Mechanisms and consequences of horizontal gene transfer in cell wall-deficient cells of Kitasatospora viridifaciens

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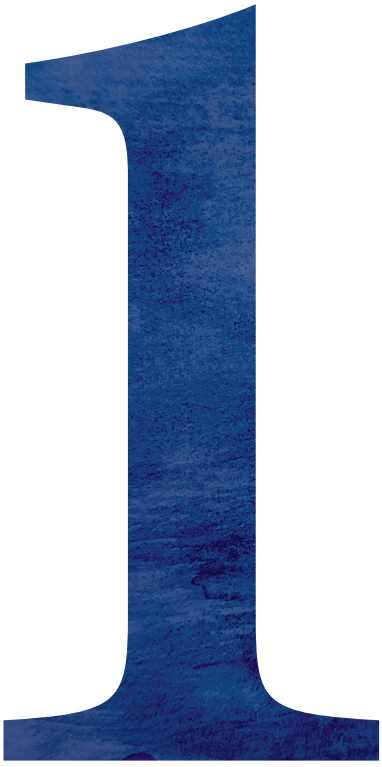

Chapter 1.

General introduction

Organisms face many challenges during their lifetime, such as injury, predation and changing environmental conditions. Bacteria are enclosed in a protective cell envelope consisting of a cell wall and at least one cell membrane. Gram-positive bacteria have a thick cell wall composed of peptidoglycan, teichoic acid polymers and capsular polysaccharides [1, 2]. Gram-negative bacteria have a thinner peptidoglycan cell wall positioned between an inner and outer cell membrane, the latter bound with lipopolysaccharides. The cell wall provides protection against chemical and mechanical stress, gives the bacterium its shape, and prevents the bursting of cells caused by osmotically-driven water influx [1, 3]. As a nearly universal component of bacterial cells, the cell wall is an important target for antibiotics [4], which are compounds that kill or inhibit the growth of bacteria [5] .

The phylum Actinobacteria (also known as Actinomycetota) is one of the largest, most diverse groups of bacteria [6-8]. Actinobacteria are Gram-positive micro-organisms that inhabit a wide variety of terrestrial and aquatic environments, and can live in close association with plants, insects and animals [6, 8]. Actinobacteria produce a plethora of bioactive compounds with antibiotic, antifungal, antiparasitic and antiviral properties, making these organisms highly relevant for human and animal healthcare as well as agriculture [6]. A major source of bioactive compounds is the genus *Streptomyces* from the *Streptomycetaceae* family. These filamentous actinobacteria form a branching network of connected hyphal cells called a mycelium [9, 10]. When the conditions are right, part of the vegetative mycelium undergoes programmed cell death (PCD) to generate nutrients for aerial hyphae to grow and develop reproductive spores [11, 12]. Antibiotics are produced during PCD to protect the valuable nutrients from consumption by nearby microbes [13, 14].

Interestingly, around 5 – 10% of filamentous actinobacteria (specifically, *Streptomyces* and the related *Kitasatospora* genus) can thrive in a cell wall-deficient state under hyperosmotic stress conditions [15]. The transition of bacteria to a wall-deficient state occurs under specific conditions such as by exposure to cell-wall targeting agents [16-18], hyperosmotic stress [15], or bacteriophages [19, 20], provided that the environment is sufficiently osmoprotective. The loss of a cell wall can provide the bacterium with new abilities, such as avoiding cell death by antibiotic treatment [21] or infection by bacteriophages [19, 20], and has been suggested to facilitate DNA uptake [22].

Horizontal gene transfer (HGT) is the transfer of DNA between organisms other than from parent to offspring [23]. This process enables bacteria to acquire new genetic information, such as DNA carrying antibiotic resistance genes (ARGs). Excessive use and misuse of antibiotics has promoted the spread of bacterial antibiotic resistance, which is itself an ancient phenomenon [24, 25] that has now become a global threat to efficient healthcare [26]. DNA has to cross the cell envelope to enter the cell and to be stably maintained in the host genome. Specialized transport systems have evolved for the selective transfer of DNA across the cell envelope during HGT [27-29]. The cell wall can thus be considered a physical barrier that

needs to be overcome to enable HGT.

We hypothesize that switching to a cell wall-deficient state can enable bacteria to participate in HGT via potentially novel mechanisms. In addition, *Streptomyces* genomes contain dozens of biosynthetic gene clusters (BGCs) that encode the information to regulate and produce bioactive molecules [7, 30, 31]. These BGCs also contain resistance genes to prevent self-killing by their own produced antibiotics. This makes filamentous actinobacteria a potential source of antibiotic resistance genes to spread into the environment, possibly facilitated by the extrusion of cell wall-deficient cells that are inherently fragile and may release DNA.The research in this thesis aims to address these questions by studying the consequences of exposure to hyperosmotic stress in the actinobacterium *Kitasatospora viridifaciens* and the role of cell wall-deficient cells in HGT.

The versatility of Actinobacteria

Actinobacteria are Gram-positive bacteria that inhabit a wide range of ecological environments [6, 8]. Actinobacteria such as *Streptomyces*, *Kitasatospora*, *Micromonospora* and *Nocardia* are a major source of enzymes and bioactive natural products with for instance, anticancer, antiparasitic, antifungal or antibacterial activity [6, 32]. These bacteria are of large significance for biotechnology, healthcare and agriculture [6, 32]. Around two-thirds of all known antibiotics are produced by actinobacteria, of which the majority is derived from *Streptomyces* [6]. *Streptomyces* is a genus of spore-forming filamentous bacteria with over 600 described species [33]. *Streptomyces* and the closely related, yet smaller *Kitasatospora* (23 validly proposed species) [34] and *Streptacidiphilus* (15 validly proposed species) [35] genera belong to the *Streptomycetaceae* family [36]. Members of the *Streptomycetaceae* will hereinafter be referred to as streptomycetes. Streptomycetes are mainly described as predominant soil-dwelling bacteria, but they can inhabit a variety of niches [8, 37], including marine and freshwater environments [38-40], deserts [41] and arctic regions [42, 43], and can live in association with animals, plants and insects [44-47].

