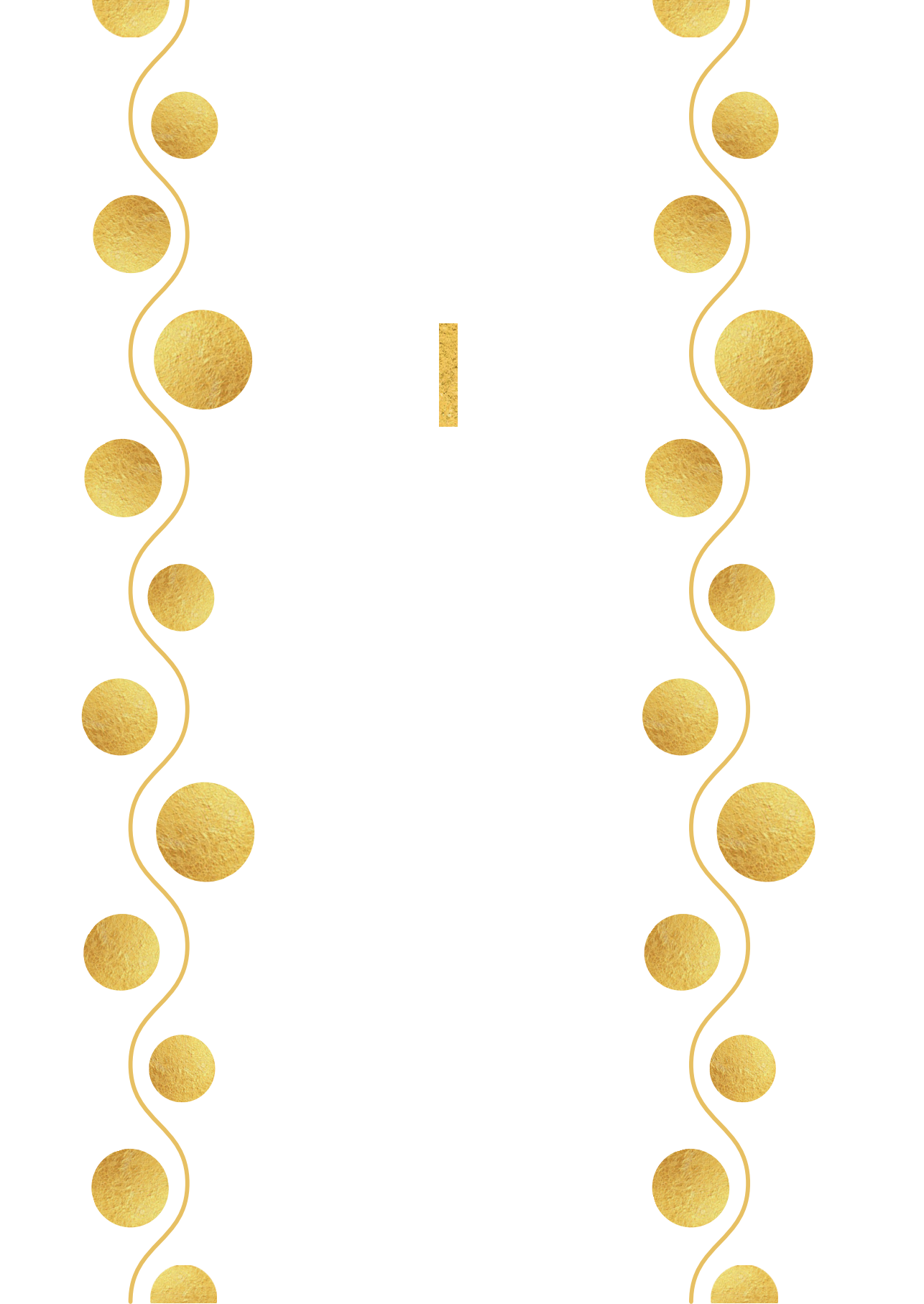
Synthetic biology approaches towards a deeper understanding of polar growth in Streptomyces

Lubbers, M.



Chapter 1

General introduction

Streptomycetes are multicellular bacteria that grow as filamentous, branching cells. These so-called hyphae form an interconnected network known as a mycelium. Following a period of vegetative growth, the substrate hyphae are degraded in a process of programmed cell death, and the remaining viable hyphae undergo compartmentalization (1, 2). Ultimately, this leads to the formation of specialized hyphae that protrude into the air. These aerial hyphae develop into chains of unigenomic spores, which can be dispersed to establish new colonies elsewhere.

