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

Ongenae, V.M.A.

Chapter 1

General introduction

Streptomyces are soil-borne multicellular bacteria, well-known for their ability to produce many clinically important antibiotics, like neomycin, streptomycin and many more [1-3]. Compared to most bacterial model species, Streptomycetes have a rather unique lifecycle, which starts from spores. If spores encounter a favorable and nutrient-rich environment, typically one or two germ tubes are formed at the distal end of each spore. These tubes form filaments, or hyphae, that grow by tip extension. Subsequently, the growing hyphae branch, leading to the formation of a vegetative mycelium that degrades organic material in the soil [4]. The vegetative hyphae contain semi-permeable cross-walls and are multinucleated [5]. When nutrients are exhausted, differentiation is initiated, leading to the formation of hydrophobic aerial hyphae that grow out of the substrate and which subsequently develop into long spore chains. These spores possess a thick cell wall, which makes them resilient to harsh conditions. Following dispersal to more favorable environments, the spores can reinitiate this lifecycle.

In their natural environment, *Streptomyces* are exposed to numerous stressors in their environment, such as heat stress, changes in acidity of the soil, competetive bacteria and bacteriophages [6-9]. The bacterial cell wall is a shape-defining and stress-bearing structure that can protect bacteria against exposure to most of these stresses. This wall is either a single layer of peptidoglycan between an inner and an outer membrane in diderm bacteria, or a thick peptidoglycan layer outside the cytoplasmic membrane in monoderm bacteria, such as *Streptomyces* [10].

Counterintuitively, *Streptomyces* can transiently lose their cell wall under hyperosmotic growth conditions or during antibiotic treatment, resulting in the formation of so-called cell wall-deficient (CWD) cells [11, 12]. These spherical cells are able to survive in an osmoprotective environment and can revert to the mycelial mode-of-growth when the stressors are no longer present. However, little was known about the relevance of these cells in nature [10].

Bacteriophages, or in short phages, recognize their hosts by binding to receptor proteins located on, or attached to, the cell wall of bacteria. Therefore, we hypothesized that the shedding of the cell wall may also protect the cells from phage infection. In this case, the phages might be unable to recognize their host when the cell wall and its associated receptor

proteins are absent. Since most research has been done on phage-host pairs in non-osmoprotective media and with unicellular model bacteria, like *Escherichia coli* and *Bacillus subtilis* [13, 14], the unique lifecycle of *Streptomyces,* requires another perspective on phage infection dynamics (Fig. 1).

