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

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Chapter 3.

Horizontal gene transfer in cell wall-deficient cells of *Kitasatospora viridifaciens*

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Part of the data presented in this chapter is published in:

Kapteijn, R., Shitut, S., Aschmann, D., Zhang, L., de Beer, M., Daviran, D., Roverts, R., Akiva, A., van Wezel, G. P., Kros, A., & Claessen, D. Endocytosis-like DNA uptake by cell wall-deficient bacteria. *Nat Commun* **13**, 5524 (2022).

Abstract

Horizontal gene transfer (HGT) is a mechanism by which bacteria exchange DNA. This is mediated via processes such as conjugation, transduction and transformation, which facilitate the passage of DNA across the protective cell wall. Here, we report that cell wall-deficient bacteria can participate in HGT via novel mechanisms. Specifically, we show that L-forms of the filamentous actinobacterium *Kitasatospora viridifaciens* can take up free plasmid DNA, leading to genetic transformation. DNA uptake is not affected by deletion of genes homologous to *comEC* and *comEA*, which encode proteins known to facilitate genetic transformation in naturally competent bacteria. In addition, co-culturing of different cell wall-deficient cell types led to DNA exchange in a DNAse-resistant manner, presumably mediated by cell-cell fusion. Our work provides insights into how the switch to a wall-deficient state enables novel routes of HGT for bacteria, that may contribute to the spread of antibiotic resistance.

Introduction

Bacteria are exposed to changing environmental conditions and rely on their cell envelope for protection. The cell envelope consists of a cell membrane and a cell wall to separate the internal from the external environment [1]. The cell membrane is a phospholipid bilayer that encloses the cytoplasm and functions as a selective barrier. The cell wall consists of a thick peptidoglycan layer for Gram-positive bacteria and a thinner peptidoglycan layer surrounded by an outer membrane for Gram-negative bacteria. The peptidoglycan layer is an important mesh-like structure that not only provides protection against mechanical stress and turgor pressure, but also defines cell shape and rigidity [1, 3].

To facilitate the selective passage of macromolecules across the cell envelope, bacteria have evolved specialized transport systems [27, 229]. For instance, naturally transformable bacteria rely on protein complexes for DNA uptake, with components similar to type IV pili or type II secretion systems. Active transport of DNA across the cell wall is facilitated by pilus structures that bind to DNA, followed by pilus retraction [129, 230, 231]. DNA-binding and pore-forming proteins are then used to translocate the DNA across the cell membrane.

Although the cell wall is a vital structure for most bacteria, some bacteria naturally lack a cell wall, or can shed their wall under specific conditions. Examples of bacteria that naturally lack a cell wall are the Mollicutes, that are mostly parasitic and live in specific osmotically protective environments such as human mucosal surfaces or the phloem sieve tubes of plants [78, 232]. Furthermore, prolonged exposure to environmental stressors such as cell wall-targeting agents can convert walled bacteria into so-called L-forms, which are cells that can proliferate without their cell wall. Reproduction of L-forms is independent of the canonical FtsZ-based division machinery [88] and is driven by an imbalance in the surface area to volume ratio of cells caused by the upregulation of membrane synthesis leading to spontaneous blebbing, tubulation and vesiculation [95, 102, 103].

Some filamentous actinobacteria, such as *Kitasatospora viridifaciens*, have the ability to transiently shed their cell wall under conditions of hyperosmotic stress, leading to the formation of so-called S-cells (Fig. 1A) [15]. Contrary to L-forms, S-cells are not able to proliferate without their cell wall, although S-cells are able to revert to the mycelial mode-of-growth after rebuilding their cell wall. Temporary cell wall-deficient cells can also be generated artificially from walled bacteria via enzymatic removal of the cell wall, for example, by the action of lysozyme that degrades peptidoglycan. This leads to the formation of protoplasts or spheroplasts, which are widely used for genetic engineering purposes, often involving the use of polyethylene glycol (PEG) to allow DNA entry into the cell [223] or to induce cell-to-cell fusion [185]. It has never been unambiguously shown whether *K. viridifaciens* walled cells, or its natural cell wall-deficient cells, are capable of natural genetic transformation without using PEG. In addition, it is unknown whether the switch to a wall-deficient state can enable

horizontal gene transfer (HGT).

In this work we show that L-forms of *K. viridifaciens* can take up DNA independent of proteins with homology to canonical DNA translocation machinery. In addition, we find that co-culturing of wall-deficient cells leads to the exchange of DNA in a DNAse-resistant manner. This study shows how the production of wall-less cells enables bacteria to participate in HGT via mechanisms that are unlikely to occur in cells with a cell wall.

Results

DNA uptake by cell wall-deficient cells

It is unknown whether walled or wall-less cells of *K. viridifaciens* are capable of natural genetic transformation. To analyze this, walled mycelial cells, temporary wall-less S-cells and protoplasts, and permanently wall-less L-forms (*alpha*) were freshly harvested and resuspended in osmotically-stable LPB medium. Subsequently, cells were incubated for 18 – 24 h with plasmid DNA (pRed*) containing an antibiotic resistance cassette and plated on selective and nonselective media to allow the detection of transformed cells. Notably, L-forms were consistently able to take up DNA, unlike mycelium, protoplasts and S-cells (Fig. 1B). DNA uptake was not restricted to one L-form line, but was observed with distinct L-form cell lines obtained from walled cells of *K. viridifaciens* grown in LPB medium with (lines *alpha* and *delta*) or without (line M1) penicillin G and lysozyme [15, 233]. No transformants were obtained with *alpha* when intact or fragmented genomic DNA was used, or when using methylated plasmid (Fig. 1C, Supplementary Fig. 1A).

While natural genetic transformation was restricted to L-forms, all wall-deficient cells were chemically transformed using PEG, with protoplasts, S-cells, and L-forms having an average transformation efficiency between 1.7 – 2.5% (Fig. 1D). The addition of PEG also enabled the transformation of *alpha* with genomic DNA, even if this was present in a crude cell extract (Supplementary Fig. 1B). However, use of methylated DNA prevented chemical transformation, indicating that PEG-based transformation is possible with different types of DNA, but is restricted when DNA is methylated. By contrast, walled cells were not transformed either with or without PEG (Fig. 1B, D). These results show L-forms can naturally take up DNA, unlike walled cells, S-cells and protoplasts.

The canonical transformation machinery is not required for DNA uptake

Naturally transformable bacteria use a specialized DNA translocation machinery with similarities to type IV pili or type II secretion systems to take up external DNA [129]. Similar components of this canonical system might also be involved in DNA uptake by L-forms. A BlastP search using the DNA-binding protein ComEA and channel-forming protein ComEC of the naturally transformable bacterium *Bacillus subtilis* str. 168 against *K. viridifaciens*

yielded two significant hits: BOQ63_029625 (helix-hairpin-helix domain-containing protein) and BOQ63_029630 (ComEC/Rec2 family competence protein), respectively (Supplementary Table 1 and Fig. 2A). The *B. subtilis* helicase/DNA translocase ComFA resulted in a hit to a putative Mfd-encoding gene (BOQ63_020315), a widely conserved bacterial protein that

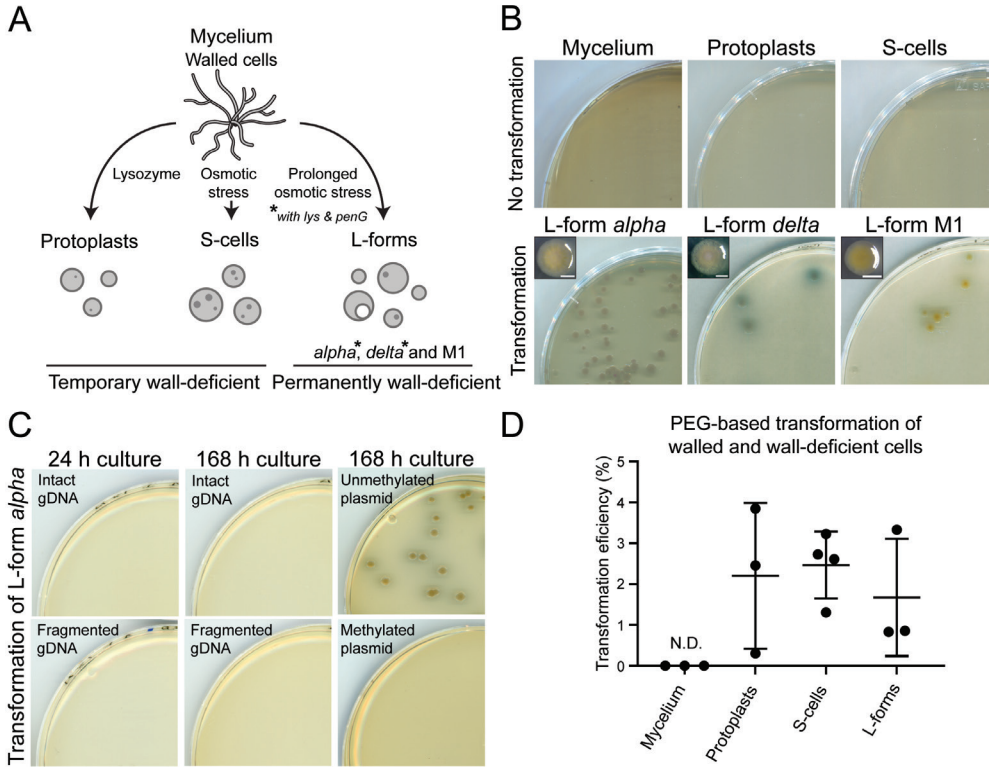


Figure 1. Cell wall-deficient cells can be transformed in a PEG-dependent and -independent manner
(A) Schematic representation of the generation of cell wall-deficient cells of *K. viridifaciens*. Temporary wall-deficient cells include protoplasts, obtained from mycelial cells by the action of lysozyme, and S-cells, extruded from the hyphal tips in a medium with high osmotic pressure. Permanently wall-deficient L-forms are generated after prolonged incubation of mycelium under a high osmotic pressure (line M1), or with the optional supplementation of lysozyme (lys) and penicillin G (penG) (lines *alpha* and *delta*). **(B)** Mycelium (n = 3), protoplasts (n = 5 from two experiments), S-cells (n = 7 from two experiments) and L-form lines *alpha* (n = 3 for both 96 h and 144 h cultures), *delta* (n = 2 for both 72 h and 168 h cultures) and M1 (n = 1 for 72 h cultures) were incubated with plasmid DNA (pRed*) for 18-24 h, plated on selective medium and incubated at 30 °C to select for transformed cells. A close-up of the colony morphology of the L-forms is given. Scale bars indicate 1 mm. Note that only L-forms show consistent DNA uptake as deduced from the emergence of transformants. **(C)** Transformation plates showing absence of transformation after 24 h incubation of 24 h and 168 h L-form *alpha* with intact or fragmented (10 ng μl^{-1}) genomic DNA (gDNA) of *alpha*Δ*ssgB* containing an apramycin resistance cassette (n = 1 for each combination). In addition, whereas transformants are obtained using unmethylated plasmid DNA (pRed*, n = 1), this is not achieved using methylated DNA (n = 3). **(D)** Polyethylene glycol (PEG)-based transformation efficiency of *K. viridifaciens* mycelium, protoplasts, S-cells and L-forms using plasmid DNA (pRed*) as given in the percentage of transformed colonies. n = 3 biological replicates except for S-cells (n = 4). N.D. : No transformants were detected.

mediates transcription-coupled DNA repair [234]. No other orthologues were found for proteins involved in DNA transport across the cell envelope for *B. subtilis*, the Gram-negative *Neisseria gonorrhoeae* [235], or for the T4SS-related DNA uptake system of *Helicobacter pylori* [236] (Supplementary Table 1). L-forms lack an intact peptidoglycan-based cell wall, and therefore, DNA only has to cross the cell membrane for internalization. In naturally transformable bacteria, ComEA and ComEC function in DNA transport across the cell membrane [235, 237, 238].

As the function of the putative *comEC* and *comEA* genes in *K. viridifaciens* was unknown, we wondered whether they could be involved in DNA uptake in L-forms. Therefore, we replaced the putative *comEC* and *comEA* genes in the L-form strain *alpha* by an apramycin resistance cassette (Supplementary Fig. 2). Strikingly, the simultaneous deletion of the *comEA* and *comEC* genes did not affect the transformation efficiency (two-sided independent t-test, $t(8) = 1.572$, $P = 0.155$), indicating that DNA uptake by L-forms occurs independently of genes homologous to this canonical DNA translocation machinery (Fig. 2B).

