phase, stationary phase and other 102 growth conditions (Nicolas et al., 2012).

The δ subunit (20.25 kDa in *B. subtilis*) is found only in Firmicutes (Weiss & Shaw, 2015). δ consists of the folded N-terminal domain and the unstructured, flexible C-terminal domain. The C-terminal domain is highly charged and consists of a K-tract (7 out of 9 amino acids are lysines) followed by the rest of the domain containing 51 negatively charged amino acids (Kubáň et al., 2019). δ increases competitive fitness of the cell and increases virulence (Rabatinová et al., 2013; Seepersaud et al., 2006). δ also enhances RNAP dissociation after termination (Wiedermannová et al., 2014). In *B. subtilis*, the δ level is increased in spores and in the transition state between

exponential and stationary phase (López de Saro et al., 1999).

The ε subunit (8.12 kDa in *B. subtilis*) is also found only in Firmicutes (Keller et al., 2014). ε binds to both αs, β and β' (Newing et al., 2020). It was believed to prevent access of putative bateriophage transcriptional factors (Keller et al., 2014), but structural data contradict this hypothesis and the function of ε subunit remains unclear (Newing et al., 2020).

4.1.3 Sigma factors

σ factors allow RNAP holoenzymes to recognise DNA promoters. Typically, the cells contain many different σ factors. Housekeeping σ factors recognise promoters that are transcribed in the exponential phase of growth. However, specific events such as sporulation or heat shock response call for specific actions (Riley et al., 2018; Straus et al., 1987). In this case a specific event calls for a specific sigma factor for transcription of specific genes. These σ factors for "specific" occasions are called alternative σ factors. The alternative σ factors recognise different promoter sequences than the housekeeping σ factor and usually a single promoter is recognised by only one σ factor (Campagne et al., 2015).

The number of σ factors varies between species. There are 7 σ factors in *E. coli*, 20 σ factors in *B. subtilis*, 109 σ factors in myxobacterium *Sorangium cellulosum* and only one σ factor in *Mycoplasma genitalium* (Burgess, 2001).

σ factors can be divided into two major families based on their homology: σ^{70} (based on *E. coli* σ^{70}) and σ^{54} (Zhang & Buck, 2015). σ^{70} is further subdivided into four groups. They have four distinct structural domains connected via flexible linkers and each sigma group is defined by the absence or presence of conserved domains (Fig. 3). Some of these domains bind promoter DNA, such as the -35 and the -10 element. (Paget & Helmann, 2003)

Group I of the σ^{70} family mostly consists of housekeeping (or primary) σ factors that initiate transcription needed in exponential phase of growth. In *E. coli* it is σ^{70}; in most bacterial species it is called σ^{A} (e.g. in *B. subtilis* or mycobacteria) but it can be called differently (e.g. HrdB in *Streptomyces coelicolor*). Group I σ factors have all 4 conserved regions, namely $\sigma_{1.1}$, $\sigma_{1.2\text{-}2.4}$, $\sigma_{3.0\text{-}3.2}$ and $\sigma_{4.1\text{-}4.2}$ that are connected via flexible linkers (total protein size is 70 kDa in *E. coli*, 42.8 kDa in *B. subtilis*) (Paget & Helmann, 2003). Of these domains the most conserved are σ_2 and σ_4 that interact with the -10 element and the -35 element, respectively. σ_4 has a helix-turn-helix motif

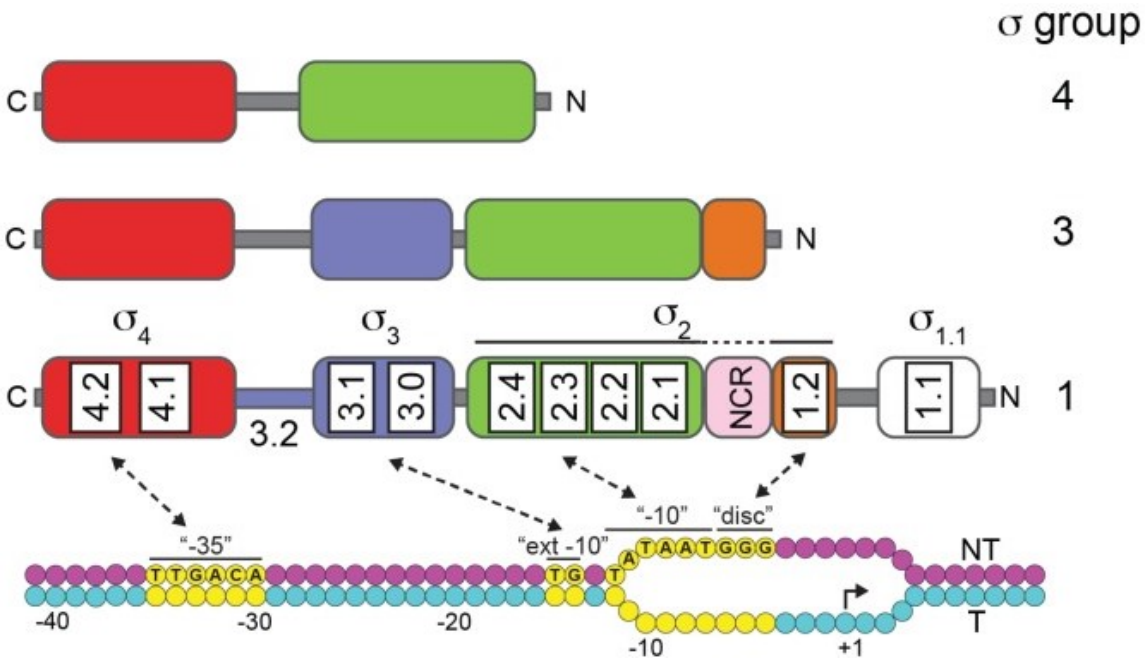

Figure 3: σ^{70} groups 1, 3 and 4 and their domain organisation. Below the σ groups is a promoter DNA with highlighted regions for σ binding (Paget, 2015).

recognising the -35 element. σ_4 also interacts with the β subunit of RNAP and this interaction contributes to the holoenzyme formation (Geszvain et al., 2004). Similarly, σ_2 interacts with the β' subunit of RNAP and recognises the -10 element, thus stabilizing the open complex in transcription (Arthur & Burgess, 1998). σ_3 in the form of three helices recognises the extended -10 element and thus facilitates transcription from promoters with weak -35 elements (Mitchell et al., 2003). $\sigma_{1.1}$ is unique to Group I. When the σ is not bound to the RNAP core, $\sigma_{1.1}$ interacts with the rest of σ so that the DNA binding sites are hidden in the protein and cannot bind to DNA (Schwartz et al., 2008). After binding of σ to the RNAP core, $\sigma_{1.1}$ lies in the RNAP primary channel and is displaced by DNA when RNAP interacts with the promoter. $\sigma_{1.1}$ stimulates isomerisation to open complex for selected promoters (Schwartz et al., 2008).

Group II lacks the $\sigma_{1.1}$ region and its σs are non-essential. Sigmas in Group II are usually involved in stress response of an organism in stationary phase such as heat, oxidative stress, nutrient stress, or high salt (Koskinen et al., 2016). Due to the high similarity between Group I and Group II some promoters recognised by Group I (e.g. σ^{70}) are also recognised by Group II σ factors (σ^S from *E. coli*) (Maciag et al., 2011). σ^S from *E. coli* is the most studied σ factor from Group II. σ^S is a general stress σ factor induced by slowed growth of bacteria (Ihssen & Egli, 2004) that controls expression of nearly 500 genes (Weber et al., 2005).

Group III is more diverse, more different from Group I than is Group II from Group I and typically contains domains σ_2, σ_3 and σ_4. Its σ_3 is able to recognise the extended -10 motif (Koo et al., 2009). These σ factors are involved in sporulation (σ^F, σ^E, σ^G and σ^K from *B. subtilis* (Hilbert & Piggot, 2004)), flagellum biosynthesis (σ^{28} in *E. coli* (Fitzgerald et al., 2018)), heat shock response and also in general stress response. The general stress response σ factor in *B. subtilis* σ^B belongs to Group III and regulates more than 160 genes (Kuo et al., 2015; Nannapaneni et al., 2012).

Group IV (also known as ECF (extracytoplasmic function) σ factors) is the largest σ group mostly comprising σ factors with extra cytoplasmatic function. After a signal from the environmnent, an anti-σ factor is degraded and an ECF σ factor is released and can bind to RNAP and initiate transcription (Fig. 4) (Mascher, 2013). ECF contain only the most essential domains: domain σ_2 and σ_4. The large diversity of ECF results in 43 phylogenetically distinct sub-groups (Staroń et al., 2009). An example from Group IV is σ^E from *E. coli* that is involved in virulence, cell viability, cell envelope integrity and is activated upon cell envelope stress (Nicoloff et al., 2017). *B. subtilis* has seven ECFs. The most studied one is σ^W, which responds to cell envelope stress, especially to antimicrobial peptides from other bacteria (Ellermeier & Losick, 2006).

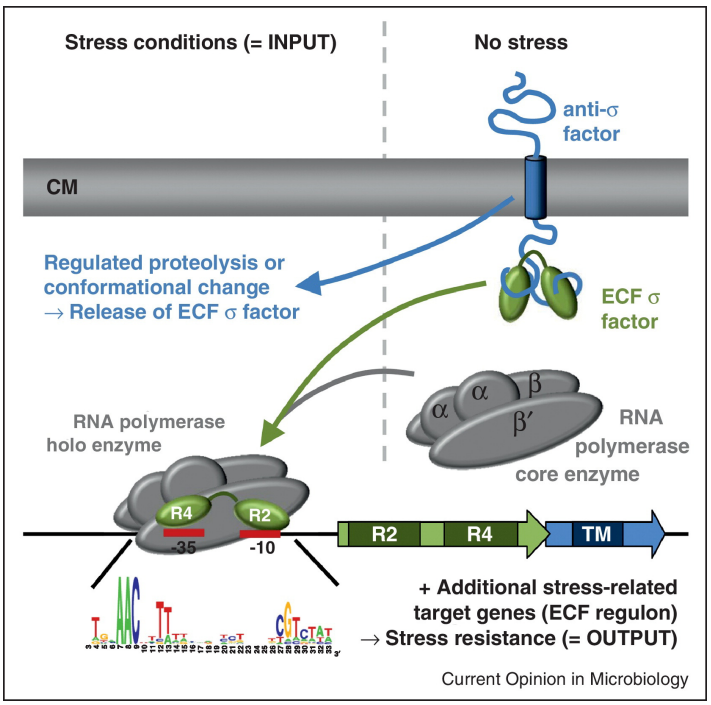

Figure 4: ECF activation after an outer stimulus by degradation of its anti-σ factor, release of ECF and binding to RNAP to initiate transcription (Mascher, 2013).

The σ^{54} family is much smaller than the σ^{70} family and its mechanism of transcription

initiation is different. It was named after the σ^{54} factor from *E. coli* that is involved regulation of nitrogen metabolism, pathogenesis and carbon utilisation (Keener & Kustu, 1988; Reitzer & Schneider, 2001). Although it is a small family, it is well conserved thoughout bacteria with the exception of endosymbionts and cyanobacteria (Riordan & Mitra, 2017). σ^{54} has three domains, in the literature called regions. An interaction between the σ^{54} Region I (N-terminal domain) and its enhancer binding protein promotes an ATP-dependent transcription initiation by formation of an open complex (Siegel & Wemmer, 2016; Taylor et al., 1996). Region II binds RNAP (Gallegos et al., 1999). Region III then recognises conservative sequences in the promoter that are positioned around -24 and -12 sites (Barrios et al., 1999; Wong et al., 1994). Also, an additional transcription activator known as an enhancer-binding protein is needed for transcription activation (Zhang et al., 2002). After σ^{54} binds to the promoter it waits for the enhancer-binding protein to bind to a DNA sequence approximately 100 nt upstream of the transcription start site.

4.1.4 Transcription

Transcription begins with the RNAP core that forms an RNAP holoenzyme by binding a σ factor (Fig. 5) (Murakami & Darst, 2003). This σ factor recognises a promoter sequence in DNA. The $\sigma_{4.2}$ domain recognises the -35 element (Kenney & Moran, 1991). Domains $\sigma_{2.3}$ and $\sigma_{2.1}$ recognise the -10 element (Feklistov & Darst, 2011) and if present, the $\sigma_{3.0}$ recognises the extended -10 motif (Koo et al., 2009). By binding of the RNAP holoenzyme to DNA, a closed complex (RP_C) where the DNA is still double-stranded is formed (Gries et al., 2010; Ruff et al., 2015). In the σ^{54} family, Region III of the σ factor recognises -24 and -12 elements in the promoter sequence to form RP_C (Wong et al., 1994). In RP_C RNAP is bound to the UP element (if present), -35 and -10 element protecting DNA from -55 to -5 nt (Ruff et al., 2015).

Transcription can only be performed on a single-stranded DNA, therefore melting of the promoter DNA is needed. σ_2 isomerises, the A_{-11} base is flipped to a pocket in σ_2 (Chen & Helmann, 1997). This is an intermediate state. Strands melt downstream of A_{-11} to +2 nt. The upstream T_{-12} base interacts with tryptophans of σ_2 (W433/W434 in σ^{70} from *E. coli*) sustaining the ds/ss junction (at -12/-11 site) (Bae et al., 2015). The melted template strand (nucleotides -9 to -5) are transferred to the RNAP active site through a tunnel between β and $\sigma_{3.2}$ (Zhang et al., 2012). The transcription bubble then expands from -11 to +2 or +3 nt and the open complex (RP_O) is formed.

In the σ^{54} family an enhancer binding protein binds to DNA upstream of the

transcription start site and its interaction with σ factor initiates the formation of a transcriptional open complex (Taylor et al., 1996).

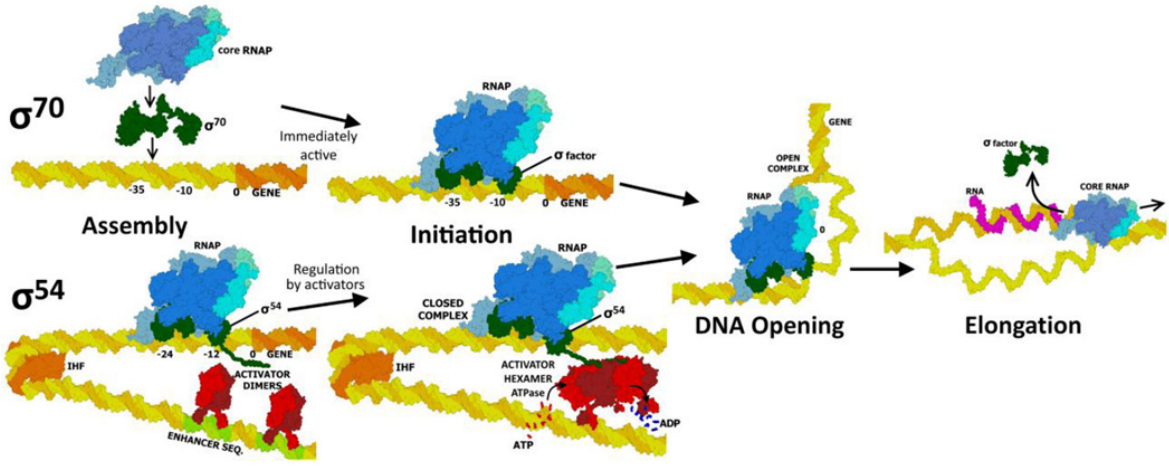

Figure 5: Transcription initiation schemes showing with differences between the σ^{70} and σ^{54} families. A) Assembly of RNAP holoenzyme and binding to promoter sequence. In the σ^{54} family, enhancer binding proteins bind to an enhancer sequence upstream of promoter. B) RP_C formation. In the σ^{54} family additional binding to enhancer binding proteins and ATP hydrolysis occur. C) Transcriptional RP_O formation and D) elongation (Siegel & Wemmer, 2016).

One molecule of initiating NTP (iNTP) enters through the secondary channel to the active site of RNAP. iNTP is the first incorporated NTP of a newly formed RNA transcript. (Zhang et al., 1999). The transcriptional bubble widens by 2 nt downstream and the iNTP binds its complementary nt at +1 (Glyde et al., 2018). Transcription proceeds and a 6-mer of RNA is formed. This stage between RP_O and transcriptional elongation complex is called initial transcribing complex. However, in the exit channel lies $\sigma_{3.2}$ that briefly pauses transcription (Murakami et al., 2002; Zhang et al., 2012). The newly-formed RNA must displace the $\sigma_{3.2}$'s loop to further proceed in transcription. If the RNA does not do it, transcription is aborted (abortive trancription) resulting in a ca 6 nt long transcript and the σ factor is released (Murakami et al., 2002). If transcription proceeds after the pause caused by $\sigma_{3.2}$, RNA reaches 8 or 9 nt, RNAP releases itself from the promoter and transcription initiation transitions to transcription elongation (Straney & Crothers, 1987; Bandwar et al., 2006).

During elongation when the correct NTP enters the secondary channel and binds to the active centre (Kettenberger et al., 2004), a conformational change occurs in the RNAP β' bridge helix and trigger loop that close the secondary channel and the NTP is delivered to the insertion site and a contact with Mg_2^+ is made (Kireeva et al., 2008).

After catalysis, a pyrophosphate is released that destabilises the closed conformation of active centre, trigger loop unfolds and opens the active centre (Brueckner et al., 2009). bridge helix then translocates DNA and RNAP moves to the next nucleotide (Mejia et al., 2015).

Termination of transcription at the ends of genes in bacteria occurs with two mechanisms: Rho-dependent and Intrinsic (also known as Rho-independent) (Fig. 6). Termination can also occur within genes and is mediated by different mechanisms. Examples of these defective terminations involve proteins such as Mfd, RNase J1 or HelD (Selby & Sancar, 1994; Mathy et al., 2007; Wiedermannová et al., 2014).

Rho factor is an ATP-dependent RNA translocase consisting of six homomeric units organised in a circle reminding of a washer (Geiselmann et al., 1992). It is involved in up to 50 % of *E. coli* transcription terminations, notably of small RNAs, tRNA, antisense transcripts. It is an essential protein (Peters et al., 2009). If the newly transcribed RNA contains the *rut* site (rho utilisation or the primary site), the Rho hexamer binds to this sequence, changes its conformation to a closed state and binds to the secondary site on RNA. Binding to the secondary site stimulates its ATPase activity (Richardson, 1982). This ATP-dependent translocation moves the Rho factor to RNAP that is paused ca 60-90 nt downstream of the *rut* site. Rho pushes RNAP from DNA and transcription is terminated (Shashni et al., 2012).

