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

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Chapter 6.

Summarizing general discussion

Bacteria are exposed to fluctuating environments, and need to adapt to survive these selective challenges. Bacteria can adapt via temporary changes, such as an altered gene expression, or by permanent changes to their genome [409, 410]. Genomic alterations can occur by point mutations or gene duplications, but new genetic information can also be acquired from other bacteria in a process called horizontal gene transfer (HGT) [127]. A variety of HGT mechanisms exist [29] that result in the transfer of genetic material from one bacterium to another, and differs from vertical gene transfer that takes place from parent to offspring [23]. Examples of horizontally-transferred genes include those involved in metabolism [116, 117], virulence, and antibiotic resistance [125]. Although not all gene transfer events may be biologically relevant, HGT can enable the acquisition of novel traits, making HGT a central player in adaptive evolution [127, 128, 411].

HGT largely occurs via mobile genetic elements (MGEs), which are segments of genetic material that have the ability to move within or between genomes, such as plasmids [120, 121]. MGEs can harbor accessory genes that provide the bacterium with a selective advantage, including antibiotic resistance genes (ARGs) [125, 126, 351]. The excessive use and misuse of antibiotics in healthcare, agriculture and aquaculture has greatly promoted the spread of antibiotic resistance [163, 334], each year resulting in more than one million lives lost worldwide due to antimicrobial-resistant infections [331]. As HGT is a major route for the dissemination of ARGs [163, 337], a better understanding of the factors involved in HGT may help to limit the spread of bacterial antibiotic resistance.

Streptomycetaceae (specifically, *Streptomyces* and *Kitasatospora*), are filamentous actinobacteria that carry dozens of biosynthetic gene clusters (BGCs) on their genome to produce bioactive compounds, such as antibiotics [31]. These clusters often contain resistance genes to prevent self-killing by these antibiotics. It has been suggested that ARGs may transfer from non-harmful soil bacteria, such as from streptomycetes, to pathogens [345, 346]. In addition, many bacterial species, including the *Streptomycetaceae*, can thrive in a wall-deficient state under specific conditions [15, 18]. During HGT, DNA has to cross the cell envelope, consisting of a cell wall and at least one cell membrane, to enter a bacterial cell. Therefore, we hypothesized that switching to a wall-less state may enable bacteria to participate in HGT due to the lack of a 'barrier' for DNA entry.

For these reasons we aimed to investigate whether wall-less cells can participate in HGT, using the actinobacterium *Kitasatospora viridifaciens* as a model. These questions were addressed within the scope of the TARGETBIO project (Transmission of Antimicrobial Resistance Genes and Engineered DNA from Transgenic Biosystems in Nature), a collaborative project that aimed to study the risks of the spread of free DNA in the environment. As wastewater treatment plants (WWTPs) are considered a hotspot for the transmission of ARGs between bacteria [163, 174, 175], TARGETBIO focused on the factors involved in spread of ARGs carried on free DNA in WWTPs.

Endocytosis-like DNA uptake in L-form bacteria

We hypothesized that wall-less bacterial cells may participate in HGT due to the lack of a cell wall, making it easier for DNA to enter the cell. To examine this, we incubated walled and wall-less cells of *K. viridifaciens* with DNA in the presence and absence of polyethylene glycol (PEG, **Chapter 3**). PEG induced DNA uptake in all types of wall-deficient cells, but not in the cell-wall containing hyphae. Strikingly, consistent transformation without the use of PEG was obtained for independent L-form lineages when using plasmid DNA, but not with intact or fragmented chromosomal DNA. No DNA uptake was observed for any of the other cell types without PEG.