High membrane fluidity is not sufficient for DNA uptake

Natural competence for transformation is a specific physiological state in which bacteria can take up genetic material from their environment, which can result in natural transformation [28]. This process is regulated differently amongst naturally transformable bacteria and can be constitutively active or restricted to a specific growth phase (reviewed by Blokesch, 2016 [28]). One of the factors controlling the development of natural competence for transformation in *B. subtilis* is the growth phase [239, 240]. To study if culture age is also affecting the DNA uptake ability of L-forms, cells from differently aged cultures were subjected to a transformation assay. Cells from 24 h cultures of *alpha* take up DNA more easily than from 72 h or 168 h cultures (one-way ANOVA, $F(2,9) = 12.16$, Tukey post hoc test, $P = 0.006$ and 0.005 respectively) (Fig. 2C). To test if shorter or longer incubation times affect DNA uptake, the transformation efficiency of 168 h *alpha* cultures was determined after 6 h, 24 h, and 48 h incubation with DNA (Fig. 2D). Transformation was detected after 6 h incubation and increased after 24 h (two-sided Kruskal–Wallis test, $H(2) = 8.769$, $P = 0.012$ and Dunn's pairwise test with Bonferroni correction gives $P = 0.010$ for 6 h and 24 h, $P = 0.233$ for 6 h and 48 h, and $P = 0.718$ for 24 h and 48 h).

It is not unlikely that differences in membrane properties that occur during cellular growth may in turn affect the DNA uptake ability. Membrane fluidity is a measure of the average viscosity of the lipid bilayer, which can affect the positioning and movement of proteins and lipids within the membrane [241]. Higher membrane fluidity is characterized by increased fatty acid disorder, lower lipid packing and higher diffusion rates, which can lead to increased membrane permeabilization [242, 243]. Analysis of the membrane fluidity of differently aged cultures indicated that the increased DNA uptake ability may correlate positively with

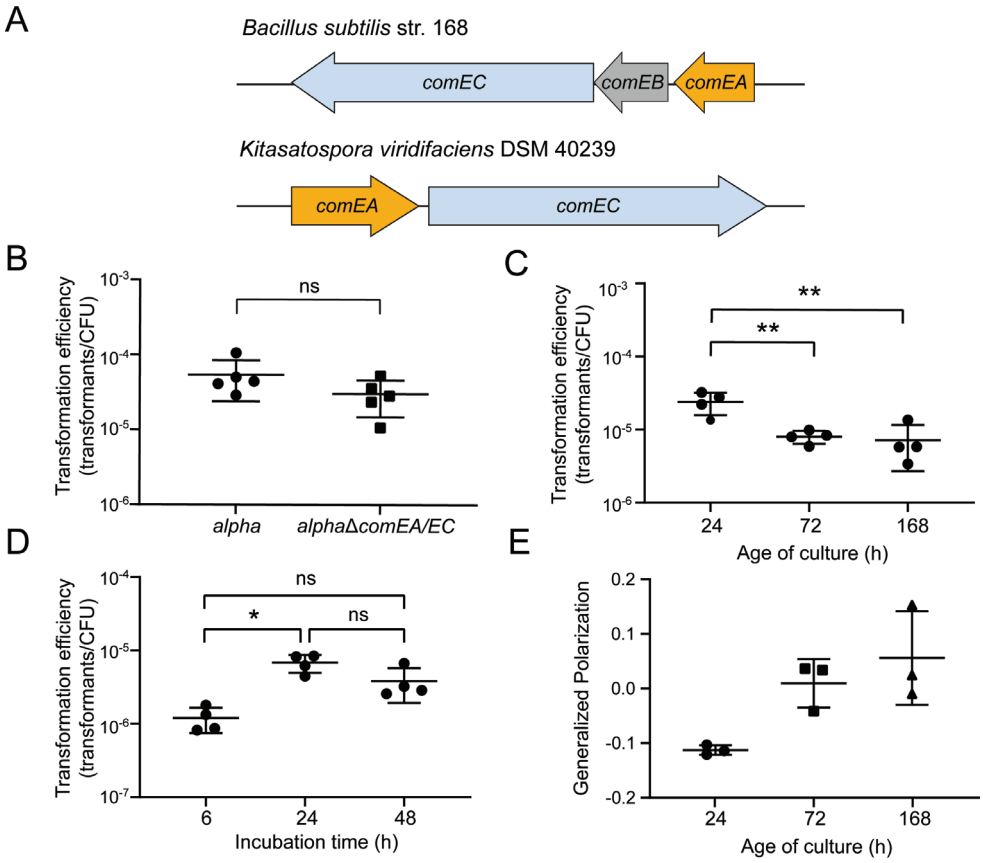


Figure 2. DNA uptake by cell wall-deficient cells is independent of homologs of the competence proteins ComEA and ComEC and correlates with membrane fluidity

(A) Localization of putative ComEA and ComEC-encoding genes (BOQ63_029625 and BOQ63_029630, respectively) on the chromosome of *K. viridifaciens* DSM 40239 as compared to *comEA* and *comEC* of naturally transformable *Bacillus subtilis* str. 168. **(B)** Transformation efficiency of 168 h *alpha* and *alpha*Δ*comEA/EC* after 24 h incubation with pFL-*ssgB*. CFU = colony forming units. ns = not significant (n = 5 biological replicates, two-sided independent t-test, $t(8) = 1.572$, $P = 0.155$). **(C)** Transformation efficiency of 24 h, 72 h and 168 h *alpha* after 24 h incubation with pFL-*ssgB*. Asterisks (**) indicate $P \leq 0.01$ (n = 4 biological replicates, one-way ANOVA, F (2,9) = 12.16, Tukey post hoc test, $P = 0.006$ (24 h – 72 h) and 0.005 (24 h – 168 h)). **(D)** Transformation efficiency of 168 h *alpha* incubated with pRed* for 6, 24, or 48 h. Asterisk (*) indicates a statistically significant difference between 6- and 24-h incubation (two-sided Kruskal–Wallis test, $H(2) = 8.769$, $P = 0.012$ with Dunn's pairwise test, including Bonferroni correction, gives $P = 0.010$ for 6 and 24 h, $P = 0.233$ for 6 and 48 h and $P = 0.718$ for 24 and 48 h). ns = not significant. n = 4 biological replicates. **(E)** Generalized polarization (GP) as measurement of membrane fluidity of 24 h, 72 h and 168 h *alpha* using the membrane dye Laurdan. Lower GP indicates a higher membrane fluidity. n = 3 biological replicates. Data in **(B – E)** are represented as mean ±SD with individual data points.

the fluidity of the membrane, as deduced from the generalized polarization [244] (GP, a lower GP indicating higher fluidity) (Fig. 2E), although no statistically significant differences were observed (Welch one-way ANOVA, $F(2, 2.798) = 13.226$, $P = 0.038$, with Games-Howell post hoc test: 24 – 72 h $P = 0.068$; 24 – 168 h $P = 0.134$; 72 – 168 h $P = 0.711$, $n = 3$). A relatively low fluidity might explain why temporary wall-deficient protoplasts and S-cells cannot take up DNA naturally. However, the fluidity of protoplasts was within the range of 24 h to 168 h *alpha* cultures as measured using a plate assay (Supplementary Fig. 3A). Subsequent analysis of the GP by fluorescence microscopy imaging showed that although protoplasts and S-cells tend to have less fluid membranes, their GP values stay within the range of the membrane fluidity of 24 – 168 h L-form cultures (Supplementary Fig. 3B). Therefore, although membrane fluidity may contribute to efficient DNA uptake, it is not sufficient to explain this process.

Co-culture of *Kitasatospora viridifaciens* wall-less cells leads to HGT

Besides facilitating the uptake of DNA, PEG has been used as an agent to induce fusion between wall-less cells, for instance protoplasts of *Streptomyces*, to perform genetic analyses or improve antibiotic production [217, 218, 245, 246]. As low levels of fusion between wall-less cells without PEG can occur [217, 233, 247], this may provide an additional route for DNA transfer between natural wall-less cells.

To test if DNA transfer can occur between wall-less cells, a co-culture experiment was set-up between *K. viridifaciens* pIJ82-GFP and L-form *alpha*Δ*ssgB* in LPB medium, a medium that induces S-cell formation and L-form growth, respectively. Each strain contains a unique antibiotic resistance cassette integrated in their linear genome (7.9 Mbp in size for the *K. viridifaciens* wild-type) at the position of ~ 3.53 Mbp (plasmid pIJ82-GFP integrated in the ϕC31 *attB* site) and ~ 5.94 Mbp (*ssgB::aac(3)IV*) (Supplementary Fig. 4). SsgB is a cell division protein required for sporulation in *Streptomyces* [248, 249], and the deletion of *ssgB* in *K. viridifaciens* results in formation of white hyphae lacking grey-pigmented spores [15]. The exchange of DNA containing an antibiotic cassette between the strains during co-culture would result in cells resistant to both antibiotics, as determined by plating dilutions of the co-culture on selective medium every 24 h for 168 h. Indeed, colonies resistant to both antibiotics, hereafter referred to as 'recombinants', were obtained after 72 h incubation (Fig. 3A, B), and not when strains were grown individually. Transfer of DNA was confirmed by PCR analysis on 15 randomly picked double-resistant colonies from three biological replicates, confirming the presence of both the hygromycin and apramycin resistance cassettes (Supplementary Fig. 5A – D). In addition, all recombinants produced cytoplasmic green fluorescent protein (eGFP) and thus express *egfp* encoded on pIJ82-GFP (Fig. 3C). Interestingly, 14 out of the 15 recombinants displayed an L-form-like phenotype (phenotype 1), as indicated by the presence of only cell wall-deficient cells and no hyphae during culture in LPB medium (Fig. 3C, recombinant #4). One recombinant displayed a mix of L-form-like and mycelial growth,

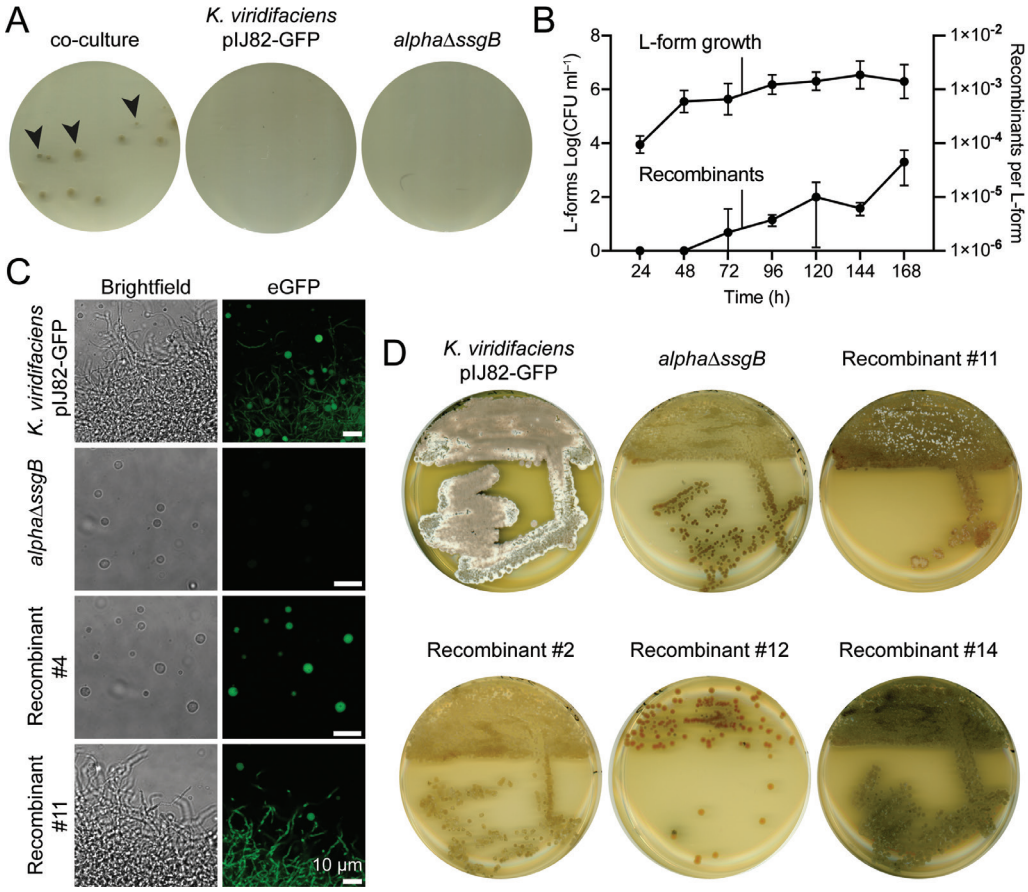


Figure 3. Co-culture between cell-wall deficient cells of *K. viridifaciens* leads to HGT

(A, B) Co-culture of *K. viridifaciens* pIJ82-GFP (Hyg^R) producing temporary wall-deficient S-cells and permanently wall-deficient L-forms *alphaΔssgB* ($Apra^R$) in LPB medium incubated for 168 h at 30 °C and 100 rpm (n = 3 biological replicates) results in double-resistant colonies on LPMA medium containing hygromycin B and apramycin selection, which is not observed for single cultures (n = 1). Representative selective medium plates are shown in **(A)**. Formation of recombinants is given as the mean double-resistant colonies per L-form CFU with error bars displaying the standard deviation in **(B)**. L-form growth is given as the mean log(colony forming units ml^{-1}) with error bars indicating the 95% confidence interval. Plates were incubated for 168 h at 30 °C. Note that after 72 h the first double-resistant recombinants were detected. **(C)** Representative fluorescence micrographs of *K. viridifaciens* pIJ82-GFP, *alphaΔssgB* and two recombinants obtained in **(B)** representing the two recombinant phenotypes, L-form-like (#4) and mycelial-like (#11). All recombinants produce cytoplasmic eGFP and contain the antibiotic resistance cassettes from both parental strains. Strains were grown for 48 h in LPB medium at 100 rpm and 30 °C. See also Supplementary Fig. 5. Scale bars indicate 10 µm. **(D)** Morphology of *K. viridifaciens* pIJ82-GFP, *alphaΔssgB* and four recombinants on MYM medium grown for 72 h at 30 °C. Note that recombinant #11 (mycelial-like) hardly produces aerial hyphae, recombinant #12 (L-form like) shows poor reversion to a mycelial-mode-of-growth and recombinant #2 and #14 (both L-form like) appear to have an changed secondary metabolite production compared to *alphaΔssgB* as deduced from the altered biomass pigmentation. See also Supplementary Fig. 6.

and could eventually be separated into an L-form-like strain (phenotype 1, recombinant #10) and a mycelial strain (phenotype 2, recombinant #11), each strain containing both resistance genes and producing eGFP (Supplementary Fig. 5C, D). The original colony thus likely consisted of two recombinants growing next to each other and were isolated together.