Intrinsic termination is defined by a GC-rich RNA hairpin followed by a run of uracils. The bond between uracils and adenines is relatively weak and this contributes to the dissociation of the newly formed RNA from the RNA:DNA transcriptional hybrid (Ray-Soni et al., 2016).

Mfd is a DNA repair protein (Roberts & Park, 2004). It recognises a stalled RNAP, binds to β' subunit and with its translocation site it binds to upstream DNA (Deaconescu et al., 2006). With its ATP-dependent activity Mfd removes RNAP from the stalled transcription complex (Park et al., 2002). Mdf can also autonomously translocate on DNA. If it "catches up" with a stalled or backtracked RNAP, it either helps RNAP to proceed in transcription or if the "obstacle" is too big, Mdf dissociates RNAP from DNA (Le et al., 2018).

RNase J1 is a 5'-to-3' exonuclease known in bacteria and archaea (Mathy et al., 2007; Condon et al., 2018; Phung et al., 2013). When an elongation complex is stalled,

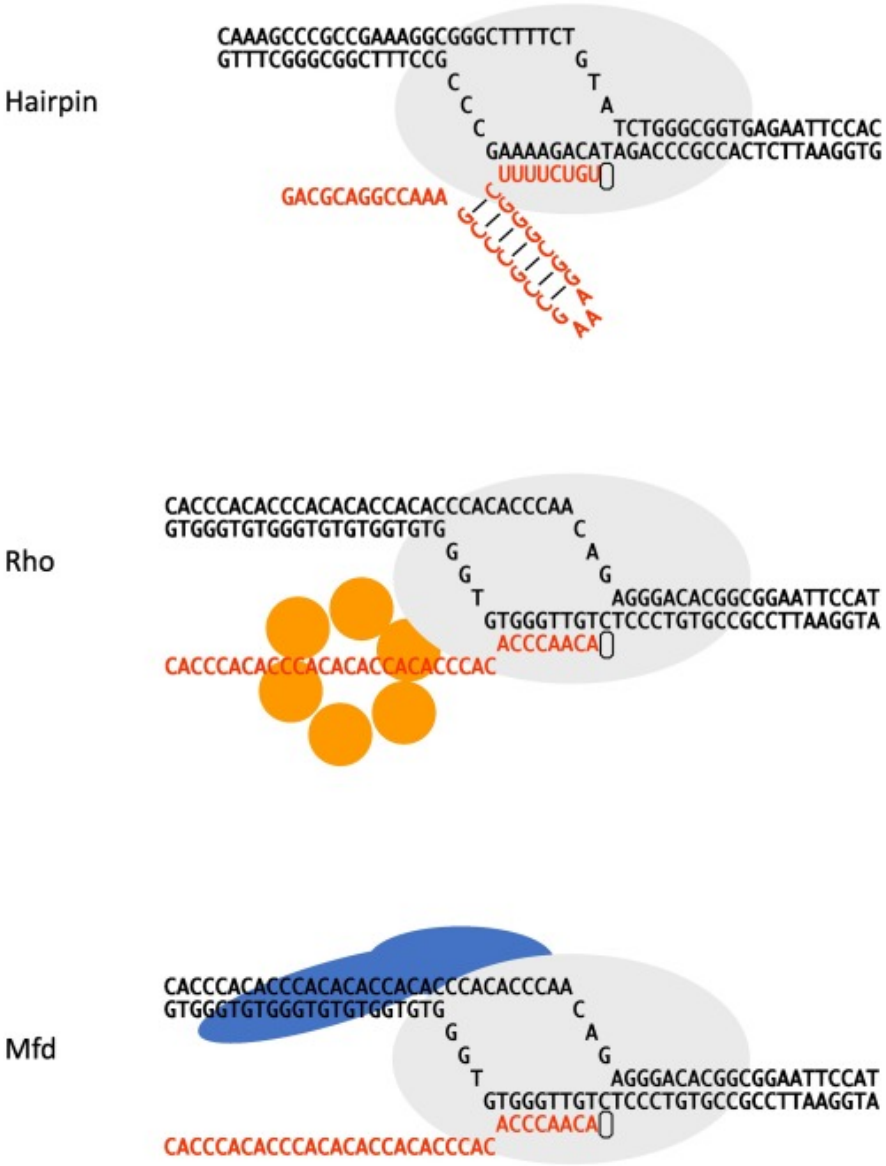

Figure 6: Types of transcriptional termination. Hairpin is an intrinsic (Rho-independent) termination that is dependent on a RNA hairpin with a following U rich sequence. Rho is Rho-dependent termination; in the figure is shown a Rho hexamer that is about to push RNAP from DNA. Mfd termination occurs in stalled elongation where Mfd, bound to RNAP and DNA, dissociates RNAP from DNA (Roberts, 2019).

RNase J1 digests RNA and upon a contact with RNAP it dissociates RNAP from the transcriptional complex by the "torpedo" mechanism (Šiková et al., 2020).

HelD is a helicase-like protein that stimulates transcription in an ATP-dependent manner. HelD binds to RNAP core and its effect can be increased by binding of an accessory RNAP subunit δ (Wiedermannová et al., 2014). HelD can approach a stalled elongation complex, "open" RNAP that results in a release of both RNA and DNA (Kouba et al., 2020).

4.2 Regulation of transcription initiation in *B. subtilis*

A highly effective way to regulate gene expression is to regulate transcription, especially its initiation phase.

4.2.1 (p)ppGpp

(p)ppGpp (guanosine 3',5' bisphosphate for ppGpp and guanosine 3',5' trisphosphate for pppGpp) is a small molecule alarmone which is produced in stress conditions (nutrient starvation, heat shock) and induces the stringent response (Cashel & Gallant, 1969; Haseltine et al., 1972). In *B. subtilis* (p)ppGpp is synthesized mostly by proteins RelA, YwaC and YjbM (Nanamiya et al., 2008). Together they are called RelA-SpoT Homologs (Atkinson et al., 2011). In stress-free conditions (p)ppGpp levels are very low (in picomolar concentration), but in stress conditions, its concentration rapidly increases to milimolar levels (Nishino et al., 1979; Ababneh & Herman, 2015). In *E. coli* (p)ppGpp directly binds to RNAP and thus influences its affinity for σ factors and overall activity (Ross et al., 2016). (p)ppGpp bound to RNAP also prevents some promoters (e.g. ribosomal) to form RP_O by affecting isomarisation of RP_C or by destabilisation of RP_O (Mechold et al., 2013). However, in Firmicutes (p)ppGpp influences transcription indirectly. (p)ppGpp is synthesized from ATP and GDP/GTP (Haseltine & Block, 1973). (p)ppGpp also inhibits enzymes for GTP synthesis both for salvation (enzyme HprT) and *de novo* synthesis (enzyme Gmk), thus changing ATP and GTP cellular concentrations (Fig. 7) (Kriel et al., 2012). The GTP concentration in the cell regulates transcription as an initiating NTP at a number of promoters (e.g. rRNA promoters in *B. subtilis*) (Krásný & Gourse, 2004).

4.2.2 Initiating NTP

High levels of iNTP are required for stabilization of RP_O during transcription initiation (Gaal et al., 1997). This regulation is especially important for rRNA synthesis that is

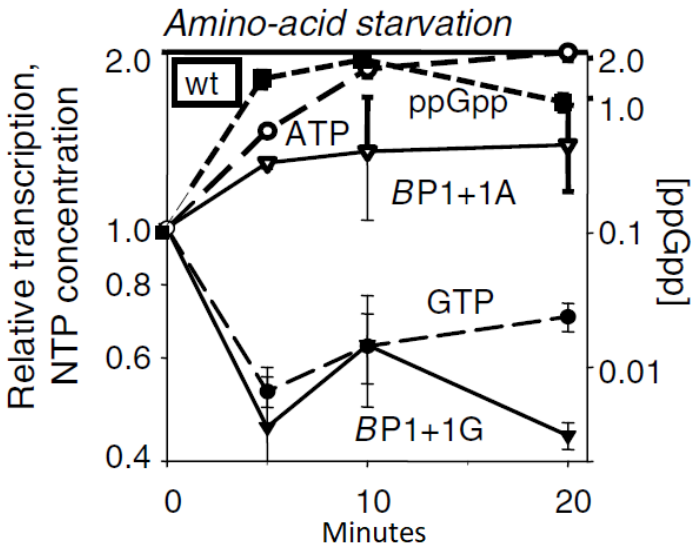

Figure 7: Amino acid starvation and its influence to NTP and (p)ppGpp concentrations and relative transcription from *B. subtilis* ribosomal promoter *rrnB*P1 with A (*B* P1 +1A) or G (*B* P1 +1G) as a initiating NTP (Krásný & Gourse, 2004).

regulated in order to respond to nutritional and environmental changes (Gourse et al., 1996). The relative levels of NTPs in the cell can decide the starting nucleotide when multiple transcriptional starts are present. This can lead to a more or less stable mRNA (Sørensen et al., 1993). Regulation by iNTP was described in bacteria such as *E. coli*, *B. subtilis* and even in yeast cells (Gaal et al., 1997; Krásný & Gourse, 2004; Kuehner & Brow, 2008).

Promoters can be divided into two groups: iNTP sensitive and iNTP insensitive (Fig. 8). iNTP sensitive promoters (such as ribosomal promoter *rrnB*P1 from *E. coli*) form relatively unstable open complexes and thus the iNTP has only a short time available to penetrate RNAP. They need high concentrations of iNTP to initiate transcription (Gaal et al., 1997). In *B. subtilis* all rRNA transcripts start with G, therefore the cellular level of GTP affects ribosomal transcription and thus influences the overall level of transcription in the cell (Krásný & Gourse, 2004; Natori et al., 2009). iNTP insensitive promoters (such as P*veg* promoter from *B. subtilis*) form relatively stable open complexes (Krásný & Gourse, 2004). Alternative σ factors in *B. subtilis* were tested with their promoters for iNTP sensitivity. All of the tested promoters were found to be NTP sensitive. Therefore it can be concluded that [iNTP] regulates various stresses and nutrient starvation (Ramaniuk, 2018).

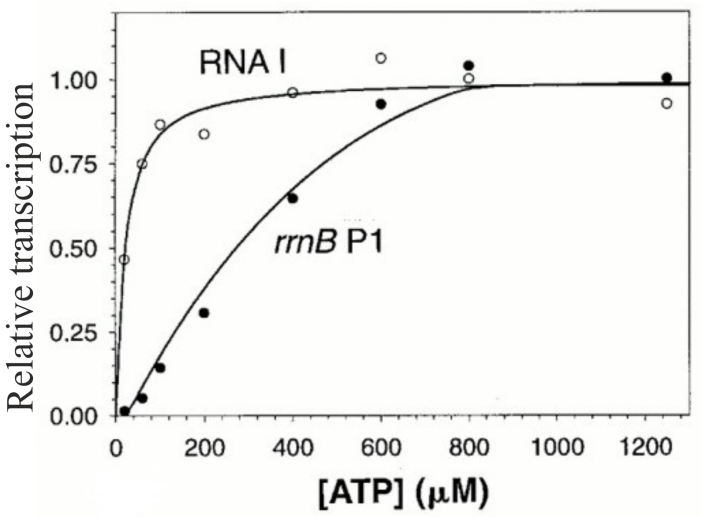

Figure 8: iNTP sensitive *rrnB P1* and iNTP insensitive promoter transcribing RNA I. The sensitive promoter does respond to increasing concentrations of iNTP, while the insensitive does not. Both promoters are from *E. coli* (Gaal et al., 1997).

4.2.3 DNA topology

The topologial state of DNA reflects the level of DNA supercoiling. Negative supercoiling, also known as the underwound DNA state, dominates in viable, stress-free cells (Bauer et al., 1980; Champion & Higgins, 2007). DNA topology regulates many mechanisms, e.g. initiation of DNA and site-specific recombination, transposition of mobile elements and transcription (Higgins & Vologodskii, 2015). DNA topology is controlled by DNA topoisomerases (Topo I, DNA gyrase, Topo III, and Topo IV) (Wang, 2002) and is influenced by nucleoid-associated proteins (Dillon & Dorman, 2010), irradiation (Rackwitz & Bald, 2018) and other forms of DNA damage (Ochs et al., 2019; Ueda et al., 1982), macromolecular crowding and many environmental factors (de Vries, 2010). Lowering of the ATP concentration (in anaerobic growth or in salt shock) affects DNA topoisomerases that decrease DNA supercoiling (Hsieh et al., 1991).

In exponential phase of growth many genes need to be highly expressed. The high expression is achieved by a more negative supercoiling of the DNA. In contrast, in stationary phase, DNA is more relaxed and transcription is suppressed (Dillon & Dorman, 2010). When DNA relaxation is induced by outer factors, the total rRNA synthesis is lowered. It is achieved by several factors, namely by decreasing the activity of the ribosomal *rrn*B P1 promoter, by lowering RNAP's affinity to iNTP in rRNA promoters and lowering RNAP's affinity for rRNA promoters in *B. subtilis* (Sudzinová et al., 2021).

4.2.4 DNA modifications

DNA modifications occur naturally in all species. Natural modifications are typically methylations. In bacteria they are the result of post-transcriptional modifications by various methyltransferases. Methyltransferases modify a nucleobase and the resulting modification destabilises the DNA helix and can lead to conformational changes (Guo et al., 1995). These modifications can either increase or decrease transcription. Naturally modified nucleobases in bacteria are N^6-methyl-adenine, C^5-methyl-cytosine and N^4-methyl-cytosine (Cheng, 1995). Also new artificial modifications are studied to either develop a completely novel DNA base pair (Zhang et al., 2017), to develop a photosensitive treatment for cancer patients (Prados et al., 1999) or to make a transcriptional switch ON/OFF system (Slavíčková et al., 2018; Vaníková et al., 2019; Chakrapani et al., 2020).

4.3 *Bacillus subtilis*

Bacillus subtilis is a soil-dwelling Gram-positive bacterium. For research purposes the model strain is often the *B. subtilis* strain 168. This strain was isolated from the *B. subtilis* Marburg strain after X-ray and UV irradiation. X-ray and UV irradiation proved to be efficient in creation of auxotrophic strains. Five auxotrophic strains were created: strain 23 (requiring threonine), strain 122 (requiring nicotinic acid) and strains 160, 166 and 168 (all tree requiring tryptophan) (Burkholder & Giles, 1947). However, the ancestral Marburg strain was lost and only the five auxotrophic strains were passed to Charles Yanofsky and thereafter to John Spizizen. Spizizen studied the strains, especially the highly-competent 168 strain (Anagnostopoulos & Spizizen, 1961). This research lead to a worldwide spread of the *B. subtilis* strain 168 (Zeigler et al., 2008).

The source of other *B. subtilis* strains is less known. After DNA analysis of various strains by DNA sequencing, the lineage became clearer (Fig. 9). Strains ATCC 6051 and NCIB3610 (3610) were found to be a more direct descendants from the Marburg strain and therefore were labeled as ancestral (Conn, 1930). Because these two strains have no nucleotide divergence, they are believed to be the Marburg strain. Strain 168 varies more from the Marburg strain and is less "wild" than the strains mentioned above (Zeigler et al., 2008).

4.4 *Bacillus subtilis* 3610

B. subtilis 3610 is an ancestral strain of *B. subtilis* whose circular chromosome is 4.2 Mbp long and which can form biofilms. Biofilms of *B. subtilis* are important for plants.

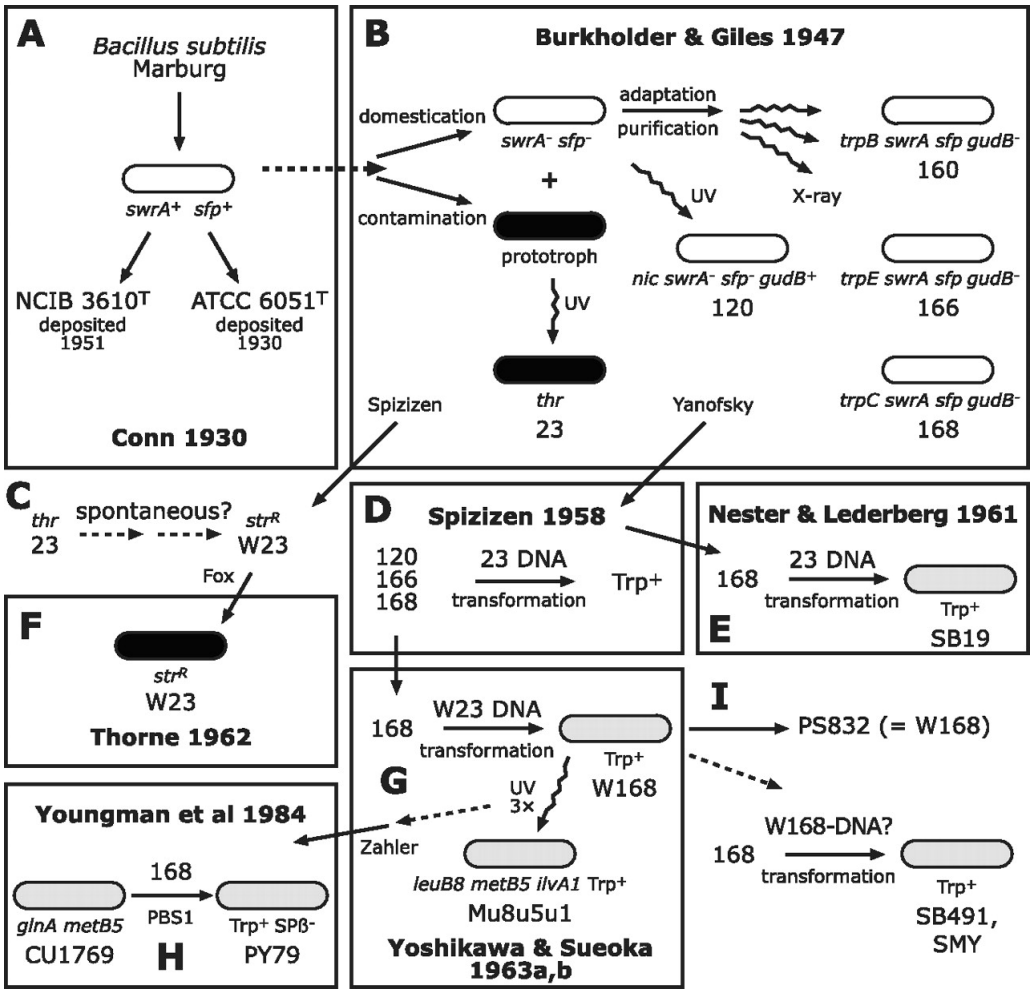

Figure 9: A model describing the lineages of *B. subtilis* strains (Burkholder & Giles, 1947).