Extracellular DNA is taken up by naturally competent bacteria in a process called natural transformation, which uses a conserved macromolecular machinery to actively transport DNA across the cell wall and cell membrane [28, 129]. We identified genes on the chromosome of *K. viridifaciens* that encoded proteins with homology to a DNA-binding protein (ComEA) and DNA channel protein (ComEC), which are part of the DNA-uptake machinery in competent bacteria. These proteins might be sufficient to transport DNA across the cell membrane of L-forms, due to the lack of a cell wall. Surprisingly, deletion of the putative *comEA* and *comEC* genes did not affect the ability of L-forms to take up plasmid DNA, suggesting an alternative uptake mechanism.

The mechanism by which L-forms take up DNA was further studied in **Chapter 4**. Microscopic examination showed that a membrane-impermeable dye stained intracellular vesicles. These vesicles did not contain eGFP that was present in the cytoplasm, strongly suggesting that these vesicles played a role in the active internalization of extracellular material. Extensive shape deformations, namely the invagination of the cell membrane and formation of internal vesicles, were observed in the L-forms. This engulfment led to the uptake of extracellular material such as fluorescently labeled DNA and polysaccharides (3 kDa fluorescent dextran). This strongly resembles the process of endocytosis, a mechanism by which eukaryotic cells can internalize external molecules, components of the cell membrane or even bacteria [289].

Lipid nanoparticles (LNPs) are non-viral particles composed of lipids that can encapsulate nucleic acids, and can be used for mRNA vaccine delivery [301]. Eukaryotic cells engulf LNPs via endocytosis, which ultimately leads to release of their cargo in the cytosol. Indeed, fluorescently labeled, 150-nm LNPs could accumulate inside intracellular L-form vesicles. Endocytosis-like uptake was suppressed when incubating cells at 4 °C or by addition of the metabolic inhibitor sodium azide, indicating this is an energy-dependent process.

To explore the ultrastructure and composition of the internal vesicles, we examined the eGFP-producing L-form cells via 3D cryo-correlative light and electron microscopy (cryo-CLEM). The use of high-pressure freezing and cryogenic focused ion beam-scanning electron microscopy (cryo-FIB-SEM) imaging without staining ensured that the L-forms could be

visualized in a near-to-native state [305-307]. The absence of cytoplasmic eGFP in an intracellular vesicle indeed correlated with the lack of cytoplasm. Cryo-FIB-SEM also revealed numerous internal vesicles and vesicle complexes lining the cell membrane, which sometimes protruded out of the cell. In addition, dark particles were observed that surrounded cytoplasmic regions with a different contrast, which are reminiscent of previously observed lipid bodies [308, 309]. As timelapse imaging seems to indicate that intracellular vesicles disappear over time, the dark particles might be interpreted as membrane degradation products.

All in all, this data indicates that extracellular liquid, containing macromolecules such as DNA, may be taken up by an active, endocytosis-like process resulting in its encapsulation in intracellular vesicles. Subsequent release of the DNA contained within the vesicles in the cytoplasm may lead to genetic transformation. Interestingly, L-forms from *Listeria monocytogenes*, a Gram-positive bacterium not known to be naturally transformable, could also be transformed after incubation with DNA [258]. As these L-forms produce similar internal vesicles [89], this suggests that endocytosis-like processes may also occur in L-forms from other bacterial species.

Endocytosis is generally considered to be restricted to eukaryotic cells [292], as only a few cases of endocytosis-like processes are known in prokaryotes [293, 294]. However, this research shows that some bacteria can perform similar processes under conditions that induce a wall-deficient L-form state. It is unknown what enables the membrane to bulge inwards to form internal vesicles in L-forms. However, the observation that shape distortions occurring during L-form proliferation are dependent on surplus membrane synthesis rather than the established cytoskeletal proteins [88, 95, 102, 103], implies that this mechanism could also be responsible for vesicle formation.