L-form *alpha*Δ*ssgB* has the ability to revert to a mycelial mode-of-growth upon removal of the osmotic stress. To analyze this for the recombinants, biomass was transferred from LPMA medium to MYM medium. Although all recombinants reverted to walled growth, differences in biomass formation and colouration were observed (Fig. 3D, Supplementary Fig. 6B), whereas mycelial biomass from random colonies of the parental strain *alpha*Δ*ssgB* was uniform in morphology (Supplementary Fig. 6A). Changes in colouration of the mycelium can be indicative of an altered production of secondary metabolites. In addition, all L-form-like recombinants had a typical 'bald' phenotype and thus lacked the ability to form aerial hyphae and spores. The mycelial recombinant could form aerial hyphae to a limited extent, but no spores were observed due to the replacement of *ssgB* by the apramycin resistance cassette (Fig. 3D, recombinant #11). These results indicate that co-culture of strains producing wall-less cells results in recombinants that contain DNA from both parental strains as indicated by the presence of both antibiotic resistance cassettes as well as eGFP production.

DNAse and cellulase affect L-form aggregation

To study the mechanism of DNA exchange between wall-deficient cells a series of co-culture experiments in six-wells plates was set-up. Growth of L-forms in six-wells plates resulted in cell aggregation which interfered with CFU analysis. DNAse I can disperse bacterial biofilms by degrading external DNA, which is an important component of the extracellular matrix [169, 250]. Indeed, the addition of DNAse I drastically reduced cell clumping in L-form cultures (Supplementary Fig. 7). DNAse I was active under these conditions as shown by the degradation of gDNA within 5 min in the culture medium (LPB medium) at 30 °C, even after incubation of DNAse I in LPB medium for 20 h before addition to gDNA (Supplementary Fig. 8). However, DNAse I may interfere with possible HGT occurring via free DNA, so an alternative enzyme was required. Another common component of the bacterial extracellular matrix is cellulose. Streptomycetes produce a cellulose-like glycan at hyphal tips via the proteins CslA and GlxA, where it forms a layer on the surface of the cell wall [251-253]. The cellulose-like glycan is thought to protect growing tips from constant cell wall remodelling [251], mediate pellet formation [254] as well as adhesion of mycelium to hydrophobic surfaces [255]. Cellulase was able to degrade the cellulose generated *in vitro* by heterogeneously produced CslA and GlxA from *Streptomyces lividans* [118]. The addition of cellulase to L-form cultures resulted in a sufficient reduction of cell clumping (Supplementary Fig. 7), indicating this glycan, at least partially, leads to L-form clumping. Therefore, cellulase was used in further experiments.

Cell wall-deficient cells exchange DNA via a DNAse-resistant mechanism

Conjugation is the most studied route of HGT for filamentous actinobacteria and occurs via the interaction of hyphae. Small hyphae were observed during co-culture of S-cells and L-forms. Conjugation is only known to occur on solid medium, but to exclude any role of the walled cells in HGT, the hyphae were removed by addition of penicillin G, which inhibits cell wall synthesis, and lysozyme, which degrades the cell wall (Supplementary Fig. 9). As expected, absence of hyphal cells did not affect the formation of recombinants, indicating that the observed HGT occurs exclusively between wall-less cells (two-sided independent t-test, $t(4)= 1.253$, $P = 0.557$ with Bonferroni-adjustment) (Fig. 4, 'penG/lys').

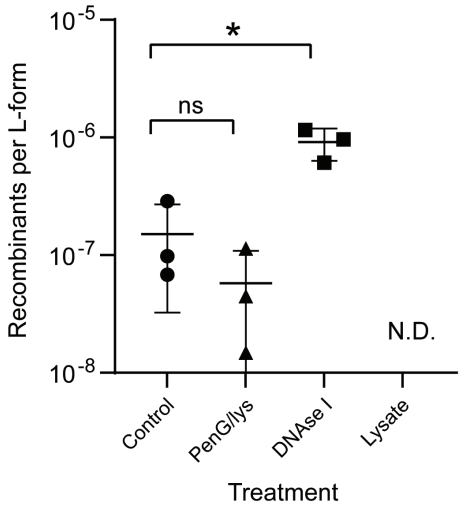


Figure 4. Horizontal gene transfer between S-cells and L-forms occurs in a DNAse-resistant manner
Effect of three treatments on HGT during co-culture of S-cells of *K. viridifaciens* pIJ82-GFP and L-form *alphaΔssgB* for 24 h at 100 rpm and 30 °C. Efficiency of HGT is given as the average number of recombinants (colonies resistant to hygromycin B and apramycin) per L-form CFU on LPMA medium. Whereas removal of walled cells by treatment with 10 mg ml^{-1} penicillin G (PenG) and 0.6 mg ml^{-1} lysozyme (Lys) did not affect HGT (ns = not significant, two-sided independent t-test, $t(4) = 1.253$, $P = 0.557$ with Bonferroni-adjustment), the presence of 1 mg ml^{-1} DNAse I increased HGT efficiency (two-sided independent t-test, $t(4) = -4.354$, $P = 0.0242$ with Bonferroni-adjustment), and no HGT was detected when co-culturing with supernatant from S-cells that were lysed by boiling. Asterisk (*) indicates $P \leq 0.05$. N.D. = No recombinants were detected. $n = 3$ biological replicates as represented by mean ±SD with individual data points shown.

L-forms take up plasmid DNA, but not gDNA, during incubation experiments. To test whether S-cells and L-forms exchange genetic material via free DNA in a co-culture setup, an excess of DNAse I was added. The presence of this enzyme increased the formation of recombinants (two-sided independent t-test, $t(4) = -4.354$, $P = 0.0242$ with Bonferroni-adjustment), possibly by further inhibiting cell clumping and improving CFU detection sensitivity (Fig. 4, 'DNAse I'). These experiments show that HGT between wall-deficient cells occurs in a DNAse-resistant manner. A spontaneous fusion event between vesicles can allow DNAse-resistant exchange of genomic DNA. To prevent cell-cell fusion, S-cells were lysed in a chemical-independent manner by boiling the S-cell filtrate for 10 min. Incubation of the sterile supernatant with L-forms prevented the formation of recombinants (Fig 4, 'lysate'). To confirm that fusion between S-cells and L-forms results in viable recombinants, fusion was artificially induced using PEG [233]. This resulted in eGFP-producing cells carrying both

resistance cassettes, showing that fusion events can lead to viable recombinants (n = 3, yield of 0.6, 11.5 and 14.9% fused cells based on L-form CFU).

In conclusion, we show that cell wall-deficient cells of *K. viridifaciens* can participate in HGT via different mechanisms. HGT may take place via the uptake of external plasmid DNA by L-forms in a manner independent of genes with homology to the canonical DNA uptake machinery. Furthermore, HGT can occur between S-cells and L-forms in a DNAse-resistant manner during co-culture, presumably via cell-cell fusion.

Discussion

The bacterial cell wall is an important protective barrier to the environment, providing stress resistance and enabling the selective passage of molecules. However, in recent years it has become clear that under some conditions, bacteria may also thrive without this layer. Prolonged exposure to environmental stresses, such as cell wall-targeting agents or a high osmotic pressure, can induce the formation of L-forms that efficiently proliferate without their cell wall [15, 17, 88, 95]. The consequences of such a wall-deficient bacterial lifestyle on their ability to participate in HGT were largely unknown.

Well-known mechanisms for HGT are natural transformation, transduction, and conjugation [127]. Such processes require sophisticated machinery to enable transport of DNA across the cell envelope. We here show that wall-deficient cells such as protoplasts, S-cells, and L-forms of *K. viridifaciens* take up DNA using PEG. Importantly, L-forms are the only wall-deficient cells that achieve consistent transformation with DNA without the addition of PEG. Specifically, while plasmid DNA was taken up without PEG, no transformation was observed when genomic DNA was used. This is likely due to the ~200-fold lower number of gDNA molecules used as compared to plasmid DNA. Furthermore, transformation of gDNA also requires a double recombination event for stable integration of the antibiotic resistance cassette, which likely also lowers the transformation efficiency.

Naturally transformable bacteria use a canonical and complex system for DNA uptake across the cell wall and cell membrane. The latter step requires the DNA-binding protein ComEA and the pore-forming channel protein ComEC, with homologs found across naturally transformable Gram-positive and Gram-negative species (e.g., ComE and ComA in *N. gonorrhoeae*). Disruption of *comEA* or *comEC* typically results in a drastic reduction or even the complete absence of transformation [237, 238, 256, 257]. However, the disruption of genes with homology to *comEA* and *comEC* in L-forms of *K. viridifaciens* had no effect on their ability to take up DNA. Interestingly, preliminary work shows that L-forms of *Listeria monocytogenes* also have the ability to take up extracellular plasmid DNA and become transformed without the use of PEG [258]. This species is not known to be naturally transformable and does not contain a functional competence system. This supports a mechanism for DNA uptake in

L-forms that is independent of the canonical natural genetic transformation machinery.

Low levels of DNA uptake have been detected in mammalian cells, and was hypothesized to occur via an endocytosis process [259, 260]. During endocytosis, external molecules or particles are internalized via vesicle formation from the cell membrane in eukaryotic cells [261]. L-forms may contain intercellular vesicles seemingly devoid of cytoplasm, suggesting the vesicles contain extracellular medium [89, 92, 262, 263]. Whether the formation of intracellular vesicles in L-form cells can act as a mechanism to capture external material such as DNA, thereby leading to transformation, is further explored in Chapter 4.

Besides the ability of L-forms to take up DNA from their environment, HGT between wall-less S-cells and L-forms was observed during co-culture. This process did not rely on free DNA, as shown by the inability of DNAse to prevent the formation of recombinant colonies, and is in line with the lack of transformation when incubating L-forms with gDNA without PEG. The most studied mechanism of HGT in *Streptomyces* is conjugation, which involves the transfer of double-stranded DNA [138] via the pore-forming FtsK-like DNA translocase TraB [139]. Conjugative interactions between streptomycetes can only occur if the donor and recipient are grown together on a solid surface [264]. In this work, the integrative plasmid pIJ82-GFP did not encode TraB and the co-cultures were performed in liquid medium. Therefore, we do not expect this process to be responsible for the observed DNA transfer between the wall-less cells.

A plausible hypothesis for the DNAse-resistant exchange of gDNA is the fusion between S-cells and L-forms. Spontaneous fusion between wall-less L-forms has been captured during time-lapse imaging of *K. viridifaciens* L-form *delta* [233] (S. Shitut, unpublished data) and *E. coli* L-forms (M. Crooijmans, unpublished data) (Supplementary Fig. 10). As cell-to-cell fusion results in a cell (temporarily) having multiple chromosomes, several recombination events may occur. The exchange of other parts of the genome, next to the markers, may explain the altered phenotypes of L-form recombinants compared to the parental strains.

Another HGT mechanism that may be involved is vesiduction [146], in which the release of DNA-containing extracellular vesicles (EVs) can lead to transformation of a recipient cell. *Streptomyces* EVs have a diameter of 20 to 400 nm and can contain proteins, RNA, DNA and metabolites such as antibiotics [72, 265, 266]. Although it is unknown if *K. viridifaciens* has the ability to produce EVs, electron microscopy imaging showed the putative detachment of small vesicle-like structures from S-cells [15] (Supplementary Fig. 11A), as well as outward cell membrane protrusions occurring in L-form cells (Supplementary Fig. 11B, Chapter 4 Fig. 3). Based on this data we cannot exclude a role for such vesicles in the observed DNA transfer events, providing that they carry (fragments of) chromosomal DNA that encode the resistance markers.

The ability of DNAse and cellulase to reduce cell aggregation in L-form cultures suggests that L-forms produce an extracellular matrix containing DNA and a cellulose-like

glycan. L-forms of *K. viridifaciens* produce peptidoglycan, which is largely detached from the cells [15], perhaps suggesting that other cell surface components can also still be produced. Production of an extracellular matrix by L-forms may offer protection and improve cell adhesion to surfaces such as medical equipment and the oral cavity via biofilm formation [111, 267-269]. Therefore, it would be interesting to further study the role of DNA, cellulose-like glycans or other components in L-form aggregation and biofilm formation.