The extensive capability of streptomycetes to produce antibiotics is reflected in the names of some species such as *Streptomyces kanamyceticus* (kanamycin) [48], *Streptomyces platensis* (platensimycin) [49] and *Streptomyces avermitilis* (avermectins) [50]. Besides producing medically relevant compounds, streptomycetes produce a myriad of industrially relevant enzymes such as amylases, lipases, cellulases, chitinases and proteases (as reviewed by Barbuto Ferraiuolo *et al.*, 2021) [51], as well as (bioactive) pigments [52]. Moreover, streptomycetes and their enzymes are utilized for the bioconversion of drug precursors [53] or for bioremediation purposes [54]. The ability of streptomycetes to produce such a diversity of enzymes and bioactive molecules is closely related to their saprophytic, multicellular lifestyle.

The complex multicellular lifecycle of streptomycetes

Streptomycetes are filamentous bacteria that reproduce via sporulation. Their mycelial morphology caused confusion among early researchers whether to identify these micro-organisms as fungi, bacteria or something in between, as evidenced by the name *Streptomyces* ('twisted fungus') [55-57]. The lifecycle of *Streptomyces* starts when favourable conditions initiate the germination of a spore, resulting in one or more germ tubes [9, 58] (Fig. 1). These structures grow by apical tip extension and branching, eventually forming a network of hyphal cells called the vegetative (substrate) mycelium. Vegetative hyphae contain semi-permeable cross-walls at irregular intervals that form connected, multigenomic compartments [10, 59]. This mycelium anchors the bacterium into the soil, where streptomycetes play an important role in the recycling of organic matter by feeding on lignin, starch or cellulose from decaying vegetation, as well as on chitin from fungal cell walls or insect exoskeletons [60-63]. Streptomycetes produce a large repertoire of specialized enzymes to degrade these complex structures, facilitating their (often) saprophytic lifestyle.

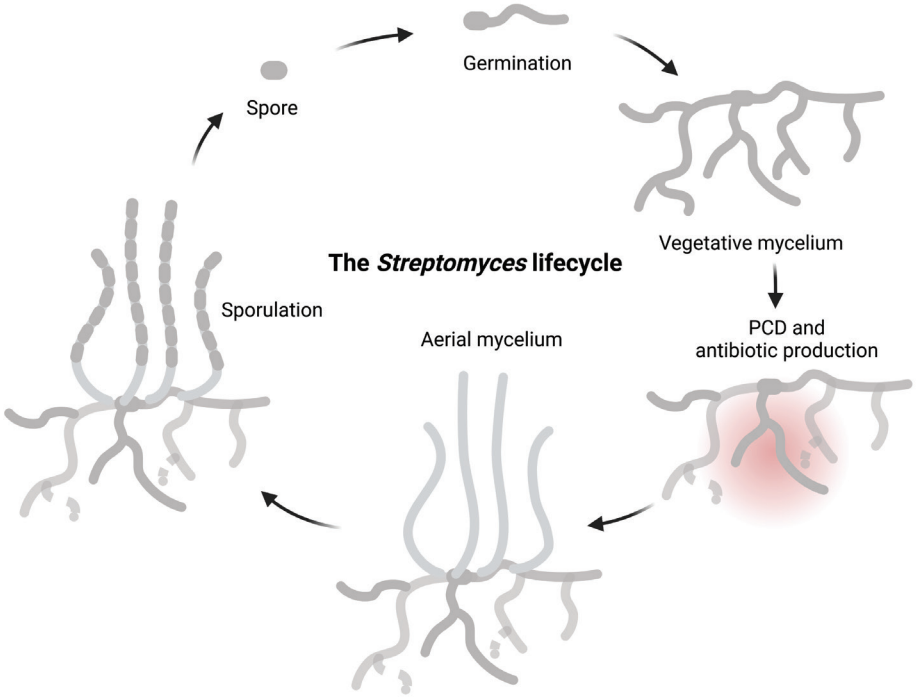

Figure 1. Schematic representation of the *Streptomyces* lifecycle
Germination of a spore is followed by construction of a network of hyphal cells, the vegetative mycelium. Nutrients for morphological differentiation are obtained by programmed cell death (PCD) of part of this mycelium, which correlates with antibiotic production (red). These nutrients are used to form aerial hyphae (aerial mycelium) that rise up in the air, eventually developing long chains of spores that can be distributed into the environment. See text for details. Created with Biorender.com

Nutrient limitation is a trigger for the onset of morphological differentiation to produce spore-bearing aerial hyphae [9]. The nutrients required for this developmental process are obtained via PCD of the vegetative mycelium [11, 12]. Streptomycetes produce antibiotics during PCD to protect the liberated nutrients against consumption by other bacteria [13, 14]. Secretion of antibiotics may also function to lyse nearby bacteria to serve as a source of nutrients [6, 7, 64]. Following PCD, a reproductive (aerial) mycelium forms by the upward growth of hydrophobic hyphae, in which extensive DNA replication takes place [9, 65]. The aerial mycelium can be observed as the formation of a white, fluffy layer on top of *Streptomyces* colonies (Fig. 2). When the hyphae stop growing, sporulation-specific cell division is initiated during which chromosome segregation and compaction occurs simultaneously with compartmentalization of the hyphae via septa formation [6]. These septa-delimited hyphal compartments will develop into spores with condensed nucleoids and a thick spore wall [9]. Streptomycetes produce long unigenomic spore chains that often contain more than 50 spores [66]. The hydrophobic, spore-bearing aerial hyphae typically have a grey, beige or green colour due to the production of spore pigments [66, 67] (Fig. 2). Distribution of spores is presumably mediated by abiotic factors such as wind and water [68], or by attachment to arthropods (e.g. mites and springtails) [69, 70] and motile soil bacteria [71].

