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Live-cell imaging of *Streptomyces* conjugation

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ABSTRACT

Time-lapse imaging of conjugative plasmid transfer in *Streptomyces* revealed intriguing insights into the unique two-step conjugation process of this Gram$^+$ mycelial soil bacterium. Differentially labelling of donor and recipient strains with distinct fluorescent proteins allowed the visualization of plasmid transfer in living mycelium. In nearly all observed matings, plasmid transfer occurred when donor and recipient hyphae made intimate contact at the lateral walls. Plasmid transfer does not involve a complete fusion of donor and recipient hyphae, but depends on a pore formed by the FtsK-like DNA translocase TraB. Following the initial transfer at the contact site of donor and recipient, the plasmids spread within the recipient mycelium by invading neighboring compartments, separated by cross walls. Intra-mycelial plasmid spreading depends on a septal cross wall localized multi-protein DNA translocation apparatus consisting of TraB and several Spd proteins and is abolished in a *spd* mutant. The ability to spread within the recipient mycelium is a crucial adaptation to the mycelial life style of *Streptomyces*, potentiating the efficiency of plasmid transfer.

1. Introduction

Conjugation, the transfer of DNA between two bacterial cells by direct cell-to-cell contact is a major route of horizontal gene transfer, a crucial process for the adaptation and evolution of genomes (Daubin and Szollosi, 2016). The complex life cycle of antibiotic producing streptomycetes poses a particular challenge for conjugative DNA transfer. Unlike unicellular bacteria that divide by binary fission, the non-motile gram-positive soil bacterium *Streptomyces* grows by apical tip extension and forms a multiply branched mycelium, which is usually septated at irregular intervals (Flärdh, 2003). Upon partial nutrient limitation, distinct non-branched hyphae, the so-called aerial mycelium, grow up into the air and become transformed into spore chains (Flärdh and Buttner, 2009). Aerial mycelium and spore chains differ from the substrate mycelium in membrane composition (Poralla et al., 2000) and an amyloidic surface layer, the rodlets, composed of hydrophobic chaplin and rodlin proteins (Claessen et al., 2004; Elliot et al., 2003). Sporulation involves the simultaneous formation of dozens of septal cross walls and the distribution of the multiple chromosomes to the prespores, yielding uninucleoic spores. After thickening of the spore walls, the ovoid spores are released by the action of peptidoglycan hydrolases (Haiser et al., 2009), giving rise to a new life cycle.

Streptomyces conjugation can only be observed, when donor and recipient grow on solid surfaces, but not in liquid culture. Conjugative plasmid transfer was initially discovered in *Streptomyces* by the formation of pock structures, visible with the mere eye (Bibb et al., 1981). Pocks are formed, when a plasmid-carrying donor was plated on a lawn of recipient spores. Then, up to 3 mm circular inhibition zones, where the differentiation is temporary retarded develop on the plate (Hopwood and Kieser, 1993; Pettis, 2018; Thoma and Muth, 2016). Replica plating on selective agar revealed that the size of the pocks exactly matched the transconjugant areas, where the recipient has obtained a plasmid by conjugation (Kieser et al., 1982; Thoma et al., 2016). The most plausible explanation for the pock-sized transconjugant area was that, following the initial transfer from the donor, the plasmid spread within the recipient mycelium thereby colonizing larger parts of the recipient (Hopwood and Kieser, 1993).

In contrast to conjugative plasmids of unicellular bacteria, even small *Streptomyces* plasmids of less than 10 kb in size, e.g. the 8830 bp pIJ101, are conjugative (Kieser et al., 1982). Such plasmids contain less than ten genes, organized in few operon structures. From various conjugative *Streptomyces* plasmids conjugation genes have been identified by subcloning and deletion analyses (Servín-González, 1993; Kataoka et al., 1991; Reuther et al., 2006b). These studies revealed four distinct loci involved in conjugation: i. *traB* (*kilA*), whose unregulated

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expression is lethal and which encodes an FtsK-like DNA-translocase. ii. *traR* (*korA*), encoding a GntR type transcriptional repressor which controls expression of *traB* (*kilA*), iii, the *cis*-acting locus of transfer (*clt*), a non-coding sequence essential for plasmid transfer, which contains series of imperfect 8-bp repeats (TRS), iv. three (or more) *spd* genes, not essential for conjugative plasmid transfer, but affecting the pock size.

