From stress to success: how actinobacteria exploit life witout a cell wall

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Chapter 7

Summary and general discussion

Streptomyces are filamentous bacteria that flourish in soil, where they encounter a wide array of environmental fluctuations. Their ability to adapt and withstand many stressors relies on their capacity to switch to different morphological states, including the formation of aerial hyphae, spores and likely cell wall-deficient (CWD) cells that can be extruded under hyperosmotic stress conditions [11, 12, 93]. One of the common stressors bacteria are exposed to are bacteriophages, as they are the most abundant biological entities on this planet [19].

In general, phages recognize their host by binding to receptor proteins attached to, or located on, the bacterial cell wall. Next, phages eject their genome, make progeny phages and eventually lyse the host. However, little is known about the infection cycle of phages in multicellular bacteria. So far, we know that *Streptomyces* phages are unable to attach to spores, and prefer replication in young vegetative mycelium [15, 16]. Interestingly, older mycelium can even protect younger germinating spores by producing molecules, like daunorubicin and actinomycin D, that kill phages [51, 187]. Recent research has shown that the viral impact on a colony can be restricted by increasing the formation of aerial hyphae and spores near the site of an infection [17]. This transient phage resistance by morphological differentiation is not only observed for spores, but also during the production of cell wall-deficient cells.

The work presented in this thesis provides new insights into the biology of phage-*Streptomyces* interactions and analyzes the ecological role of CWD cells in nature.

Cell wall-deficiency as an escape mechanism from phage infection

For phages to complete a successful infection cycle, the first step is to recognize and attach to a suitable bacterial host. The receptor-binding proteins (RBPs) on the phages' tail fibers, tail spike or even head fibers, play a crucial role in viral attachment and subsequent genome delivery in the host bacterium [188]. The RBPs recognize surface associated macromolecules on the cell envelope, like lipopolysaccharides, transporter proteins or substrate receptors for diderm bacteria and peptidoglycan and (lipo)teichoic acids on monoderm bacteria. However, some phages can also recognize receptor proteins on structures like flagella, pili and capsule layers [35]. Many defense

systems have been discovered recently that interfere with this initial step of phage infection [189]. In **Chapter 2**, we hypothesized that shedding of the cell wall might be an efficient strategy for transient resistance against phages. Many bacteria are known to shed their cell wall in response to environmental stressors, and most receptor proteins that phages are able to recognize are usually located, associated with, or attached to the bacterial cell wall [190]. Therefore, we reasoned that CWD bacteria could temporarily render immunity from phage attachment and infection by losing the cell wall, before reverting to the walled mode-of-growth.

To test this hypothesis, we exposed several bacterial strains under hyperosmotic stress to bacteriophages in **Chapter 5**. As we predicted, the mycelium of *Streptomyces* strain MBT86 was entirely converted into cell wall-deficient cells after infection with phage LA7 (which was later named Pablito, in **Chapter 3**) in osmoprotective medium. These CWD cells could revert to the mycelial mode-of-growth and significantly more colony forming units were obtained after phage infection, indicating that a greater fraction of this multicellular population could survive an infection in osmoprotective medium compared to the standard *Streptomyces* growth medium (see Fig. 1). Not only this strain, but all tested *Streptomyces* strains became CWD after infection with a susceptible phage, although some strains seemed to make more CWD cells compared to others. This might be explained by the different phage-host pairs and therefore differences in infection mechanisms, or the timing of phage infection, since there seems to be an ecological trade-off between phage reproduction rate and progeny release [191]. Some phages quickly assemble and release new progeny phages, leading to early lysis of the host and perhaps the bacteria have less time to grow into long hyphae, resulting in less CWD cells.

Not only multicellular bacteria, but also *Escherichia coli* and *Bacillus subtilis* were able to shed their cell wall after phage infection. Therefore, we expected that many other bacteria are capable of shedding the cell wall after phage infection, which was later also shown in detail for *Listeria monocytogenes* and *Enterococcus faecalis* [153]. The interaction between *E. coli*, *B. subtilis* and their respective phages was also imaged using cryo-electron microscopy. This revealed that phage T4 could no longer bind to *E. coli* CWD cells, probably because the receptor proteins that phage T4 recognizes are located on the

outer membrane [31]. Interestingly, phage φ29 could still attach and infect CWD cells of *B. subtilis*. The RBPs of this phage are likely recognizing lipoteichoic acids, which are anchored in the membrane, and remnants could still be present in CWD cells and exposed to the environment [142].