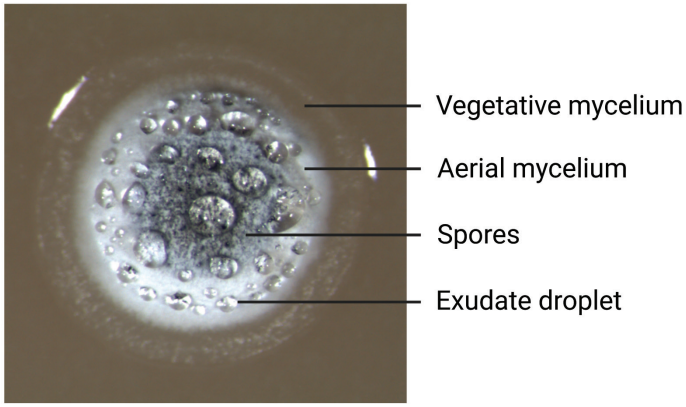

Figure 2. *Streptomyces* colony morphology
Stereomicroscopy image of *Streptomyces* colony (~ 2 cm in diameter) with vegetative (substrate) mycelium (brownish colour) grown into the solid medium (MS medium), and aerial mycelium (aerial hyphae) with spores (dark colour) forming on top. This colony also produces exudate droplets on top of the aerial hyphae, which may contain extracellular vesicles and antibiotics [72]. Created with Biorender.com.

Analysis of the first published *Streptomyces* genome, from the model organism *Streptomyces coelicolor* A3(2), revealed the presence of more than 20 gene clusters on its linear chromosome [73]. These BGCs encode enzymes for the production and regulation of, as well as resistance to, secondary metabolites such as antibiotics, siderophores and pigments. Sequencing of

more than 30 *Streptomyces* genomes indicated the presence of between 20 to 45 BGCs per strain [31]. Some of these BGCs are inactive under laboratory conditions ('silent'), while others are active, but cannot yet be linked to a specific product ('cryptic') [7]. A major focus of *Streptomyces* research revolves around the activation of silent gene clusters to elicit the production of novel bioactive molecules [7, 74].

A changing morphology under hyperosmotic conditions

Growth of streptomycetes under hyperosmotic conditions led to the discovery of a novel cell type in *Kitasatospora* and *Streptomyces* [15]. Cultivation of *K. viridifaciens* in liquid medium with high concentrations of sucrose resulted in delayed growth, hyperbranching, excess membrane formation and condensed nucleoids. Furthermore, the presence of high levels of sucrose led to a temporary arrest in hyphal tip growth and the subsequent formation of spherical cells containing DNA (Fig. 3). Closer inspection by microscopy revealed that these cells did not contain an intact cell wall and were sensitive to lysis by exposure to water. These cell wall-deficient cells were coined 'S-cells' due to their formation under stressful conditions (stress-induced cells). Whereas S-cells cannot proliferate in their wall-deficient state, they have the ability to revert to a mycelial mode-of-growth. Prolonged exposure of *K. viridifaciens* to hyperosmotic stress leads to the formation of proliferating cell wall-deficient cells, also known as 'L-forms' (Fig. 3), which are further discussed in the next section. Formation of cell wall-deficient cells is hypothesized to be an adaptive strategy to ensure bacterial survival under environmental challenges, such as hyperosmotic stress or antibiotic treatment [15, 75, 76].

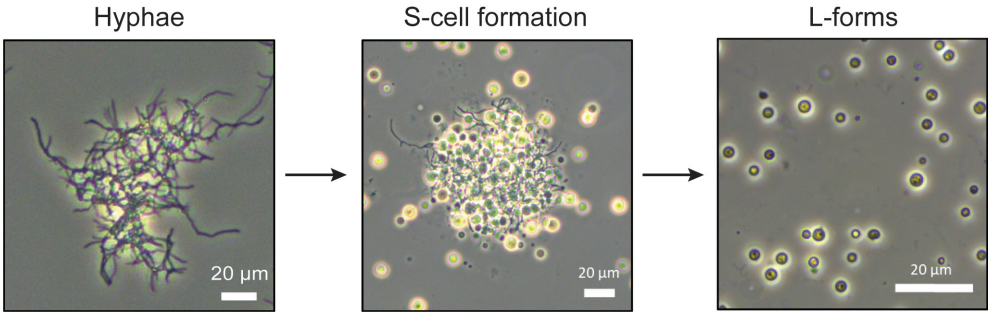

Figure 3. Formation of cell wall-deficient cells by *Kitasatospora viridifaciens*
In standard culture medium (TSBS medium), *K. viridifaciens* grows as walled cells (hyphae) whereas exposure to hyperosmotic stress (20% (w/v) sucrose in L-phase broth) induces the formation of wall-deficient S-cells, which are extruded from the hyphal cells [15]. Prolonged incubation under hyperosmotic conditions, either with or without the presence of penicillin G and lysozyme, induces the switch to an L-form state, in which proliferating wall-deficient cells are produced. Note that both S-cells and L-forms can revert to growth with a cell wall.

Cell wall-deficient bacteria and the discovery of L-forms

Whereas the cell wall serves as an important protective structure, some bacteria can thrive without a cell wall. A distinction can be made between bacteria that do not have a cell wall and those that can switch between a walled and a wall-deficient lifestyle. The Mollicutes belong to a class within the phylum of Mycoplasmatota (originally named Tenericutes) that is composed of permanently cell wall-deficient bacteria [77, 78]. Mollicutes (often called mycoplasmas) often have a parasitic lifestyle as intracellular symbionts of animals, insects, plants, or humans. These bacteria are thought to have evolved from walled, Gram-positive bacteria by reductive evolution and typically have small genomes (around 0.5 to 2 Mbp).

In the 1930s, dr. Emmy Klieneberger-Nobel studied mollicutes that were associated with disease, known at that time as 'pleuropneumonia-like organisms', at the Lister Institute of Preventive Medicine in England [79]. During this work, she discovered large round 'bodies' or 'granules' in cultures of *Streptobacillus moniliformis*, and initially suggested that these were symbiotic bacteria [80]. These bacteria were designated 'L-form' or 'L-phase' strain L1 (the L standing for the Lister Institute). L-forms were later identified as a morphological transition of bacteria into a cell wall-deficient state, occurring in multiple bacterial species (Fig. 4) [81-84]. Since their discovery in 1935, much insight has been gained on L-form physiology, the natural occurrence of wall-less bacteria, and how to induce bacteria to switch to an L-form state.