TraB of the *S. venezuelae* plasmid pSVH1 was purified and characterized as a membrane-associated ATPase that assembled to hexameric rings with a central ~30 Å pore, highly resembling the structure of *Pseudomonas aeruginosa* FtsK. Purified $TraB_{pSVH1}$ was able to spontaneously insert into artificial membranes and form characteristic voltage dependent pore structures in lipid bilayers (Vogelmann et al., 2011). TraB interacts with the plasmid DNA by binding to the *clt* locus. DNA binding is achieved by the c-terminal winged helix-turn-helix fold of TraB, with helix α3 recognizing the 8-bp TRS repeat. Domain swapping experiments with TraB proteins of different *Streptomyces* plasmids revealed that exchange of only 13 aa corresponding to helix α3 was sufficient to switch specificity of TraB towards recognition of a different *clt* region (Vogelmann et al., 2011).

Detailed mutant analyses, excluding polar effects, revealed three *spd* genes affecting pock size in plasmids pIJ101, and pSVH1 (Kendall and Cohen, 1988; Thoma et al., 2015). These genes are translationally coupled with overlapping stop and start codons and encode membrane proteins. The predicted Spd proteins of plasmid pIJ101 and pSVH1 do not show any sequence similarity and none of the predicted proteins contains a functionally characterized Pfam domain. Bacterial two-hybrid analyses revealed a complex interaction pattern of the Spd proteins, TraB, and other plasmid encoded proteins with no obvious function (Thoma et al., 2015, 2016).

2. Possible cellular sites of conjugative plasmid transfer in *Streptomyces* matings

Streptomyces grows by incorporating peptidoglycan (PG) precursors mainly at the hyphal tip. This characteristic type of growth is directed by a protein assembly, the “polarisome” or “Tip Organizing Center (TIPOC), which includes the coiled-coil proteins DivIVA and Scy, and the stress-bearing FilP cytoskeleton (Holmes et al., 2013; Fuchino et al., 2013). New sites of growth arise by hyphal branching. Such lateral branches are formed by small DivIVA cluster remains at the lateral walls, which were generated by splitting off and separation from the main DivIVA assembly at the extending tip (Flärdh et al., 2012).

Hyphal growth by apical tip extension raises the question, which part of the mycelium is involved in conjugation (Fig. 1). Since a TraB-eGFP fusion protein was localized to the hyphal tips (and putative branching points), it was suggested that *Streptomyces* conjugation involves the tips (Reuther et al., 2006a). Tip fusion was a plausible model for two reasons: First, *Streptomyces* grows as a non-motile mycelium by apical tip extension. Therefore, the only possibility of mating pair formation is that a tip extends until it meets the tip or the lateral wall of a mating partner. The lateral walls of *Streptomyces* mycelium are unable to make contact by their own. Second, hyphal tips are equipped with the complete cell wall remodeling machinery to promote growth by tip extension. Since *Streptomyces* plasmids usually do not encode a PG-hydrolase, they depend on host encoded PG-hydrolases to penetrate cell envelopes of donor and recipient.