B. subtilis forms biofilms and produces surfactin on plant roots that protects plants from pathogens (Chen et al., 2013). Environmental strains (such as 3610) form robust biofilms, whereas laboratory strain 168 does not (Fig. 10) (Mielich-Süss & Lopez, 2015). The loss of the robust biofilm formation in 168 is due to mutations in genes *sfp* (a gene for a production of surfactin), *epsC* (for exopolysaccharide production), *swrA* (regulator for *fla/che* operon) and in a promoter region of *degQ* (facilitates transfer of a phosphate from DegS to DegU, where DegS is a sensory kinase and DegU-P controls diferentiation through stabilization of ComK production).

Additionally, *B. subtilis* 3610 contains the *rapP* gene, which is present on the plasmid pBS32 (that is absent from strain 168). RapP is a biofilm inhibitor and is antagonized by coexpressed Phr (Pottathil & Lazazzera, 2003). Phr together with Rap is a quorum sensing system for response regulators, such as ComA, Spo0F, or DegU (Mutlu et al., 2020). However, RapP on pBS32 has a single nucleotide mutation, that prevents Phr binding (Bendori et al., 2015). Together introduced *rapP* with corrected

sfp, *epsC*, *swrA* and promoter of *degQ* to strain 168, the produced biofilm is similar to a biofilm of strain 3610 (McLoon et al., 2011).

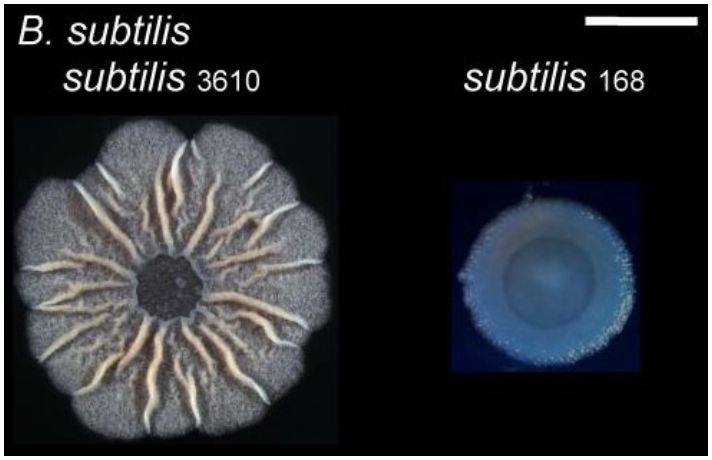

Figure 10: Biofilms of *B. subtilis* strains 3610 and 168 (Mielich-Süss & Lopez, 2015).

Natural competence is the ability to acquire extrachromosomal DNA from the environment (Hamoen et al., 2003). In contrast to biofilm formation, environmental strains of *B. subtilis* are less competent than laboratory strains. Moreover, *B. subtilis* biofilm competent cells and matrix producers are believed to be mutually exclusive cell types (She et al., 2020). ComI on pBS32 decreases competence in cells 100-fold (Konkol et al., 2013). DegQ negatively regulates competence in *B. subtilis* 3610 and its unmutated presence is conducive for biofilm formation (Kobayashi, 2007; McLoon et al., 2011).

Competence is initiated when a peptide pheromone from ComX is sensed by membrane sensory kinase ComP (Fig. 11). Response regulator ComA then activates the *srf* operon and surfactin and ComS is produced. ComS releases ComK from MecA, which normally delivers ComK for proteolysis. Released ComK then accumulates in the cell (Kalamara et al., 2018). ComK activates more than 100 genes (Ogura et al., 2002) and these activate competence, induce growth arrest and persistance, also known as the K-state (Burton & Dubnau, 2010; Yüksel et al., 2016; Berka et al., 2002). Out of these components, ComX negatively impacts biofilm formation (Špacapan et al., 2020) and ComK negatively regulates *sinI*, which leads to a halt in biofilm formation. The SlrR protein, which is active in biofilm, negatively regulates the *srf* operon, which is necessary for competence activation (She et al., 2020).

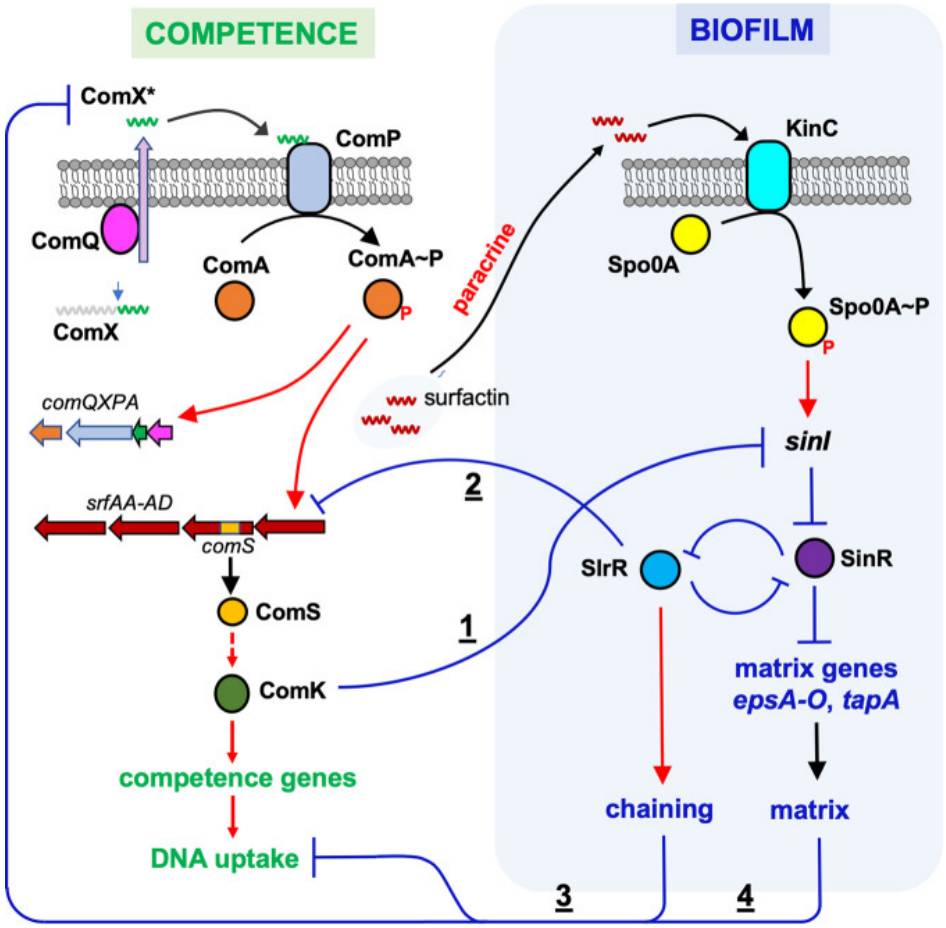

Figure 11: A scheme of competence and biofilm regulation in *B. subtilis* (She et al., 2020).

4.4.1 Plasmid pBS32

Plasmids in *B. subtilis* are not abundant and from this small group only a handful of them are larger than 50 kb. One of them is pBS32. pBS32 is a large, 84 kbp plasmid encoding 102 genes. The copy number of pBS32 is 1 or 2 per cell, but the number can increase 100-fold after mitomycin C treatment (Myagmarjav et al., 2016). pBS32 bears a very high similarity to plasmid pLS32 from the *B. subtilis* subspecies *natto* (Tanaka et al., 1977). Replication of pBS32 was not extensively studied but because of its high similarity to pLS32 it is believed to have a similar mechanism of replication.

pLS32 is replicated through the theta mechanism (Tanaka & Ogura, 1998). The pLS32 replication (Tanaka & Ogura, 1998) and the copy number (Tanaka et al., 2005) are regulated by RepN. RepN is a 287 amino acid long protein encoded by the plasmid. The *repN* gene contains in its coding region a replication initiation origin *oriN* (Tanaka & Ogura, 1998). The *repN* gene contains five 22 bp tandem repeats (iterons) (Tanaka et al., 2005) that are common among replication origins in low copy plasmids in both Gram-positive and Gram-negative bacteria (Ubeda et al., 2012). The first three iterons

regulate copy number and incompatibility without binding of RepN. Incompatibility is a phenomenon when two plasmids with the same replication or partitioning cannot coexist within the cell. RepN binds to the last two iterons to initiate replication by initiation of melting of an AT-rich region downstream of the iterons (Tanaka et al., 2005).

Plasmid segregation to daughter cells is directed by AlfA and AlfB proteins. They are encoded near the *ori* site. AlfB binds to the plasmid *parN* gene. AlfA forms two protofilaments that unidirectionally polymerise to opposite sides of the cell. AlfB with bound DNA interacts with an AlfA protofilement and the AlfA protofilament polymerisation pushes AlfB-DNA to the cell pole (Becker et al., 2006).

As described above, pBS32 encodes the *rapP* gene that negatively impacts biofilm formation (Bendori et al., 2015). pBS32 also encodes the already mentioned ComI, which is a competence inhibitor. Since the predicted structure of ComI is a transmembrane protein it is believed to interact with and inhibit some proteins in the competence apparatus (Konkol et al., 2013).

pBS32 contains a putative prophage whose genes make up one third of the plasmid. The prophage lies between putative integrase genes *zpaO* and *zpbV*. The putative prophage also encodes phage terminase subunits (ZpbL and ZpbK), a capsid protein (ZpbH), tail proteins (ZpbB, ZpaY, ZpaW), a head-tail adaptor protein (ZpbE), a DNA packaging protein (ZpbF), a portal protein (ZpbJ) and a tail tape measure protein (ZpaZ). The majority of genes are encoded clockwise, but prophage genes are encoded counter-clockwise (Konkol et al., 2013).

pBS32 also encodes a relatively novel sigma factor of RNAP called σ^{N}. Further information on σ^{N} can be found in the following chapter (Myagmarjav et al., 2016; Burton et al., 2019).

4.4.2 σ^{N}

σ^{N} is a plasmid encoded σ factor in *B. subtilis* 3610 (Fig. 12). It lies in the *zpdN* (*sigN*) operon close to *oriZ*. σ^{N} is 199 amino acids long with a mass of 23.15 kDa. It belongs to Group IV (ECF) of the σ^{70} family (Myagmarjav et al., 2016).

σ^{N} was found after treatment of cells with mitomycin C (final concentration 1 $\mu g.ml^{-1}$). Mitomycin C is an anti-cancer agent, induces prophage expression, excision and amplification (Okamoto et al., 1968; Mauël & Karamata, 1984). However, Mito-

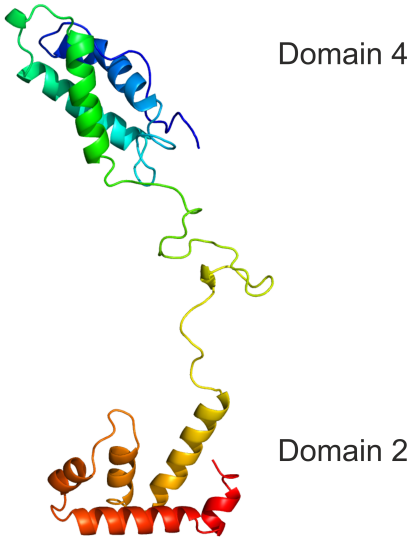

Figure 12: Prediction of σ^N protein folding by Phyre2 (Kelley et al., 2015).

mycin C also leads to DNA strand scissions by DNA alkylation (Lee et al., 2006) and therefore to DNA linearisation (Ueda et al., 1982). This Mitomycin C treatment leads to activation of σ^N, transcription of σ^N-dependent genes on pBS32 and eventually to cell death. σ^N activation also leads to release of defective phage-like particles enveloped in ZpbH capsid protein (Myagmarjav et al., 2016).

σ^N has three identified promoters: P*sigN1*, P*sigN2* and P*sigN3* (Fig 13). P*sigN1* is a σ^A-dependent promoter. To this promoter binds a LexA dimer, its binding site overlapping with the P*sigN1* promoter sequence (Burton et al., 2019). These promoters are thus inaccessible to RNAP (Raymond-Denise & Guillen, 1991). DNA damage response (SOS response) leads to polymerisation of the RecA protein alongside ssDNA (Cox, 2007). When RecA encounters LexA, RecA stimulates autoproteolysis of LexA and LexA dissociates from DNA thus enabling RNAP to start transcription (Little, 1984).

Downstream P*sigN1* lies the P*sigN2* promoter. It is also σ^A-dependent, but its transcription is weaker. The P*sigN3* promoter is a σ^N-dependent promoter. From its consensus sequence of -35 (TTACG) and -10 element (GATATA) 17 promoters were predicted on the pBS32 plasmid, but none on chromosomal DNA. The σ^N-dependent promoters do not have extended -10 elements and have short 15 bp spacers (Burton et al., 2019).

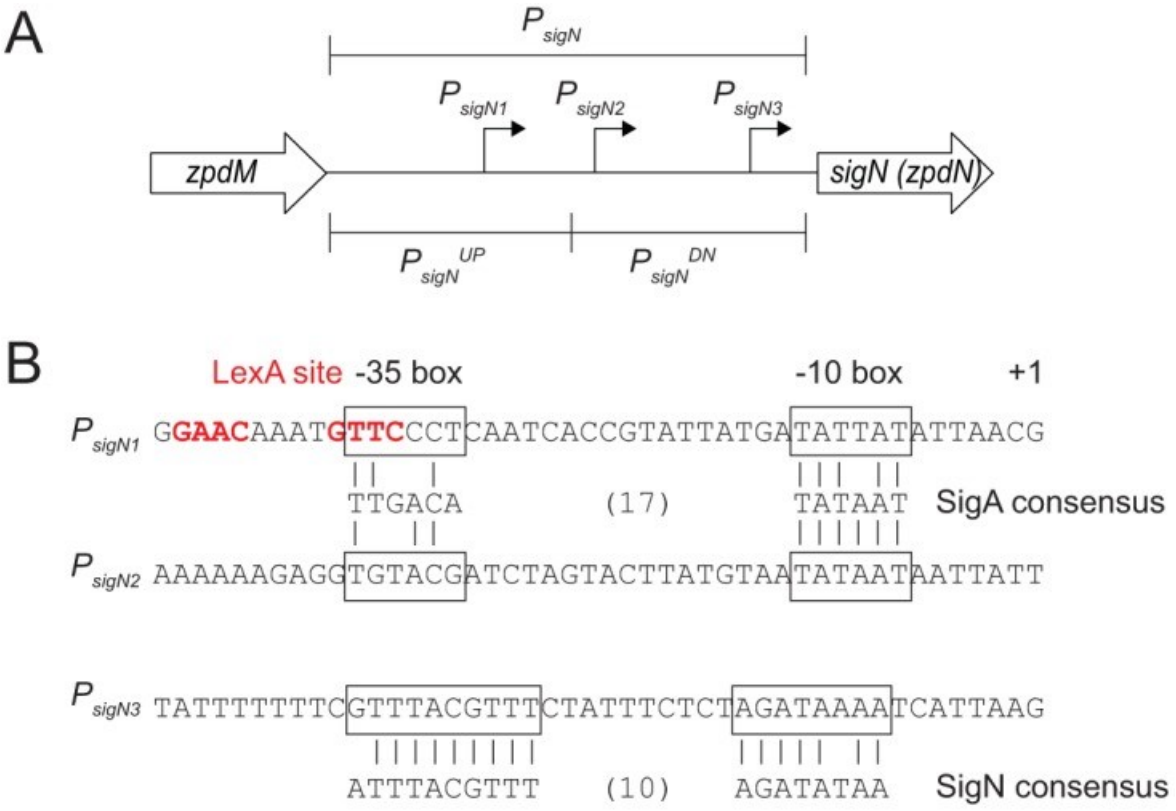

Figure 13: σ^N promoter sequences P*sigN1*, P*sigN2* and P*sigN3* with highlighted -35 and -10 elements. Vertical lines below promoters depict a consensus sequence. On the P*sigN1* promoter is in red highlighted a consensus sequence for a LexA dimer (Burton et al., 2019).

5 Materials

This Chapter are summarises materials used in this Thesis.

5.1 Laboratory Equipment

Laboratory equipment used for a realisation of this Thesis.