All in all, this work shows that the switch to a cell-wall deficient state enables HGT in *K. viridifaciens*. DNA uptake without the use of PEG occurs independently of proteins homologous to canonical DNA translocation machinery found in naturally transformable bacteria, and is further studied in Chapter 4. In addition, DNAse-resistant transfer of DNA takes place during co-culture of cell-wall deficient cells, yielding viable recombinants. Although the mechanism remains unclear, we speculate that this process occurs via spontaneous cell-cell fusion, possibly involving membrane vesicles. The switch to a wall-deficient state has been described for an increasing number of bacterial species, including pathogens such as *Mycobacterium sp.* and *Staphylococcus aureus* [21, 88, 115, 270, 271]. Future studies are therefore required to understand the implications of this research for bacterial evolution, especially with regard to antibiotic resistance. This should involve elucidation of the mechanism and the occurrence of HGT by cell-wall deficient cells in a wider range of bacterial species, including pathogens.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Supplementary Table 2 and 3, respectively. *Kitasatospora viridifaciens* DSM40239[221] was grown confluently on maltose-yeast extract medium (MYM) to obtain spores, which were harvested after 72 h – 96 h incubation [222]. In brief, spores were resuspended in MilliQ using a cotton swab and filtered through a syringe filled with cotton wool. Spores were resuspended in 20% (v/v) glycerol (Duchefa Biochemie) and stored at − 80 °C until use. For mycelial growth in liquid cultures, *K. viridifaciens* was grown overnight at a density of 1×10^6 spores ml^{-1} in L-phase broth (LPB) without sucrose at 200 rpm, or in tryptone soy broth medium (TSBS) consisting of 30 g L^{-1} Tryptone Soy Broth powder and 10% (w/v) sucrose [223] for gDNA isolation. LPB medium contains 0.25% bacto-peptone, 0.15% yeast extract, 1.5% oxoid tryptone soy broth powder, 0.15% oxoid malt extract, 0.5% glucose (all w/v), 0.64M sucrose and 25 mM $MgCl_2$. Strains were grown for 48 h in LPB medium at 100 rpm to induce the formation of S-cells[15].

L-forms were grown on solid L-phase medium agar (LPMA) or liquid LPB medium[15] to allow wall-less growth, or on MYM medium for 72 h to induce reversion to a mycelial-mode-of-growth. Liquid cultures were inoculated with 1×10^6 spores ml^{-1} for *K. viridifaciens*

strains or with a frozen aliquot of a 24 – 48 h L-form culture in case of L-form strains, unless stated otherwise. L-forms were grown in liquid culture for 72 – 96 h for chemical transformation and co-culture assays, and were grown for 168 h for all other experiments unless stated specifically. L-forms were adjusted to 5 – 7.5 × 10^7 CFU ml^{-1} for transformation assays (based on OD_{600} of 3 for 72 h and 168 h cultures and 0.2 for 24 h cultures), and 2.5 – 5 × 10^7 CFU ml^{-1} (OD_{600} of 2) for all other experiments with 168 h cultures. *Kitasatospora* cultures were grown at 30 °C.

Escherichia coli was grown in liquid LB medium cultures (shaking at 250 rpm) or on solid LB medium at 37 °C. When required, antibiotics (5 μg ml^{-1} thiostrepton, 25 μg ml^{-1} chloramphenicol, 50 μg ml^{-1} apramycin, 100 μg ml^{-1} ampicillin or 100 μg ml^{-1} hygromycin B with the exception of LB medium which contained 200 μg ml^{-1} hygromycin B) were added to the culture medium. *E. coli* JM109 [272] was used for plasmid cloning and *E. coli* ET12567/pUZ8002 [273] was used to obtain methylation-deficient DNA for conjugation to *K. viridifaciens* and to extract DNA for transformation assays.

Construction of plasmids

All PCRs were performed using PFU or Q5® High-Fidelity DNA polymerase (NEB). The primers used in this study are listed in Supplementary Table 4. GeneRuler DNA Ladder Mix (SM0334, Thermo Scientific) was used to confirm the size of DNA molecules via gel electrophoresis. To create pFL-*ssgB* (Supplementary Table 3), a hygromycin resistance cassette was amplified using primer pair Hyg_F-231_EEV and Hyg_R + 1237_HEV with pMS82 [274] as the template. The PCR products were digested with EcoRV and cloned into pWHM3-oriT [275] to generate pWHM3-oriT-hyg (Supplementary Table 3). The 3' flank of *ssgB* was digested from pKR1[15] and cloned into pWHM3-oriT-hyg using XbaI and HindIII to generate the final plasmid. All restriction enzymes were ordered from New England Biolabs.

pRK1 (Supplementary Table 3) was created by amplifying the upstream flanking region of *comEA* by PCR with primers FL1-comEA/comEC-FW and FL1-comEA/comEC-REV, thereby introducing unique EcoRI and XbaI restriction sites, while the downstream flanking region of *comEC*, made by gene synthesis (Baseclear, Leiden, the Netherlands), was flanked by XbaI and HindIII sites. The flanking regions and apramycin cassette were cloned in pWHM3-oriT using the EcoRI, HindIII restriction sites interspersed with an apramycin resistance cassette containing flanking XbaI sites, thereby creating the final plasmid. The *comEA/comEC* deletion mutant was created in L-form strain *alpha* [15] using pRK1, which replaced the nucleotides +58 relative to the start codon of *comEA* (BOQ63_029625) until +2489 relative to the start codon of *comEC* (BOQ63_029630) with an apramycin resistance cassette. Note that the gene annotation of *Streptomyces viridifaciens* ATTC11989 (accession CP023698) was used to determine the putative correct start and stop codons for *comEC*, resulting in the genome location 5,041,836 to 5,044,433 on CP090841. To create pIJ82-GFP,

the region containing the *eGFP* gene with a *gap1* promoter was amplified from pGreen [276] using primer pair gap1_FW_BglII and egfp_RV_EcoRI. The resulting PCR product was cloned into pIJ82 using BglII and EcoRI to generate the final plasmid.

Construction of bacterial strains

Strain *K. viridifaciens* pIJ82-GFP (Supplementary Table 2) was generated by intergeneric conjugation between *K. viridifaciens* DSM40239 with the methylation-deficient *E. coli* ET12567/pUZ8002 carrying the integrative pIJ82-GFP plasmid (Supplementary Table 3), respectively, based on the method as described in Kieser *et al*., (2000) [223]. In brief, *E. coli* was grown to an OD600 of 0.4 – 0.6 in LB medium at 37 °C containing kanamycin, chloramphenicol and hygromycin B. Cells were washed twice and resuspended in LB medium. Around 10^8 spores were added to 500 µl 2 × YT broth (containing 16 g Bacto tryptone, 10 g Bacto yeast extract and 5 g NaCl per L) before performing a heat shock for 5 min at 42 °C for *K. viridifaciens*. After cooling down the spores to room temperature, *E. coli* cells were mixed with spores in a 1:1 volume, and after brief centrifugation the majority of the supernatant was removed and the cells were resuspended the remaining liquid. Cells were diluted in MilliQ and plated on SFM containing 10 mM $MgCl_2$ followed by incubation at 30 °C for 16 – 20 h, after which a selective overlay was performed with P-buffer [186, 224] containing nalidixic acid to a final concentration of 20 µg ml^{-1} and hygromycin B to select for strains with the conjugated plasmid. Plates were incubated at 30 °C until colonies appeared. Single colonies were grown on MYM medium containing hygromycin B, and acquisition of pIJ82-GFP was further confirmed by fluorescent microscopy of liquid cultures, before generating confluent plates for spore preps.

Strain *alpha*Δ*comEA/EC* (Supplementary Table 2) was obtained by chemical transformation of *alpha* with pRK1 followed by selection for apramycin. Subsequent growth on a nonselective medium allowed for double homologous recombination leading to the replacement of the *comEA/EC* region by an apramycin resistance cassette, yielding thiostrepton-sensitive, apramycin-resistant cells. The strain was verified by PCR using primer pair ComEA_Apra_check_FW and ComEC_Apra_check_RV (Supplementary Table 4) to confirm the replacement of the region by the apramycin cassette. To further confirm the deletion of this region, PCR was performed using primer pairs ComEC_Presence_Check_1_FW/RV and ComEC_Presence_Check_2_FW/RV (Supplementary Table 4), which amplify parts of *comEC* only if this genomic region is still present. Strain *alpha*Δ*ssgB* (Supplementary Table 2) was obtained via a similar process using chemical transformation of *alpha* with pKR1[15] by Van der Meij (2014) [277]. Confirmation of replacement of *ssgB* with the apramycin cassette was performed using primer pairs SsgB_Presence_FW and SsgB_Presence_RV (Supplementary Table 4).

Genomic DNA preparation

Genomic DNA for transformation was isolated from a 120 h culture of *alpha*Δ*ssgB* (Supplementary Table 2) or from a 24 h TSBS medium (containing 30 g L^{-1} Tryptone Soy Broth powder and 10% (w/v) sucrose) culture of *K. viridifaciens* wild-type or *K. viridifaciens* pIJ82-GFP for PCR using phenol:chloroform extraction [223]. After removal of supernatant, walled cells were resuspended in 10 mg ml^{-1} lysozyme (lysozyme from chicken egg white, 70,000 U mg^{-1}, Sigma-Aldrich) for 1 – 2 h. This step was not required for wall-less cells. Next, the cell pellet was resuspended in 10.3% (w/v) sucrose containing 0.01 M ethylenediamine tetraacetic acid (EDTA, VWR Chemicals BDH) pH = 8, following lysis with 10% (w/v) sodium dodecyl sulfate (SDS, 20765.02, Serva). Extraction with phenol:chloroform (1:1 mix of phenol, Fisher BioReagents™ and chloroform, Honeywell) was performed and the nucleic acids were precipitated using isopropanol (Honeywell). The pellet was dissolved in Tris-EDTA buffer (Trizma® base, Sigma-Aldrich), followed by RNase A (EN0531, Thermo Fisher) and Proteinase K treatment (19131, Qiagen). The gDNA was isolated using phenol:chloroform extraction and precipitated using absolute ethanol (Biosolve) before resuspension in nuclease-free water. Fragmented gDNA was obtained by beat-beating the intact gDNA for 12 min using 2 mm diameter glass beads in a Mikro-Dismembrator U (Sartorius) at 2,000 rpm. Chromosomal DNA concentrations were verified using the Quant-IT™ Broad-Range dsDNA Assay Kit (Q33130, Invitrogen) for use in transformation assays.

Preparation of protoplasts from *Kitasatospora*

K. viridifaciens strain DSM40239 was inoculated at a density of 5×10^6 spores ml^{-1} in liquid TSBS:YEME (1:1) medium cultures with 0.5% (w/v) glycine [223] (Duchefa Biochemie) and 5 mM $MgCl_2$ (Duchefa Biochemie). Per litre, YEME medium (Yeast extract-malt extract medium) contains 3 g Difco yeast extract, 5 g Difco Bacto-peptone, 3 g Oxoid malt extract, 10 g glucose, 340 g sucrose, 5 mM $MgCl_2 \times 6H_2O$ and 0.5% (w/v) glycine. The culture was grown for 48 h while shaking at 200 rpm, after which protoplasts were prepared [223]. Cells were washed with 10.3% (w/v) sucrose before lysozyme treatment was performed by the addition of 10 mg ml^{-1} of chicken egg-white lysozyme (~ 70,000 U mg^{-1}, Sigma-Aldrich). The cells were incubated for 2 – 3 h at 100 rpm and 30 °C, after which mycelial fragments were separated from the protoplasts by filtration through a cotton wool filter. Cells were concentrated by centrifugation at 1000×*g* if required.

Isolation of S-cells from *Kitasatospora*

S-cells were isolated from LPB cultures by filtration [15]. In short, the culture was filtered through a sterile EcoCloth™ filter (Contec) and subsequently passed through a 5 µm Isopore™ membrane filter (Merck). The cells were concentrated by gentle centrifugation at 1000×*g* for 20 min, after which 90% of the supernatant was removed. The cell pellet was resuspended

carefully in the remaining liquid. For testing a high concentration of S-cells for spontaneous DNA uptake, *K. viridifaciens* was inoculated at 1×10^7 spores ml^{-1}, and filtration was only performed through the EcoCloth™ filter.

Chemical transformation

Freshly prepared protoplasts, S-cells, L-forms, or mycelial fragments were kept on ice prior to transformation. For chemical transformation, 50 µl of cells were mixed with 1 µg pRed* [278] (Supplementary Table 3), 150 ng gDNA of strain *alpha*Δ*ssgB*, filter-sterilized salt-lysed cells (35 ng DNA from *alpha*Δ*ssgB*), or MilliQ. Then, 200 µl of 25% (w/v) PEG 1000 (NBS Biologicals) in P-buffer was added to the cells, followed by gently mixing and diluting the suspension in P-buffer. Serial dilutions were plated on LPMA medium and after 16 – 18 h incubation, an overlay was performed with 1 ml of P-buffer containing antibiotics. Colony forming units (CFU) were counted after 168 h for L-forms and mycelium or after 336 h for S-cells and protoplasts. Transformants were verified by streaking on a selective medium and microscopy.