Horizontal gene transfer in cell wall-deficient cells

HGT is mediated by a variety of gene transfer processes, of which some depend on free DNA, cell-cell contact, or extracellular vesicles (EVs) [127]. In **Chapter 3** we examined if HGT between wall-less cells can occur without the explicit addition of free DNA. To this end, *K. viridifaciens* and its L-form derivative were tagged with different, chromosomally-located resistance genes. The co-culture of these strains, under conditions that allowed S-cell formation and L-form proliferation, resulted in recombinants containing both antibiotic resistance cassettes. Further examination revealed that the formation of recombinants did not depend on the presence of hyphal cells and was resistant to DNAse treatment. Gene transfer could, however, be inhibited by disrupting S-cells prior to use in the co-culture. A HGT mechanism that does not dependent on free DNA but on the presence of intact wall-less cells is cell-cell fusion, a process that can be induced in wall-less protoplasts.

Protoplast fusion by chemicals such as PEG is a commonly used technique in strain development [185, 218, 412]. Cell-cell fusion results in cells temporarily containing distinct

chromosomes, which may facilitate DNA recombination and the generation of cells with altered characteristics [413]. Most recombinants obtained after co-culturing were L-form-like, and some recombinants had different phenotypes compared to the parental strain upon reversion to walled growth. This may suggest that genetic changes have occurred, possibly mediated by spontaneous cell fusion. The addition of PEG to a mixture of S-cells and L-forms could reproduce the formation of recombinant strains, and L-form fusion without the presence of PEG has been observed via timelapse imaging (**Chapter 3**). Therefore, cell-cell fusion may have contributed to HGT between wall-less cells during co-culture. However, we also noticed the outward blebbing of cell membranes in electron microscopy images of S-cells and L-forms of *K. viridifaciens* (**Chapter 4**) [15]. This may indicate the formation of EVs, which are known to mediate HGT via vesiduction [146]. It is therefore possible that the observed HGT is a result of cell-cell fusion and/or vesiduction.

A glance at the past: L-forms as model for early lifeforms

It is estimated that around 2.5 – 4 billion years ago, during the Archean Eon, the first forms of life emerged on earth [414, 415]. These lifeforms were likely simple cells without a cell wall. There are similarities between ancient microfossils discovered in Australia (2.4-3.5 billion years old) and wall-less bacterial cells [324]. Different model systems are used to simulate early lifeforms, such as artificial giant lipid vesicles and L-forms, which can undergo strikingly similar morphological transitions [93, 94]. The lack of a cell wall may have enabled massive HGT events, for example by cell fusion and lightning-triggered electroporation [319-321], which contributed to cellular evolution. The HGT processes observed for wall-less cells in **Chapter 3 and 4** further demonstrate how HGT may have taken place between early lifeforms. Moreover, the non-specific engulfment of extracellular material would have enabled uptake of particles such as food. Evolution of a primitive cell wall would have necessitated the evolution of selective uptake machinery, which at the same time would reduce the chance of uptake of potentially harmful substances.

Hyperosmotic stress results in large genomic rearrangements

K. viridifaciens is a soil-inhabiting bacterium that forms a mycelial network of connected, multi-genomic hyphal cells. Exposure to hyperosmotic stress results in the formation of colonies with an aberrant phenotype, in addition to the formation of wall-less cells [15]. In **Chapter 2** we isolated and further examined these aberrant colonies to study the cause and consequences of this phenotypic diversity. We show that these isolates have a permanently altered morphology, such as delayed sporulation and reduced formation of aerial hyphae and/or spores on solid medium, and some strains were auxotrophic for arginine. Such changes can be caused by loss of genomic regions, as is often observed in members of the closely related *Streptomyces* genus [191, 204].

To examine this, in total 25 isolates (encompassing colonies resembling the wild-type and those displaying irregular morphologies) were subjected to whole-genome sequencing (WGS) using Illumina short-read sequencing technology. Mapping of the sequencing reads against a reference genome can reveal the presence of large deletions or amplifications. WGS analysis showed that 15 out of 20 isolates with an aberrant phenotype indeed contained genomic rearrangements (**Chapter 2**). The *K. viridifaciens* DSM40239 genome consists of a linear chromosome and a linear megaplasmid KVP1 (1.7 Mbp). However, fifteen isolates had lost part of, or the entire megaplasmid, with nine of these isolates carrying additional deletions on one or both outer chromosomal arms. In some cases, the affected regions were remarkably similar.