- Owl™ EasyCast™ B1A Mini Gel Electrophoresis Systems (Thermo Scientific)
  - Horizontal electrophoresis for analysis of DNA fragments
- Dual plate vertical electrophoresis unit SC20-CDC (Sigma-Aldrich)
  - Vertical electrophoresis for analysis of RNA fragments
- XCell SureLock™ Mini-Cell (Invitrogen)
  - Vertical eletrophoresis for running Novex minigels for analysis of proteins
- NuPAGE® Novex 4–12% Bis–Tris gel (Invitrogen)
  - Precast polyacrylamide gradient gels for protein analysis having 10 - 15 wells
- PowerPac 3000 Electrophoresis Power Supply (Bio Rad)
  - Voltage source for electrophoreses
- MINI ROCKER MR 1 (Biosan)
  - Shaker with a rocking motion for staining SDS-PAGE gels
- BAS-MS2040 (FUJI)
  - Phosphor screen for imagining radioactive phosphorus ^{32}P
- GD-4534 (Scie-Plas)
  - Vaccuum gel dryer
- Mini 900EP15 Contamination and Radiation Monitors (Thermo Scientific)
  - Geiger-Müller portable radiation counter
- Molecular Imager FX (Bio Rad)
  - System imaging radioactive phosphorus and fluorescence

- UV-1601PC UV-Visible (Shimadzu)
  - UV and visible light double beam spectrophotometer
- UVT-20M (Herolab)
  - Transluminator for UV irradiation of stained DNA and RNA gels
- NanoDrop Lite
  - UV sectrophotometer for measuring DNA, RNA and protein concentration
- Avanti J–26XPI (Beckman Coulter)
  - Centrifuge with a cooling system. Maximum speed for 500 ml cuvettes is 17 700 g and for 50 ml cuvettes is 25 000 g
- UNIVERSAL 320 R (Hettich)
  - Centrifuge with a cooling system. Maximum speed for 1.5 ml eppendorf tubes is 21 382 g and for 50 ml cuvettes is 9509 g
- EG 2200 (Kern)
  - Analytical scales
- pH/ION 510 (Oakton Instruments)
  - pH meter
- UP 200S (Hielscher)
  - Sonicator
- Bio RS-24 Mini-Rotator (Biosan)
  - Rotator with a vertical rotation of a platform
- KAR-230 (Kartell)
  - Vacuum desiccator

5.2 Chemicals and Enzymes

- ^{32}P-UTP – M. G. P.
- *Eco*RI (15 U.µl^{-1}) – Takara
- *Hind*III (15 U.µl^{-1}) – Takara
- *Pst*I (15 U.µl^{-1}) – Takara
- 1× NuPAGE MES SDS Running buffer – Invitrogen
- 2-mercaptoethanol (ME) – Serva
- Agarose for Molecular Biology – Amresco
- Agarose for molecular biology – Amresco
- Agarose for solid soil – Lachema
- Amino acids – AppliChem
- Ammonium persulfate – Sigma
- Ampicillin – Biotika
- Aqua pro injection (iH_2O) – Braun
- Boric acid (H_3BO_3) – Penta
- Bovine serum albumin (BSA) – Sigma
- Bromphenol blue – Dr. G. Gruber & Co.
- Buffer PE – Qiagen
- Buffer QG – Qiagen
- Calcium chloride ($CaCl_2$) – Lach-Ner
- Cell Lysis Solution – Promega
- Cell Resuspension Solution – Promega
- Chlorophorm – Penta
- Column Wash Solution – Promega
- Coomassie Brilliant Blue G-250 – Serva

- Disodium phosphate (Na_2HPO_4) – Lach-Ner
- Dithiotreitol – Serva
- Erythromycin – Serva
- Ethanol 96% – Penta
- Ethylenediaminetetraacetic acid (EDTA) – Lachema
- Expand High Fidelity PCR System Buffer with $MgCl_2$ – Roche
- Expand High Fidelity PCR System Polymerase – Roche
- Formamide – Penta
- Gel Red – Biotium
- GeneRuler Low Range DNA Ladder marker – Thermo Fisher
- Glycerol – Sigma
- H buffer for RE (10x) – Takara
- Imidazole – Sigma
- Isopropanol – Lach-Ner
- Isopropyl β-D-1-thiogalactopyranoside (IPTG) – Sigma
- Lincomycin – Serva
- M buffer for RE (10x) – Takara
- Magnesium chloride ($MgCl_2$) – Penta
- Magnesium sulfate ($MgSO_4$) – Lachema
- Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7\,H_2O$) – Penta
- Monopotassium phosphate (KH_2PO_4) – Penta
- Monosodium phosphate (NaH_2PO_4) – Lachema
- NTP (ATP, GTP, CTP, UTP) – Roche
- Neutralization Solution – Promega
- NuPAGE™ LDS Sample Buffer (4X) – Invitrogen

- Phosphoric acid (H_3PO_4) – Penta
- Polyacrylamide – Serva
- Potassium chloride (KCl) – Lachema
- SAP Promega – NEB
- SAP buffer – NEB
- SUMO Protease (1 U.µl^{-1}) – Invitrogen
- SUMO Protease Buffer + Salt (10X) – Invitrogen
- SUMO protease – Invitrogen
- Slide A Lyzer Dialysis Cassette – Thermo Fisher
- Sodium acetate (CH_3COONa) – Lachema
- Sodium chloride (NaCl) – Lach-Ner
- Sodium hydroxide (NaOH) – Penta
- Spectinomycin – Sigma
- Tetrametyletylendiamin – Serva
- Tris(hydroxymethyl)aminomethane (Tris-acetate) – Serva
- Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) – Sigma
- Tryptone – Oxoid
- Wizard Midipreps DNA Purification Resin – Promega
- Xylene cyanol – Sigma-Aldrich
- Yeast extract – Difco
- dNTP (dATP, dCTP, dGTP, dTTP) – Roche

5.3 Buffers and Solutions

- LB medium
  - 10 g Tryptone
  - 5 g Yeast extract
  - 10 g NaCl
  - distilled water to final volume 1 litre
- 50xTAE
  - 2 mol.l^{-1} Tris-acetate
  - 50 mmol.l^{-1} EDTA (pH 8)
  - 2 mol.l^{-1} Tris-acetate
  - 50 mmol.l^{-1} EDTA (pH 8)
- 10 × TBE
  - 0.9 mol.l^{-1} Tris-HCl (pH 8)
  - 0.02 mol.l^{-1} EDTA (pH 8.0)
  - 0.9 mol.l^{-1} H_3BO_3
- Bradford's reagent
  - 100 mg Coomassie Brilliant Blue G-250
  - 50 ml 96% EtOH
  - 100 ml 85% H_3PO_4
  - distilled water to final volume 1 litre
- Z-buffer
  - 0.06 mol.l^{-1} Na_2HPO_4
  - 0.04 mol.l^{-1} NaH_2PO_4
  - 0.01 mol.l^{-1} KCl
  - 0.001 mol.l^{-1} $MgSO_4$
  - Adjust to pH 7.0 using NaOH
- 10x PBS
  - 1.37 mol.l^{-1} NaCl

  - 27 mmol.l^{-1} KCl
  - 100 mmol.l^{-1} Na_2HPO_4
  - 18 mmol.l^{-1} KH_2PO_4
- 20 x transcription buffer
  - 800 mmol.l^{-1} Tris-HCl (pH 8)
  - 200 mmol.l^{-1} $MgCl_2$
  - 20 mmol.l^{-1} Dithiotreitol
- STOP solution
  - 95% formamide
  - 0.05% bromphenol blue
  - 20 mmol.l^{-1} EDTA (pH 8)
  - 0.05% xylene cyanol
- SPI
  - 15 mmol.l^{-1} $(NH_4)SO_4$
  - 80 mmol.l^{-1} K_2HPO_4
  - 44 mmol.l^{-1} KH_2PO_4
  - 4 mmol.l^{-1} Sodium citrate
  - 2 mmol.l^{-1} $MgSO_4 \cdot 7\,H_2O$
  - 0.02% Casamino acids
  - 0.1% Yeast extract
  - 50 mg.l^{-1} Phenylalanin
  - 50 mg.l^{-1} Tryptophan
  - 0.5% Glucose
- SPII
  - 15 mmol.l^{-1} $(NH_4)SO_4$
  - 80 mmol.l^{-1} K_2HPO_4
  - 44 mmol.l^{-1} KH_2PO_4
  - 4 mmol.l^{-1} Sodium citrate

- 2 mmol.l^{-1} $MgSO_4 \cdot 7\,H_2O$
- 50 mg.l^{-1} Phenylalanin
- 50 mg.l^{-1} Tryptophan
- 0.5% Glucose
- 0.5 mmol.l^{-1} $CaCl_2$
- 2.5 mmol.l^{-1} $MgCl_2$

5.4 Markers

Markers were used for DNA and protein gels.

DNA markers:

- GeneRuler Low Range DNA Ladder (25-700 bp) – Thermo Fisher
- GeneRuler DNA Ladder Mix (100-10 000 bp) – Thermo Fisher
- GeneRuler 1 kb DNA Ladder (250-10 000 bp) – Thermo Fisher
- GeneRuler 1 kb Plus DNA Ladder (75-20 000 bp) – Thermo Fisher

Protein markers:

- Novex™ Sharp Pre-stained Protein Standard (3.5-260 kDa) – Invitrogen

5.5 Bacterial strains

Bacterial strains used in this Thesis (Tab. 1).

5.6 List of primers

Primers used in this Thesis (Tab. 2).

Table 1: Bacterial strains used in this Thesis

*These plasmids were kindly provided by D. Kearns, Indiana University.

**These strains were also used in publication (Sudzinová et al., 2021)

Strain	**Genotype**	**Origin**
DH5α	*E.coli* supE44; ΔlacU169(Φ80 lacZΔM15); hsdR17; recA1; endA1; gyrA96; thi–1; rel–A1	Invitrogen
BL21	*E.coli* strain B F^{-} *ompT gal dcm lon* $hsdS_{B(rB-mB-)}$ $[malB^{+}]_{K\text{-}12}(\lambda^{S})$	Thermo Fisher
RLG 6903	DH5α pDG1663	(Guérout-Fleury et al., 1996)
RLG 7555	BSB1 amyE::P*veg*-lacZ -38/-1, +1G	(Krásný & Gourse, 2004)
LK 180	DH5α p770	(Ross et al., 1990)
LK 615	*B. subtilis* strain 3610, undomesticated	J. D. Wang
LK 1177	DH5α p770/P*veg*	(Krásný & Gourse, 2004)
LK 1723	*B. subtilis* with RNAP C-ter. His10x; MH 5636	(Qi & Hulett, 1998)
LK 2530	DH5α pBM05*	This Thesis
LK 2531	BL21 pBM05*	This Thesis
LK 2608	DH5α p770/P*zpbY*	This Thesis**
LK 2609	DH5α p770/P*zpdG*	This Thesis**
LK 2672	DH5α p770/*sigN P2 + P3*	This Thesis**
LK 2673	DH5α p770/P*zpaB*	This Thesis**
LK 2712	DH5α p770/*PsigN3 core*	This Thesis
LK 2867	DH5α pMP199* amyE::Physpank-RBSdownsigN spec amp	This Thesis
LK 2870	DH5α pDG1663/P*zpbY-lacZ*	This Thesis
LK 2883	BSB1 amyE::Physpank-RBSdownsigN spec	This Thesis
LK 2885	DH5α pDG1663/P*sigN3-lacZ*	This Thesis
LK 2887	BSB1 amyE::Physpank-RBSdownsigN spec thrC::P*sigN3-lacZ*	This Thesis
LK 2891	BSB1 amyE::Physpank-RBSdownsigN spec thrC::P*zpbY-lacZ*	This Thesis
LK 2902	BSB1 amyE::Physpank-RBSdownsigN spec thrC::*lacZ*	This Thesis

Table 2: List of primers used in this Thesis

Number	**Sequence**	
27	CTTCCACAGTAGTTCACCAC	R Primer for pDG3661
999	GCGCTACGGCGTTTCACTTC	R Primer for p770
1000	CCACCTGACGTCTAAGAAACC	L Primer for p770
3109	GCGAATTCCGTGTCGGTCAACATAATAAAGG	*sigN* P2 P3 F
3110	GCAAGCTTCGGCAAAAATCTTTCTCTCACC	*sigN* P2 P3 R
3111	GCGAATTCGCGATGAATGAAGAGACACGG	P*zpaB* F
3112	GCAAGCTTAGTCCATCTCGAAGATCTGGT	P*zpaB* R
3113	GCGAATTCGACTCCAACATTTCTATTCC	P*zpbY* F
3114	GCAAGCTTGGTCTTCTTCACTTAATTCA	P*zpbY* R
3115	GCGAATTCCCAGAGTGGTTTGAGTTCCC	P*zpdB* F
3116	GCAAGCTTTTCCCGAACGATCAACACCT	P*zpdB* R
3117	GCGAATTCTCAAAGATCTTCTAACTTGT	P*zpdG* F
3118	GCAAGCTTGGCAGTAATCAATCAATTCT	P*zpdG* R
3447	AATTCTTTTCGTTTACGTTTCTATTTCTCTA-GATAAAATCATTAAGTCTA	P*sigN3* F
3448	AGCTTAGACTTAATGATTTTATCTAGAGAAATA-GAAACGTAAACGAAAAG	P*sigN3* R
3451	GCGAATTCGAACCATTCAATACTTCTTG	P*ydcJ* F
3452	CGTTCGAATGGGATCGTGTTCCATATCG	P*ydcJ* R
3453	GCGAATTCCGGAATCTAGAGCGACGGCT	P*yybN* F
3454	CGTTCGAAACGGAAATTGCTCTTCAGGA	P*yybN* R
3455	GCTTCGAACATATCCTCGTATAGAGCCA	P*dpoL* F
3456	CGCTTAAGGCTGCCATTAGGGTTGGAAG	P*dpoL* R
3457	GCTTCGAAGTCATCTGCTGCATCATCGC	P*phyC* F
3458	CGCTTAAGCTCTACCATGCATCATATGT	P*phyC* R
3459	GCGAATTCGTCAGCATCGTTGACCATCA	P*nosA* F
3460	CGCTTAAGCAAGGTGTGGGCTTTCGCTA	P*nosA* R
3669	AATTCTATTTGACAAAAATGGGCTCGTGTTGTA-CAATAAATGTGTCTA	Pveg F
3670	AGCTTAGACACATTTATTGTACAACACGAGCC-CATTTTTGTCAAATAG	Pveg R

6 Methods

In this Chapter some of the Methods are taken from my Bachelor Thesis Kambová, 2019.

6.1 Electrophoresis

6.1.1 Horizontal agarose gel electrophoresis

Horizontal agarose electrophoresis is used to separate and visualise DNA.

In an erlenmayer flask prepare agarose solution; for DNA < 1000 bp use 1.5% agarose, for > 1000 bp use 1% agarose. Dissolve agarose in 1x TAE buffer in a microwave oven. Add Gel Red (intercalation agens) at a ratio of 1:10 000. Pour the agarose solution into a gel electrophoresis apparatus and add a comb. Let the gel solidify. Pour 1x TAE buffer into the electrophoresis apparatus for the anode and cathode to be submerged in the buffer and remove the comb. Mix DNA with 6x TriTrack Loading buffer at 6:1. Add 5 µl of DNA ladder marker into the first well. In this Thesis was used GeneRuler Low Range DNA Ladder, GeneRuler 1 kb DNA Ladder, GeneRuler 1 kb Plus DNA Ladder and GeneRuler DNA Ladder Mix. Load the DNA samples into the gel. Connect the electrophoretic apparatus to a source of current and turn on the power for 5 $V \cdot cm^{-1}$ for roughly an hour. Visualise the gel with a UV lamp. Analyse the gel in the Quantity One programme with the Volume Rect Tool.

6.1.2 Vertical polyacrylamide gel electrophoresis

Vertical polyacrylamide electrophoresis was used to visualise the RNA transcripts.

Clamp together two electrophoretic glasses with two spacers on the sides and tape the bottom with PVC tape. In a fume hood prepare polyacrylamide solution; 35 ml 7% polyacrylamide with urea, 350 µl 10% ammonium persulfate and 35 µl tetrametyletylendiamin. Gently mix and pour between the glasses. Add the comb to protrude approximately 1 cm into the gel. Let the gel solidify. Remove the clamps and tape and put the glasses into the electrophoretic apparatus. Pour 1x TBE buffer between the glasses and outside of them for the anode and cathode to be submerged in the buffer and remove the comb. After transcription add 10 µl of each transcribed sample into the electrophoretic wells. Connect the cables from the electrophoretic apparatus to a source of current and turn on the power for 200 V for ca 2 hours. Dismantle the electrophoretic apparatus and remove the gel on filter papers. Put the

gel into the dryer for 80℃, 40 min, then let it cool down for 30 min in the dryer. Put the gel onto a ^{32}P-sensitive screen into a cassette and let it expose overnight. Scan the screen with Biorad Molecular Imager FX. Analyse the gel in programme Quantity One.

6.1.3 SDS-PAGE

Method sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate proteins based on their molecular weight.

Assemble the XCell SureLock™ Mini-Cell with NuPAGE Novex 4–12% Bis-Tris gel. Pour 1× NuPAGE MES SDS Running buffer inside the apparatus. Prepare the protein samples: add 3 µl NuPAGE™ LDS Sample Buffer (4X), 3-5 µl protein solution and add iH_2O to 12 µl. Denature the sample for 5 min at 95℃. Load the samples into the gel, and include 5 µl of Novex™ Sharp Pre-stained Protein Standard. Connect the electrophoretic apparatus to a source of current and run the gel at 200V for 35 min. Visualise proteins by staining with SimplyBlue™ SafeStain.

6.1.4 Protein gel staining using SimplyBlue™ SafeStain

Put the disassembled gel into a plastic box. Pour 100 ml distilled water, microwave for 1 min, put the sample on a shaker with a rocking motion for 1 min, then discard the water and repeat two times. Add 20 ml SimplyBlue™ SafeStain, microwave for 1 min, put the sample on a shaker with a rocking motion for 5 min, then discard SimplyBlue™ SafeStain. Pour 100 ml distilled water and put the sample on a shaker with a rocking motion for 10 min. Discard the water, pour 100 ml distilled water and put in a fridge for storage. Scan the gel.

6.2 Dialysis

This method is used to replace one buffer with another in a protein solution, using Slide A Lyzer Dialysis Cassette containing two cellulose membranes which have pores of a defined size.

Insert the dialysis cassette with desirable volume and pore size into a plastic float and hydrate for 5 min in 500 ml of cooled dialysis buffer (4℃). Remove the cassette from buffer and inject protein solution with a syringe. Using the syringe, remove air from the cassette. Place the cassette into a plastic float and into the dialysis buffer with a magnetic stirrer. Dialyse over night (12-16 hours) at 4℃. Replace the used dialysis buffer with a fresh dialysis buffer. After 4 hours remove the cassette from the dialysis

buffer and remove the protein solution from the cassette and store in a new eppendorf tube.

Dialysis buffer for cleavage with SUMO Protease: 1x P buffer with 3 mM 2-mercaptoethanol (ME).

Storage buffer after protein isolation: 50 mM Tris-HCl (pH 8), 150 mM NaCl, 5 mM $MgCl_2$, 5% glycerol.

6.3 Determination of protein concentration

Protein concentration in this Thesis was determined by the Bradford method. The protein standard for protein calibration was BSA (stock solution 400 $mg.ml^{-1}$).

Prepare cuvettes for samples. Into each cuvette add distilled water; 100 µl into blanks and into other cuvettes add 100 µl distilled water minus volume of protein sample. Protein standard concentrations and volumes for calibration are listed in Table 3. Add proteins into each cuvette. Start a reaction by mixing in 900 µl Bradford reagent. After 5 minutes measure the absorbance at 595 nm by a spectrophotometer. Make a calibration curve and calculate protein concentration.

Table 3: Calibration table for protein concentration

Sample	**BSA (µl)**	**BSA (µg)**	**iH_2O (µl)**
1	0	0	100
2	1.50	0.60	98.50
3	3	1.20	97
4	6	2.40	94
5	12	4.80	88
6	24	9.60	76

6.4 beta-galactosidase assay

Beta galactosidase asssay is used to measure activity of β-galaktosidase, a protein encoded by the *lacZ* gene. In this Thesis I used beta-galactosidase assay to measure promoter activity.

