Off the wall: characterisation and exploitation of a cell wall-deficient life style in filamentous actinomycetes

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Chapter 1
General Introduction

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Filamentous actinomycetes

Actinobacteria constitute one of the largest phyla within the bacterial domain. This phylum contains Gram-positive species with a high GC content and a range of different morphologies, including cocci, rods, or mycelia[1,2]. The fragmented hyphal shape was used to name the Actinomycetales order as the term derives from the Greek words, *aktis* (ray) and *mukēs* (fungi)[2]. Actinomycetes species that form sporulating aerial hyphae are classified within the *Streptomycetaceae* family[3]. Novel taxonomic markers and the increasing number of genome-sequencing projects have helped in the classification of the different actinomycetes belonging to the *Streptomycetaceae* family. Recent phylogenetic analysis have validated *Kitasatospora*[4,5] and *Streptacidiphilus*[6] as sister genera of *Streptomyces*, which collectively comprise this family. All members of the *Streptomycetaceae* share a canonical life cycle[7] (see Fig. 1).

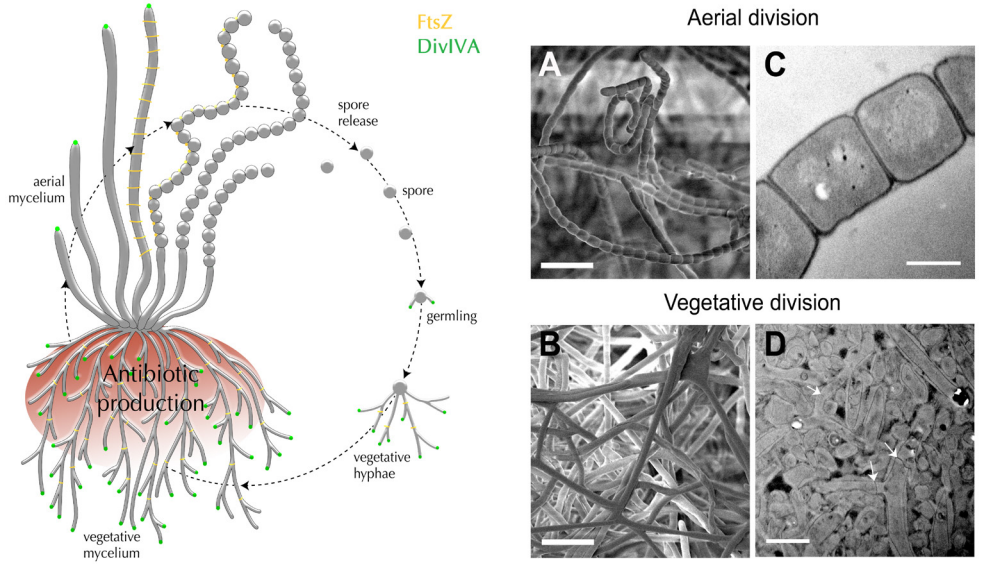

Figure 1. The developmental life cycle in the *Streptomycetaceae* family. The cycle starts with the germination of spores and the formation of germ tubes that grow by tip extension, a process coordinated by DivIVA. After a period of vegetative growth, aerial hyphae are formed, which will ultimately sepatate into chains of spores. Sporulation requires FtsZ, which assembles into rings that uniformly compartmentalize the aerial hyphae. Following their formation, spores are dispersed to establish colonies elsewhere. The scanning electron micrographs shows the aerial (**A**) and the vegetative (**B**) hyphae of *Kitasatospora viridifaciens* and depict the two distinct modes of cell division, namely septation during sporulation (**C**) and cross-wall formation during vegetative growth (**D**). Scale bar represents 5 µm (**A, B**), 500 nm (**C**) and 2 µm (**D**). Schematic life cycle adapted with permission of Lizah van der Aart. The scanning electron micrographs were taken with the assistance of Joost Willemse.