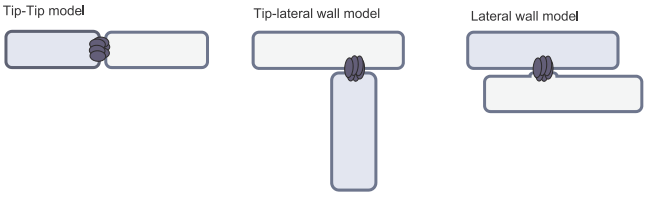

Fig. 1. Theoretical models of donor-recipient contact formation during *Streptomyces* matings. Due to the mode of growth by apical tip extension, tips are the most plausible sites of DNA transfer. Tips seem to be the only compartments of the *Streptomyces* mycelium, able to make contact to a mating partner and already contain the enzymatic equipment for cell wall remodeling. In the tip-tip model (left), DNA transfer directed by the hexameric TraB (blue) occurs at fused tips. In the tip-lateral wall model, tip-localized TraB transfers the DNA at the lateral wall, whereas in the lateral wall model, DNA transfer proceeds via the lateral walls, maybe at an emerging branching point (as indicated).

Mating of filamentous ascomycete fungi occurs by hyphal fusions, resulting in cytoplasmatic intermixing. Hyphal fusions are essential in the sexual development but are also common in all stages of the ascomycete life cycle. Live-cell imaging of hyphal fusions in *Neurospora crassa* showed that the hyphae are able to fuse tip-to-tip, tip-to-side, and also side-to-side (involving the induction of branches) (Hickey et al., 2002; Glass et al., 2000).

3. Detection of plasmid transfer at the lateral walls via a mating pore

To visualize the sites of conjugative DNA transfer in *Streptomyces*, matings with differentially labelled donor and recipient strains were performed (Thoma et al., 2016). To label plasmid-carrying hyphae, eGFP was inserted in a pIJ101 derivative (pLT303) and introduced into *Streptomyces lividans* TK23. The recipient was labelled by the integration of the *mCherry* gene into the chromosomal PhiC31 attachment site. In this experimental set up, the donor lights up in green and the recipient in red, when irradiated with light of the appropriate wave length. If the plasmid has been transferred to the recipient, the transconjugant hyphae show fluorescence in both channels, appearing yellow in the overlay (Thoma et al., 2016). Whenever plasmid transfer was observed, donor and recipient were in contact via the lateral walls, but not at the tips (Thoma et al., 2016). This indicates that conjugative plasmid transfer proceeds via the lateral walls and not by tip fusion, as it was suggested previously (Reuther et al., 2006a). However, contact via the lateral walls does not exclude involvement of the hyphal tip. When mycelia grow on agar and hyphae come into close contact, the hyphal tips usually avoid each other and continue to grow side by side. Therefore, one could speculate that the impact of the donor tip hitting the recipient mycelium results in the collapse of the TIPOC. As a consequence a new tip would be established in a distance too short to be resolved by light microscopy. Such an arrest of growth, collapse of the tip, and redistribution of polarisome assemblies to new sites, close to the original tip has been shown to occur in *Streptomyces* mycelium after recovering from hyperosmotic shock (Fuchino et al., 2017).

At the contact sites of donor and recipient, red fluorescence of the recipient was never observed on the donor side (Thoma et al., 2016). This demonstrated that donor and recipient compartments do not completely fuse, which would have resulted in the intermixing of the cytoplasms. In contrast, a conjugation pore, specific for the transfer of DNA but excluding the diffusion of larger proteins, like mCherry is more likely. Thus, DNA transfer between two *Streptomyces* hyphae is clearly discriminated from the mating process of filamentous ascomycete fungi.

4. Visualization of plasmid transfer during *Streptomyces* matings by time lapse microscopy

The ability to distinguish donor and recipient allowed the observation of conjugative DNA transfer by time lapse microscopy. Donor and recipient spores were spotted onto sterile uncoated μ-dishes (IBIDI) to allow gas exchange (Jyothikumar et al., 2008) and covered with minimal agar supplemented with growth factors and low amounts of thiostrepton (2.5 μg ml^{-1}) to induce *egfp* expression. The samples were incubated at 30 °C and observed with a Nikon Eclipse Ti-E inverted optical microscope (unpublished results, Fig. 2). Images were taken at 20 min intervals. When a donor hypha accidentally met the recipient