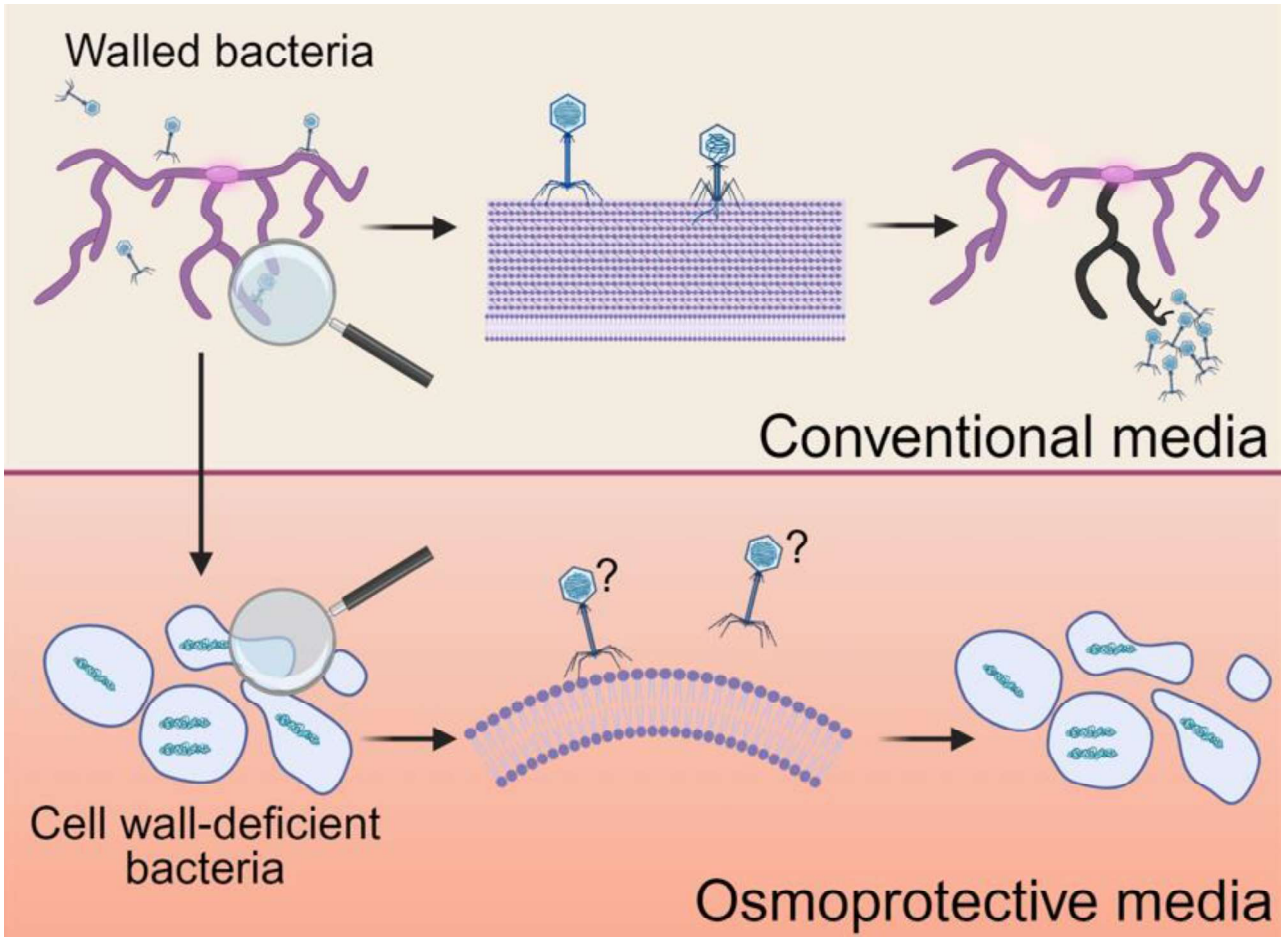

Figure 1. Schematic overview of phage infection in Streptomycetes in conventional and osmoprotective medium. In normal medium, phages recognize the RPBs on the bacterial cell wall and the hyphae lyses. In osmoprotective medium, the mycelium can become cell wall-deficient and phages no longer recognize their usual host, which therefore stays uninfected.