Grow over-night cultures in 5 ml of medium with appropriate antibiotics. Next morning measure OD_{600}, inoculate to initial $OD_{600} = 0.03$ in 25 ml medium and incubate at 37℃. At $OD_{600} = 0.5$ start a reaction. At times 0, 10, 20, 30 and 40 min measure OD_{600} and collect 1 ml samples, cool on ice for 30–60 min. Centrifuge at 14 000 rpm, 10 min, 4℃. Discard the supernatant and freeze the pellets at -20℃.

Prepare Z-buffer with 0.05 $mol.l^{-1}$ ME (2 ml per reaction + 2x blank). Dissolve ONPG in Z-buffer with ME (200 µl per reaction + 2x blank; final concentration 4 $mg.ml^{-1}$). Resuspend pellets with 500 µl Z-buffer with ME. Centrifuge at 14 000 rpm, 10 min, 4℃. Discard the supernatant. Resuspend pellets with 500 µl Z-buffer with ME. Sonicate 5 x 20 s, 1 min on ice. Centrifuge at 14 000 rpm, 10 min, 4℃. In 2 ml eppendorf tube pipette 800 µl Z-buffer with ME + 200 µl sonicate. In 2 blanks pipette 1 ml Z-buffer with ME. Put the samples in a thermoblock and preheat them at 30℃ for 5 min. At 20 s intervals add 200 µl ONPG, record the start time and keep the samples in the thermoblock. When the sample turns yellow, add 500 µl Na_2CO_3 to stop the reaction and record the time. Measure all samples at OD_{420} and OD_{550}. For blank use Z-buffer + ME + ONPG + Na_2CO_3. If the samples are diluted in water, use water as a blank. Calculate total protein concentration in sonicate using the Bradford method. Calculate AU (Miller units) using equation 1.

$$AU = \frac{1000\,(OD_{420} - 1.75OD_{550})}{V\,(ml)\,s\,(min)\,c\,(g.l^{-1})} \tag{1}$$

OD_{420} = yellow from O-Nitrophenol; OD_{550} = OD from cells; V (ml) = volume of the culture in reaction; s (min) = reaction duration; c ($g.l^{-1}$) = protein concentration of sonicate

6.5 σ^N and RNAP isolation

SUMO-σ^N fusion protein in an expression plasmid pBM05 was generously provided by Professor Daniel Kearns from Indiana University Bloomington. RNAP was purified from strain LK 1723 from our laboratory. In this construct RNAP has a His10-tag and is located on a chromosomal DNA.

σ^N: Transform plasmid pBM05 by the heat shock method (described in detail in the Chapter *E. coli* strain construction, Transformation) into competent DH5α *Escherichia*

coli (LK 2530). DH5α is known to have a high transformation efficiency. From this strain isolate plasmid DNA by QIAprep Spin Miniprep Kit. Transform plasmid by the heat shock method to a competent BL21 *E. coli* (LK 2531), which is used for protein overproduction.

Incubate the cells at 37℃ in 30 ml LB medium with appropriate antibiotics (final concentration 100 µg.ml^{-1} ampicillin for σ^N and 25 µg.ml^{-1} chloramphenicol for RNAP) over night. Measure OD_{600} and inoculate to initial OD_{600} = 0.03 in 1 l LB medium with ampicillin (final concentration 100 µg.ml^{-1}), or in 2 l LB medium with chloramphenicol (final concentration 25 µg.ml^{-1} chloramphenicol) and incubate at 37℃. Grow to late exponential phase (OD_{600} = 0.6–0.8). For induction at room temperature, add isopropyl β-D-1-thiogalactopyranoside (IPTG) to final concentration 0.3 mM. After 3 hours (2 hours for RNAP), cool down the cells for 20 min, then centrifuge at 6000 rpm, 10 min, 4℃. Discard the supernatant and resuspend the pellet in 10 ml 1x P buffer. For the entire σ^N isolation use 1x P buffer pH 9.5, for RNAP isolation use 1x P buffer pH 9.5 only. Centrifuge the solution at 4750 rpm, 15 min, 4℃, discard the supernatant and froze the pellet at -40℃.

Defrost the pellet in conical tube and resuspend in 10 ml 1x P buffer with 3 mM ME and vortex. Sonicate the conical tube in a beaker filled with ice 12 times for 10 seconds with a 1 minute pause in between. After sonication, centrifuge at 27 000 *g*, 10 min, 4℃. Meanwhile, rinse 1 ml of Ni-NTA agarose beads with 10 ml 1x P buffer with 3 mM ME and centrifuge at 2000 *g*, 3 min, 4℃ and repeat. Pour the supernatant from centrifugation through a gauze to rinsed Ni-NTA agarose beads and let proteins bind to beads for 1 hour on an ice while gently shaking. After binding, pour the solution to a Poly-Prep® Chromatography Column (BIO-RAD) at 4℃ and let the solution flow through the column. Add 30 ml of 1x P buffer with 3 mM ME to the column and let it flow through. Afterwards, pour 30 ml of 1x P buffer with 3 mM ME and 30 mM imidazole to the column. Let the liquid flow through. Add 3 ml 1x P buffer with 3 mM ME and 400 mM imidazole. Collect the eluate in 5–6 eppendorf tubes in 0.5 ml aliquotes.

Analyse the eluted protein solution by SDS-PAGE. Into each well pipette 3–10 µl protein sample, NuPAGE™ LDS Sample Buffer (stock solution 4x) and adjust with water to 12 µl. Run the SDS-PAGE according to the SDS-PAGE instructions above.

At this step, RNAP is fully isolated and can be stored in storage buffer at -20℃. σ^N is still linked to SUMO-tag, which can interefere with its transcription efficiecy.

Therefore, cleavage of SUMO-tag is needed.

Prepare 1 l of 1x P buffer with 3 mM ME. Dialyse the protein samples using Slide A Lyzer Dialysis Cassettes, 3 500 MWCO according to a dialysis instructions above. Analyse the protein concentration after dialysis using the Bradford method. Cleave the SUMO tag using SUMO protease and SUMO protease buffer + Salt. Cleave over night at 30℃. Verify with SDS-PAGE.

Prepare 1 ml Ni-NTA agarose beads - rinse them twice with 10 ml 1x P buffer with 3 mM ME and centrifuge at 2000 *g*, 3 min, 4℃. Add cleaved proteins to Ni-NTA beads. Let proteins bind to the beads for 1 hour at 4℃. Centrifuge 3 min, 4750 *g*, 4℃. Transfer the supernatant to a clean eppendorf tube and add 300 µl 1x P buffer with 3 mM ME to the beads. Centrifuge the beads again and transfer the supernatant into another clean eppendorf tube. Verify that in the supernatant contains cleaved σ^N by SDS-PAGE. Store proteins in storage buffer at -20℃.

6.6 Plasmid pBS32 isolation

pBS32 is a large plasmid of 84 kbp. Therefore, it is isolated alongside chromosomal DNA. For isolation, High Pure PCR Template Preparation Kit from Roche was used.

Cultivate strain LK 615 over night at 37℃ in 3 ml LB medium without antibiotics. Add 1 ml of overnight culture into a clean eppendorf tube. Centrifuge at 3000 *g* for 5 min at RT. Pour the supernatant out and resuspend the pellet in 200 µl 1x PBS. Add 5 µl 10 $mg.ml^{-1}$ lysozyme (lysozyme is dissolved in 10 mM Tris-HCl, pH 8) and incubate 15 min at 37℃. Add 200 µl Binding buffer and 40 µl proteinkinase K. Immediately mix and incubate for 10 min at 70℃. Add 100 µl isopropanol. Pipette solution to a High Pure Filter Tube and insert it into a Colletion Tube. Centrifuge 1 minute at 8000 *g*. Assemble the Filter Tube with a new Collection Tube and discard the flow through liquid. Add 500 µl Inhibitor Removal Buffer to the Filter Tube and centrifuge 1 min at 8000 *g*. Discard the flow through liquid and assemble the Filter Tube with a new Collection Tube. Add 500 µl Wash Buffer to the Filter Tube and centrifuge 1 min at 8000 *g*. Discard the flow through liquid and assemble the Filter Tube with a new Collection Tube. Repeat (adding 500 µl Wash Buffer and centrifuging) but after removing the flow through liquid, place the Filter Tube back to the Collection Tube. Centrifuge 10 s at 8000 *g*. Assemble the Filter Tube with a clean, sterile eppendorf tube. Add 200 µl Elution Buffer (heated to 70℃) to the Filter Tube. Centrifuge 1 min at 8000 *g*. Eluted DNA is in an eppendorf tube and is stored at 4℃.

Measure the DNA concentration by nanodrop. Analyse on 0.8% agarose gel.

6.7 Plasmid isolation using midiprep

For isolation of $<$ 20 kbp plasmids the Wizard *Plus* Midipreps DNA Purification System from Promega is used.

Grow 100 ml culture in LB medium with required antibiotics over night at 37℃. In the morning, pellet the cells by centrifugation at 4750 *g*, 10 min, 4℃. Pour off the supernatant. Resuspend the pellet in 3 ml of Cell Resuspension Solution by vortex. Add 3 ml of Cell Lysis Solution and mix by gently inverting the tube. Add 3 ml of Neutralization Solution and mix by gently inverting the tube. Centrifuge at 27 000 *g*, 10 min, 4℃. Decant the supernatant through some gauze to a new tube.

Preheat iH_2O at 65-70℃. Assemble a Midicolumn with a vacuum pump. To the supernatant from the last centrifugation add 7.5 ml of thoroughly resuspended Wizard Midipreps DNA Purification Resin. Turn on the vacuum pump. Pour the DNA with resin to the Midicolumn. Let the solution pass through the column. Add 3 times 10 ml of Column Wash Solution to the Midicolumn and let the solution pass through the column. Dry the resin by continuing to draw a vacuum for maximately 30 s. Remove the vacuum pump, turn it off and separate the reservoir in the Midicolumn by cutting it with sharp scissors or a scalpel. Transfer the Midicolumn reservoir to a 1.5 ml eppendorf tube. Centrifuge at 10 000 *g*, 2 min, room temperature. Transfer the Midicolumn reservoir to new a 1.5 ml eppendorf tube and add 300 µl preheated iH_2O. Wait 1 min and then centrifuge at 10 000 *g*, 20 s, room temperature. Remove the Midicolumn and centrifuge again at 10 000 *g*, 5 min, room temperature. Transfer the supernatant to a new eppendorf tube.

To 300 µl of supernatant, add 200 µl iH_2O and 500 µl phenol. Extract DNA by using a rotator for 5 min. Centrifuge at 10 000 *g*, 5 min, room temperature. Transfer the aqueous phase (upper phase) to a new eppendorf tube and add 250 µl phenol and 250 µl chloroform. Repeat the extraction and centrifugation and transfer the water phase to a new eppendorf tube. Add 400 µl chloroform, repeat the extraction and centrifugation and transfer the aqueous phase to a new eppendorf tube while measuring the volume of aqueous phase.

To the aqueous solution, add 0.1 volumes of 3 M CH_3COONa. To this volume add 2.2 volumes of cooled 96% ethanol. Mix and put to -80℃ for 10 min. Centrifuge at

5000 *g*, 5 min, 4℃. Discard the supernatant and add 100 µl 70% ethanol. Centrifuge at 5000 *g*, 5 min, 4℃. Discard the supernatant and put the open eppendorf tubes into a dessicator for 15 min. Dissolve DNA with 30 µl iH_2O over night. Measure the DNA concentration by nanodrop. Analyse on 1% agarose gel.

6.8 Cleavage of a supercoiled plasmid to a linear plasmid

In this Thesis the p770 plasmid is used. It contains a single restriction site for *Pst*I (CTGCAG). This cleavage thus produces a linear form of the plasmid.

Mix a solution (Tab. 4) and digest for 3 hours at 37℃. Inactivate the enzyme with CH_3COONa and ethanol. Use the same procedure as at the end of the midiprep plasmid isolation.

To the solution add 0.1 volumes of 3 M CH_3COONa. To this volume add 2.2 volumes of cooled 96% ethanol. Mix and put to -80℃ for 10 min. Centrifuge at 5000 *g*, 5 min, 4℃. Discard the supernatant and add 100 µl 70% ethanol. Centrifuge at 5000 *g*, 5 min, 4℃. Discard the supernatant and put the open eppendorf tubes into a dessicator for 15 min. Dissolve DNA with 15 µl iH_2O over night. Measure the DNA concentration by nanodrop. Analyse on 1% agarose gel.

Table 4: Cleavage of supercoiled plasmid for linear (final volume 50 µl)

Component	**Volume (µl)**
H buffer (10x)	5
*Pst*I	1
Plasmid (10 µg)	
iH_2O	

6.9 PCR

PCR was used to amplify studied promoter regions. The used primers are listed in the primer section. DNA Polymerase and buffer were from Expand High Fidelity PCR System from Roche.

Mix a solution for a PCR reaction (Tab. 5). Insert eppendorf tubes with PCR mixes into a thermocycler and amplify DNA with a programme (Tab. 6). Analyse

Table 5: PCR mix for 1 reaction

Component	**Volume (µl)**
iH_2O	40.25
Buffer with $MgCl_2$(10x)	5
dNTP mix (each dNTP 10mM)	1
Forward primer (100 pmol. μl^{-1})	1
Reverse primer (100 pmol. μl^{-1})	1
DNA template (1 µg)	1
DNA polymerase (3.5 U.μl^{-1})	0.75

the outcome by horisontal agarose electrophoresis. Mix 5 µl of PCR sample with 2 µl Loadyng Dye and run a 1.5% agarose gel.

Table 6: PCR programme

Number of cycles	**Temperature (°C)**	**Time (s)**
1	95	120
1	95	15
	56	30
	72	45
5	95	15
	52	30
	72	45
5	95	15
	48	30
	72	45

6.10 Primer annealing

Primer annealing is used for construction of short dsDNA. Mix the components (Tab. 7) and put it into a thermocycler. Begin at 95°C and every 6 s decrease the temperature by 0.1°C until it reaches 71°C. Analyse on 1.5% agarose gel.

Table 7: Primer annealing

Component	Volume (µl)
High Fidelity Expand Polymerase Buffer with $MgCl_2$	5
Forward primer	2.50
Reverse primer	2.50
iH_2O	40

6.11 Transcription *in vitro*

Transcription *in vitro* was used for both linear DNA templates (amplified by PCR) and supercoiled DNA templates (plasmids). The only difference is in the amount of DNA put in the reaction. For linear DNA templates the amount was 70 ng DNA, for supercoiled DNA templates the amount was 50 ng.

Dilute DNA templates to 70 ng $\cdot$ µl^{-1} (DNA from PCR), respectively 50 ng $\cdot$ µl^{-1} (plasmid DNA) with iH_2O. Reconstitute RNAP with σ^Aor $\sigma^N$for 30 min in 30°C. Final concentration of RNAP is 30 nM and the final concentration of σ^A or σ^N is 160 nM. Add 1 µl of DNA template into each eppendorf tube. Prepare Master mix containing 1x transcription buffer, 100 mM KCl, 0.1 mg.ml^{-1} BSA, 1 mM GTP, 0.2 mM ATP, 0.2 mM CTP, 10 µM non-radioactive UTP and 2 µM radioactive ^{32}P-UTP. Concentrations of components are listed as final concentrations. Add Master mix to eppendorf tube with DNA templates and heat the samples at 37°C for 5 min. Initialise the reaction with RNAP reconstituted with sigma factor. The reaction volumes are 10 µl of reaction solution and the reactions are carried out at 37°C. After 15 min halt the reaction with 10 µl STOP solution and place samples on ice. Visualise the RNA transcripts by vertical polyacrylamide gel electrophoresis and analyse it with programme Quantity One.

6.12 *E. coli* strain construction

Cleavage with restriction endonucleases was used to ligate specific sequences to plasmid p770 and then transferred to DH5α (*E.coli*) strain.

6.12.1 Cleavage of inserts with restriction endonucleases

Prepare desired DNA fragments with PCR using chromosomal DNA or plasmid as templates. Set up the restriction reaction (Tab. 8). Digest for 1-2 hours at 37°C. Inactivate the enzymes for 15 min at 80°C.

Table 8: Cleavage reaction for inserts (final volume 20 µl)

Component	Volume (µl)
M buffer (10x)	2
Insert (350 ng)	
*Eco*RI	0.30
*Hind*III	0.30
iH_2O	

6.12.2 Digestion of a plasmid with restriction endonucleases

Isolate a plasmid by midiprep (see above). Set up the restriction reaction (20 µl total volume) (Tab. 9). Digest for 1-2 hours at 37°C. Inactivate the enzymes by loading the sample on a 1% agarose gel with an appropriate marker. As the concentration of marker is determined by the producer, compare the band intensities of the digested plasmid with marker. Estimate the plasmid concentration by determining which band has the closest intensity to the digested plasmid.

Table 9: Digestion reaction for a plasmid (final volume 20 µl)

Component	Volume (µl)
M buffer (10x)	2
Plasmid (5 µg)	
*Eco*RI	0.80
*Hind*III	0.80
iH_2O	

6.12.3 Isolation of DNA from agarose gel

Extract and purify DNA from agarose gel using QIAquick Gel Extraction Kit Protocol from Qiagen.

Inspect at the gel under UV lamp and excise the DNA fragment using a scalpel. Weigh the gell slice in an eppendorf tube. Add 3 volumes of Buffer QG to 1 volume of gel. Dissolve the gel at 50°C for 10 min. Add 1 gel volume of isopropanol to the solution and mix. Transfer the solution to a QIAquick spin column in a 2 ml collection

tube and centrifuge at 17 900 *g*, 1 min, room temperature. Discard flow-through and put QIAquick spin column back to the collection tube. Add 0.5 ml Buffer QG to the QIAquick spin column and centrifuge at 17 900 *g*, 1 min, room temperature. Discard the flow-through and place the QIAquick spin column back. Add 0.75 ml Buffer PE to QIAquick spin column, let the column stand for 2–5 min and then centrifuge at 17 900 *g*, 1 min, room temperature. Discard the flow-through, place the QIAquick spin column back and repeat the centrifugation. Place the QIAquick spin column into a new eppendorf tube. Add 50 µl iH_2O, let the column stand for 1 min and then centrifuge at 17 900 *g*, 1 min, room temperature. Analyse on 1% agarose gel.

6.12.4 Plasmid dephosphorylation

The cleaved plasmid is dephosphorylated on 5' ends to prevent self-ligation. This is needed for ligations with insert from PCR reactions that were prepared by cleavage with restriction enzymes and still contain 3'phosphates. However, when ligating plasmids with annealed linear DNA, the annealed DNA does not have phosphates on 5' end. Therefore, for ligation with an annealed DNA dephosphorylation is counterproductive.