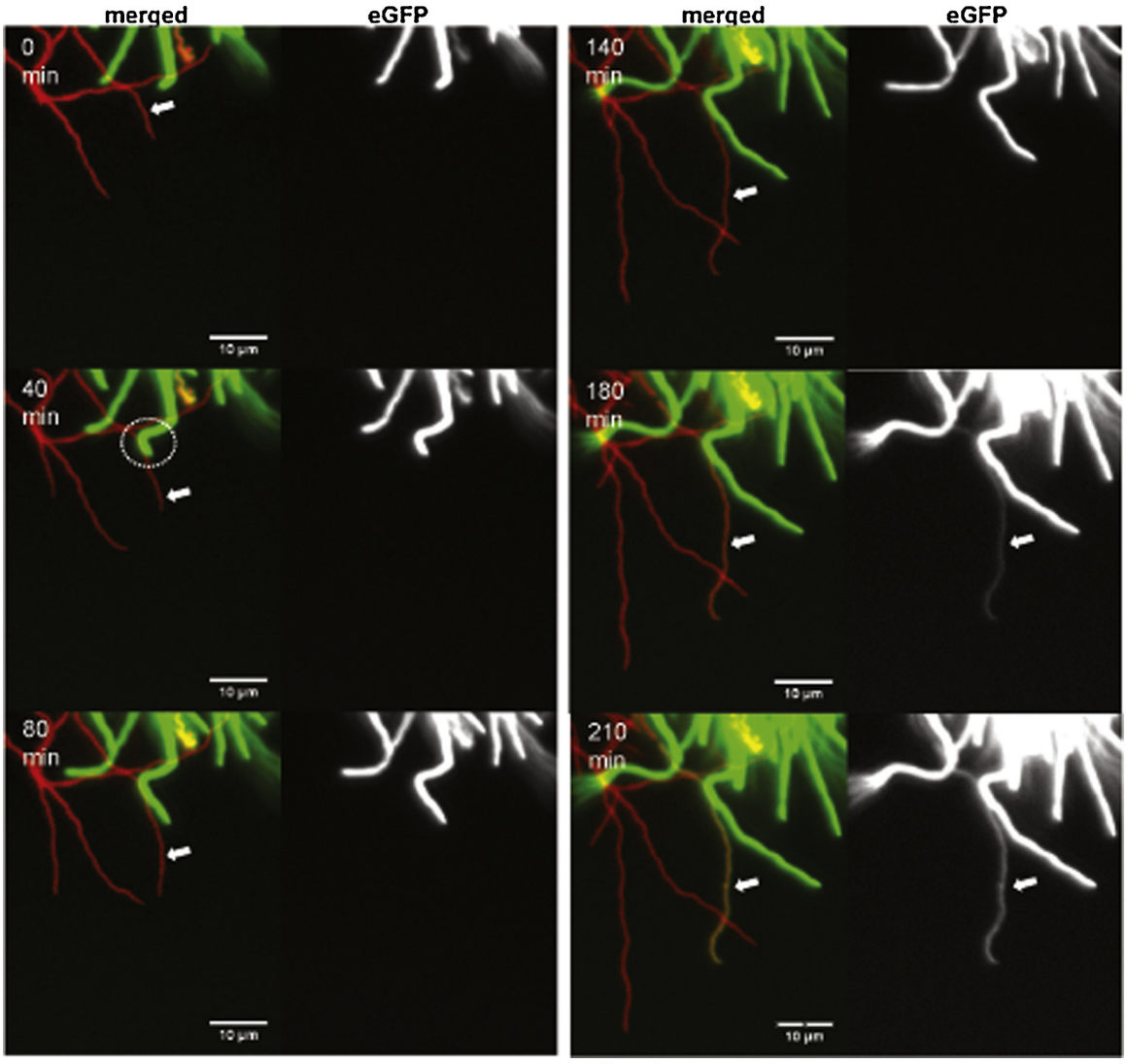

Fig. 2. Detection of conjugative plasmid transfer by time lapse fluorescence microscopy. Spores of the donor *S. lividans* TK64, carrying the eGFP encoding conjugative plasmid pLT303 (green) and *S. lividans* LT23-mCherry (red) were spotted onto µ-dishes (IBIDI, uncoated) and covered with supplemented minimal agar. At 20 min intervals, pictures were taken. Images on the right side show the eGFP-channel, while on the left side, the merged eGFP/mCherry images are displayed. The dashed circle marks the contact site of donor and recipient, where plasmid transfer takes place. The white arrow marks the recipient hyphae that obtains the plasmid (yellow in the overlay). Microscopy images are representative of different conjugation events.