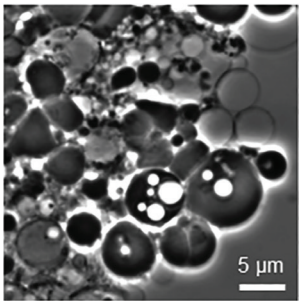

Figure 4. L-forms of *Streptobacillus moniliformis*
Phase-contrast micrograph of *S. moniliformis* L-forms suspended in nutrient broth after induction by ampicillin treatment on nutrient agar containing 20% calf serum [83]. Image reproduced from Roberts *et al*., (2021) [83] , Fig. 3 panel B under terms of the Creative Commons Attribution 4.0 International License: https://creativecommons.org/licenses/by/4.0/legalcode.Copyright 2021, The Author(s), published by Oxford University Press on behalf of FEMS.

Physiology and proliferation of L-form bacteria

L-forms are wall-less derivatives of Gram-positive or Gram-negative bacteria that can reproduce indefinitely without their cell wall [18]. Electron microscopy of L-forms from different bacterial species indicated that the cell wall is either largely or completely lacking [18, 85, 86]. Due to the absence of an intact cell wall, L-forms have a more-or-less spherical cell morphology. Cells are around 0.5 – 50 µm in diameter, can contain multiple chromosomes [87-90] and intracellular vesicles [89, 91, 92], and increase in size over time [88, 93]. L-forms require osmoprotective conditions (e.g. by the presence of sucrose or NaCl) to prevent bursting by the influx of water, which would occur in standard culture media that are generally hypotonic [20]. Some L-form lines ('unstable' L-forms) have the ability to revert to a walled state, though

often at a low frequency, in contrast to 'stable' L-forms, which can no longer rebuild their cell wall [93-95].

Proliferation of L-forms is in stark contrast with canonical bacterial cell division (Fig. 5). Cell division in walled bacteria is a carefully regulated process that mediated by a macromolecular complex, the divisome [96]. In the early stage of cell division, filaments of FtsZ (a tubulin homolog) associate with the cytoplasmic side of the cell membrane in a diffuse region around mid-cell. These filaments bind membrane-associated proteins such as FtsA (an actin homolog) and other factors, and condense into a mature, focused Z-ring [97, 98]. The FtsZ filaments move around the circumference of the division plane in a treadmilling motion, while recruiting and guiding the peptidoglycan synthesis machinery [99]. New cell wall material is inserted at discrete sites that move along the division plane, thereby building sequentially smaller rings that form the septum to divide the cell in two [100].

L-form proliferation appears to depend on biophysical processes and does not require any of the canonical cell division proteins [91, 93, 95, 101-103]. Instead, L-forms proliferate via extensive shape deformations (Fig. 5) including extracellular budding (blebbing, the pinching-off of one or more progeny cells) [88, 89, 104], intracellular budding (vesiculation, progeny cells form in the cytoplasm or within intracellular vesicles and are released by the bursting of the parental cell) [17, 89, 91], tubulation (pearling or extrusion-resolution, formation of a tubular protrusion that develops vesicles that are transiently connected by membrane or a membranous tube) [88, 104] as well as binary fission-like processes (division of a cell resulting in two cells with nearly equal volumes) [91, 105]. Notably, not all released vesicles are viable [89].

L-forms require excess membrane synthesis to undergo such extensive shape deformations [95, 103]. This is achieved via mutations that lead to increased synthesis of membrane fatty acids, or via mutations that inhibit peptidoglycan precursor synthesis. Excess membrane synthesis leads to an increase in the cell surface area relative to the cell volume. This imbalance can lead to torsional stress and spontaneous shape deformations of the cell membrane, generating new progeny cells. In addition, mutants of *Bacillus subtilis* L-forms with an altered content of cell membrane branched-chain fatty acids had a reduced membrane fluidity, and could not undergo membrane scission and formation of progeny cells [102]. Excess membrane production and the biophysical properties of the cell membrane are therefore key to L-form proliferation.

L-forms also require a countermeasure to high levels of reactive oxygen species (ROS). The lack of normal cell wall synthesis in L-forms likely alters their cellular metabolism, leading to an increased flux trough the electron transport chain (ETC) and increased ROS production [106]. As increased ROS levels can damage cellular components such as DNA, enzymes and lipids [107], this needs to be prevented by acquisition of mutations that repress ETC activity, or by growth of L-forms in anaerobic cultures or addition of ROS scavengers [106].

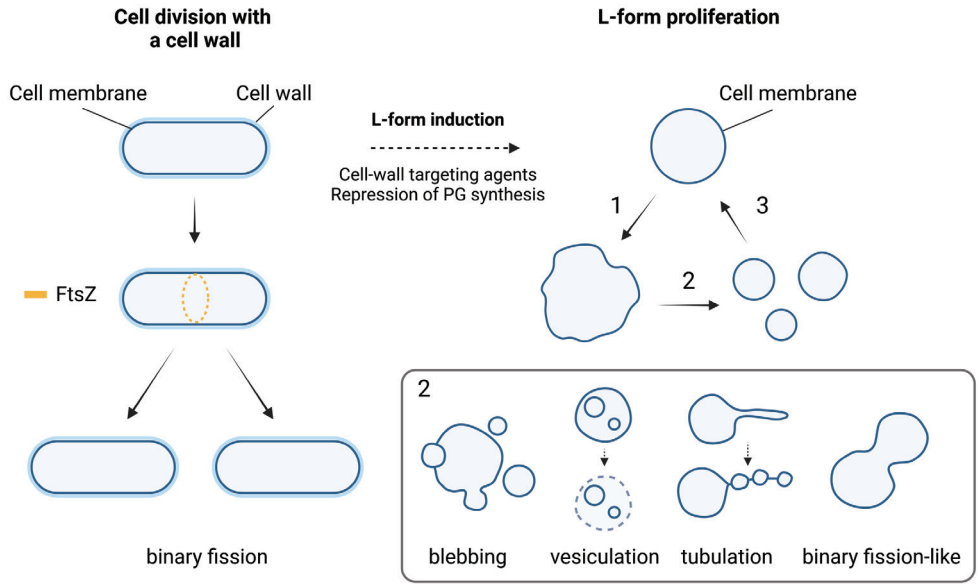

Figure 5. Schematic representation of canonical cell division of unicellular walled bacteria versus proliferation of cell wall-deficient L-form bacteria