Set up the reaction (Tab. 10). Dephosphorylate for 1 hour at 37℃. Inactivate the enzyme at 65℃ for 15 min.

Table 10: Dephosporylation reaction for a plasmid (final volume 60 µl)

Component	**Volume (µl)**
Plasmid from gel isolation	
SAP buffer	6
SAP Promega	3
iH_2O	

6.12.5 Ligation

Set up the ligation reaction (Tab. 11). As a negative control, prepare also one sample with all components but without any insert. Ligate over night (12-16 h) at 16℃.

6.12.6 Transformation

For *E. coli* was used heat shock transformation.

Table 11: Mix for ligation (final volume 20 µl)

Component	**Volume (µl)**
Plasmid	100 ng
T4 DNA Ligase (25 U.µl^{-1})	1
T4 DNA Ligase Buffer	2
Insert 5-10x molary more than plasmid	
iH_2O	

Take a tube with competent cells from -80℃ freezer and thaw them on ice. Put clean eppendorf tubes for transformation on ice to cool down. In each eppendorf tube pipette 10 µl of ligation mixture and 90 µl competent cells (DH5α). Make a control with only competent cells. Mix and incubate on ice for 30 min. Incubate at 42℃ for 90 s, then incubate for 5 min on ice. In each eppendorf tube put 1 ml LB media without antibiotics. Incubate with shaking at 37℃ for 1 hour. Centrifuge the transformation solution at 13 000 rpm, 1 min at room temperature. Quickly pour off the supernatant and resuspend the pellet in the remaining supernatant. Plate the cells on a dry LB agar supplemented with appropriate antibiotics. Incubate at 37℃ overnight.

If the transformation is successful, the number colonies on the control plate with only competent cells is zero. The number of colonies of competent cells with plasmid without insert is low. The number of colonies of cells with plasmid with an insert is high.

Pick individual colonies, make midipreps and sequence them with forward and/or reverse primers, at least two colonies per plate with insert to verify the construct.

6.12.7 Strain storage

Mix 850 µl of overnight grown cells with 150 µl glycerol and store at -80℃.

6.13 *B. subtilis* strain construction

B. subtilis strins were constructed for experiments observing *in vitro* promoter activity. First, transform an integrative plasmid pMP199 with σ^N with a weak Shine-Dalgarno sequence from σ^D under the hyperspank promoter into competent *B. subtilis* trp+ (strain LK 2504). Thaw the competent cells in 37℃ for 10 minutes. To 100 µl com-

petent cells add 1 µg plasmid pMP199. Incubate with shaking at 37℃ for 1 hour. Plasmid pMP199 should integrate into the *amyE* site. Spread the cells on a dry LB agar plate with spectinomycin (100 $mg.l^{-1}$). Incubate at 37℃ overnight. Grow strains from single colonies in 2 ml LB medium with spectinomycin (100 $mg.l^{-1}$) over night at 37℃. Mix 850 µl of overnight grown cells with 150 µl glycerol and store at -80℃.

6.13.1 *B. subtilis* competent cells

To integrate another plasmid containing a promoter of interest with the reporter *lacZ* gene, ligate first the promoter PCR product into the pDG1663 plasmid and transform into DH5α *E. coli*. pDG1663 is a plasmid that integrates at *thrC* in *B. subtilis*. Verify transformation with sequencing (primer 27) and isolate plasmid DNA. To integrate this plasmid into a construct with integrated pMP199 plasmid, prepare competent cells from *B. subtilis* strains with integrated pMP199.

Grow cells in 10 ml LB with spectinomycin (100 $mg.l^{-1}$) over night at 37℃. Next morning measure OD_{600}, inoculate to initial OD_{600} = 0.03 to 2x50 ml SPI medium and incubate at 37℃. Measure OD_{600} only from one erlenmayer flask. At OD_{600} = 1 (stationary phase) inoculate 10 ml from the erlenmayer flask that was not used for absorbance measuring to a clean erlenmayer flask with 90 ml SPII. Grow at 37℃ for 90 minutes. Halt the growth by placing the culture on ice for 10 minutes. Centrifuge for 5 minutes, 4000 rpm, 4℃. Pour the supernatant to a clean sterile flask. Take 9 ml of supernatant to another sterile flask, add 1 ml glycerol and mix. In the mix of supernatant and glycerol resuspend the bacterial pellet. Froze the cells in 500 µl aliquotes at -80℃.

To these competent cells transform plasmids with promoters of interest. The strains have spectinomycin and mls resistance (erytromycin 1 $mg.l^{-1}$ and lincomycin 25 $mg.l^{-1}$). Store in glycerol solution at -80℃.

7 Results

7.1 σ^N purification

σ^N was purified according to a protocol described in the Chapter "Materials and Methods". Fig. 14 shows various stages of σ^N purification on SDS-PAGE gels. After elution with imidazole, fractions 3 - 5 were dialysed together and fraction 2 was dialysed separately (Fig. 14A). The first fraction was discarded. After dialysis, protein concentration was measured by Bradford method. The pooled fractions 3 - 5 had 0.15 µg.µl^{-1} and the second fraction had 3.66 µg.µl^{-1}. Therefore, the pooled fraction were discarded and the second fraction was cleaved with SUMO-protease. For cleavage, 30 µl of SUMO-protease was used (Fig. 14B). The proper binding od SUMO proteins was verified by SDS-PAGE (Fig. 14C). σ^N was stored in storage buffer (Fig. 14D).

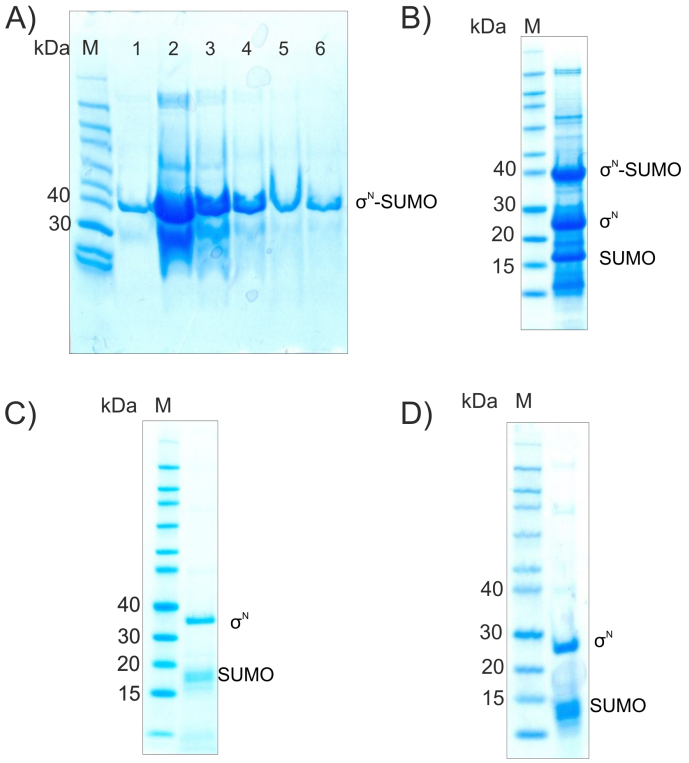

Figure 14: SDS-PAGE gels depicting various stages of σ^N purification. M stands for protein marker. (A) Protein fractions after elution with imidazole. The second eluate shows the most amount of protein. (B) σ^N after SUMO-protease cleavage. (C) σ^N after binding of SUMO to Ni-NTA beads. (D) σ^N after final dialysis to storage buffer.

7.2 RNAP isolation

RNAP was isolated according to a protocol described in Chapter "Materials and Methods" (Fig. 15).

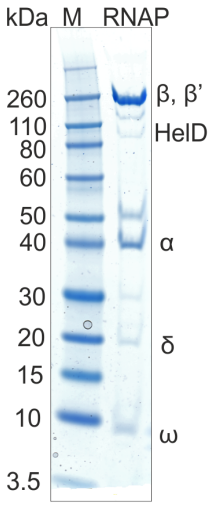

Figure 15: SDS-PAGE gel depicting isolated RNAP with its subunits. M stands for protein marker.

7.3 Linear PCR templates for transcription

Linear DNA templates for transcription were prepared by PCR using the pBS32 plasmid (Fig. 16). DNA templates consisted of the predicted σ^N-dependent promoter and approximately 200 bp upstream and 150 bp downstream from the promoter. From the 17 predicted promoters (Burton et al., 2019) the following promoters were selected: P*sigN* (P*zpdN*), P*zpaB*, P*zpbY*, P*zpdB* and P*zpdG*.

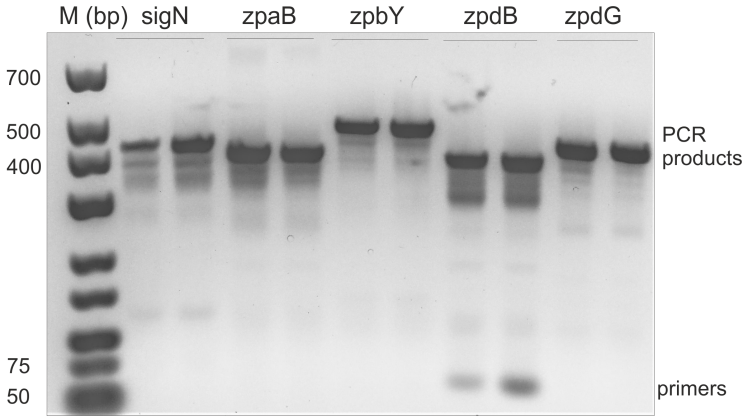

Figure 16: Linear PCR templates with various promoters on a 1.5% agarose gel. M stands for Gene Ruler Low Range Marker.

Ing. Marek Schwarz from Institute of Microbiology used σ^N promoter consensus sequences to predict σ^N-dependent promoters on chromosomal (genomic) DNA (gDNA) in *B. subtilis* 3610. As the consensus sequence he used TTTAC followed by a 15 (or

16) bp spacer and GATATAA or GATAAAA. In all pBS32 predicted σ^N-dependent promoters save one was the spacer 15 bp long. In one case the spacer was 16 bp long. Based on this consensus he found 10 promoters in gDNA. From these we selected five promoters for further studies. P*ydcJ* lies upstream the *ydcJ* gene that produces a hypothetical prophage protein alpha/beta hydrolase. P*yybN* lies upstream the *yybN* gene whose function is unknown. P*dpoL* lies in the *yorK* gene upstream the *yorL* gene that encodes DNA polymerase from prophage SPβ.

Because mitomycin C induces prophage expression (Okamoto et al., 1968), P*dpoL* seemed like a worthy candidate to study even though prophage expression is insufficient in decrease optical density after mitomycin C treatment (Myagmarjav et al., 2016). Promoter P*phyC* is upstream of the *phyC* gene that encodes a protein involved in inositol phosphate metabolism. The last promoter, P*nosA*, drives transcription of a putative antisense RNA (to the *nosA* gene). The *nosA* gene encodes nitric oxide synthase. These five predicted σ^N-dependent promoters located in the gDNA were prepared by PCR using gDNA of *B. subtilis* 3610 (Fig. 17). The architecture of these promoter fragments was the same as for the plasmid-defined promoter fragments: 200 bp of upstream DNA followed by the promoter followed by 150 bp of downstream DNA.

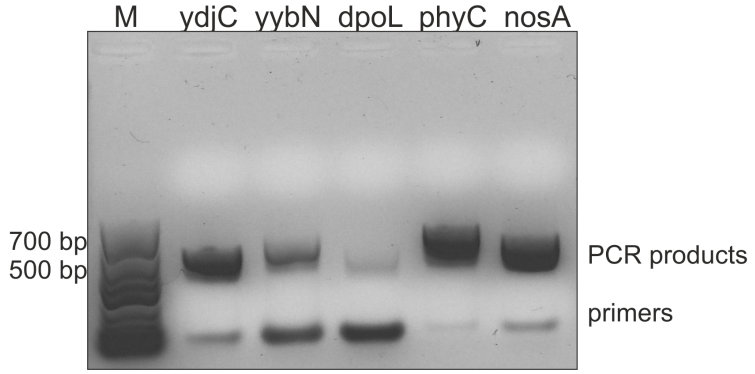

Figure 17: Linear PCR templates with various promoters on a 1.5% agarose gel. M stands for Gene Ruler Low Range Marker.

7.4 Promoters on supercoiled or linear plasmid

Selected σ^N-dependent promoters were ligated into plasmid p770 (pRLG770) and transformed to competent *E. coli* DH5α cells. Plasmids with selected promoters were isolated and a part was linearised (Fig. 18).

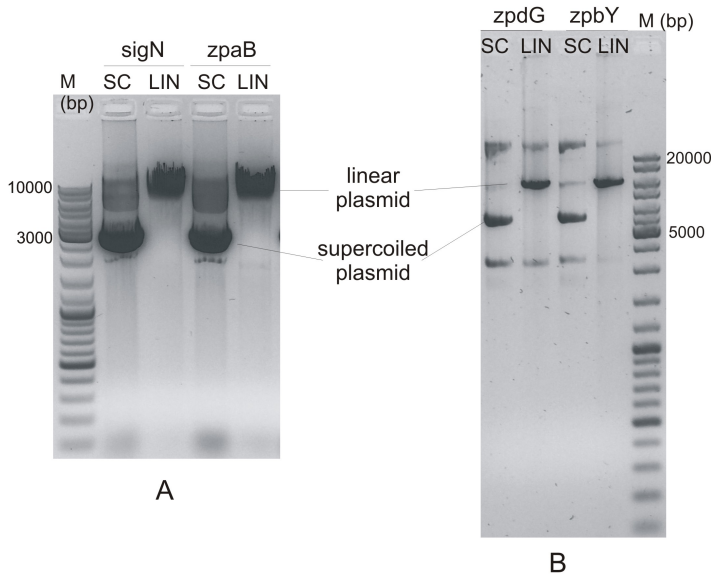

Figure 18: Supercoiled and linear plasmids with various promoters on an agarose gel. (A) sigN and zpaB promoters with upstream and downstream sequences in a supercoiled (SC) and a linearised (LIN) plasmid p770. Marker GeneRuler DNA Ladder Mix, on a 1% agarose gel. (B) zpdG and zpbY promoters with upstream and downstream sequences in a SC and a LIN plasmid p770. Marker GeneRuler 1 kb Plus DNA Ladder, on a 0.8% agarose gel.

7.5 Verification of predicted σ^N-dependent promoters

To test the activity of the predicted σ^N-dependent promoters (Burton et al., 2019) we used *in vitro* transcription on linear templates using RNAP reconstituted with σ^N. For each reaction we used 80 ng of DNA. To determine whether the promoters are also σ^A-dependent, each promoter was tested also with RNAP reconstituted with σ^A. As a positive control, we used the strong P*veg* promoter (Fig. 19). All tested promoters from plasmid pBS32 were active. The experiment was performed three times.

Next, we tested the predicted σ^N-dependent promoters identified in gDNA (Fig. 20). The same experiment setup was used as above. As a positive control was selected the P*zpdG* promoter. None of the gDNA-encoded putative σ^N-dependent promoters supported transcription. Therefore, it can be concluded that these sequences are not σ^N-dependent promoters, at least not in our *in vitro* system. They may require an extra transcription factor, which was not present in our *in vitro* system.

7.6 [iNTP] titration

The concentration of iNTP regulates the rate of transcription initiation (Gaal et al., 1997). Therefore, *in vitro* transcriptional experiments with the [iNTP] titration were

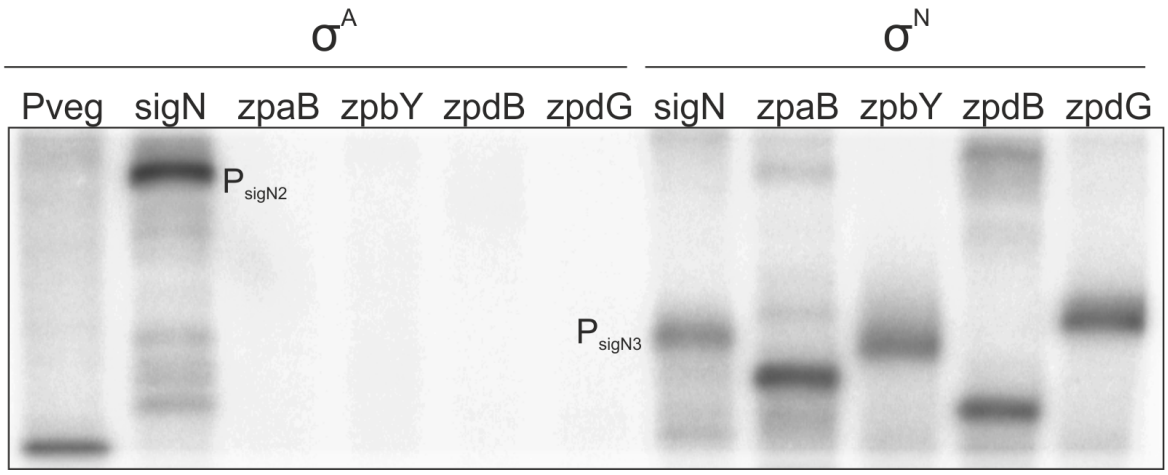

Figure 19: Transcription verification on predicted σ^N-dependent promoters on pBS32. Promoters P*sigN*, P*zpaB*, P*zpbY*, P*zpdB* and P*zpdG* were tested with either σ^A or σ^N. P*sigN* construct contained both σ^A-dependent P*sigN2* and σ^N-dependent P*sigN3*. All promoters but P*sigN2* were exclusively σ^N-dependent, while P*sigN2* was exclusively σ^A-dependent. σ^A-dependent promoter P*veg* served as a strong positive control. The experiment was performed three times; in the picture are results of one representative experiment.