The life cycle starts with the germination of spores, leading to the formation of germ tubes that grow by tip extension. New tips are formed by lateral branching, which generates a vegetative mycelium[8]. The vegetative mycelium contains multinucleated compartments, formed by widely spaced cross-walls[9]. Upon nutrient depletion, a complex developmental program initiates both the reproductive growth phase and antibiotic production[10]. During this stage, programmed cell death (PCD) induces the degradation of the vegetative mycelium, causing the release of building blocks required for formation of aerial hyphae[2]. Ultimately, the aerial hyphae develop into thick-walled and grey-pigmented spores[11]. These spores can be dispersed again to establish new colonies elsewhere.

The developmental transitions from vegetative to aerial growth and sporulation are tightly controlled. For instance, the *bld* (bald) genes regulate the onset of aerial growth (Table 1). Null mutations in these genes give a comparable phenotype: a smooth ("hairless") colony surface where formation of aerial hyphae is blocked[12,13]. Likewise, spore formation is controlled by the *whi* (white) genes (Table 1), as sporulation-defective mutants are unable to form the characteristic grey-pigmented spores and remain white[14,15]. The *bld* and *whi* genes encode mostly regulatory proteins[16]. In addition, several structural proteins have been characterized that are required for the formation of aerial hyphae: the spore-associated protein B (SapB), the chaplins and the rodlins[17-22], which collectively render aerial hyphae hydrophobic. Additionally, a surface-associated glycan is required for aerial growth. The proteins responsible for synthesis of this glycan are encoded in the *cslA-glxA* operon, which act in conjunction with a series of copper-related proteins[23-28]. Table 1 provides a selected overview of genes that cause defects in morphological development in *Streptomyces*, genes required for cell division and cell wall synthesis are discussed in the following sections.

Table 1. List of genes affecting development in *Streptomyces*. Please note that the list only shows a selection of genes known to influence morphogenesis.

Locus	Gene product	Reference
Cell division genes		
ftsZ	Tubulin-like protein	29,30
ftsQ (divIB)	Small membrane protein required for sporulation septa	31
*ftsW**	Integral membrane protein required for sporulation septa	32,33
*ftsI**	Penicillin binding protein	32
*ftsL**	Membrane protein required for sporulation septa	34
ssgA	Cytoplasmic protein that activates septum formation	35,36
ssgB	Cytoplasmic protein that recruits FtsZ during sporulation	37-39
***bld* genes**		
bldA	Leucyl tRNA	40,41
bldB	Small DNA-binding protein	42-44
bldC	Transcriptional regulator of the MerR family	45,46
bldD	Small DNA-binding protein that binds to promoters of *whiG, bldN* and *sigH*	47-49
bldG	Anti-anti-sigma factor	50,51
bldH	AraC-like regulator (also called *adpA*)	13
bldK	Oligopeptide ABC transporter	52-54
bldJ	Extracellular signaling molecule imported by BldK	52
bldM	Two component response regulator (BldM-WhiI)	55,56
bldN	ECF sigma factor	57-59
***whi* genes**		
whiA	Transcription factor with WhiB	60,61
whiB	Transcription factor with WhiA	61,62
whiD	Transcription factor (also called *wblB*)	63,64
whiE	8 gene locus for grey polyketide spore pigment	65
whiG	RNA polymerase sigma factor	66,67
whiH	Transcriptional repressor of the GntR family	68
whiI	Response regulator	69,70
whiJ^{+}	DNA-binding protein, repressor of development.	71,72
Hydrophobic sheath components		
ramCSABR	Gene cluster involved in the synthesis of the SapB precursor	73-75
chpA-H	Chaplin proteins	19-21
rdlAB	Rodlin proteins	18,22
Cell surface polymers		
cslA	Cellulose synthase-like protein	24,26
glxA	Radical-copper oxidase	25,26
*sco**	Copper metallochaperone	76
*dtpA**	DyP-type Peroxidase A	77

$^{+}$Grey phenotype, *Defective sporulation is medium dependent, ECF: extracytoplasmic function