Cell division of bacteria with a cell wall (here shown as Gram-positive bacteria) is mediated via the conserved FtsZ (Filamenting temperature-sensitive mutant Z) protein. FtsZ filaments (with other factors) move around the circumference of the division plane in a treadmilling motion, guiding cell wall synthesis machinery, ultimately leading to binary fission. Walled bacteria can be induced to grow in a cell wall-deficient L-form state under osmoprotective conditions. L-form proliferation does not depend on FtsZ and is mediated via excess membrane synthesis (1) that leads to a variety of shape deformations of the L-form cell and formation of progeny cells (2, see box for proliferation mechanisms), that expand in size overtime and repeat the growth cycle (3). See text for details. Created with Biorender.com.

Induction of L-form bacteria and their occurrence in natural environments

The generation of L-forms under laboratory settings is generally performed by long serial passages of bacteria under osmoprotective conditions (for weeks up to several years). Cell wall synthesis inhibitors, such as beta-lactam antibiotics and glycopeptides, are required to prevent the expansion of the peptidoglycan layer [15, 16, 108]. Lytic enzymes help the escape of the cell from the cell wall sacculus [109]. A faster method to generate L-forms relies on the inhibition of early steps in cell wall synthesis, namely the formation of peptidoglycan precursors. This is achieved via transcriptional repression of cell wall-synthesis genes [88] or inhibition of essential enzymes via antibiotics such as fosfomycin or D-cycloserine [95].

Besides generating and maintaining L-forms under laboratory conditions on solid medium or in liquid culture, much research has focused on determining whether L-forms can exist in natural environments. Wall-less cells have been detected in samples from plants, animals and humans [18] (Fig. 6), and is further discussed below. It should be noted that in many experiments, bacteria were deliberately introduced (sometimes injected) in these tissues

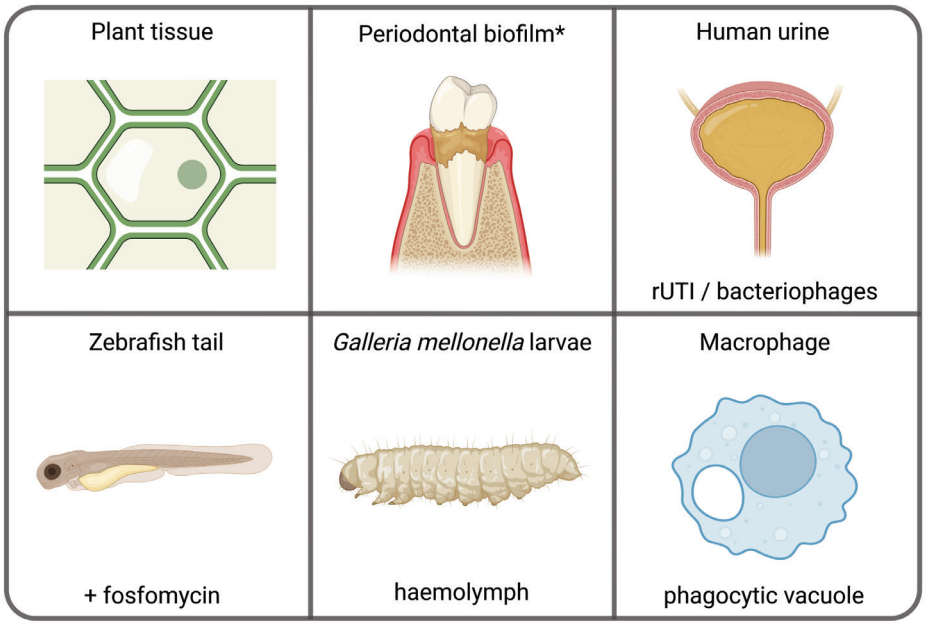

Figure 6. Natural environments that can sustain L-form bacteria or enable the transition from walled to wall-less bacterial cells

L-forms, or L-form-like cells, have been detected inside plant cells after incubation of seeds with L-forms [110], in periodontal biofilms of the oral cavity from patients with periodontitis [111], in urine from patients suffering from recurrent urinary tract infections (rUTI)[21] as well as after exposure of *Enterococcus faecalis* to bacteriophages in urine [20], inside tail fins of zebrafish larvae injected with walled *Escherichia coli* cells exposed to Fosfomycin [21], in haemolymph of *Galleria mellonella* after injection of walled *Staphylococcus aureus* or *B. subtilis* [112], and after incubation of murine macrophages with *B. subtilis* [112]. Asterisk (*) indicates that cell wall-deficient cells were detected that could be either Mollicutes or L-forms. Created with Biorender.com.

to study whether L-forms would survive, transfer within the tissue or if walled cells would switch to an L-form state. Associations of L-forms with plants, sometimes intracellularly, can be obtained after incubation of plant cell suspensions with L-forms, direct injection of L-forms in plant tissue [113, 114] or by soaking plant seeds in an L-form suspension [110]. L-forms could be detected in strawberry plants up to 42 cm away from the injection site after 168 h incubation, indicating that bacteria may have entered the plant vascular system [114]. Plant tissue may therefore be sufficiently osmoprotective, possibly enabling L-form bacteria to enter or transfer within plant tissue due to their flexible morphology.