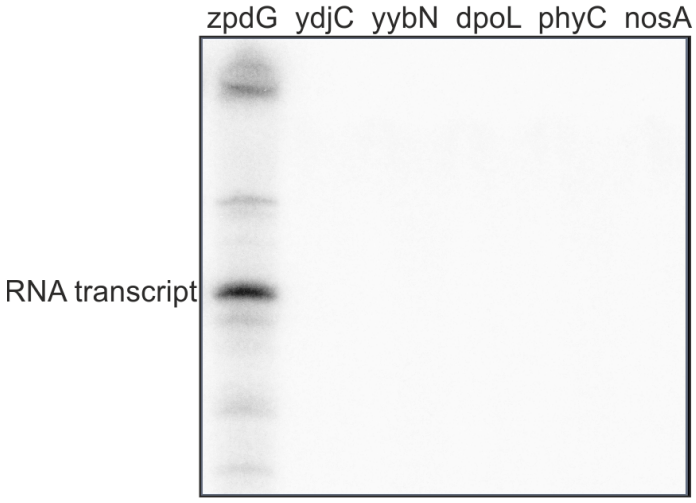

Figure 20: Transcription verification on predicted σ^N-dependent promoters on gDNA. Promoters P*ydcJ*, P*yybN*, P*dpoL*, P*phyC* and P*nosA* were tested with σ^N. σ^N-dependent promoter P*zpdG* served as a positive control. The experiment was performed three times; in the picture are results of one representative experiment.

performed. [iNTP] titration is used to determine the affinity of RNAP for its iNTP. [iNTP] titration was at first done with linear PCR products (promoter sequence with approximately 200 bp upstream and 150 bp downstream). [iNTP] titration was performed at least three times.

K_{NTP} is a value indicating the iNTP concentration that is required for at 50% transcription. Typically, iNTP insensitive promoters have K_{NTP} below 100 µM, while iNTP sensitive promoters have K_{NTP} above 100 µM. The higher the K_{NTP}, the wider the range of the iNTP to which the promoter responds (Krásný & Gourse, 2004).

The *sigN* (or *zpdN*) gene has three promoters: two σ^A-dependent promoters P*sigN1* and P*sigN2*) and one σ^N-dependent promoter (P*sigN3*). Based on a prediction, P*sigN2* should have UTP as the iNTP and P*sigN3* should start with GTP (Burton et al., 2019). As we were interested in P*sigN3*, [GTP] titrations were performed. The PCR construct included both P*sigN2* and P*sigN3* promoters (Fig. 21). As the expression on P*sigN2* was affected by the GTP concentration (even though it should not, having predicted +1 T), it is therefore included in my results. The K_{GTP} value of P*sigN2* is 178 µM and K_{GTP} value of P*sigN3* is 131 µM and they can be considered to be moderately iNTP sensitive.

The K_{GTP} value of P*zpaB* is 363 µM, which makes P*zpaB* the most iNTP sensitive promoter tested here. The K_{ATP} value of P*zpbY* is 290 µM which makes this promoter iNTP sensitive. For P*zpdB*, the K_{GTP} value is 138 µM and for P*zpdB* 138 µM. Both P*zpdB* and P*zpdG* are therefore moderately iNTP sensitive.

7.7 The effect of DNA topology on transcription *in vitro*

Next, we tested the effect of DNA topology, i.e. the supercoiled (SC) and linearised (LIN) states on σ^N-dependent transcription. We tested the RNAP affinity for promoter DNA, the RNAP affinity for iNTP, and the overall transcriptional activity of different promotes on SC versus LIN templates.

7.7.1 RNAP affinity for the promoter DNA

The affinity of RNAP for the promoter DNA can be performed by DNA titration, i.e. increasing of DNA concentration in *in vitro* transcription. Titration was performed three times. RNAP affinity was slightly higher for the LIN promoter (Fig. 22). As apparent from Fig. 22, after reaching a maximal value, further increases in DNA concentration

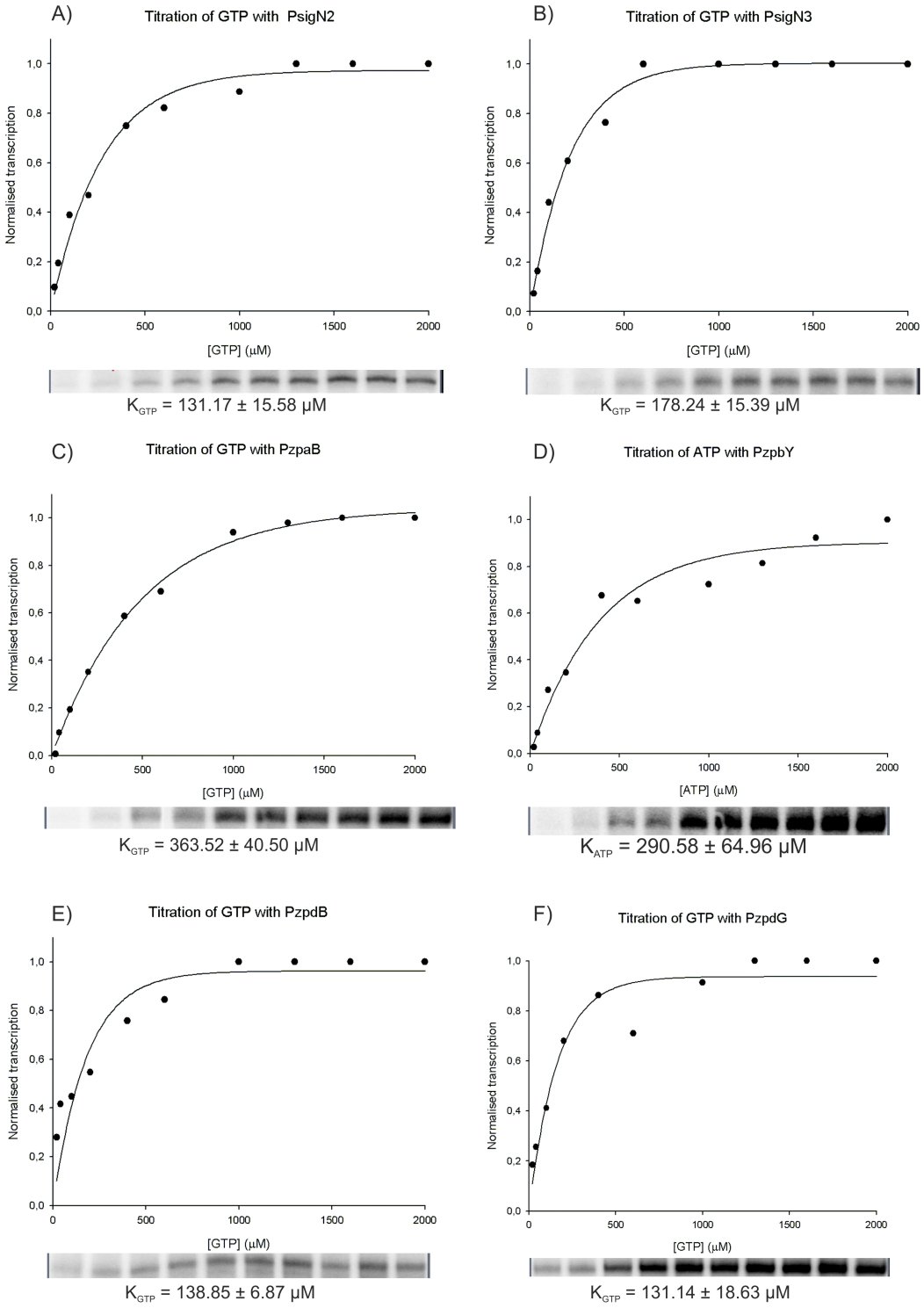

Figure 21: A representative graph of the [iNTP] titration on various promoters. Relevant primary data (radiolabeled transcripts) and calculated K_{NTP} (from three experiments) are shown below graph. A) Transcription on P*sigN2* promoter with increasing concentration of GTP. B) Transcription on P*sigN3* with [GTP]. C) Transcription on P*zpaB* with [GTP]. D) Transcription on P*zpbY* with [ATP]. E) Transcription on P*zpdB* with [GTP]. F) Transcription on P*zpdG* with [GTP]. The experiment was performed three times; in the picture are results of one representative experiment.

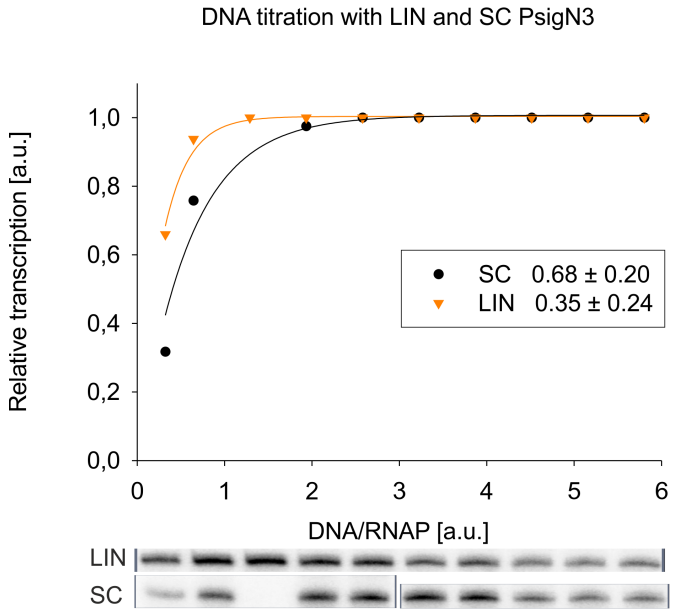

Figure 22: A representative graph of DNA titration of either SC or LIN P*sigN3* promoter on a plasmid. DNA/RNAP is a molar ration of DNA to RNAP. Below the graph are relevant primary data and in the graph are statistically calculated values (from three experiments) describing how much promoter DNA is required for transcription to work at 50 %. The SC primary data were electronically assembled from two parts present in the same gel. The third SC sample has a degraded RNA. The experiment was performed three times; in the picture are results of one representative experiment.

led to decreases in transcription, possibly to non-specific binding of RNAP to DNA. For the calculations of the DNA/RNAP ratio for the 50% effective transcription, the values after the maximum were taken as 1.

7.7.2 [iGTP] titration on LIN and SC plasmid

To test whether RNAP affinity for iNTP changes between LIN and SC plasmids, *in vitro* transcriptional experiments were performed with a promoter construct containing both P*sigN2* + P*sigN3* on the plasmid. The plasmid with the promoter construct was either supercoiled (SC) or linearised (LIN). The iGTP effect on transcription was calculated solely from the P*sigN3* transcripts (Fig. 23). K_{GTP} of the LIN template was slightly higher than on the SC template, but not by much (206.77 µM for LIN vs. 173.90 µM for SC). Both LIN and SC transcripts were loaded on one PAA gel, therefore the intensities of transcription between LIN and SC are comparable. Transcription from linear templates was stronger than from SC, which is surprising, because the linearised templates typically hinder transcription (Dillon & Dorman, 2010; Sudzinová et al., 2021).

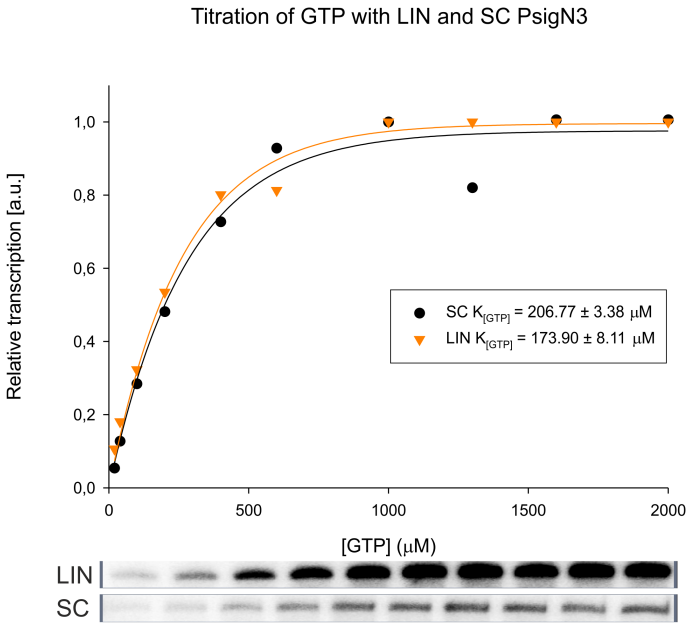

Figure 23: A representative graph of the [GTP] titration of either SC or LIN P*sigN3* promoter on a plasmid. Below the graph are relevant primary data. K_{GTP} values are in the inset. The experiment was performed three times; in the picture are results of one representative experiment.

7.7.3 The effect of DNA topology on transcription from various promoters

The effect of DNA topology was tested with various σ^N-dependent promoters. Transcriptions were performed with 150 mM KCl and were repeated at least three times. All promoters were tested with both σ^A and σ^N. For transcriptions with σ^N we used both SC and LIN templates (Fig. 24). P*zpaB* and P*zpdG* promoters displayed small decreases in transcription with the LIN template compared to SC templates. Transcription from the DNA construct with the P*zpbY* promoter increased more than 3 times in LIN DNA. A long construct containing both P*sigN2* and P*sigN3* displayed a decrease in transcription from LIN DNA for the σ^A-dependent P*sigN2* promoter but transcription increased from P*sigN3* from LIN DNA (Fig. 24B). Transcription from the core promoter sequence P*sigN3* was non-existent from LIN DNA (Fig. 24C).

7.8 The effect of DNA topology on transcription *in vivo*

The *in vivo* activity of σ^N-dependent promoters was tested in *B. subtilis* trp+ (strain LK 2504) with integrated σ^N-encoding gene, preceded by a weak Shine-Dalgarno sequence from *sigD* and under the control of the hyperspank promoter (inducible by IPTG) integrated at the *amyE* locus; and the *lacZ* gene under the control of the tested promoter sequence integrated at the *thrC* locus. Promoter activity was determined by

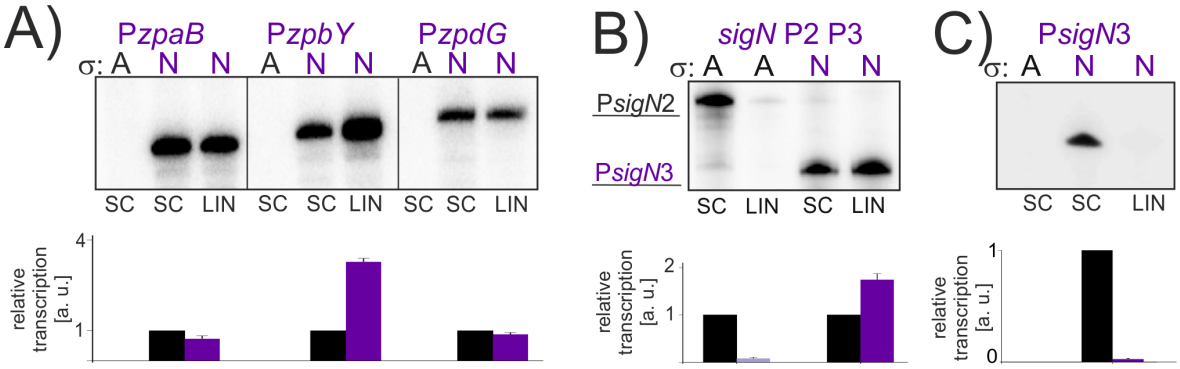

Figure 24: Transcriptions on σ^N-dependent promoters. A) σ^N-depentent promoters in *in vitro* transcriptions. B) Transcription on a longer promoter construct containing both σ^A-dependent promoter P*sigN2* (*sigN* P2) and σ^N-dependent promoter P*sigN3* (*sigN* P3). C) Transcription on a P*sigN3* core promoter. The experiment was performed three times; in the picture are results of one representative experiment. The figure was adapted from Sudzinová et al., 2021.

beta-galactosidase assays.

First, I confirmed that σ^N is induced by IPTG and that beta-galactosidase assays are working (Fig. 25). Strain LK 2887 with σ^N under the IPTG-inducible hyperspank promoter together with β-galactosidase under P*sigN3-lacZ* promoter fusion was grown. At OD_{600} = 0.5 (mid exponential phase) and at time 0 min were cells treated by IPTG (1 mM). As a positive control, the σ^A-dependent P*veg* promoter (strain LK 7555) was used. The activity of the P*sigN3* promoter increased 4 times in the presence of IPTG. However, a basal P*sigN3* activity was observed also in the absence of IPTG indicating that the hyperspank promoter was leaky (Huang et al., 2015). P*veg*, as a σ^A-dependent promoter, did not respond to the induction of σ^N.

Next, the *in vivo* activity of P*zpbY* (LK 2891) was measured four times (Fig. 26). P*zpbY* was selected due to its high *in vitro* activity on relaxed DNA. As a negative control we used a strain expressing σ^N and containing promoterless *lacZ* (strain LK 2902). 1 mM IPTG was added at the start of bacterial growth and 5 µg/ml of novobiocin (to induce DNA relaxation (Gellert et al., 1976)) was added at OD_{600} = 0.5 (mid exponential phase). Samples were collected at 0, 10 20, 30 and 40 minutes after novobiocin treatment and β-galactosidase activities were determined. While the activity of P*zpbY* on supercoiled DNA (untreated cells) steadily decreased by 60 %, its activity on relaxed DNA (treated by novobiocin) remained approximately the same as at time 0 (addition of novobiocin) and even slightly increased at the latest time point. When comparing the absolute activity of promoter P*zpbY* at 40 min after novobiocin or mock

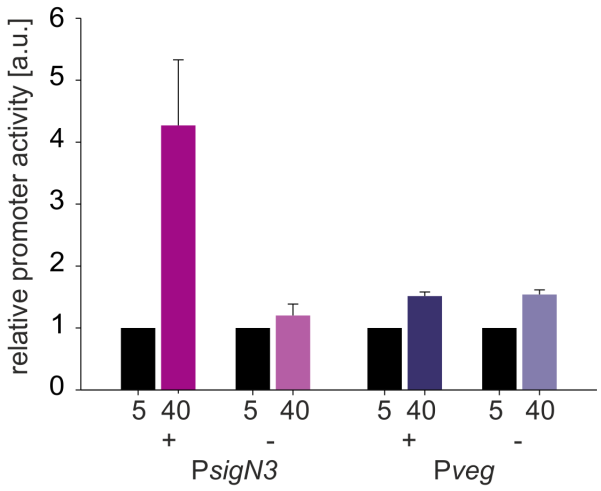

Figure 25: Activities of P*sigN3* and P*veg* promoter with (+) or without (-) IPTG. The experiment was performed two times. The columns show averages and the error bars.

treatment, the activity of relaxed P*zpbY* was 3-4 times higher than the activity of supercoiled P*zpbY*. LK 2902 displayed no promoter activity as there was no promoter upstream the reference *lacZ* gene.