Filamentous growth and cell division in actinomycetes

The cell wall is a highly dynamic structure. In order for cells to grow, incorporation of new cell wall material into the pre-existing PG needs to be spatially and temporary controlled. Filamentous actinomycetes grow by apical tip extension, in which nascent PG is incorporated at the hyphal tip[78]. Tip growth relies on DivIVA (Green foci in hyphae Fig. 1), which is a coiled-coil protein preferring negatively-curved membranes and in actinobacteria it is an essential protein[78-81]. In *Streptomyces coelicolor*, DivIVA forms a multiprotein complex together with cytoskeletal proteins Scy and FilP, called the polarisome. This complex guides PG synthesis at existing hyphal tips and induces new tips during branching[82,83]. This makes the role of DivIVA in actinobacteria strikingly different from that in firmicutes, where DivIVA is not essential and mostly involved in correct placement of the septum[84,85].

Cell division in filamentous actinomycetes is markedly different between the vegetative and reproductive growth phases. During vegetative growth cell division leads to the formation of permeable cross-walls (Fig. 1) that allow diffusion of molecules[86-88] between the compartments, which remain physically connected to one another. Although the deletion of the conserved cell division gene *ftsZ* abolishes cross-wall formation, it is not lethal[29]. How mycelial networks are organized in the absence of cross-walls is under debate, but may involve membranous structures that compartmentalize the vegetative mycelium[86,87]. FtsZ has a second important role in actinomycetes during sporulation (Fig. 1), where it is necessary for assembling the cell division machinery driving constriction and separation[9]. In this way FtsZ has a more similar function to that in unicellular bacteria, where cell division starts with the positioning of FtsZ at midcell[89,90]. In *Escherichia coli* and *Bacillus subtilis*, FtsZ localization is controlled by the activity of the Min system and nucleoid exclusion[91-93]. These systems exert their effect on FtsZ positioning by preventing polymerization at unwanted sites in the cell. Contrary to these mechanisms in unicellular bacteria, positioning of FtsZ is positively controlled in actinomycetes. Actinomycetes use SsgB to localize FtsZ to the sites where cell division septa will be formed[39]. Following assembly of the other required divisome members, a thick cell wall is synthesized.

Peptidoglycan synthesis

An important constituent of the cell wall is peptidoglycan (PG). The PG forms a meshwork-like structure that envelopes the cell, and which in essence forms a single gigantic molecule called the murein sacculus[94]. Peptidoglycan strands are composed of monomers of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc). These strands are covalently cross-linked via peptide stems that emerge from the MurNAc subunits. The composition of the peptide stem and the degree of cross-linking varies tremendously among bacterial species and can also vary along with changes in environmental conditions[95,96].

The PG biosynthetic pathway can be spatially divided into three phases: a cytoplasmic phase, a membrane-associated phase and a periplasmic phase. In the cytosolic phase, precursor molecules UDP-GlcNAc and UDP-MurNAc-pentapeptide are formed. All steps are regulated by a cascade of mainly Mur enzymes: a family of transferases (MurA), dehydrogenases (MurB), amino acid ligases (MurC, MurD, MurE, MurG, and Ddl), racemases (Alr, DadX, and MurI) and glycosyltransferases (MraY, MurG)[97,98]. In the next phase, the cytoplasmic product UDP-MurNAc-pentapeptide is tethered to the lipid membrane via the transport lipid bactoprenol (C55- isoprenoid undecaprenyl phosphate), thereby forming the precursor lipid I. Attachment of UDP-GlcNAc to lipid I via MurG leads to the formation of lipid II. Lipid II is then transported to the other side of the cytoplasmic membrane. The identity of the transporter or flippase in the PG biosynthesis pathway remains controversial, although studies highlight FtsW[99], MurJ[100,101] and/or Amj[102] as possible candidates.