Although the mechanism of shedding the cell wall after phage infection in osmoprotective medium still remains unclear, it was later shown that the endolysins released from lysed *L. monocytogenes* bacteria cause degradation of the cell walls of uninfected cells [153], where this transition to cell wall-deficiency was also observed. However, when we added spent medium of a CWD culture after phage infection to an uninfected culture of *B. subtilis*, we did not observe formation of CWD cells (**Chapter 5**). This suggest that either

the concentration of endolysins was too low, or that *B. subtilis* does not become CWD due to endolysin release of neighboring bacteria. These results emphasize that every phage-host pair might react differently and that not all research on model organisms can be extrapolated to other bacteria.

Identifying new *Streptomyces* phage species

Characterizing and sequencing more phages that infect multicellular bacteria to increase our knowledge on this viral dark matter is of extreme importance. In **Chapter 3** and **4** we have isolated three new phage species. *Streptomyces* phage Pablito could be characterized as a new species in the genus *Janusvirus*, while *Streptomyces* phage Verabelle and Vanseggelen were classified as new species in the genus *Camvirus*. All three phages have a dsDNA genome with a relative high G+C content of over 65% and are predicted to have a temperate lifestyle. Compared to other phages from model organisms like *E. coli*, relatively few *Streptomyces* phages have been accurately sequenced, classified and characterized [173]. Additionally, mostly dsDNA phages have been isolated and ssDNA, ssRNA and dsRNA *Streptomyces* phages are highly underrepresented [76]. This bias could be due to the standardized isolation methods and difficulties sequencing phages with a single-stranded genome [98, 130, 192]. However, new high-throughput isolation methods could really benefit this field, for example by using the Mimetas microfluidics platform [193]. Here, 96 different *Streptomyces* strains can be tested simultaneously for susceptibility to phage infection in environmental samples, speeding-up the usually time-consuming protocol of phage enrichment, isolation and microscopic characterization of the phage-host interaction.

Isolating and characterizing more *Streptomyces* phages could in the long run lead to a BASEL-like collection for these multicellular bacteria. The BASEL collection [194] is a phage library consisting of *E. coli* phages that are all well-sequenced, phenotypically characterized and for which host receptors proteins are known. This collection is broadly used and facilitates the discovery of the molecular mechanisms of phage-host interactions [195-197]. For *Streptomyces* phages, there is the Actinobacteriophage database [76], but this consists solely of phage genomes and is not readily available for experiments. Since we only know the tip of the iceberg of the interaction

between phages and their multicellular bacterial hosts, a library of sequenced and characterized *Streptomyces* phages would greatly benefit the field and hopefully contribute to more open science and collaborations between different research groups worldwide.

Mechanism of phage infection in multicellular bacteria is largely unknown

Since a commonly used *Streptomyces* phage library is not readily available, we have infected 29 different *Streptomyces coelicolor* mutant strains lacking distinct cell wall-associated proteins, with 46 phages of our own collection to begin to understand the molecular mechanism of phage infection in multicellular bacteria. In **Chapter 6**, we identified that phage CF3 could no longer successfully infect *S. coelicolor* lacking YidC2. This protein is predicted to be a membrane protein insertase in *E. coli* and *B. subtilis*, but little is known about its function in *Streptomyces* [155, 156]. Unlike its parent, *S. coelicolor* Δ*yidC2* did not form CWD cells after phage infection. CWD cells of the Δ*yidC2* mutant could only be formed by exposing the strain to lysozyme, indicating that YidC2 plays an important role in shedding of the cell wall after phage infection. Interestingly, phage CF3 could still attach to *S. coelicolor* Δ*yidC2*. However, using biochemistry and cryo-electron microscopy, we found that the progeny phages could no longer be released into the environment due to a block in degradation of the host cell wall.