Cells with an L-form or L-form-like morphology have also been detected in human and animal samples. Cell wall-deficient cells were detected in all periodontal biofilm samples from patients with aggressive periodontitis [111] and in urine of patients with recurrent urinary tract infections (rUTI) [21]. Urine-derived *Escherichia coli* isolates could readily transition between a walled and a wall-less L-form state in absence or presence of fosfomycin, respectively [21]. Switching to an L-form state may not only enable bacterial pathogens to

evade killing by cell wall-targeting antibiotics, but also switch back to a walled state after the antibiotic treatment has finished, thereby causing recurrence of the disease [21, 94, 112, 115]. Indeed, walled *E. coli* cells can transition to a wall-less morphology in zebrafish larvae in the presence of fosfomycin, and switch from L-form to a walled state in absence of the antibiotic [21].

Endogenous lytic enzymes may also induce bacteria to switch to a wall-less state inside host tissue or host cells. For example, after injection of walled *B. subtilis* or *Staphylococcus aureus* cells in the wax moth *Galleria mellonella*, spherical cells were observed in the insect haemolymph (a fluid tissue analogous to blood) [112]. In addition, incubation of *B. subtilis* walled cells with murine macrophages led to the presence of wall-less cells inside the phagocytic vacuoles of the macrophage. Lastly, bacteriophages can induce L-form transition in *Enterococcus faecalis*, a pathogen causing urinary tract infections, in human urine, a process that is mediated by endolysins released from phage-infected cells [20]. Switching to a wall-less state may therefore enable bacteria to enter and transfer within plant tissue, escape cell wall-targeting antibiotics or host lytic enzymes, and survive bacteriophage infection.

Horizontal gene transfer

Horizontal gene transfer (HGT), also known as lateral gene transfer, is defined as '*the non-sexual movement of genetic information between genomes*' [23]. This is distinct from vertical gene transfer, during which genetic information is transferred from parent to offspring. HGT is a widespread natural phenomenon can that can occur between bacteria from the same species, related bacteria (e.g. between *Synechococcus* and *Prochlorococcus* within the order Synechococcales), but also between members of more distantly related taxa that live in similar environments (e.g. between members of the Clostridia class and Euryarchaeote archaea), as inferred from gene sharing network analysis [116]. Although relatively few eukaryotic genomes have been investigated for genes of bacterial origin, there is evidence for HGT between eukaryotes and bacteria [23]. For example, for rumen-inhabiting ciliates that live in close contact with bacteria, around 4% of their genes is predicted to have a bacterial origin [117]. In *S. coelicolor*, the gene encoding GlxA, an enzyme involved in cellulose-like glycan biosynthesis at the hyphal tips [118], is predicted to be acquired from fungi by HGT [119].

The main agents by which HGT takes place are so-called 'mobile genetic elements' (MGEs), a broad term for segments of genetic material that move within a genome or between bacteria [120, 121]. Examples are plasmids (conjugative and non-conjugative), transposable elements (nonconjugative and conjugative transposons) and bacteriophages. Some mobilizable elements utilize the conjugative functions of other conjugative MGEs. The collection of MGEs is known as the prokaryotic mobile gene pool or 'mobilome' [120]. MGEs contain core genes required for their transmission, such as for independent replication, recombination (homologous or non-homologous), packaging (phages), or extrusion (conjugation) of DNA [121]. Accessory genes on MGEs can provide the host with a selective advantage such as

antibiotic resistance genes (ARGs), virulence factors or genes involved in metabolism.

HGT can result in the acquisition of novel traits, given that the internalized nucleic acids are stably maintained in the genome and not used as a food source [28, 122, 123]. Genes that are overrepresented amongst transferred genes in bacteria are those involved in metabolism [116, 117]. Bacteriophage-driven HGT resulted in the transfer of beneficial genes from resident to invading *E. coli* strains in the mouse gut [124]. This provided the invader with a fitness advantage that may aid in gut colonization. HGT can also lead to exchange of virulence and resistance genes [125], which is mainly driven by MGEs [126]. HGT can thereby contribute to genome plasticity (the alterable nature of genomes) and bacterial adaptation [127, 128].

Mechanisms of HGT between bacteria

A variety of mechanisms mediate HGT between bacteria, of which natural transformation, conjugation and transduction are the most characterized [127] (Fig. 7). Less explored mechanisms include vesiduction, gene transfer agents and transfer via nanotubes. These HGT mechanisms are briefly discussed below.

Natural transformation is the active uptake of exogenous DNA during a transient physiological state called 'competence', which ultimately leads to genetic transformation [28, 129, 130]. Around 80 species are known to be naturally competent for transformation under laboratory conditions [131]. This occurs via a largely conserved set of proteins encoded on the bacterial genome (DNA uptake in *Helicobacter pylori* [132] and *E. coli* [130] being the exception). DNA is captured and transferred as dsDNA across the cell wall (and outer membrane for Gram-negative bacteria) via a transformation pilus that shares similarities with type IV pili and type II secretion systems. A DNA receptor (ComEA) and DNA translocase (membrane channel ComEC) transfer ssDNA across the cell membrane. In the cytosol, ssDNA is then bound by proteins such as DprA, SsbB and RecA [133] to protect it from degradation, and after a homology search the DNA is integrated in the chromosome.

Bacterial conjugation is a contact-dependent process that requires conjugative plasmids or chromosomally-located integrative and conjugative elements (ICEs) that encode the DNA transfer machinery, and is extensively studied in Gram-negative bacteria [29, 134]. A pilus (or surface adhesin for Gram-positive bacteria) attaches to a recipient cell and a conjugative pore is formed. Some pili can retract, which might function to bring the donor and recipient cell together [135, 136]. A multi-protein complex, the relaxosome, binds to DNA at the origin of transfer (*oriT*). The DNA is nicked at the *oriT* and unwinded by a relaxase, after which this complex is recruited to the conjugative pore and single-stranded DNA is transferred to the recipient cell. DNA is replicated in both donor and recipient cell ensuring both cells contain a dsDNA version of the transferred DNA. Whereas unicellular Gram-positive bacteria likely use homologous proteins for conjugation, a distinct process takes place in *Streptomyces* [137]. Conjugation in *Streptomyces* involves the transfer of double-stranded DNA [138] via the pore-

forming FtsK-like DNA translocase TraB [139]. Spd (spread) proteins mediate the intra-mycelial transfer of DNA across the septal cross walls [140-142]. Even cell wall-deficient mycoplasma can perform conjugation via a simpler conjugative channel [78].