In the final phase of PG synthesis, lipid II monomers are polymerized into PG strands by glycosyltransferases in the periplasmic space[98]. These glycan strands are then incorporated into the pre-existing sacculus structure and cross-linked by transpeptidases. These glycosyltransferases and transpeptidases are referred to as penicillin binding proteins (PBPs) after their affinity for the antibiotic penicillin, which inhibits the activity of these PG synthases[103,104]. Recently, a new family of PG synthases was described: the SEDS (shape, elongation, division and sporulation) proteins[105-107]. In contrast to the PBPs, the SEDS proteins are relatively insensitive to antibiotics known to inhibit PBPs. Instead, the SEDS are affected by a different class of bioactive compounds, and thus may be an interesting target for novel antibiotics[108].

Adaptive morphogenesis

The cell wall is a highly dynamic structure, which can be altered in response to the cell's requirement. For example, alpha-proteobacteria, like *Caulobacter crescentus* can synthesize a single stalk as part of their dimorphic life cycle[109]. Other bacterial cells can undergo morphological changes

to move to new environments. For instance, when motile bacteria are challenged by dry surroundings or increased viscosity, they can differentiate into swarmer cells, which are elongated and have typically an increase number of flagella[110,111].

Sudden temperature shocks, changes in the pH or changes in the amount of osmolytes can also stress the cell envelope and lead to changes[112]. In both Gram-positive and Gram-negative bacteria, perturbations in the integrity of the cell envelope often initiate a so-called cell envelope stress response (CESR)[113,114]. In Gram-negative bacteria, the CESR is also triggered when cell envelope proteins in the periplasm are damaged or malfunctioning[114]. Upon activation, the CESR-linked regulatory systems will try to counteract the induced damage. The regulatory systems underlying this stress response are conserved, and the best-known ones are two component systems (TCS) and extracytoplasmic function (ECF) sigma factors. TCS are comprised of a membrane-associated sensor kinase that autophosphorylates before transferring the phosphoryl group to its cognate response regulator[115]. A good example of how TCS affects cell wall homeostasis, and thereby cellular morphology, has been described for *Corynebacterium glutamicum* . Under the influence of hyperosmotic conditions, the *mtrAB* two component system is involved in the regulation of cell wall metabolism and osmoregulation. Depletion of *mtrAB* caused a morphogenic switch from rod-shape to segmented cells and conferred resistance to ethambutol[116]. This two-component system is also present in *Mycobacterium tuberculosis*, where it is involved in the virulence response of this bacterium[117,118].

Microorganisms inhabiting environments where sudden environmental changes occur often have specific adaptation mechanisms. For instance, microorganisms in snow and ice habitats have to cope with fluctuating seasonal changes, which include variations in temperatures, UV radiation and availability of nutrients and water[119]. These physical and chemical conditions may lead to desiccation caused by rapid loss of cytoplasmic water and the increase in intracellular ions, which affects cell size homeostasis and compromises membrane integrity[120]. An example of a specific adaptation to cope with this stress is found in *Arthrobacter* strain A3 isolated from the alpine permafrost. Upon cold shock, this strain accumulates the non-reducing disaccharide trehalose that acts as an intracellular osmoprotectant[121]. The OstA protein is involved in the synthesis of trehalose but also acts as an osmotic stress-sensing morphogenetic protein. Depletion of OstA not only reduced the intracellular content of trehalose but also arrested the morphological switch from rod-shaped to myceloid cells[122].

Another specific adaptation mechanism is observed in microorganisms which are abruptly exposed to acid

stress. Bacteria used as probiotics entering the stomach get challenged with extreme acid stress and require a fast response to survive the sudden exposure to hydrochloric acid. For instance, when *Propionibacterium freundenreichii* strain SI41 was exposed to an acidic environment of pH 2, the cells underwent severe mortality, and changed their morphology from rod-shaped to segmented cells. If transient exposure to pH 5 in a chemically defined medium is allowed, the acid tolerance response takes place and there is no significant loss of viability or dramatic change in morphology[123].