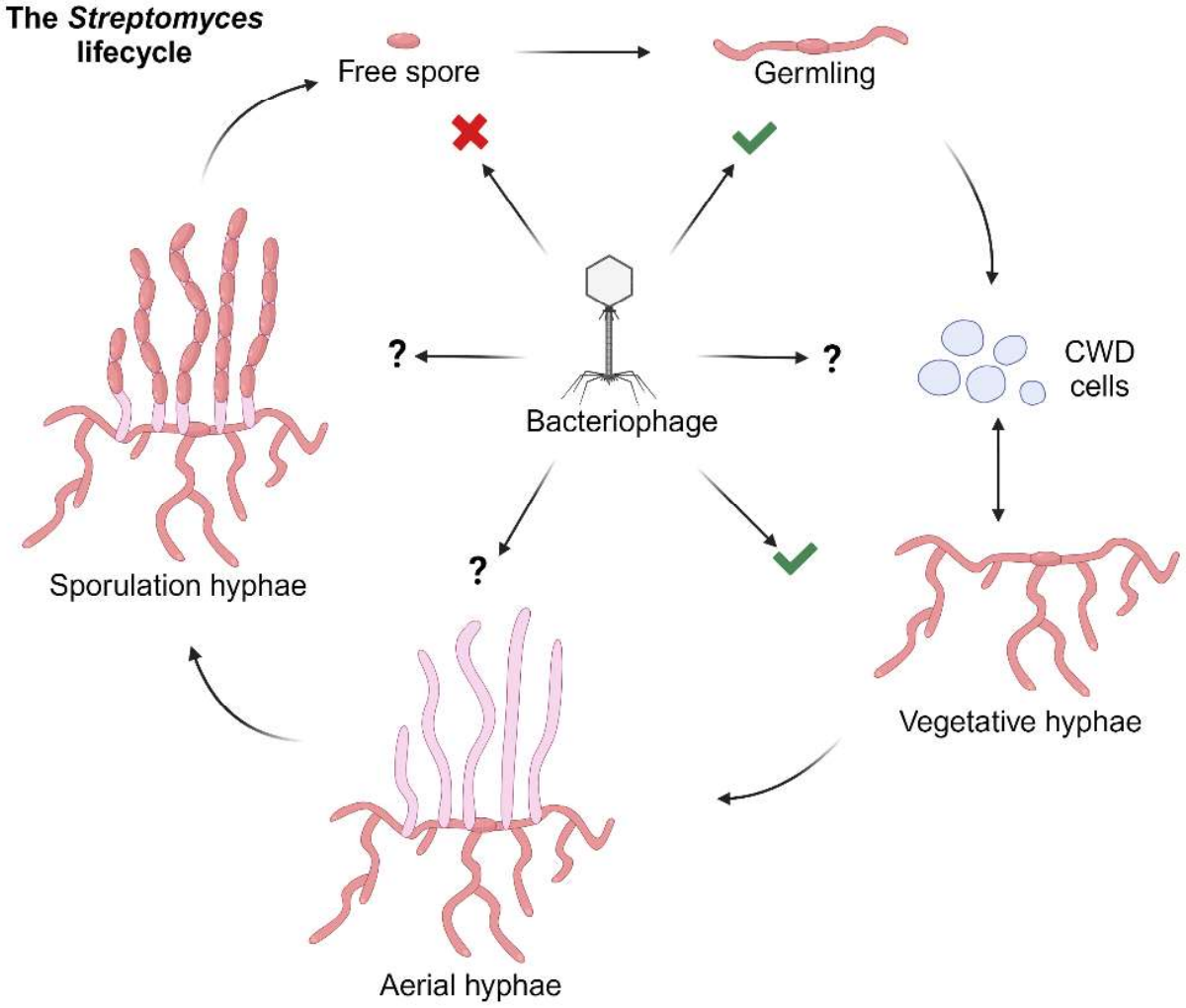

Figure 1. Schematic representation of phage infection in the *Streptomyces* lifecycle. The *Streptomyces* lifecycle starts as a free spore, which in turn grows into a germling. These germlings form a network of mycelium with vegetative hyphae, which under osmoprotective stress can form cell wall-deficient cells. Phages cannot infect the dormant spores and prefer young germlings and vegetative hyphae. After nutrient depletion, aerial hyphae are formed, which produce spores. It is unknown whether phages can attach and infect aerial hyphae, sporulation hyphae and CWD cells.

In general, little is known about the interaction of phages with *Streptomyces* bacteria during their different morphological states, from multicellular hyphae to cells after shedding their cell wall. So far, it has been shown that phage infection is optimal in young germlings and vegetative mycelium [15, 16]. Additionally, we know that phages cannot infect spores, probably due to their metabolic inactivity or different cell surface, and that the production of aerial hyphae can prevent spreading of phage infection [17]. However, the infection dynamics during the cell wall-deficiency stage remain largely unexplored.

The aim of this thesis is to study phage infection in *Streptomyces*, focused on cell wall-deficient cells. The results of this thesis contribute to a better understanding of phage infection dynamics in multicellular bacteria.

In **Chapter 2**, I review and discuss the hypothesis that bacteria can evade phage infection by shedding the cell wall. Phages recognize and attach to receptor proteins on, or attached to, the bacterial cell wall, like peptidoglycan and (lipo)teichoic acids in Gram-positive bacteria. Gram-negative bacteria on the other hand, have a wide range of structures on the outer membrane that phages can recognize, like lipopolysaccharides, transporter proteins and pores. However, some bacteria are known to shed their cell wall in response to environmental stressors. Without the protection of the cell wall, bacteria become spherical, only enclosed by a membrane and very fragile, but also lack most of the receptor proteins necessary for successful phage recognition.

Since little is known about the infection dynamics in multicellular bacteria, I sequenced and characterized three new bacteriophage species in **Chapter 3 and 4**. When compared to unicellular bacteria like *E. coli* or *Pseudomonas aeruginosa*, relatively few *Streptomyces* phages have been properly sequenced and characterized. Therefore, these phages increase our knowledge on the viral dark matter and are now called *Streptomyces* phage Pablito, belonging to the genus *Janusvirus,* and *Streptomyces* phage Vanseggelen and *Streptomyces* phage Verabelle that belong to the genus *Camvirus*. All three new phage species are used in future studies and their availability will contribute to expand the knowledge on phage-host dynamics in multicellular bacteria.

In **Chapter 5**, I show that phage infection converts the mycelium of most *Streptomyces* species into cell wall-deficient cells in osmoprotective medium, which we hypothesized in **Chapter 2**. *Streptomyces* spp. MBT86 was infected with *Streptomyces* phage Pablito and the presence of cell wall-deficient cells was quantified. The response to shed the cell wall was independent of the tested bacteriophage; several other phages could also induce this reaction. The wall-less cells were protected from phage infection and could revert to their canonical mycelial mode-of-growth, demonstrating that a significant fraction of the population survived the phage infection. To our surprise, not only strains MBT86, but all other tested *Streptomyces* strains could form cell wall-deficient cells after infection with a susceptible phage. Interestingly, we also found this behavior in the unicellular bacteria *E. coli* and *B. subtilis,* which was shown with fluorescent microscopy, phage growth curves and cryo-electron microscopy.

Since the mechanism behind shedding the cell wall in *Streptomyces* and the overall phage infection dynamics in multicellular bacteria remains largely unknown, we tested a knock-out library of 28 *Streptomyces coelicolor* cell wall mutants for their ability to be infected by our in-house phage collection. In **Chapter 6**, I describe the discovery of the role of the membrane insertase YidC2 in *S. coelicolor* during phage infection. With adsorption assays and cryo-electron microscopy, we discovered that phage CF3 could not lyse *S. coelicolor* Δ*yidC2* and the phages remained inside the infected bacterium, while the wildtype was killed. We showed that YidC2 plays an important role during the insertion of the holin-endolysin system from the phage inside the bacterial membrane, which subsequently leads to lysis of the infected bacteria.

The main findings of this thesis are summarized and discussed in **Chapter 7**. Here, I give my perspective for future directions in the research field of cell wall-deficiency associated with bacteriophages.