The filamentous cells elongate through polar growth, in which new cell wall material is added at the extending tips (3). Tip extension and branching require DivIVA (3), a membrane-associated protein that localizes to these growing regions (4) (Fig. 1B). DivIVA interacts, amongst others, with the cytoskeletal proteins Scy and FilP, to form a dynamic complex at the tip, regulating the insertion of new cell wall material (5-7). While the peptidoglycan-synthesizing enzymes in *Streptomyces* have not been identified yet, DivIVA also interacts with CslA, a cellulose synthase-like protein that is involved in the biosynthesis of β-glucan at mycelial tips (8-10).

DivIVA is an essential protein, and partially depleting DivIVA results in the formation of abnormally shaped hyphae and irregular branching (11). In contrast, overexpressing DivIVA causes peptidoglycan to be added at multiple new sites, triggering excessive lateral branching (4, 12). In *Streptomycetaceae*, DivIVA consists of four domains: an N-terminal domain consisting of a coiled-coil segment bearing an N-terminal membrane-targeting sequence, followed sequentially by an intercoil region, a larger coiled-coil, and a C-terminal domain (12) (Fig. 1A). The N-terminal domain and the second coiled-coil are highly conserved among *Streptomycetaceae*, in contrast to the variable intercoil region and C-terminal domain (12). Computer simulations predicted that the N-terminal domain binds to the membrane, with its affinity dependent on membrane lipid composition (13). Furthermore, DivIVA forms a multimeric complex in *Streptomycetaceae* and long filaments *in vitro* (11).

In non-polar growing bacteria, such as Bacillota, DivIVA is non-essential and plays an important role in cell division. For instance, in *Bacillus subtilis*, DivIVA targets the division septum and cell poles, contributing to the control of the topological specificity of cell division (14). DivIVA associates with negatively curved membranes, possibly through molecular bridging of the curvature by DivIVA multimers (15). DivIVA also plays a role in sporulating

Bacillus cells, interacting with the chromosome segregation machinery to assist in positioning the oriC region at the cell pole (16). Structural models based on crystallography indicate that the N-terminal membrane-binding domain of *B. subtilis* DivIVA forms a dimer with crossed-over loops, likely interacting with the membrane surface (17). Further, models based on the crystal structures suggest that the C-terminal coiled-coil region is involved in oligomerization and forms a tetramer (17).

A comprehensive understanding of DivIVA's function requires the ability to delete the gene, which has not been feasible in *Streptomycetaceae* due to its essentiality (12). However, this limitation can be circumvented by using L-forms. These are bacterial cells that can proliferate in the absence of the cell wall and cell division machineries (18). Their proliferation is driven by biophysical processes, particularly increased membrane synthesis, leading to an imbalance in the cell's surface-to-volume ratio (19, 20). Previously, an L-form strain of *K. viridifaciens* was generated under laboratory conditions and named alpha (21). *Alpha* only proliferates in its cell wall-deficient state when grown in osmoprotective media. When transferred to media with lower osmolyte concentrations, it reverts to walled, filamentous growth resembling that of the parental strain (21). Strain Δ*divIVA* was generated by deleting the *divIVA* gene, disabling the strain from reverting to walled, filamentous growth (22). Introducing the native *divIVA* gene into the Δ*divIVA* strain restored walled growth (22), demonstrating successful complementation *in trans*.

Outlook on the thesis

DivIVA plays a central role in polar growth, making it crucial to gain a deeper understanding of its structure and function. The aim of this thesis is to enhance our understanding of polar growth in *Streptomyces*. By expressing homologous and heterologous DivIVA proteins in strain Δ*divIVA*, we have built numerous novel constructs to contribute to our understanding of the growth and mycelial morphology of *Streptomyces* (Fig. 1C).

In **Chapter 2**, we explore a spectrum of morphological engineering techniques, offering insights and providing inspiration for future advancements in bioproduction. Furthermore, we propose L-forms as a platform for morphology engineering of *Streptomyces*, as we believe this chassis can lead the way in optimizing morphology in challenging microorganisms and thus improve their exploitability in biotechnology.

To have a better understanding of the switching of L-forms to a walled state, one must be able to quantify this in a reproducible and predictable manner. Therefore, in **Chapter 3**, we employ gradient agar plates to investigate the reproducible transition between walled and cell wall-deficient states in actinomycetes. To facilitate the transition between these two states and reduce cell lysis, we apply an osmolyte gradient.