In response to a large variety of environmental stresses, several bacteria transit into a so-called viable but non-culturable (VBNC) state[124]. In this state, the cells remain metabolically active but do not proliferate when transferred to nutrient-rich environments. Bacteria in environmental reservoirs can transition into the VBNC state, but also pathogenic bacteria can change into the VBNC within their host[125]. For instance, the gastric disease and ulcer-causing bacterium *Helicobacter pylori* transitions from its characteristic spiral shape to a coccoid VBNC state, under starvation conditions, aerobiosis and acid stress[126]. Some studies report structural modifications of the peptidoglycan that accompany this morphological transformation. Analysis of PG architecture revealed a substantial accumulation of the dipeptide monomers (N-acetylglucosaminyl-N-acetylmuramyl–L-Ala–D-Glu), and a decrease of tripeptide monomers (N-acetylglucosaminyl-N-acetylmuramyl–L-Ala–D-Glu-mesoDAP) in coccoid cells compared to the spiral form[127]. The PG hydrolase AmiA is responsible for the dipeptide monomer accumulation. This PG modification provides an escape from the immune sensor (nucleotide oligomerization domain) Nod1, which only senses murein tripeptides or longer and is unable to recognize dipeptides[128].

Adaptive morphogenesis is not restricted to bacteria. Also in Archaea modification of the cell envelope is a common strategy to cope with extreme environmental changes. Several species from the *Methanosarcina* genus adapt to elevated osmolarity, during which they transition from multicellular aggregates to spherical single cells as they lose the methanochondroitin outer layer[129]. Also, *Haloarchaea* species from the Deep Lake community in Antarctica adapt to cold-water temperatures, by changing the N-acetylation of the S-layer and forming biofilms[130]. Altogether, these examples demonstrate that cell envelope changes are common in microorganisms when they are exposed to stress-causing conditions.

Life without a cell wall

Although the cell wall provides mechanical protection to virtually all bacteria[98,104], several species can adapt to a transient or permanent life style without this protective envelope. For instance, the multidrug resistance pathogen *Pseudomonas aeruginosa* can transition from rod-shaped to spherical cell wall-deficient (CWD) cells when treated

with β-lactams, which provides a mechanism to escape from the bactericidal effect. When the antibiotic is removed, the entire population of cells reverts to the walled state[131]. Likewise, the rod-shaped bacterium *Mycobacterium bovis* undergoes a dramatic morphological transition when exposed to harsh conditions such as cryogenic stress and nutrient starvation, leading to the formation of giant filamentous structures that produce and release spherical CWD cells[132]. These morphological variances offer an alternative route for the cell to survive and reproduce whilst exposed to stress.

Interestingly, several bacterial species evade the stress of cell wall-targeting antibiotic by adopting a cell wall-deficient life style as so-called L-forms. The first L-forms were described in 1935 and named in honor of the Lister Institute where they were discovered[133]. Following the first studies on L-forms, many terms have been used interchangeably to describe morphologically similar cells such as L-phase bacteria, L-variants, and CWD forms. L-forms can be broadly described as mutant forms of walled bacteria that are able to grow and divide indefinitely without a cell wall. L-form bacteria require an isotonic environment since they lack the turgor-withstanding cell wall. A few clinical reports have described L-form cells in patient samples[134], and some studies have reported bacterial L-forms in plant tissue[135].