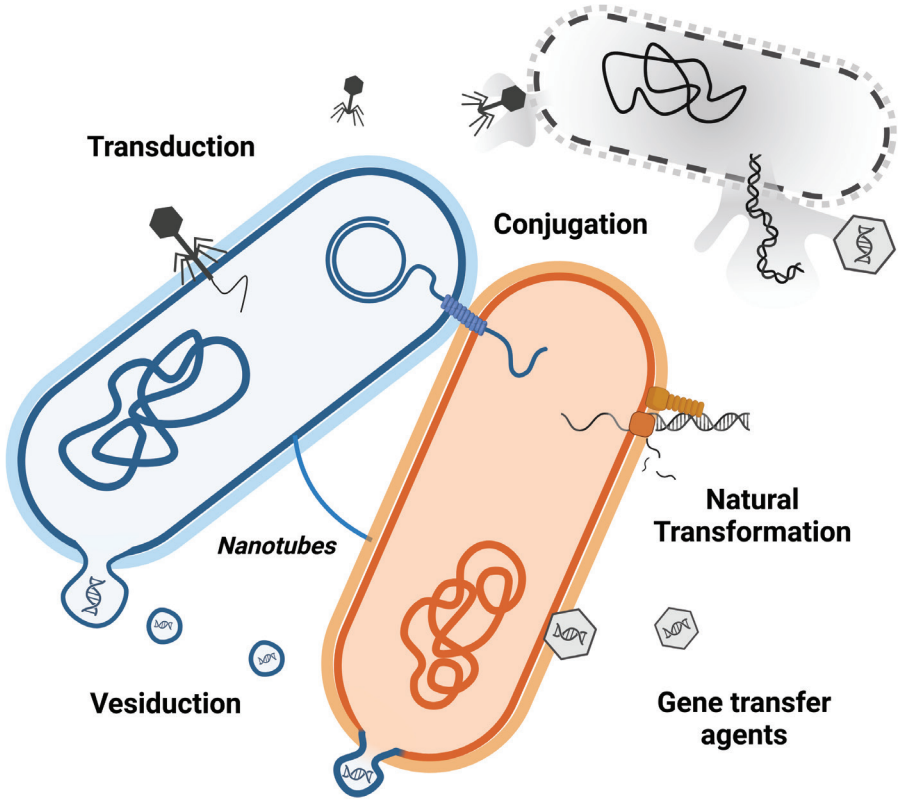

Figure 7. Schematic representation of horizontal gene transfer mechanisms between bacteria
Cell lysis (grey, dashed cell) can result in release of DNA, gene transfer agents or bacteriophages. Naturally competent cells can import extracellular DNA via a specialized natural transformation machinery. Transduction is the transfer of bacterial DNA via bacteriophages. Gene transfer agents (phage-like particles) can package bacterial DNA and deliver this to the recipient cell. Conjugation involves cell-cell contact mediated by a surface adhesin or conjugative pilus from a donor cell (blue) and subsequent transfer of ssDNA via a pore to the recipient cell (orange). Vesiduction involves the transfer of DNA via extracellular vesicles (EVs) released from a donor cell (blue), and subsequent delivery via fusion of EVs with the membrane of the recipient cell (orange). Italics indicate that the existence and biological relevance of nanotubes, membranous structures that may transfer plasmid DNA, is unclear. See text for details. Created using Biorender.com.

Gene transfer mediated by bacteriophages (bacterial viruses) is called transduction. During bacteriophage infection, phage genetic material is injected in a host cell, followed by replication and generation of new phage particles (lytic cycle) or genetic material is integrated into the bacterial genome as a prophage (lysogenic cycle). During the packaging of new phage particles, bacterial DNA can become encapsidated in a phage particle [143, 144]. Bacterial DNA can be mis-recognized as phage DNA (generalized transduction), or DNA adjacent to prophage DNA is mistakenly excised (specialized transduction). In addition, bacterial DNA of up to several hundred kbp can be packaged in phage particles in a process called lateral transduction. This DNA is subsequently transferred to a new host cell via the phage particle. Transduction can lead to the transfer of genes involved in metabolism, toxin production, drug resistance, virulence as well as DNA containing pathogenicity islands (MGEs that encode virulence factors) [144, 145].

Vesiduction involves the transfer of DNA via small, non-replicating membrane vesicles (< 400 nm) also known as extracellular vesicles (EVs) [146]. Bacterial EVs are derived from the cell membrane, outer cell membrane (OMV) or both the outer and inner cell membrane (O-IMV) for Gram-negative bacteria [147]. EVs can contain enzymes (e.g. beta-lactamase) [148], sugars, lipids, RNA and DNA, and can mediate the transfer of virulence genes and ARGs [149-151]. EVs are increasingly recognized to play an important role in cell-to-cell interactions, structural integrity of biofilms and can serve as antibiotic decoys [147, 152].

Gene transfer agents (GTAs) are 'phage-like entities' that are encoded on the bacterial genome (reviewed by Lang *et al*., 2012 [153]). Unlike phages, GTAs are not excised, but expressed from the chromosome. The resulting particles contain random pieces of host DNA of around 4 – 14 kb in size, which is too small to carry all genes required to encode the GTA itself, and the particles are likely released via cell lysis. Despite their small size, GTAs can mediate the transfer of ARGs between bacteria [153-155].

Bacterial nanotubes are described as membranous structures that transfer cytoplasmic content between adjacent bacteria [156]. Such connections would enable the transfer of fluorescent proteins, nonconjugative plasmids, mRNA or proteins that confer antibiotic resistance. Nanotubes are hypothesized to be a form of bacterial communication by functioning as a network for the exchange of molecules. However, there is controversy regarding the existence and relevance of these structures due to the observation of nanotubes forming exclusively from dying cells and the possibility of the structures being artefacts [157].