Transformation assay

Freshly prepared cells were incubated with 30 ng $µl^{-1}$ unmethylated DNA (pRed* or pFL-*ssgB* as indicated) or MilliQ for 18 – 24 h at 100 rpm unless stated otherwise. A final concentration of 100 or 10 ng $µl^{-1}$ intact gDNA and 10 ng ul^{-1} for fragmented gDNA isolated from *alpha*Δ*ssgB* was used in combination with both 24 h and 168 h *alpha* cultures. Dilutions were plated on selective and non-selective LPMA medium after careful resuspension. Mycelial cells were diluted similarly on MYM medium. Colony forming units were determined after 168 h incubation at 30 °C for L-forms and mycelium and up to 336 h for protoplasts and S-cells. Transformants were verified by growth on a selective medium and by PCR (using primers Tsr_Hyg_FW1 and Tsr_Hyg_RV1 for pFL-*ssgB*) or microscopy. Cells were prepared from at least five replica cultures to compare transformation efficiencies between strains. DNA uptake of S-cells was tested using filtrate obtained via the standard procedure, as well as more concentrated filtrate that was obtained via inoculation of 1×10^7 spores ml^{-1} and filtration of the bacterial culture through the EcoCloth™ filter only. Colony plates were imaged using the Epson Perfection V600 Photo scanner with Epson Scan Utility v3.9.2.0 software.

Membrane fluidity

Three replicate cultures of 24 h, 72 h and 168 h L-form cultures or freshly prepared protoplasts were subjected to a Laurdan dye assay as a measure for membrane fluidity [244]. About 1 ml of each culture was first centrifuged at 1000×*g* for 10 min to remove any traces of the culture media. Cells were resuspended in 1 ml P-buffer and adjusted to an OD_{600} of 0.6. 10 mM Laurdan (6-Dodecanoyl-2-Dimethylaminonapthalene, Invitrogen) stock solution was

prepared in 100% dimethylformamide (DMF, Sigma-Aldrich) and stored at −20 °C in an amber tube. To each 1 ml OD-adjusted culture, 1 µl of Laurdan dye was added to a final concentration of 10 µM. The cultures were then incubated in the dark at 30 °C for 10 min, while shaking at 100 rpm. The cells were washed three times with P-buffer containing 1% dimethyl sulfoxide (DMSO, Sigma-Aldrich) to remove unbound dye molecules before the cells were resuspended in P-buffer. About 200 µl of this resuspended culture was aliquoted into a 96-well black/glass bottom SensoPlate™ (655892, Greiner Bio-One). Three technical replicas were measured per culture, as well as one replica per culture condition without dye to measure background fluorescence.

Sample excitation was performed at 350 nm followed by fluorescence emission capture at 435 and 490 nm, determined using a Spark® multimode microplate reader (Tecan) with Sparkcontrol V3.1 software. After subtracting the background fluorescence, the generalized polarization (GP) value was calculated using Equation (1):

$$GP = \frac{I_{435} - I_{490}}{I_{435} + I_{490}} \quad (1)$$

Values obtained after calculation lie in the range of −1 to +1 with those closer to −1 indicating greater fluidity.

Preparation of cells for quantification of membrane fluidity by microscopy was performed as following. Cells were washed and OD-adjusted as mentioned above. Laurdan dye (stock concentration 10 mM) was added to 100 µl of culture to get a final concentration of 100 µM. The culture was placed at 30 °C for 5 min, while shaking at 100 rpm in the dark. 900 µl of prewarmed P-buffer containing 1% DMSO was added and the culture was centrifuged (1000×*g*, 10 min) to remove any unbound dye molecules. The cells were finally resuspended in 100 µl of P-buffer for microscopy analysis. Cells treated similarly but without Laurdan dye were used as control for microscopy measurements.

DNAse I activity test

The activity of DNAse I (Roche, grade II, from bovine pancreas) was tested by incubation of around 1 µg gDNA (as determined by NanoDrop 2000 Spectrophotometer) of *Streptomyces coelicolor* M145 with 1 mg ml^{-1} DNAse I in LPB medium. The DNAse I solution in LPB was either prepared fresh or was incubated for 20 h at 30 °C prior to use to determine the stability of the enzyme. Incubation with gDNA was performed for 5 and 15 min at 30 °C followed by the immediate addition of 6X DNA Gel Loading Dye (Thermo Scientific) and subsequent agarose gel electrophoresis. As a control, gDNA was incubated in LPB at 30 °C for 15 min without DNAse I.

Co-culture experiments

The 168 h co-culture experiment between S-cells and L-forms was performed as following. *K. viridifaciens* pIJ82-GFP to a final density of 1×10^6 spores ml^{-1} and a 72 h colony of *alpha*Δ*ssgB* grown on LPMA medium were inoculated together (n = 3) or separately (n = 1) in 20-ml liquid cultures containing LPB medium and were incubated for 168 h at 100 rpm. Every 24 h the cultures were homogenized by gentle swirling and a 0.5 ml sample was plated on LPMA medium containing apramycin and hygromycin B. At the same time, dilutions were plated on LPMA medium containing only one of the antibiotics. Colony forming units (CFU) were determined after incubation for up to 168 h. The recombination frequency was calculated as the CFU ml^{-1} resistant to both antibiotics per CFU ml^{-1} of the L-form strain (LPMA medium containing apramycin). The presence of the apramycin cassette in *ssgB* and the integrative pIJ82-GFP in recombinants was confirmed as following. Recombinant colonies were grown on double selection medium (LPMA medium) and the production of cytoplasmic eGFP encoded on the pIJ82-GFP plasmid was confirmed by fluorescence microscopy after growth in LPB medium containing both antibiotics. Lastly, PCR was used to amplify the hygromycin resistance cassette as located on pIJ82-GFP (primer pair Hyg_FW2/ RV2) and to confirm the replacement of *ssgB* by the apramycin resistance cassette (primer pair SsgB_Presence_FW/ RV).

Co-culture experiments in six-wells plates were carried out using a final volume of 4 ml LPB medium. Cells from 72 h cultures of *alpha*Δ*ssgB* inoculated from a colony were collected by gentle centrifugation at 1000×*g* for 10 min and resuspended in fresh LPB medium. *K. viridifaciens* pIJ82-GFP was grown at a density of 1×10^7 spores ml^{-1} in 50 ml LPB without antibiotics for 72 h before collection of S-cells, and the supernatant was replaced by 10 ml LPB medium. Co-cultures were performed at a final concentration of $1 - 5 \times 10^7$ ml^{-1} S-cells (as measured with a haemocytometer) and an OD_{600} of 0.175 of L-forms (~ 1×10^7 CFU ml^{-1} on LPMA medium corresponding to ~1.3×10^7 cells ml^{-1} as measured by a haemocytometer). The strains were also grown individually as control for antibiotic resistance for each condition.

Cellulase (*Aspergillus niger*, Sigma) was added to a final concentration of 10 U ml^{-1} to reduce cell clumping. The concentration of required cellulase was determined by incubation of *alpha*Δ*ssgB* under the same conditions as used for the co-culture assay (but without S-cells), supplemented with or without 5, 10 or 20 U ml^{-1} cellulase and incubated for 24 h at 100 rpm, followed by visual assessment of clumps by microscopy. S-cell filtrate was boiled in a waterbath for 10 min to lyse the cells, followed by pelleting at maximum speed for 2 min, after which the supernatant was kept on ice prior to use. The resulting S-cell lysate was sterile as no growth was detected on LPMA medium. DNAse I (Roche, grade II, from bovine pancreas) was used at a final concentration of 1 mg ml^{-1}. S-cells were incubated with DNAse I for 15 min at 30 °C prior to use in the co-culture. Chicken egg-white lysozyme (Sigma, 70,000 U

mg^{-1}) and penicillin G (Roth, Potassium salt, 1,600 U mg^{-1}) were used at a final concentration of 10 mg ml^{-1} and 0.6 mg ml^{-1}, respectively [15]. This was confirmed by absence of mycelial cells after incubation of *K. viridifaciens* pIJ82-GFP S-cells with lysozyme and penicillin G for 48 h at 30 °C and 100 rpm compared to a control culture without supplements.

The six-wells plates were incubated at 100 rpm at 30 °C for 24 h, after which the cultures were resuspended by pipetting and dilutions were plated in duplo on LPMA medium containing apramycin, hygromycin B or both antibiotics. CFU were determined after incubation for up to 168 h at 30 °C. The recombination frequency was calculated as the number of colonies resistant to both antibiotics relative to the CFU of *alpha*Δ*ssgB*.

PEG-induced cell fusion assay

Cell-cell fusion assays were based on the method by Shitut *et al.*, (2022) [233]. In short, filtered S-cells were adjusted to 75 cells per 4×10^{-6} ml (~1.9×10^8 cells ml^{-1}). L-forms were adjusted to $OD_{600} = 0.7$ corresponding to ~ 1×10^8 cells ml^{-1}. The supernatant of the cells was replaced with P-buffer containing 1 mg ml^{-1} DNAse I after gentle centrifugation for 10 min at 1000×*g*. Excess DNAse I was present during all steps to prevent possible PEG-mediated DNA uptake. Equal volumes of cells were mixed and incubated at room temperature for 10 min, followed by the addition of liquid 100% PEG 1000 (NBS Biologicals) to a final concentration of 10% (v/v) and another incubation at room temperature for 5 min. Cells were pelleted for 10 min at 1000×*g* to replace the supernatant with P-buffer containing DNAse I. Dilutions were plated on LPMA medium containing appropriate antibiotics. As a control, cells from single cultures were subjected to the PEG-fusion assay, as well as mixing different strains briefly and plating immediately on LPMA medium containing selection for both antibiotic resistance markers, which both did not yield recombinant colonies.

Bioinformatic search for putative competence genes and ϕC31 *attB* locus

Protein sequences from *Bacillus subtilis* str. 168, *N. gonorrhoeae,* and *H. pylori* strain P12 were obtained from the UniProt database or literature and are provided in Supplementary Data 1 (published in Kapteijn *et al.,* 2022 [279]). Protein BLAST was run for these sequences against the translated coding sequence database of *Streptomyces viridifaciens* strain DSM40239 (also known as *K. viridifaciens* strain DSM40239), with sequence accession numbers CP090840, CP090841, and CP090842, using the offline BLAST software (v. 2.12.0). Hits with an E-value of 1×10^{-6} or lower were collected (Supplementary Table 1). To determine the location of the ϕC31 *attB* locus in *K. viridifaciens* DSM 40239, the *S. coelicolor* A3(2) strain M145 *attB* sequence (5'CGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTACTCACC 3') [280] was used to perform a BlastN search on the NCBI website against the genome of *K. viridifaciens*. Two putative *attB* sites, namely in BOQ63_022735, encoding a putative pirin family protein, and in BOQ63_033305, encoding a putative chaplin protein, were identified.

To confirm integration of pIJ82-GFP in this site, a touchdown-PCR was performed using Q5 Polymerase to amplify a 400 bp (primer pair Attb_pirin_FW/RV) and 517 bp (primer pair Attb_chaplin_FW/RV) region covering the putative *attB* loci, using gDNA from *K. viridifaciens* wild-type and *K. viridifaciens* pIJ82-GFP as a template. Amplification of the pirin family protein-encoding region resulted in a near-complete loss of PCR product for *K. viridifaciens* pIJ82-GFP (Supplementary Fig. 4), likely due to the integration of pIJ82-GFP in this site that makes the target region too long to amplify. No loss of PCR product was observed for the putative chaplin protein-encoding region. As the *attB* site is also located in a pirin family protein in *S. coelicolor*, this strongly suggests that BOQ63_022735 contains the preferred *attB* integration site.