L-forms are generated in laboratory settings by cultivation of walled cells in osmoprotective media together with agents that degrade or interfere with cell wall synthesis, such as lysozyme and penicillin G[136-138]. Prolonged exposure in such media can cause bacterial cells to accumulate mutations, which promote a cell wall-deficient (CWD) life style[136,138-140]. These mutations fall in two separate classes. In the first class of mutation the cell membrane formation is stimulated either by blocking PG synthesis or directly activating the synthesis of fatty acids. In the tractable *B. subtilis* L-form system the expression of the *murE* operon was repressed, and in combination with penicillin treatment yielded cells that were able to proliferate without the cell wall[139]. Successful conversion to L-forms was also possible by deleting individual genes (*murC* and *dal*), which also blocked the PG precursor pathway[141]. Proliferation of L-forms was also benefited by a single point mutation in *accDA* or the overexpression of the AccDA protein. Both *accA* and *accD* are part of the protein complex acetyl-CoA carboxylase, which catalyze the first step in fatty acid synthesis[142]. Overexpression of these subunits increased the membrane lipid accumulation, benefiting the L-form mode-of-proliferation. The second class of mutations is important to reduce the oxidative stress caused by repression of cell wall synthesis. Kawai *et al* (2015) demonstrated that inhibition of PG synthesis stimulates the flux through the electron transport chain, which increases reactive oxygen species (ROS). This was based on three observations: 1) ROS levels are abnormally elevated in CWD cells, 2) repres-

sion of antioxidant systems in L-forms severely inhibited their proliferation and 3) anaerobic cultures or addition of ROS scavengers promote L-form growth in Gram-positive and Gram-negative bacteria[143].

Other studies have shared insights on how the transition to the L-form state requires the designated step of escape from the cell wall sacculus. For instance, the isolation of a *B. subtilis* strain that efficiently switches between the L-form and rod-shape state led to the identification of two mutations required for L-form escape. The first one mutation was identified in the *walR* transcription regulator, while a second mutation was identified in the cell division gene *sepF*[144].

Unlike walled bacteria, L-forms are able to propagate independently of the FtsZ-based division machinery[139]. Instead, these cells use a so-called extrusion-resolution system for proliferation, during which numerous progeny cells are more or less simultaneously released from the mother cell. Additionally, L-form cells may propagate by means of blebbing, tubulation and vesiculation, which give rise to a morphologically heterogeneous population of cells[139,140,145-149]. The mode by which L-forms proliferate is conserved in Gram-positive and Gram-negative bacteria and appears to be based solely on biophysical principles. The excessive membrane production in L-form cells drives an imbalance between the growth of the cell surface area and internal volume, leading to vesicle extrusion[140].

Although the biology of L-forms has been addressed in considerable detail, little is known about the relevance of these cells in wild bacteria, and whether the numerous examples of wall-less bacteria identified in patient[150-155] and plant[135,156-158] samples have accumulated mutations or contained a natural adaptation response system.

Outline of thesis chapters

Filamentous actinomycetes have spurred much interest due to their ability to synthesize a wide diversity of natural products. Although these microorganisms have a canonical life cycle (Fig.1) and a characteristic multicellular mode-of-growth, they are able to transition to a unicellular, CWD state. In this thesis, the filamentous actinomycetes *Kitasatospora viridifaciens* DSM 40239 was chosen to study three distinct types of CWD cells: protoplasts, stress-induced S-cells (see below) and L-forms (Fig. 2). As a source to analyse genetic changes caused by the switch from a walled to a wall-deficient state, the genome sequence of *K. viridifaciens* was determined (**Chapter 2**). *In silico* analysis combined with PFGE approaches revealed that *K. viridifaciens* contains a linear chromosome and a megaplasmid KVP1. Unexpectedly, protoplast formation and regeneration often caused dramatic chromosomal rearrangements and loss of KVP1, which potentially provide a source for creating genetic diversity.

Chapter 3 describes the response of filamentous actinomycetes to high levels of osmolytes in the environment. Such elevated osmolyte concentrations causes hyperosmotic stress, leading to a reduction in growth, an excess in membrane synthesis and hypercondensation of DNA. Strikingly though, were the spherical CWD S-cells that were extruded under these conditions in the culture. S-cell formation was found in many filamentous actinomycetes, inferring that this response is common. These S-cells are only transiently CWD and eventually establish new mycelia. Notably, prolonged exposure to hyperosomotic stress converted S-cells to mutant L-form variants that proliferate without a wall.