To enhance our understanding of YidC2's role in the infection process, we hypothesized that the phage might exploit this protein to insert some of its own proteins into the membrane. Since most known phage holins are membrane-associated proteins, we closely examined the holin-endolysin system of phage CF3. Previous studies showed that holin proteins disrupt the membrane by making holes, through which the endolysin protein can exit and subsequently break down the peptidoglycan layer [182]. However, some holins do not form the typical "holes", but can be used for non-lytic protein translocation, by temporarily permeabilizing the membrane, so that endolysins can passage [183]. Using Alphafold3 combined with a bacterial two-hybrid assay and characterization of endolysin secretion mutants, we could show that YidC2 interacts with the holin-endolysin system of phage CF3. We propose that YidC2 facilitates insertion of the holin and endolysin of phage CF3 into the

membrane. Through non-lytic protein translocation, the endolysin is flipped across the membrane, allowing it to enzymatically degrade the peptidoglycan layer. This mechanism allows the cell membrane to remain intact and the bacteria will form CWD cells.

Outlook and future directions

The results of this thesis are a promising starting point to further improve our understanding of the ecological value of cell wall-deficient bacteria in nature and the interaction between phages and their multicellular hosts. The combination of several techniques, like confocal microscopy, electron microscopy, genetics and many more, made this work possible and allowed for the discovery of unprecedented new insights. However, several questions remain to be explored. For example, can phages traverse though mycelial hyphae? This hypothesis could be investigated by live-imaging of a fluorescent *Streptomyces* phage during infection [198]. However, deleting or adding genes in *Streptomyces* phages is relatively complicated, probably due to the high G+C content, low burst sizes and small capsid heads. One way to circumvent these disadvantages is by integrating temperate *Streptomyces* phages into their host, and manipulating the phage genome inside the bacteria with genome editing systems like CRISPR-Cas9 [162, 199], after which the engineered phages can be isolated from their host with mitomycin C, which induced excision of temperate phages from the bacterial genome [200]. Live-imaging of a fluorescent phage during infection in *Streptomyces* could resolve whether phages can actually travel though hyphae and may also shed a light on the mechanism-of-action of cell wall-deficiency after phage infection in a multicellular host.

Since most research on phage-host interactions has been done in unicellular bacteria [19, 48, 54], multicellular bacteria might respond with a completely different mode-of-action. In phage research, the ratio between phages and bacteria during infection, or Multiplicity of Infection (MOI) is an important concept. An MOI of 1, for example, is calculated as one bacterium getting infected by one phage, and an MOI of 0.1 means that for every 10 bacteria, only one is infected by a phage. *Streptomyces* spores can make one or two germ tubes and further in the lifecycle, the mycelial network gets counted as one colony forming unit. So, MOI calculations with multicellular bacteria loses

its significance, once a spore has germinated [98] and when *Streptomyces* are infected with phages at low MOI, only one or two hyphae of a whole mycelium will be infected. Perhaps, the multicellular mycelial colony signals to uninfected hyphae to produce CWD cells in order to save the population, similar to abortive infection in unicellular bacteria [46]. For these reasons, calculating MOI in multicellular bacteria like *Streptomyces*, should be optimized and a new and more accurate formula for MOI in multicellular bacteria would benefit this research field.

However, the most urgent question that remains to be resolved would be to answer the impact of shedding the bacterial cell wall in phage therapy. Phage therapy is a promising strategy for treating antibiotic-resistant bacteria. However, my work has shown that bacteria can readily become CWD upon exposure to phages, which provides a protective mechanism for the population of bacteria. Interestingly, previous research has shown that cell wall-deficient bacteria were found in blood samples and in patients with recurrent urinary tract infections [66, 201, 202]. This indicates that urine and blood can sustain the transient morphological state of CWD cells and that clinical trials testing phage therapy should take this morphological switch into consideration. For example, phage therapy shows promising outcomes when treating mycobacterial infections, but there never is a 100% success rate [203, 204], which might be explained by the ability of many mycobacterial strain to form viable cell wall-deficient cells [205]. Alongside conventional diagnostics tools, a next step would be to also monitor the formation of CWD cells after phage infection during experimental procedures and clinical trials for phage therapy treatments, which have likely been overlooked in the past [206]. This would increase our knowledge on the occurrence and impact of CWD cells after phage therapy and will contribute to our battle against antibiotic-resistant bacteria.