Imaging and microscopy

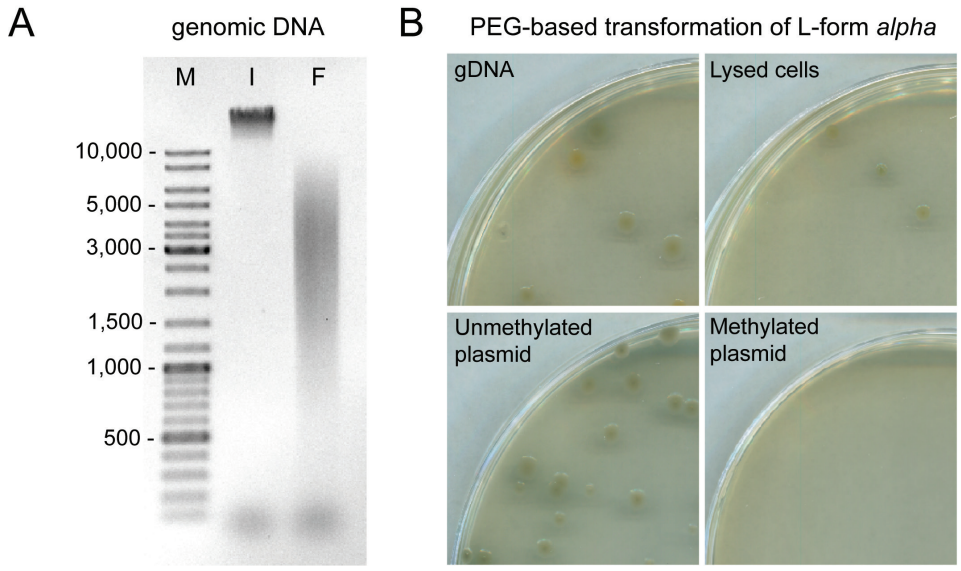
Bacterial growth on solid medium and liquid culture in six-wells plates were imaged using the Epson Perfection V600 Photo scanner with Epson Scan Utility v3.9.2.0 software. Single colonies were imaged using a Zeiss SteREO Discovery v. 8 equipped with a Schott VisiLED Ring Light S80-55 and Bresser MikroCam SP5.0. Bresser MikroCamLabII software was used to capture images. Light microscopy was performed using the Axio Lab A1 Microscope (Zeiss) equipped with an Axiocam 105 color camera (Zeiss) and ZEN 2.5 software (blue edition, Carl Zeiss Microscopy GmbH). Fluorescence microscopy was performed using a Zeiss LSM 900 confocal microscope with Airyscan 2 module and Zeiss Zen 3.1 software (blue edition, Carl Zeiss Microscopy GmbH). For eGFP an excitation wavelength of 488 nm was used and emission was captured between 490-620 nm.

To measure the membrane fluidity, 10 µl of cells were imaged on an µ-Slide 8 Well Ibidi® slide coated with 0.1% (w/v) poly-L-lysine (Sigma-Aldrich; excess poly-L-lysine was removed and the slide was allowed to dry prior to applying the sample). Samples were excited using a 405 nm laser and images were captured at emissions of 430 and 500 nm. GP values were calculated using the Calculate GP plugin in Fiji [281] to obtain a histogram of pixel counts over the range of −1 to +1. Briefly, the image is split into individual channels followed by background subtraction and setting the non-significant pixels to zero. The images are then assigned letters A and B to calculate A − B and A + B using the image calculator. Finally, a ratio of (A − B)/(A + B) is shown as an image where minimum pixel values are set to −1 (red) and maximum pixel values set to +1 (blue). Using the 'analyze histogram' function, a list of values is obtained and used for plotting the distributions of different samples. All image analysis was performed using Fiji (ImageJ) software [282].

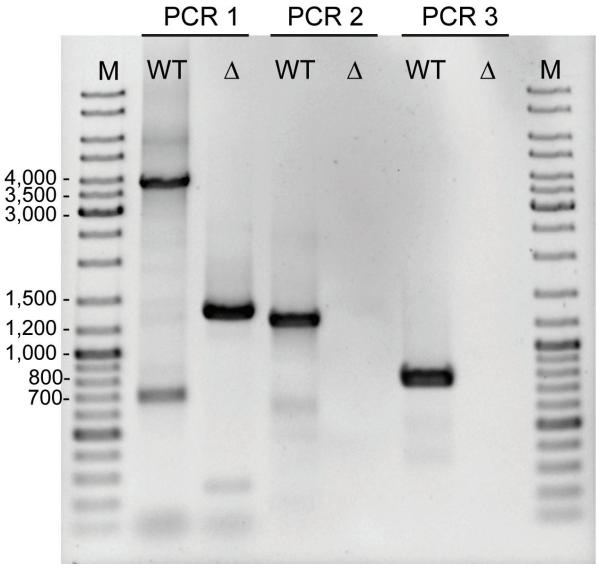
Statistics

All statistics were performed using SPSS statistics software (IBM, version 27.0), with a significance level of 0.05. Normality of data was tested using Q-Q plots, Shapiro-Wilk test and Kolmogorov–Smirnov where applicable. Homogeneity of variances was tested using Levene's test. Bonferroni adjustments were made to *P*-values when multiple individual tests were performed. Means, standard deviations and 95% confidence intervals were calculated and plotted via Excel (version 2016) or Graphpad Prism v. 9.0.0. Graphs were generated using Graphpad Prism v. 9.0.0 or using R version 3.6.1, and other graphical images were generated using Adobe Illustrator v. 26.3.1).

Supplementary Data

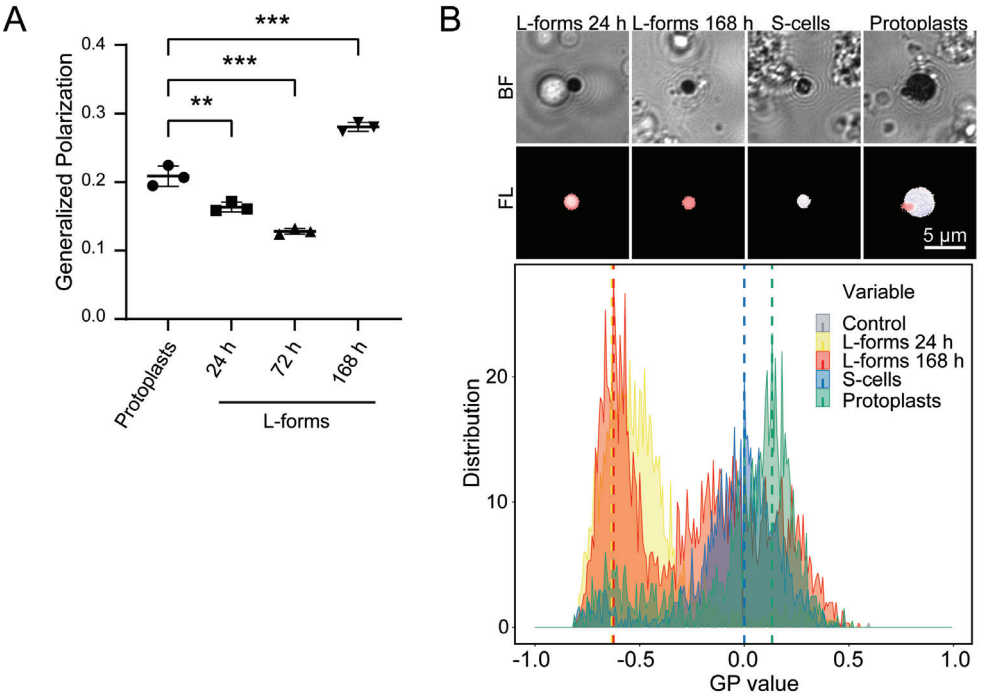


Supplementary Figure 1. gDNA shearing and PEG-based transformation of L-forms
(A) Gel electrograph of 100 ng intact (I) or fragmented (F) gDNA of *alphaΔssgB* as used in the transformation assay in Fig. 1C. One batch of gDNA was prepared, analyzed and used for transformation. M = GeneRuler DNA Ladder Mix (Thermo Scientific) with fragment size indicated in bp. **(B)** PEG-based transformation of *alpha* using gDNA, filter-sterilized salt-lysed cells from mutant line *alphaΔssgB*, and unmethylated or methylated plasmid DNA (pRed*) (n = 1), showing transformation plates. Note that use of methylated DNA inhibited transformation as compared to unmethylated DNA.



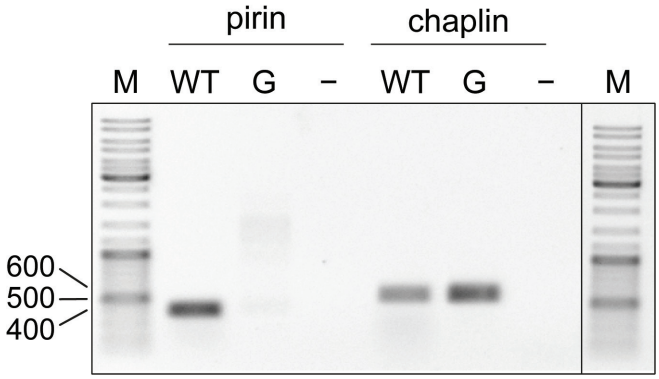
Supplementary Figure 2. PCR verification of *alphaΔcomEA/EC* mutant

Gel electrograph of PCR products from three different PCR mixes to confirm the replacement of *comEA* and *comEC* by an apramycin resistance cassette. M = GeneRuler DNA Ladder Mix (Thermo Scientific) with DNA fragment size indicated in bp; PCR template used is indicated by WT = gDNA *alpha* and Δ = gDNA *alphaΔcomEA/EC*. Expected products: PCR 1 WT = 3676 bp, mutant = 1294 bp; PCR 2 WT = 1197 bp, mutant = no amplification, PCR 3 WT = 745 bp, mutant = no amplification.



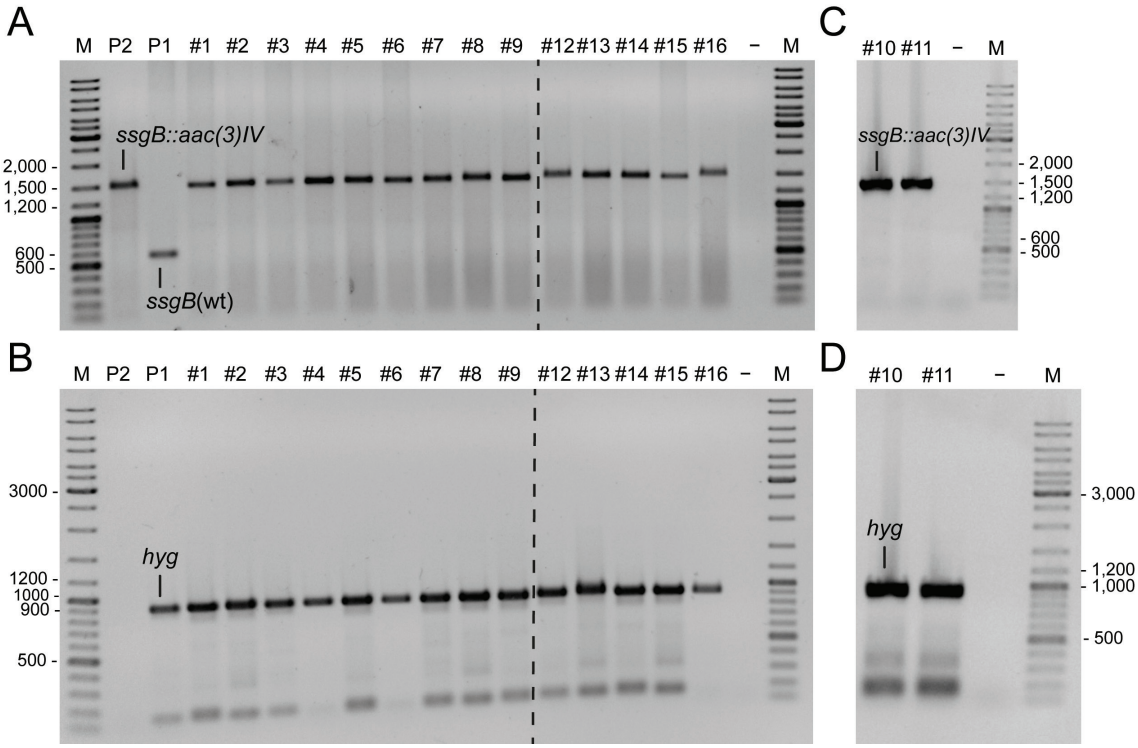
Supplementary Figure 3. Membrane fluidity of cell wall-deficient cells

(A) Generalized Polarization (GP) as measure of membrane fluidity of *K. viridifaciens* protoplasts and 24 h, 72 h and 168 h L-form cultures (*alpha*). Lower GP indicates a higher fluidity. All cell types were significantly different from each other, but this graph only highlights the difference between protoplasts and L-forms. Asterisks (** and ***) indicate $P \leq 0.01$ and $P \leq 0.001$, respectively (one-way ANOVA, F (3,8) = 154.81, P = 2.01e-07, Tukey post-hoc test: protoplasts and L-forms is P = 0.0014 (24 h); P = 2.30e-05 (72 h); P = 5.20e-05 (168 h) and L-form comparison between 24 h –72 h (P = 0.0064); 24 h – 168 h (P = 1.00e-06); 72 h – 168 h (P = 1.65e-07). Data are represented as mean ±SD with individual data points, n = 3 biological replicates. **(B)** Membrane fluidity of L-form *alpha* (24 h and 168 h cultures), S-cells and protoplasts of *K. viridifaciens*. Cells from at least two cultures were combined during cell preparation, and three cell samples were imaged per cell type except for protoplasts (two samples) in one experiment. Top rows show brightfield images (BF) and heatmap of fluorescence emission (FL) (red to blue colour indicate GP values of -1.0 to 1.0 respectively) of representative cells stained with a Laurdan dye for quantifying the membrane fluidity. Bottom panel shows frequency distributions of the Generalized Polarization (GP) with the dashed line indicating the mode GP value. Lower GP values correspond to higher membrane fluidity indicating that L-forms have more fluid membranes compared to S-cells and protoplasts. Control = cells imaged and analyzed without Laurdan staining.