Two isolates retained a small part of the megaplasmid. A similar observation was made for a protoplast revertant of *K. viridifaciens* that was generated from walled cells grown under hyperosmotic stress conditions [198]. Whole-genome alignment of *K. viridifaciens* to *Streptomyces viridifaciens* ATTC11989, both listed as the same bacterial species, revealed that the outer chromosomal arm of *S. viridifaciens* was homologous to a region in KVP1. This indicates that there is a possibility for genetic recombination to take place between linear chromosomes and megaplasmids. As a consequence of such recombination events, a segment of the megaplasmid could become integrated into the chromosome structure.

The genome of *K. viridifaciens* contains many genes encoding for proteins associated with transposable elements (TE), which are a form of mobile genetic elements (MGE). MGEs are segments of genetic material that encode all proteins required for transmission within a genome or to a different bacterium [121]. For some isolates, genes associated with TEs were present at the site of a large deletion or flanked putatively amplified genomic regions. TEs are known to be activated by environmental stressors [214, 215] and are associated with genome instability such as chromosomal rearrangements [196, 197, 211]. We therefore hypothesize that hyperosmotic stress activates TEs in hyphal cells, leading to genetic instability and aberrant colony phenotypes in *K. viridifaciens*. The extrusion of wall-deficient cells may enable loss of the megaplasmid and further genomic recombination if multiple chromosome copies end up in one cell.

All isolates were still sensitive to hyperosmotic stress, as observed by the formation of wall-less cells, but some isolates seemed to grow slower than the parental strain under these conditions. Although it is not known whether the altered phenotypes serve an adaptive purpose, it could be that slower growth may help the bacterium to persist under stressful conditions.

Hyperosmotic stress leads to enhanced genome plasticity

Streptomycetes are subjected to widespread intra- and interspecies gene exchange [416, 417] and are known for their high genetic instability [188, 189, 191, 204]. **Chapter 2** shows that hyperosmotic

stress leads to genome instability, most likely via activation of TEs, leading to genomic rearrangements. **Chapter 3 and 4** show how formation of wall-less cells under hyperosmotic stress can lead to HGT. These processes can result in an altered genome or acquisition of new genetic information. Therefore, the results in this thesis indicate that growth under hyperosmotic stress conditions can lead to intra- and intergenomic gene transfer in *K. viridifaciens*, which enhances its genome plasticity (Fig. 1), the alterable, dynamic nature of genomes [128, 418, 419].