hypha (white arrow), the tip of the donor hypha bended and grew next to the recipient hyphae for about 5 µm (dashed circle), before separating again. About 2 h later, eGFP was visible in the complete recipient hyphae, indicating that the plasmid had been transferred and already spread. At later time points, expression of eGFP further increased. The time delay in the detection of plasmid transfer in this experimental setup might reflect the time period the plasmid requires for transfer, replication/ establishment in the recipient and expression of the *egfp* gene.

A complete overview on the time scale of the mating process, starting from the germination of donor and recipient spores is depicted in Fig. 3 (unpublished results). When spotted on IBIDI µ-dishes, spores started to germinate and develop germ tubes after ˜ 7–8 h. The extending hyphae of donor and recipient came in contact after ˜ 16–17 h. At the contact sites plasmid transfer was taking place, although eGFP was detectable in the recipient only 4.5–5 h later. Following the transfer from the donor, the plasmid spread in the recipient hyphae in both directions. After ˜ 30 h, large areas of the recipient mycelium were colonized by the plasmid (Fig. 3, lower panel, orange/yellow hyphae).

5. Association of pock formation with the spreading of the plasmid in the recipient mycelium

Apparently, the newly transferred plasmid spread in the recipient mycelium, visualized by the appearance of orange/yellow hyphae (Figs. 2 and 3). The plasmid was not only distributed to newly growing filaments, but also travelled backwards to older mycelial compartments, sometimes even reaching the spore compartment, from which the recipient mycelium started to grow (Thoma et al., 2016). This intramycelial spreading implies that the plasmid was able to pass vegetative cross walls, which occur under these growth conditions in irregular distances of 10–20 µm. Visualization of plasmid spreading in the recipient hyphae is in agreement with the concept of intra-mycelial plasmid spreading within the pock area.

spd genes on *Streptomyces* plasmids have been originally discovered by their effect on the size of the developing pock structures. Inactivation of a single *spd* gene or deletion of all *spd* genes does not interfere with conjugative transfer of the plasmid from the donor to the recipient, but it results in hardly visible pocks and tiny transconjugant regions after replica plating on selective agar. Fluorescence microscopy