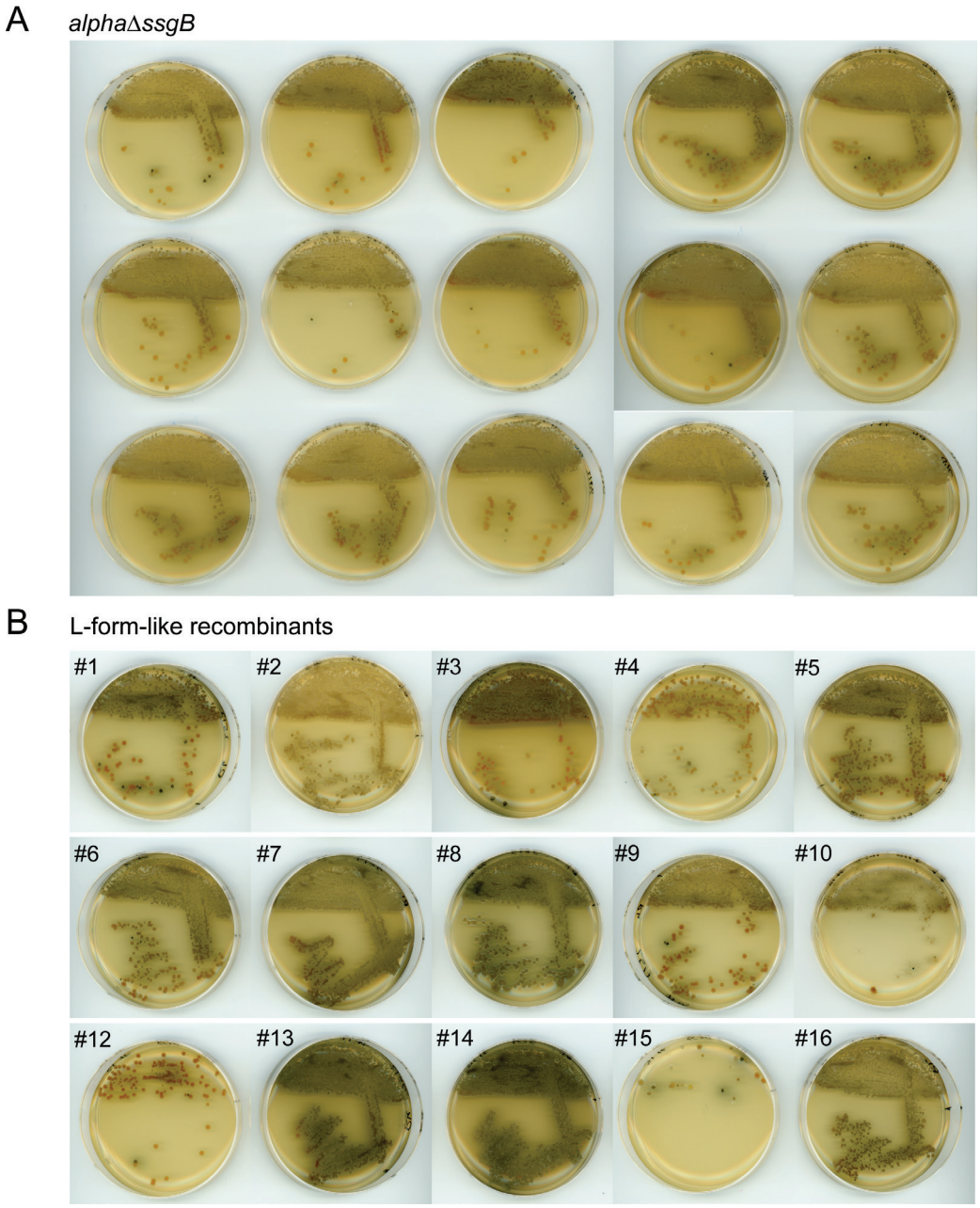
Supplementary Figure 4. Location of *attB* site for integration of pIJ82-GFP

Gel electrograph of PCR products from two PCR mixes to analyze the integration site of pIJ82-GFP in two putative chromosomal *attB* sites. Pirin: PCR amplification of a 400 bp-region of BOQ63_022735 encoding a putative pirin family protein-encoding gene. Chaplin: PCR amplification of a 517 bp-region of BOQ63_033305 encoding a putative chaplin protein. PCR template used is indicated by WT = gDNA *K. viridifaciens* and G = *K. viridifaciens* pIJ82-GFP, - : MilliQ negative control. The lack of amplification for *K. viridifaciens* pIJ82-GFP in the pirin-encoding gene suggests pIJ82-GFP (~ 7 – 8 kbp in size) has integrated in this site. M: GeneRuler DNA Ladder Mix (Thermo Scientific) with DNA fragment size indicated in bp.

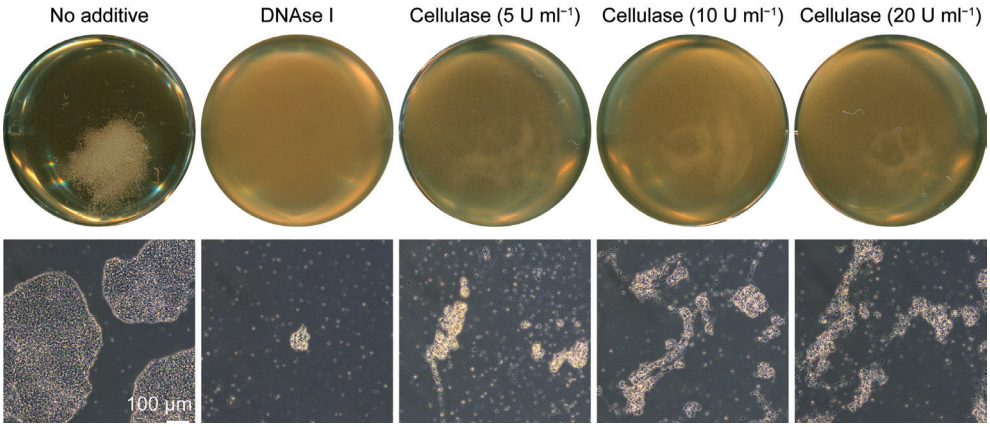


Supplementary Figure 5. Verification of antibiotic resistant cassettes in recombinants

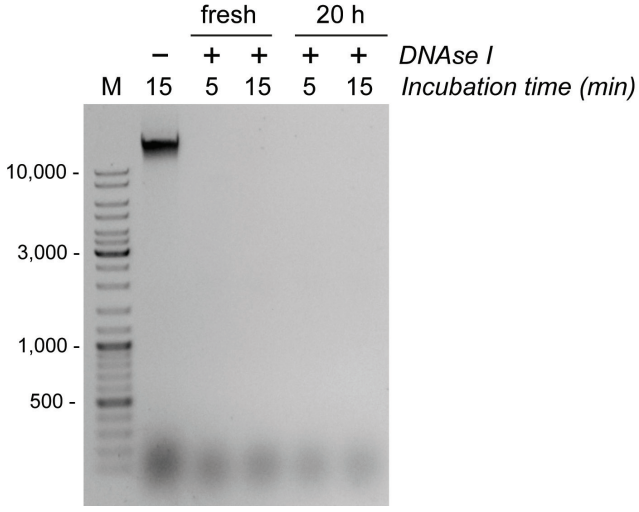
(A – D) Gel electrographs of PCR products to confirm the presence of the apramycin (*aac(3)IV*, **A, C**) and hygromycin resistance cassettes (*hyg*, **B**, **D**) in 15 recombinants, five from each replica experiment from different timepoints. P2: *alphaΔssgB,* P1: *K. viridifaciens* pIJ82-GFP, recombinants nr 1 to 15, – : No template control, M = GeneRuler DNA ladder Mix (Thermo Scientific) with DNA fragment size given in bp. **(A, C)** PCR amplification of the *ssgB* locus (BOQ63_33950), containing either the wild-type (wt) *ssgB* gene (550 bp) as present in *K. viridifaciens* pIJ82-GFP or the *aac(3)IV* resistance cassette (1460 bp) as present in *alphaΔssgB*. **(B, D)** PCR amplification of a 916 bp product of *hyg* to detect the presence of the hygromycin resistance cassette as present in *K. viridifaciens* pIJ82-GFP but not in *alphaΔssgB*.



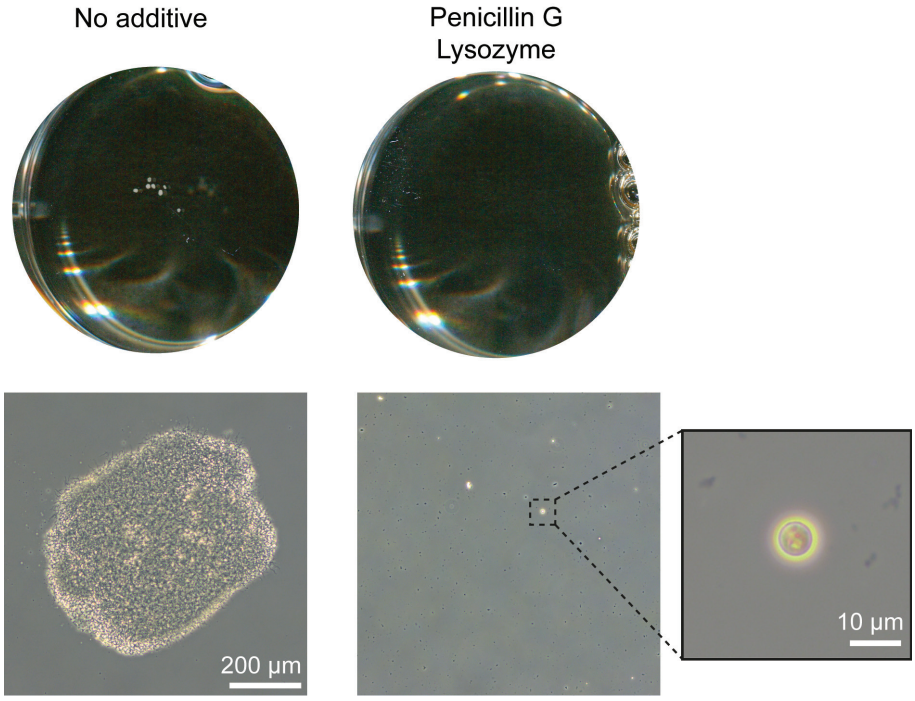
Supplementary Figure 6. L-form recombinants have different phenotypes upon reversion
(A – B) Morphology of 15 random colonies of the parental strain *alphaΔssgB* **(A)** and 15 recombinant colonies displaying L-form-like growth (recombinants #1 to 10 and #12 to 16)**(B)** plated from LPMA medium onto MYM medium to induce mycelial growth, and incubated for 72 h at 30 °C. Note the differences in colouration and biomass of the recombinants compared to the parental strain *alphaΔssgB*.



Supplementary Figure 7. Cellulase as anti-clumping agent during L-form growth
L-form *alphaΔssgB* incubated in LPB (no additive) or in LPB supplemented with 1 mg ml^{-1} DNAse I or 5, 10 or 20 U ml^{-1} cellulase for 24 h at 30 °C and 100 rpm. Top row displays scan images of the six-wells plate, bottom row displays representative brightfield micrographs to indicate cell clumping. Scale bar = 100 µm.

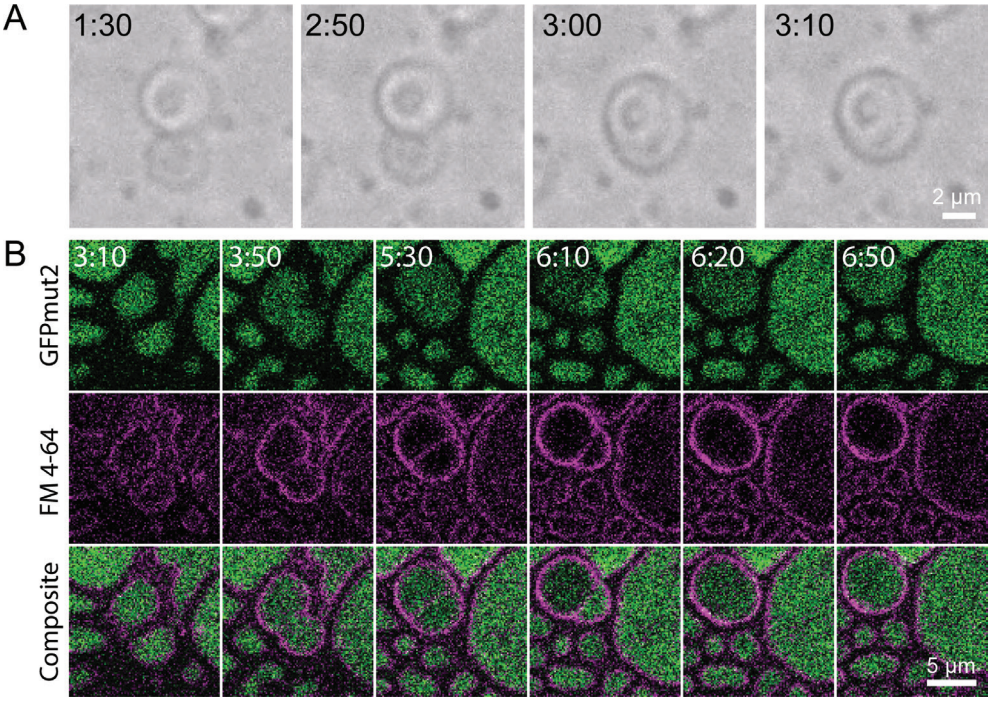


Supplementary Figure 8. DNAse I activity in LPB medium
Gel electrograph indicating DNAse I activity by degradation of gDNA. Around 1 µg gDNA from *Streptomyces coelicolor* M145 was incubated with (+) or without (–) freshly prepared DNAse I (1 mg ml^{-1}, Roche, grade II, from bovine pancreas)('fresh') in LPB medium at 30 °C for 5 or 15 min, prior to performing gel electrophoresis. The same incubation was performed with DNAse I that was incubated in LPB medium for 20 h at 30 °C prior to use ('20 h'), indicating the stability of the enzyme. M: GeneRuler DNA ladder Mix (Thermo Scientific) with DNA fragment size indicated in bp.