A deeper understanding of each of the four regions of DivIVA can provide new insights into their functions. In **Chapter 4**, we study the minimal domain requirements to facilitate walled growth in filamentous Actinomycetota by expressing truncated variants of DivIVA in the Δ*divIVA* strain. Various microscopy techniques, such as cryo-electron microscopy, transmission electron microscopy, and scanning electron microscopy, were used to observe the morphological effects of domain deletions. Furthermore, we replace DivIVA of *K. viridifaciens* with those from the unicellular bacteria *Mycolicibacterium smegmatis* and *Bacillus subtilis* to investigate whether these proteins could facilitate reversion of Δ*divIVA* to a walled state.

Computer simulations predicted that the N-terminal domain of DivIVA binds to the membrane, with its affinity dependent on membrane lipid composition (13). In **Chapter 5**, this domain is further investigated. Amongst others, we examine whether chimeric *K. viridifaciens* DivIVA with membrane binding domains from other Actinomycetota could facilitate reversion of Δ*divIVA*. By expressing an alanine screening library in Δ*divIVA*, our goal is to determine the essential amino acids in the N-terminal membrane-binding domain of DivIVA in *Streptomyces*. Furthermore, we investigate the link between DivIVA and the phospholipid cardiolipin.

In **Chapter 6**, we investigate whether DivIVA functions through phase separation, which is the ability of molecules to self-organize into membrane-less compartments. Although phase separation is well characterized in eukaryotes, its significance in bacteria is not well understood (23). The phase-separating potential of DivIVA is first evaluated using the predictive algorithm PScore. Based on these predictions, *in vitro* and *in vivo* approaches are employed to examine the phase-separating properties of distinct DivIVA domains in detail. The work presented here aims to establish a new model for the role of DivIVA in coordinating hyphal branching in *Streptomycetaceae*, thereby providing new insights into bacterial morphogenesis.

In **Chapter 7**, the results obtained in this thesis are discussed, and ideas are shared to provide pointers for future research directions.

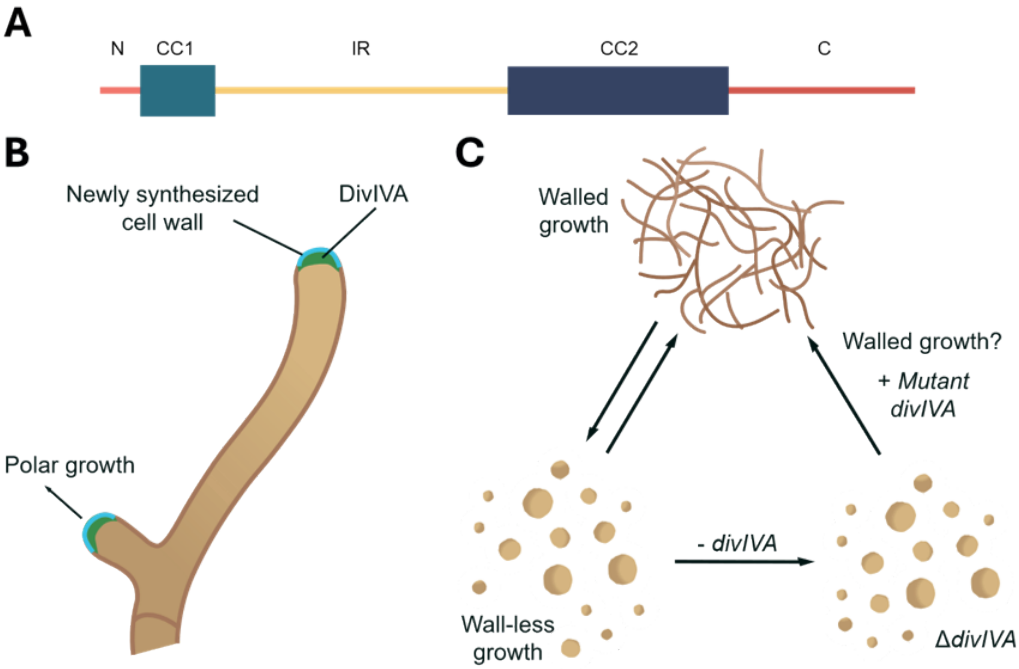


Figure 1. The engineering platform applied in this thesis. A) Schematic overview of DivIVA, which contains an N-terminal domain, consisting of a coiled-coil segment (CC1) bearing an N-terminal membrane-targeting sequence (N), followed sequentially by an intercoil region (IR), a larger coiled-coil (CC2), and a C-terminal domain (C). B) DivIVA orchestrates polar growth at the tips of *Streptomycetaceae*. C) Reintroducing divIVA restored the ability to support walled growth in Δ*divIVA*.