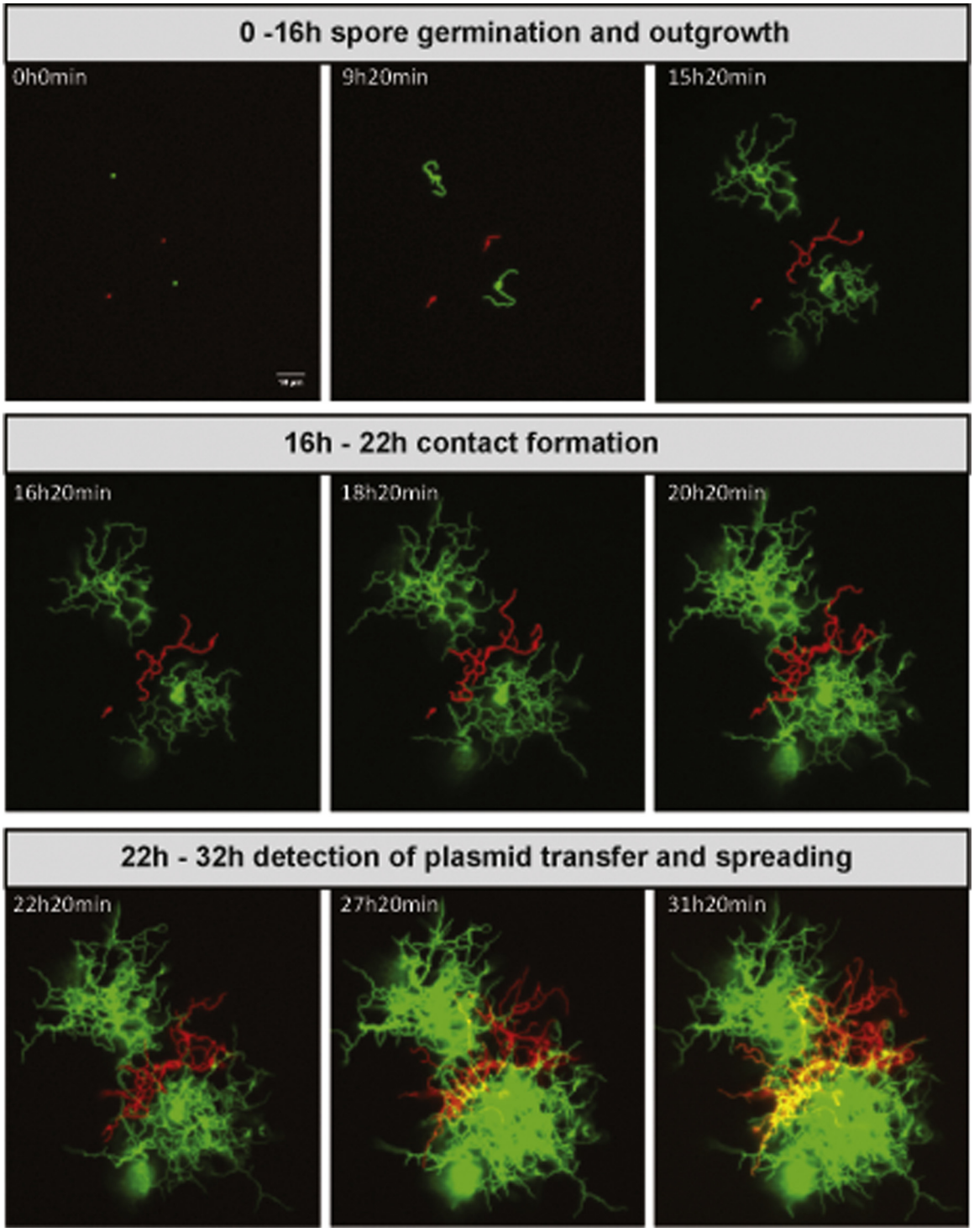

Fig. 3. Overview of conjugative plasmid transfer during *Streptomyces* conjugation. The experimental set up was as described in the legend to Fig. 2. Starting from the germination of spores, the outgrowing mycelium was observed at 20 min intervals for 32 h with a Nikon Eclipse Ti-E inverted microscope, equipped with a 40x air objective and appropriate filter sets. Contact between donor and recipient hyphae was formed after about 16 h. Plasmid transfer and intramycelial spreading of plasmid pLT303 (yellow in the overlay) was visible after 22 h. Microscopy images are representative of different conjugation events.

now demonstrated that the *spd* genes are in fact required for the successive spreading of the plasmid to the older compartments of the recipient. When the *spd* genes of plasmid pLT303 were deleted, transfer of the resulting plasmid pLT303ΔSpd was restricted to the recipient compartment contacting the donor and the hyphal compartments extending thereof (Thoma et al., 2016).

Intramycelial plasmid spreading depends on the interaction of the DNA-translocase TraB with the Spd proteins (Thoma et al., 2016). The complex protein-protein interaction pattern of the Spd proteins suggested that the Spd proteins assemble to a septal cross wall traversing multi-protein complex (Thoma et al., 2015).