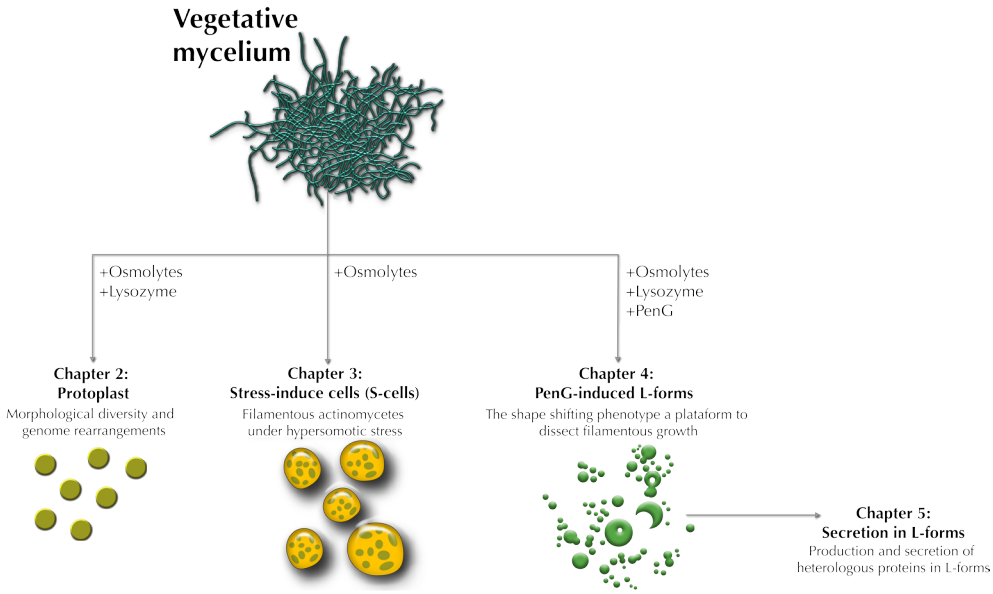

Figure 2. Schematic outline of this thesis and the three types of CWD cells investigated. The three types of CWD cells were formed in a osmoprotective environment with sucrose and magnesium chloride. Protoplast were obtained by lysozyme treatment which generates cells uniform in size. The Stress-induced cells formed as consequence of the hyperosmotic stress caused by excess of osmolytes, these CWD cells are heterogeneous in size. L-forms are mutants created after prolonged exposure to Penicillin G and lysozyme, they are not homogeneous in size.

Chapter 4 investigates the use of a PenG-induced L-form strain of *K. viridifaciens* as a platform to identify genes required for the switch to the walled state. This strain, called *alpha*, has the unique ability to switch between a CWD and walled state, depending on the growth conditions. Using this strain allowed us to disrupt the polar growth determinant *divIVA* or part of the *dcw* gene cluster, which contains a number of genes involved in cell division and cell wall synthesis, including the essential gene *murG*. The absence of *divIVA* or the *dcw* gene cluster prevented *alpha* from switching to the walled state. Strikingly, complementation of the *dcw* mutant with solely the *divIVA* gene restored the switch to filamentous mode-of growth. An *in silico* analysis identified a *murG*-like gene in the genome of *K. viridifaciens*, hereinafter named *murG2*. Reduction of *murG2* in the absence of *murG* dramatically reduced the ability of *alpha* to switch to the filamentous mode-of-growth, implying that MurG2 provides lipid II synthase activity in the absence of MurG.

Chapter 5 explores the capacity of *alpha* for the secretion of antibiotics and enzymes. We introduced a reporter plasmid with a cellulase gene, and we exploited the shape shifting capacity of *alpha* to compare the secretion in the L-form and mycelial state. The results demonstrate that secretion of secondary metabolites is not affected by the absence of the cell wall, and that DivIVA is not essential for secretion. Altogether these results are the first indications that *alpha* can be further improved as production platform.

A general conclusion to this thesis is provided in **Chapter 6**.