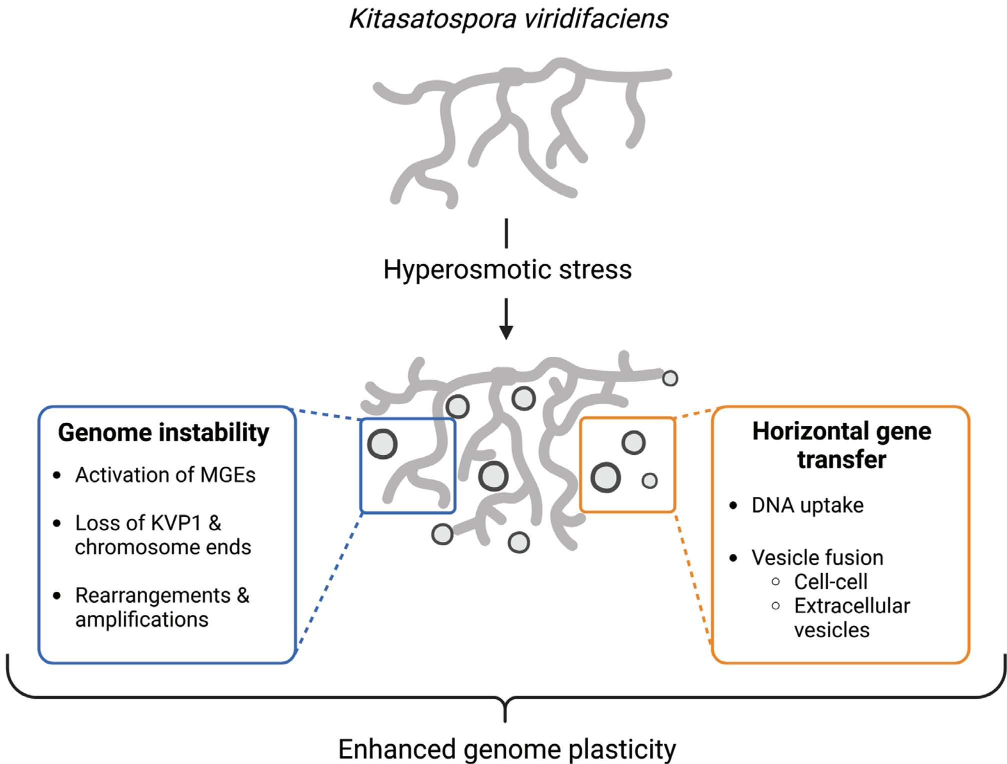

Figure 1. Hyperosmotic stress enhances genome plasticity in *Kitasatospora viridifaciens*.
Hyperosmotic stress leads to genome instability in *K. viridifaciens*, as observed by loss of the megaplasmid KVP1, deletions on the chromosomal arms and putative DNA rearrangements and amplifications. This is potentially mediated by activation of mobile genetic elements (e.g. transposable elements) which are abundantly present on the chromosome and megaplasmid. Hyperosmotic stress also induces the formation of cell wall-deficient cells that can participate in horizontal gene transfer (HGT). HGT can occur via endocytosis-like DNA uptake and vesicle fusion, the latter which may involve direct cell-cell fusion and/or mediated by extracellular vesicles. Genome instability and HGT are processes that can alter the bacterial genome and thus contribute to genome plasticity.

Exposure to environmental stress, for example by changing weather conditions, may contribute to genome plasticity and adaptation of streptomycetes to environmental challenges or new environmental niches. Genetic instability and HGT mostly affect the terminal regions

of linear chromosomes, rather than affecting the highly conserved, centrally located region [58, 216, 420]. The terminal chromosomal regions often contain BGCs responsible for biosynthesis of secondary metabolites [421-423]. Genomic alterations are therefore likely to affect BGCs and may thereby contribute to the diversification of bioactive molecule production in streptomycetes.

A possible role for wall-deficient cells in ARG dissemination and biofilm structure

An environment that is generally considered a hotspot for dissemination of ARGs are WWTPs [163, 174, 175]. WWTPS use microbes to break down organic waste during biological treatment. Small, biofilm-like microbial aggregates (flocs) containing a high diversity of bacteria are mixed with contaminated water during the activated sludge process to break down organic matter. Environments with a high density of bacteria, such as biofilms, are known to facilitate HGT [165, 171, 172]. In addition, wastewater contains free-floating DNA, large amounts of bacteriophages and low concentrations of chemicals such as antibiotics. This may enable bacteria to switch to a wall-deficient state, which can contribute to DNA transfer via the HGT mechanisms explored in this thesis (**Chapter 3**, **4**), as well as via release of DNA upon contact with water.