A prominent Spd protein is the coiled-coil integral membrane protein SpdB2. SpdB2 of plasmid pSVH1 was co-purified with TraB and interacted with SpdA and Spd79. It was shown to form oligomers, bind PG and non-specifically to ds-DNA, but not to a single-stranded one (Tiffert et al., 2007). SpdB2 formed membrane pores *in vivo*, promoting

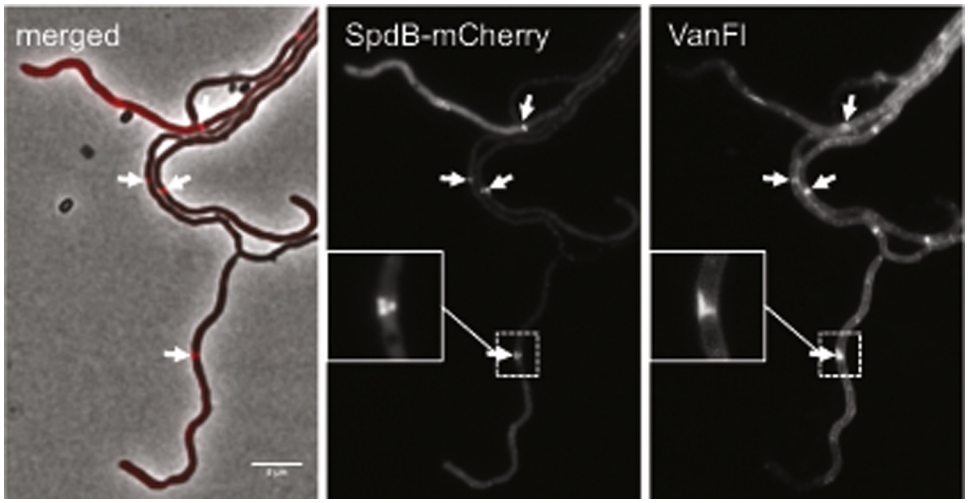

Fig. 4. Localization of SpdB$_{pIJ101}$-mCherry to vegetative cross walls of *S. lividans* mycelium. *spdB* of plasmid pIJ101 was amplified and fused to a mCherry encoding sequence by Gibson assembly and inserted into the NdeI-linearized expression vector pGM1190 (Vollmer and Muth, unpublished results). *S. lividans* (pGM1190-SpdB-mCherry) was grown on coverslips inserted into R5-agar supplemented with thiostrepton (12.5 µg/ml) to induce expression of *spdB-mcherry*. After 63 h of growth, the coverslip was stained with BODIPY FL Vancomycin (VanFl) (right image) to visualize sites of PG synthesis and subjected to fluorescence microscopy. Red fluorescence channel (middle image) and the overlay (left image) of red channel (SpdB-mCherry) and green channel (VanFl) are shown. The inserts show a zoomed-in view of the SpdB-mCherry localization to the cross wall.