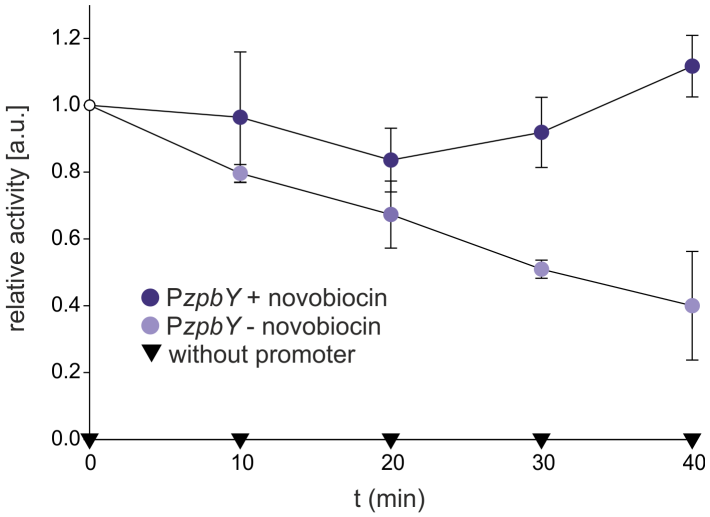

Figure 26: Activity of the P*zpbY* promoter and strain LK 2902 (*lacZ* without promoter) with (+, relaxed DNA) or without (-, supercoiled DNA) novobiocin as measured by beta-galactosidase assays. The experiment was performed three times; the symbols show averages and the error bars.

8 Discussion

σ^N is an unusual σ factor from *B. subtilis* 3610. It is encoded on plasmid pBS32 and activated by mitomycin C treatment that induces the SOS response. Activated σ^N induces transcription from σ^N-dependent promoters on pBS32. Overall, the effect of mitomycin C treatment and activation of σ leads to cell death (Myagmarjav et al., 2016; Burton et al., 2019).

In this Thesis I studied transcription from σ^N-dependent promoters including regulation by [iNTP] and DNA topology.

8.1 Experimental confirmation of σ^N-dependent promoters

First, *B. subtilis* σ^N and RNAP were purified, DNA promoter constructs were prepared by PCR, and *in vitro* transcriptions were used to verify whether the selected predicted promoters were indeed σ^N-dependent. Two types of promoters were tested: promoters encoded on pBS32 (the same plasmid that encodes σ^N) and promoters encoded on gDNA. Both types of promoters were from *B. subtilis* 3610.

All five selected σ^N-dependent promoters from plasmid pBS32 were active in *in vitro* transcription (Fig. 19). Transcriptional activity of promoters was tested with either σ^A or σ^N. The relative σ^N-dependent transcriptional activity of P*sigN3* was the lowest and of P*zpaB* the highest. Transcription with σ^A was successful only from the construct containing both P*sigN3* and a σ^A-dependent P*sigN2*.

Table 12: Selected σ^N-dependent promoters tested in this Thesis

Promoter	**Operon**	**Protein function**
P*sigN2*	*sigN*	Sigma factor
P*sigN3*	*sigN*	Sigma factor
P*zpaB*	*zpaB*	ATP-dependent DNA gyrase
P*zpbY*	*zpbYZzpcABCD*	*zpbZ*: NTP phosphohydrolase
P*zpdB*	*zpdBCDEF*	Nucleotide synthesis
P*zpdG*	*zpdG*	DNA polymerase III

Not all of the genes in σ^N-dependent operons have known functions. However, the

putative or confirmed functions of tested σ^N-dependent genes are summarised in Tab. 12 (Konkol et al., 2013). All σ^N-dependent operons encode proteins that either synthesize or hydrolyse NTPs or have functions related to DNA topology, replication or synthesis (Konkol et al., 2013). They may be involved in the build up of pBS32 after mitomycin C treatment where the plasmid number grows 100-fold (Myagmarjav et al., 2016). Under normal circumstances plasmid replication is directed by RepN that is encoded on plasmid. However, RepN expression is not induced while σ^N is overexpressed (Burton et al., 2019). Interestingly, the *zpaB* gene encodes an ATP-dependent DNA gyrase that generates negative supercoiling (Gellert et al., 1976).

The putative σ^N-dependent promoters from gDNA were predicted by Ing. Marek Schwarz based on σ^N promoter consensus (Burton et al., 2019). However, none of these promoters was active in *in vitro* experiments. Hence, it is likely that these predicted promoters are not in fact σ^N-dependent. Promoters P*ydjC*, P*yybN* and P*nosA* have a 16 bp spacer while the spacer length of all but one σ^N-dependent promoters from pBS32 is 15 bp (Burton et al., 2019). Moreover, the σ^N-dependence of promoter from pBS32 with the 16 bp spacer has not been confirmed yet. The longer spacer could be a hindrance to σ^N-dependent transcription. The lack of promoter activity can also mean that our *in vitro* transcription system lacked some factors to support their activity.

8.2 Regulation of σ^N-dependent promoters by the concentration of the iNTP

Promoter regulation by [iNTP] is an important mechanism of transcription control (Krásný et al., 2008). The higher the affinity for [iNTP] is, the lower is the required [iNTP] for transcription initiation. The concentration requirements for the initiating iNTP of σ^N-dependent promoters can be found in Tab. 13. It came as a surprise when P*sigN2* was sensitive to [GTP] when it was predicted to have T at its +1 site. In *B. subtilis*, the +1 nt is in 94% A or G, only in 4.5% the transcription starts with U (T on a non-template strand) (Krásný et al., 2008). It is therefore likely that the predicted +2 nt is in fact the first transcribed nucleotide.

The vast majority (11 out of 17) of the predicted σ^N-dependent promoters have G at their +1 position. Four promoters have as their transcriptional start A, two promoters have +1 T. Out of the tested promoters all but one have +1 G. The exception is P*zpbY*, which encodes +1 A.

In the stringent response to stress conditions, the alarmone ppGpp causes the GTP level to decrease, while the ATP level slightly increases (Krásný & Gourse, 2004). The stringent response can be also activated by the SOS response (Strugeon et al., 2016). Hence, the mitomycin C treatment that induces the SOS response also activates the stringent response leading to a decrease in [GTP] and an increase in [ATP]. After the decrease in [GTP] and increase in the [ATP] levels, the iGTP-sensitive promoters should reduce their transcription. However, the [GTP] in exponentially growing *B. subtilis* cells is 1 - 3 mM (Lopez et al., 1979). After the stringent response, the [GTP] drops to 60%, to about 0.6 - 1.8 mM (Krásný & Gourse, 2004). This is apparently still sufficient for a strong expression from σ^N-dependent promoters and we predict that expression from these promoters in the stringent response should be unaffected by the decrease of [GTP].

Table 13: K_{NTP} values for linear DNA templates with various promoters

Promoter	**K_{NTP} (µM)**	**Sensitivity to [iNTP]**
P*sigN2*	178.24	Moderate
P*sigN3*	131.17	Moderate
P*zpaB*	363.52	Sensitive
P*zpbY*	290.58	Sensitive
P*zpdB*	138.85	Moderate
P*zpdG*	138.85	Moderate

8.3 The *in vitro* effect of DNA topology on σ^N-dependent promoters

DNA topology is an important transcriptional regulator. DNA relaxation negatively affects effectivity of promoters from *E. coli* (Lim et al., 2003), *B. subtilis* (Sudzinová et al., 2021), viruses (Giladi et al., 1992) and other organisms. In my laboratory Petra Sudzinová conducted a research focused on the effects of DNA topology on transcription in *B. subtilis* (Sudzinová et al., 2021). She studied the effects of DNA topology primary on σ^A-dependent transcription, looking at the affinity of RNAP for promoter DNA and [iNTP]. She discovered that these effects were promoter-dependent. The ribosomal *rrnB P1* promoter's activity was reduced by DNA relaxation while the constitutive P*veg* promoter's activity was less reduced. Furthermore, she showed that RNAP

complexed with alternative σ factors displayed reduced activities on relaxed templates. To summarise, a decrease in promoter activity with decreased supercoiling was the prevailing trend.

To complete this study, complementary experiments using σ^N-dependent promoters were conducted in this Thesis and some of the results were included in the publication (Sudzinová et al., 2021) that can be found in Supplement 1.

My previous experiments were conducted on linear PCR DNA templates. To test DNA topology, promoter constructs were inserted into a plasmid that was left in the supercoiled form or relaxed (linearised) with a restriction enzyme.

First, the RNAP affinity for relaxed and supercoiled promoter DNA was tested. RNAP affinity for relaxed promoter DNA was tested with the P*sigN3* promoter by transcription. The affinity of RNAP to the relaxed promoter was twice as high as for the supercoiled promoter. This is similar to the behaviour of the σ^A-dependent P*veg* promoter and is opposite to ribosomal promoters *rrn BP1* and *rrn BP2* from *B. subtilis* (Sudzinová et al., 2021).

Second, the effect of DNA topology on RNAP affinity for [iNTP] was tested. Transcriptions from the P*sigN3* promoter were performed using increasing iNTP levels. The K_{GTP} value on relaxed plasmid (K_{GTP} = 173.90 µM) was similar to the K_{GTP} value on linear PCR product (K_{GTP} = 131.17 µM). This means that the sensitivity of RNAP to [iNTP] is similar between promoters on tested PCR products and relaxed plasmids. Therefore, only iNTP titration with P*sigN3* was performed. The affinities of RNAP for [iNTP] on other σ^N-dependent promoters are assumed to not deviate from already measured data on PCR products.

Overall, the RNAP affinity on relaxed supercoiled P*sigN3* for [iNTP] was slightly higher than on supercoiled DNA. In contrast, K_{NTP} values of P*veg* and *B. subtilis* ribosomal promoters were higher on supercoiled DNA than on relaxed (Sudzinová et al., 2021). This means that RNAP on both P*veg* and ribosomal promoters have higher affinity for iNTP on supercoiled DNA while RNAP at σ^N-dependent promoters has higher affinity for iNTP on relaxed DNA.

Third, the overall effect of DNA topology on various σ^N-dependent promoters was tested. Transcriptions with σ^N-dependent promoters in relaxed or supercoiled states

were performed (Fig. 24). Promoter activities of P*sigN3* (on the long sigN*P2 + P3* construct), P*zpaB* and P*zpdG* were the same from relaxed and supercoiled DNA. P*zpbY* had a significant increase in activity on relaxed DNA. The long sigN*P2 + P3* construct also showed an increase in P*sigN3* activity on relaxed DNA and in the same time showed a sharp decrease in the σ^A-dependent P*sigN2* promoter activity on relaxed DNA. In comparison, σ^A- and alternative σ-dependent promoters decrease their activity on relaxed DNA (Sudzinová et al., 2021). This shows that P*sigN2* behaves as other σ^A-dependent promoters, while P*sigN3* behaves differently.

Finally, to test whether the promoter activity on relaxed DNA is influenced by the promoter core or the sequence surrounding it, transcription from core promoter P*sigN3* was carried out. However, the activity of P*sigN3* decreased on relaxed DNA. This result means that the promoter core is not the deciding factors in the relatively high activity of σ^N-dependent promoters on relaxed DNA. My promoter constructs contained approximately 200 bp upstream and 150 bp downstream from the promoter. This indicates that somewhere in this sequence lies the key factor or factors for the relatively high activity of σ^N-dependent promoters on relaxed DNA. Upstream -35 element in σ^N-dependent promoters is a relatively AT-rich sequence (ca -70 position) that could be an UP element stimulating transcription (Condon et al., 1995). Analogously, in the *lacZ* operon operator sequences (that bind proteins and influence transcription) are both upstream (O_3) and downstream (O_2) the promoter core sequence (Oehler et al., 1990).

8.4 The *in vivo* effect of DNA topology on σ^N-dependent promoters

To determine whether the relatively high activity of σ^N-dependent promoters in relaxed DNA was also present in an *in vivo* system, *B. subtilis* strain with σ^N and the *lacZ* gene under the control of the promoter P*zpbY* (promoter with the highest *in vitro* activity) was grown in the presence or absence of novobiocin, an agent relaxing DNA (Gellert et al., 1976). It was confirmed that although the activity of P*zpbY* steadily decreased on supercoiled DNA after mid-exponential phase, the activity on relaxed DNA remained mostly the same (Fig. 26). As the promoter activity dramatically decreases for most known promoters on relaxed DNA (Lim et al., 2003; Sudzinová et al., 2021), the fact that P*zpbY* keeps its activity makes this promoter relatively highly active in the relaxed state of DNA. Furthermore, the difference in promoter activities at the latest time

point was great between relaxed and supercoiled DNA. Relaxed P*zpbY* had 3-4 times higher activity than supercoiled P*zpbY*. Further *in vivo* experiments need to be carried out to test whether all relaxed σ^N-dependent promoters have relatively high activities .

σ^N is activated by mitomycin C treatment (Myagmarjav et al., 2016). Mitomycin C can induce double strand DNA breaks (Dusre et al., 1989). When double stranded DNA breaks are introduced, DNA becomes relaxed. As σ^N-dependent promoters are transcribed only with σ^N that is activated by mitomycin C, and DNA in this situation is relaxed, σ^N-dependent promoters have perhaps evolved to withstand relaxed DNA.

Promoter consensus sequence of σ^N-dependent promoters differs from σ^A-dependent promoters (Fig. 27). First, the spacer length of σ^A promoters is 17 bp (Helmann, 1995), while the spacer length of σ^N promoters is 15 bp with one exception with 16 bp (Burton et al., 2019). σ^A promoters are more active in exponential phase, where DNA is more supercoiled (Dillon & Dorman, 2010), while σ^N promoters are more active in relaxed DNA. Also, σ^S from *E. coli* that is active in stationary phase with more relaxed DNA, does not require 17 bp spacers. Instead, it needs either 18 or 19 bp spacers with the TG motif or smaller 15 or 16 bp spacers without the TG motif (Kusano et al., 1996; Bordes et al., 2003; Typas & Hengge, 2006). Perhaps when DNA is more relaxed, σ already recognises a more relaxed promoter with a 15 bp spacer that is about the same length as a more supercoiled promoter with a 17 bp spacer. Second, the -10 element starts with G_{-12}. G at position -12 has not been reported yet (Djordjevic, 2011). This nucleotide could be the nucleotide that is σ^N-selective.

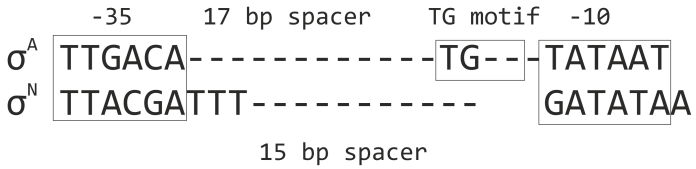

Figure 27: Consensus promoter sequences of σ^N and σ^A promoters. Highlighted are -35 element, spacer, TG motif and -10 element.

Out of the σ^N-dependent tested promoters the P*zpbY* promoter had the highest activity on relaxed DNA. This promoter has A as its +1 site, whereas other tested promoters have +1 G (Burton et al., 2019). Downstream of the -10 element is a 5 bp A-rich area ($_{-5}$AATAACA$_{+1}$). In other predicted σ^N-dependent promoters the area is also AT-rich, but several bp longer than in P*zpbY* (Burton et al., 2019). This combination could contribute to the relatively high activity of the P*zpbY* promoter on

relaxed DNA.

8.5 Biological role of σ^N

It remains unclear which σ^N-dependent operons/genes and how, cause cell death. σ^N may influence a toxin-antitoxin system by controlling its expression. A toxin-antitoxin system is composed of a toxin (protein) and an antitoxin (protein or RNA). When antitoxin is present, toxin cannot harm the cell. When the antitoxin levels decrease, toxin is activated and subsequently kills the cell (Hayes & Van Melderen, 2011). Some toxin-antitoxin systems are encoded on low-copy plasmids and kill plasmid-free daughter cells because antitoxin is less stable than toxin; in the cell without the plasmid antitoxin degrades faster, toxin is then activated and the cell is killed (Gerdes et al., 1986).

Another example of a toxin-antitoxin system is the ToxIN system from *Erwinia carotovora*. This system protects the bacteria from bacteriophage infection and inhibits bacterial growth (is bacteriostatic) (Fineran et al., 2009). I speculate that σ^N in the presence of mitomycin C may affect (repress) a toxin-antitoxin system that subsequently kills some cells and the surviving state are in a bacteriostatic state.

8.6 Potential utilisation of σ^N-dependent transcription in biotechnologies

Recently, advances in cell-free systems using linear DNA were made. Cell-free systems are not burdened by sustaining life and are therefore used as precise and effective system to manipulate gene expression and metabolism (Silverman et al., 2020). Linear DNA is much easier to construct and can contribute to protein toxin expression and analysis (Sun et al., 2014). Since transcription from linear templates is less effective (Sudzinová et al., 2021), σ^N-dependent promoters (such as P*zpbY*) can be used in such a system to improve the effectivity of expression. It can be both used in an unchanged form or be modified to an even stronger promoter. The promoters should include the 15 bp spacer and G_{-12}. Further research needs to be conducted to determine the importance of upstream and downstream sequences of promoter.

9 Conclusions

- σ^N, *B. subtilis* RNAP and promoter constructs were successfully prepared.
- Selected σ^N-dependent promoters were experimentally confirmed to be indeed σ^N-dependent and at the same time not σ^A-dependent.
- The influence of the concentration of regulating iNTP on various promoters was determined. RNAP on most σ^N-dependent promoters had a moderate affinity to iNTP.
- Effects of DNA topology in *in vitro* experiments was investigated.
  - Relaxed σ^N-dependent promoters had the same or higher activity than their supercoiled versions, which is an opposite trend than displayed promoters rocognised by other σ factors (Sudzinová et al., 2021).
  - This relatively high activity was not dependent on the promoter core sequence.
  - RNAP on relaxed σ^N-dependent promoters had ahigher affinity for iNTP.
  - RNAP affinity for promoter sequence was higher on relaxed DNA.
- In *in vivo* experiments, the activity of promoter relaxed P*zpbY* was slightly higher compared to supercoiled DNA.

Future experiments should focus on *in vivo* analysis of the activity of σ^N-dependent promoters in relaxed and supercoiled DNA. Also, it will be highly interesting to determine the sequences and factors causing a relatively high promoter activity on relaxed DNA. Application of these studies could improve the *in vitro* expression from cell-free systems.

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10 Supplement 1

The following publication contains a subset of the results in this Thesis. In the publication, my results are in the Chapter "The Effect of Supercoiling on Transcription In Vitro with Alternative Sigma Factors". These results can be found in this Thesis in Chapter "The *in vitro* effect of DNA topology on σ^{N}-dependent promoters".