It is unknown whether bacteria capable of switching to a wall-deficient state are present in WWTPs. Therefore, in **Chapter 5** I studied the presence of filamentous actinobacteria capable of producing cell wall-deficient cells in WWTPs. Using different selective media, 62 actinobacteria were isolated from wastewater, the majority originating from activated sludge samples. Based on 16S rDNA sequencing, 58 isolates belonged to *Streptomyces*, three to *Micromonospora* and one belonged to the *Pseudonocardia* genus. Exposure of these isolates to hyperosmotic stress, known to induce a wall-deficient state in streptomycetes [15], indeed resulted in the formation of large vesicles in a quarter of the isolates. Staining with fluorescent dyes indicated the presence of nucleic acids inside these large vesicles, most likely being genomic DNA, which thereby resemble wall-deficient S-cells.

One of the vesicle-forming strains was a *Micromonospora* isolate, suggesting that hyperosmotic stress can induce formation of wall-less cells in bacteria outside of the *Streptomycetaceae* family. The ability to extrude vesicles, or wall-less cells, may be linked to the polar mode-of-growth of these filamentous bacteria [59, 181]. Indeed, extrusion of cell wall-deficient cells is also observed in polar-growing mycobacteria [270]. New cell wall material is synthesized and incorporated at the cell poles, most likely making this region a weak spot, allowing vesicle extrusion.

EVs, also known as membrane vesicles, exert a significant influence on both the establishment and architecture of biofilms [424, 425]. Within biofilms, instances of cells lacking cell walls have been documented [111, 267, 268]. Wall-deficient cells could potentially fulfil a role akin to that of EVs in structuring biofilms or biofilm-like aggregates. Notably, cells devoid of cell walls

are susceptible to disruption upon exposure to water, leading to the release of cellular contents, including DNA – an essential biofilm component as highlighted in Whitchurch *et al.* (2002) [169].

Given this connection, it becomes intriguing to explore the potential presence of wall-deficient bacteria within activated sludge flocs or other biofilm structures. Investigating this aspect would entail inducing structural disruption of the biofilm under osmoprotective conditions, followed by filtration stages designed exclusively for the passage of wall-less bacteria. Such an inquiry would yield deeper insights into the contributions of wall-less cells to the architecture of biofilms, HGT, and the dissemination of ARGs in the environment.

Outlook

Transfer of DNA containing antibiotic resistance genes via HGT is a major factor in the spread of antibiotic resistance amongst bacteria. Further understanding of how, where and when HGT between bacteria occurs could help to minimize the spread of resistance genes.

The work in this thesis shows that the shift to a wall-less state can enable bacteria to participate in HGT. Some of these gene transfer mechanisms cannot occur in cells with an intact cell wall. This thesis identified a potential role for EVs in HGT via wall-less cells. A next step would be to further explore this hypothesis by incubation of wall-less cells with DNA-containing EVs, and to study whether the formation of wall-less cells correlates with EV production.

In this thesis, the study of HGT between wall-less cells was limited to the Gram-positive soil bacterium *K. viridifaciens*, although it is likely that wall-less cells from other bacteria may behave similarly. It would, however, be of interest to study whether L-forms from Gram-negative bacteria are capable of engulfing extracellular material via the endocytosis-like mechanism reported here, or whether the presence of an outer membrane would prevent this.

One of the environments where wall-less cells may reside are biofilm(-like) environments, which are associated with infections, antibiotic resistance and HGT. Further studies into the occurrence of wall-less cells, and their contribution to gene transfer and biofilm structure, may offer important insights to help reduce the spread of antibiotic resistance and to improve treatment of bacterial infections. For example, treatment with cell-wall targeting antibiotics may not be the preferred option if this induces the pathogen to switch to a wall-deficient state. This switch may enable the pathogen to survive the antibiotic treatment and cause recurrence of the infection [21], and may enable the acquisition of resistance genes and/or biofilm formation, thereby leading to increased antibiotic resistance. Future studies into HGT should therefore include wall-less cells derived from pathogenic bacteria. Increasing the knowledge on the formation and occurrence of bacterial wall-less cells and their role in HGT will contribute to the fight against bacterial antibiotic resistance.