Environments conducive to the spread of antibiotic resistance genes

Conjugation is generally considered the predominant mechanism of gene transfer between bacteria [158-160]. Genes encoding for resistance against all major classes of antibiotics have been identified on conjugative plasmids [161, 162], and ARGs are often associated with conjugative elements [163]. However, from a list of top priority antibiotic resistant pathogens, 11 out of 12

are known or predicted to be naturally transformable [164]. Therefore, transfer via free DNA should not be overlooked as contributing to the spread of antibiotic resistance.

Natural environments identified as 'hotspots' for HGT are those with a high bacterial density and a high chance of cell-cell contact [165]. Plant-associated niches (e.g. between epidermal cells, in sub-stomatal cavities [166] and on the hypocotyl and roots [167]) allow the formation of dense bacterial communities that can enable gene transfer. Biofilms are structured, surface-associated microbial communities that are held together by a matrix of self-produced polymeric substances including DNA [168, 169]. Biofilms also exist as non- attached aggregates [170]. Biofilms identified to mediate HGT are located in the respiratory, intestinal and urogenital tract, on plant leaf surfaces and in the rhizosphere, in aquatic systems and in wastewater (as reviewed by Sørensen *et al*., 2005 [165]). Biofilms promote plasmid stability and enhance gene transfer compared to planktonic communities [171, 172].

Wastewater treatment plants (WWTPs) are facilities that collect wastewater from households, companies, industries and healthcare facilities that function to remove and/or reduce pollutants in this water to an acceptable level, prior to discharge into the environment [173]. WWTPs are considered a hotspot for the spread of antibiotic resistance due to the utilization of a large diversity of bacteria during biological treatment of wastewater. This wastewater can contain free-floating DNA and a wide range of chemicals including antibiotics, which are constantly mixed with bacteria, thereby potentially enabling the spread of ARGs [163, 174, 175].

A better understanding of the factors involved in HGT in these environments may aid in limiting the spread of antibiotic resistance amongst bacteria.

Scope of this thesis

The increase in antibiotic resistant bacteria is a global problem that threatens effective healthcare. One of the mechanisms how bacteria acquire antibiotic resistance is via HGT, a process in which DNA conferring antibiotic resistance transfers from one bacterium to another. Environments that promote HGT are those with a high bacterial density, such as found in WWTPs. However, little is known about the factors contributing to the spread of DNA in these environments. The work in this thesis is part of the project 'Transmission of Antimicrobial Resistance Genes and Engineered DNA from Transgenic Biosystems in Nature' (TARGETBIO), which aimed to address this knowledge gap by studying the fate of free DNA carrying antibiotic resistance in wastewater systems, and the role of antibiotic-producing filamentous actinobacteria, such as *Streptomyces*. These ubiquitous soil bacteria produce antibiotics as part of their lifecycle and carry ARGs on their genome, which makes them a potential source of resistance determinants. In addition, streptomycetes such as *K. viridifaciens* are capable of producing cell wall-deficient cells. The lack of an intact cell wall

may enable cell wall-deficient cells to participate in HGT via potentially novel mechanisms. This drastic morphological switch is observed after exposure to cell-wall targeting compounds, hyperosmotic stress or bacteriophages, external stressors that may be present WWTPs. It is, however, unknown whether cell wall-deficient cells can participate in HGT and if the bacteria capable of producing cell wall-deficient cells are present in wastewater systems. Exposure of *K. viridifaciens* to hyperosmotic stress also results in drastic phenotypic diversity, which may be due to genetic instability commonly observed in streptomycetes. Hyperosmotic conditions might therefore contribute to bacterial genome plasticity via cell wall deficiency-mediated HGT and genomic rearrangements.

The aim of this thesis is to study the consequences of the exposure of *Kitasatospora viridifaciens* to hyperosmotic stress and the role of the resulting cell wall-deficient cells in horizontal gene transfer in nature.

The results in this thesis contribute to identifying the consequences and potential risks of bacteria transitioning to a cell wall-deficient state in relation to HGT. Ultimately, the results from this research help to make predictions which environmental conditions can enable bacteria to more easily acquire or spread DNA, thereby contributing to a better understanding of the spread of antibiotic resistance.

Growth of *K. viridifaciens* under hyperosmotic conditions leads to the formation of phenotypically diverse colonies. In **Chapter 2** I investigated the cause and consequences of this diversity. Whole genome sequencing was applied to detect large genomic deletions and rearrangements in colonies with a mutant phenotype. These changes are potentially caused by genome-encoded transposable elements activated by the hyperosmotic stress.

Growth under hyperosmotic conditions also results in the formation of cell wall-deficient cells, that may participate in HGT due to their lack of an intact cell wall. In **Chapter 3** the ability of cell wall-deficient cells from *K. viridifaciens* to take up DNA with and without use of chemicals is studied. Surprisingly, L-forms could naturally take up DNA, which was not affected by the deletion of genes encoding proteins with homology to the DNA uptake machinery of naturally transformable bacteria. In addition, the transfer of DNA between wall-less cells of *K. viridifaciens* during co-culture is investigated.

Spontaneous DNA uptake in L-form cells occurs via a mechanism independent from proteins homologous to the canonical natural transformation machinery. In **Chapter 4** various microscopy techniques were employed to study the uptake of extracellular material in L-form cells. An endocytosis-like process is described that allows the nonspecific uptake of polysaccharides, DNA and even 150-nm lipid nanoparticles (LNPs), which may reflect an ancient uptake mechanism of early lifeforms that did not yet evolve a cell wall.

Wastewater treatment plants are a potential environment for the transfer of DNA between bacteria as well as for the formation of wall-less cells. Biofilm and activated sludge present in WWTPs may offer a protective environment where cell-wall deficient cells may reside. In **Chapter 5** I investigated the occurrence of filamentous actinobacteria capable of formation of cell wall-deficient cells, confirming the production of nucleic acid-containing vesicles under laboratory conditions. The main findings of this work are summarized and discussed in **Chapter 6**.