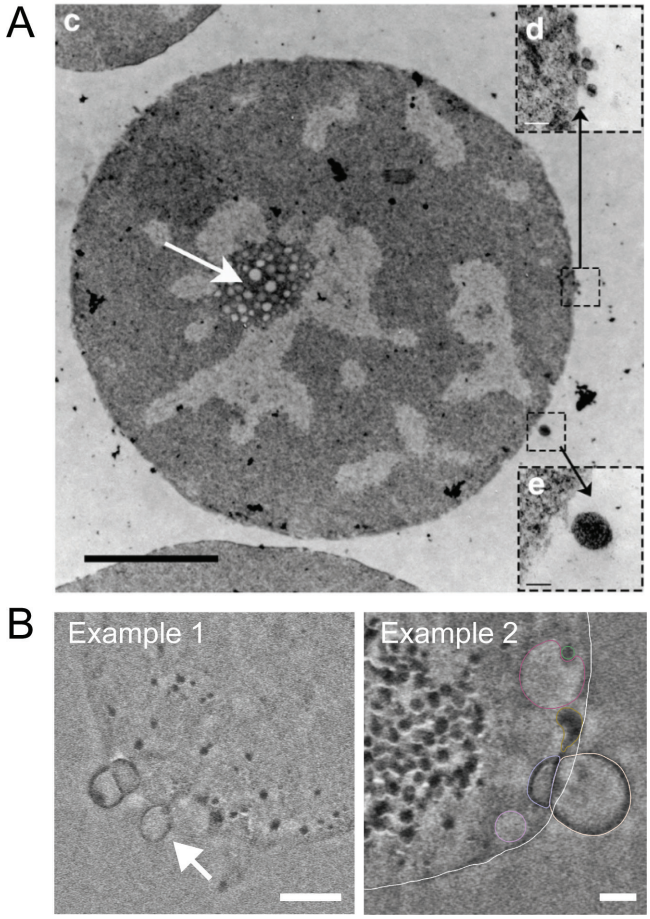


Supplementary Figure 9. Removal of walled cells by penicillin G and lysozyme

S-cell filtrate from *K. viridifaciens* pIJ82-GFP in LPB incubated in the presence or absence of penicillin G (0.06 mg ml^{-1}) and lysozyme (10 mg ml^{-1}) at 30 °C and 100 rpm for 48 h. The additives prevented formation of large clumps consisting of walled and wall-less cells, whereas S-cells were still present. Top row shows scan images of the six-wells plate, bottom row shows representative brightfield micrographs of (absence of) clumps.



Supplementary Figure 10. Spontaneous fusion between L-form cells.
(A) Stills from time-lapse imaging of L-form *delta* in LPB showing fusion between two cells (S. Shitut, unpublished results). Elapsed time is indicated in h:min. Scale bar = 2 µm. **(B)** Stills from time-lapse imaging of an *Escherichia coli* K-12 MG1655 L-form expressing cytoplasmic GFPmut2 (green) in LPB containing 400 µg ml^{-1} penicillin G covered with an LPMA medium agar pad, showing cell-cell fusion (M. Crooijmans, unpublished results). Membrane is visualized using FM 4-64 (magenta). Elapsed time is indicated in h:min. Scale bar = 5 µm.



Supplementary Figure 11. Disorganized cell surface of S-cells and L-forms
(A) Release of small, vesicle-like structures from S-cells of *K. viridifaciens* as imaged using transmission electron microscopy[15]. Scale bar indicates 2 µm (panel 'c') or 100 nm (panels 'd' and 'e' in magnified sections). Panel A is reproduced from Ramijan *et al.*, (2018) [15], Fig. 4 panel c-e (https://doi.org/10.1038/s41467-018-07560-9) under terms of the Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/legalcode). Copyright 2018, The Authors, published by Springer Nature. **(B)** Membrane protrusions visible on the cell membrane of L-form *alpha* of *K. viridifaciens* as imaged using FIB-SEM (Chapter 4, Fig. 3 as published in Kapteijn *et al.*, 2022 [279]). Scale bar indicates 500 nm (Example 1) or 200 nm (Example 2).

Supplementary Table 1. Overview of protein BLAST results

Significant hits (gene locus tag given) (E-value < 1e-06), E-value and percent identity of the indicated protein sequences used as query against *K. viridifaciens* DSM40239. See Supplementary Data 1 from Kapteijn *et al.* (2022) [279] for the full query protein sequences and accession numbers.

Organism	Protein(s)	Hits	E-value	Percent identity
Bacillus subtilis strain 168	ComEA	BOQ63_029625	5.28e-24	34.90%
	ComEC	BOQ63_029630	1.78e-09	26.81%
	ComFA	BOQ63_020315	1.03e-07	24.83%
	ComGA, ComGB, ComGC, ComGD, ComGE, ComGF, ComGG, ComC	–	–	
Neisseria gonorrhoeae	ComE	BOQ63_029625	4.05e-10	46.43%
	ComA, ComP*, PilC, PilD, PilE1, PilV, PilQ, PilF, PilG, PilT	–	–	
Helicobacter pylori strain P12	ComE3, ComB2-4, Com6-10	–	–	

*Protein sequence obtained from Wolfgang *et al.*, (1999) [283]

Supplementary Table 2. Strains used in this study

Strain	Description	Notes/references
Escherichia coli JM109	*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17, *supE*44, *relA*1, λ⁻, Δ (*lac-proAB*), [F', *traD*36 *proAB*, *lacI*^{q}ZΔM15]	272
Escherichia coli ET12567/ pUZ8002	Methylation-deficient strain (F⁻, *dam*-13::*Tn*9, *dcm*-6, *hsdM*, *hsdR*, *recF*143, *zjj*-202::Tn10, *galK*2, *galT*22, *ara*14, *lacY*1, *xyl*-5, *leuB*6, *thi*-1, *tonA*31, *rpsL*136, *hisG*4, *tsx*-78, *mtl*-1, *glnV*44) carrying the non-transmissible pUZ8002 plasmid	273
Kitasatospora viridifaciens DSM40239	Wild-type	DSMZ, 221
Kitasatospora viridifaciens pIJ82-GFP	*K. viridifaciens* DSM40239 containing pIJ82-GFP	This work
alpha	L-form derivative of DSM40239 obtained after exposure to penicillin G and lysozyme	15
M1	L-form derivative of DSM40239 obtained after exposure to hyperosmotic stress	15
delta	L-form derivative of DSM40239 obtained after exposure to penicillin G and lysozyme	233
alphaΔssgB	*ssgB::aac(3)IV*	277
alphaΔcomEA/EC	*(comEA-comEC)::aac(3)IV*	This work

Supplementary Table 3. Plasmids used in this study

Plasmid	Features	Notes/Ref.
pRed*	pIJ8630-derivative expressing *mCherry* under control of the *S. coelicolor A3(2) gap1* promoter	278
pGreen	pIJ8630-derivative expressing *eGFP* under control of the *S. coelicolor A3(2) gap1* promoter	276
pSET152	*E. coli-Streptomyces* shuttle vector; high copy number in *E. coli* and integrating in the ϕC31 *attB* site in *Streptomyces*	284
pIJ82	pSET152-derivative carrying a hygromycin resistance cassette	Kindly provided by Dr. B. Gust (JIC)
pIJ82-GFP	pSET152-derivative expressing *eGFP* under control of the *S. coelicolor gap1* promoter	This work
pMS82	*E. coli-Streptomyces* shuttle vector integrative in the ϕBT1 *attB* site for genomic integration in *Streptomyces*	274
pWHM3-oriT	Self-replicating, multi-copy, unstable plasmid harboring *oriT,* used as *E. coli/Streptomyces* shuttle vector	275
pWHM3-oriT-hyg	pWHM3-oriT-derivative carrying a hygromycin resistance cassette inserted into the *tsr* gene in the EcoRV site	This work
pFL-*ssgB*	pWHM3-oriT-hyg-derivative containing a hygromycin resistance gene and a downstream flanking sequence of *ssgB* derived from pKR1 to enable integration into the genome	This work
pRK1	pWHM3-oriT containing both flanks of the *comEA-comEC* region interspersed with the *apra-loxP* cassette conferring resistance to apramycin	This work
pKR1	pWHM3-based construct used to replace *ssgB* by *aac(3)IV*	15

Supplementary Table 4. Primers used in this study

Primer	Sequence (5' – 3')	Notes	Ref.
Hyg_F-231_EEV	ctgaGAATTCGATATCGATCGG CGGGGCCTGGCGGCG	Amplification of the hygromycin resistance cassette from pMS82	This work
Hyg_R+1237_HEV	ctgaAAGCTTGATATCGGATCC TTGCCGAGCTGGGAT	Amplification of the hygromycin resistance cassette from pMS82	This work
FL1-comEA/comEC-FW	GACGAATTCAGGACCGGATG CACCGGTTC	Amplification of flank 1 of *comEA-comEC* locus	This work
FL1-comEA/comEC-REV	GAATCTAGACCGCACCGTCT CGTTGATCG	Amplification of flank 1 of *comEA-comEC* locus	This work
ComEA_Apra_check_FW	CACTCGTGTGAGTGACCGTT	Amplification of *comEA* region in PCR1 mix	This work
ComEC_Apra_check_RV	AACGGCAAGGGTGGACG	Amplification of *comEA* region in PCR1 mix	This work
ComEC_Presence_Check_1_FW	TACGACACCGAGTCCGCAG	Amplification of *comEC* region 1 in PCR2 mix	This work
ComEC_Presence_Check_1_RV	CGCAAGGGCCAACATGTCTC	Amplification of *comEC* region 1 in PCR2 mix	This work
ComEC_Presence_Check_2_FW	AGACCCTCCTCACCGTCAAG	Amplification of *comEC* region 2 in PCR3 mix	This work
ComEC_Presence_Check_2_RV	GACAGCAGGAAACCGAAGGA	Amplification of *comEC* region 2 in PCR3 mix	This work
gap1_FW_BglII	GATTACAGATCTCCGAGGGC TTCGAGACC	Amplification of the region containing the *gap1* promoter and *eGFP* gene from pGreen	This work
egfp_RV_EcoRI	TAAGCAGAATTCTTACTTGTA CAGCTCGTCCA	Amplification of the region containing the *gap1* promoter and *eGFP* gene from pGreen	This work
SsgB_Presence_FW	GGCGGGTACTCCGTGATGAT TC	Confirm *ssgB* replacement by *apra-loxP* cassette	277
SsgB_Presence_RV	AGCTTTCGGCGAGGATGTGG	Confirm *ssgB* replacement by *apra-loxP* cassette	277
Tsr_Hyg_FW1	AAGGCCAAGACATTCGGCAT	Confirm presence of pFL-*ssgB* in natural transformants	This work
Tsr_Hyg_RV1	CGAGCGACGTGCGTACTATC	Confirm presence of pFL-*ssgB* in natural transformants	This work
Attb_pirin_FW	GTACTGACCGTCGCCACC	Check integration of pIJ82-GFP in putative ϕC31 *attB* locus, 400 bp product in wild-type	This work
Attb_pirin_RV	ACTTCGGGGCGATCATCTTG	Check integration of pIJ82-GFP in putative ϕC31 *attB* locus, 400 bp product in wild-type	This work
Attb_chaplin_FW	TGCGTTGTCCATACAGGAGG	Check integration of pIJ82-GFP in putative ϕC31 *attB* locus, 517 bp product in wild-type	This work
Attb_chaplin_RV	ATATGCGGCAATCACGCAAG	Check integration of pIJ82-GFP in putative ϕC31 *attB* locus, 517 bp product in wild-type	This work
Hyg_FW2	CCCTGTGAATAGAGGTCCGC	Confirm presence of hyg. B resistance gene	This work
Hyg_RV2	AGTCGTGCAGGAAGGTGAAG	Confirm presence of hyg. B resistance gene	This work