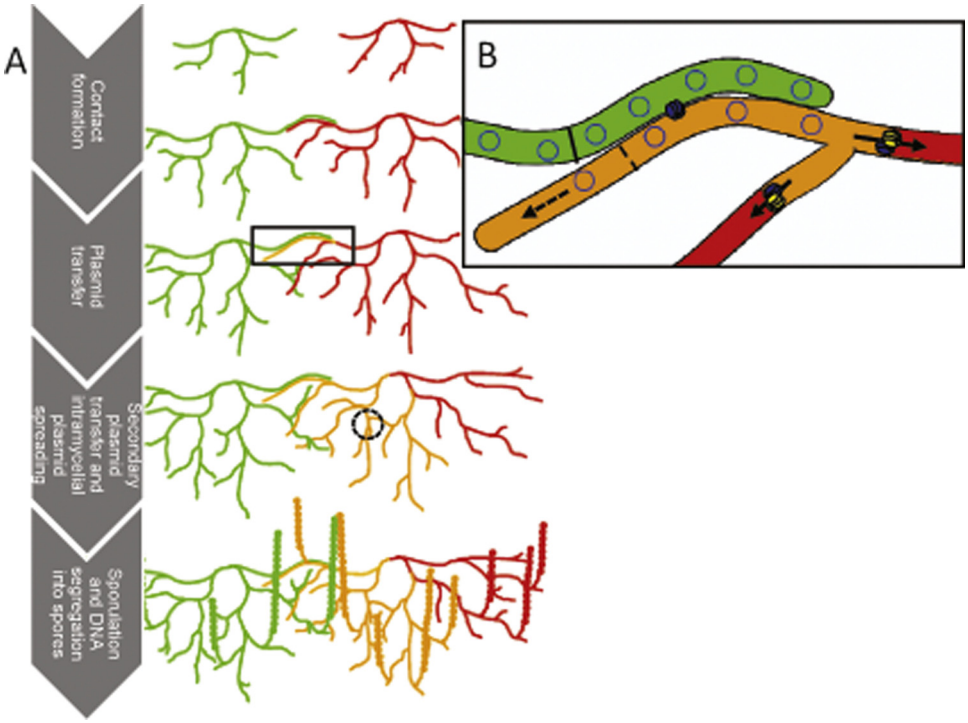

Fig. 5. Colonization of a *Streptomyces* colony by a conjugative plasmid. Colonization of *Streptomyces* mycelium by a conjugative plasmid involves three distinct routes. First, the plasmid propagates in extending hyphae, before formation of septal cross walls (dashed arrow, Fig. 5B). Second, each transconjugant compartment is a potential donor, able to transfer the plasmid to plasmid-free recipient hyphae (dashed circle, Fig. 5A). Third, the Spd-apparatus (blue and yellow ellipses, 5B) directs intra-mycelial DNA-translocation events across preformed cross walls (arrows, 5B). The combined action of these distinct processes ensures the efficient colonization of the recipient mycelium before sporulation.

spheroplast formation of *E. coli* BL21 expressing the T7 lysozyme, and *in vitro*, as demonstrated by single channel recordings in planar lipid bilayers (Thoma et al., 2015).

To study localization of Spd proteins, the *spdB2* homologue of plasmid pIJ101 (*spdB*) was fused to *mCherry* and cloned under control of the thiostrepton inducible P_{tipA} promoter, yielding pGM1190-SpdB-mCherry (Vollmer and Muth, unpublished results). *S. lividans* (pGM1190-SpdB-mCherry) transformants were grown on a coverslip inserted into R5 agar supplemented with 12.5 µg ml^{-1} thiostrepton. After 63 h incubation at 30 °C, the mycelium attached to the coverslip was stained with BODIPY FL Vancomycin (VanFl) to visualize vegetative cross walls. Besides a diffuse irregular fluorescence in parts of the mycelium, which was the dominating signal at early time points (< 27 h), specific red fluorescence foci were detected in the mycelium with a preference to sites where cross walls have been formed, as revealed by VanFl staining (unpublished results, Fig. 4, right panel). Although this indicates localization of SpdB to the vegetative cross walls in the absence of other Spd proteins and Tra, these data have to be carefully interpreted. First, the functionality of the SpdB-mCherry fusion construct could not be tested. Second, expression of *spdB-mCherry* under control of the P_{tipA} promoter probably does not represent the native expression level of the KorA-controlled *tra-spd* operon (Stein et al., 1989). This might be reflected by the irregular, diffuse SpdB-mCherry fluorescence in parts of the mycelium. Nevertheless, preference of SpdB-mCherry to the vegetative cross walls in the absence of any other Spd protein or TraB suggests that SpdB might recruit its interaction partner to the cross walls. This supports the model that intramycelial plasmid spreading is mediated by a cross wall traversing multi-protein apparatus, with the DNA translocase TraB pumping the plasmid to the neighboring compartment (Thoma et al., 2015).

6. Conclusion: *Streptomyces* conjugation, a process highly adapted to the *Streptomyces* life style

Conjugative DNA transfer in *Streptomyces* is a distinct and unique process (Thoma and Muth, 2016) differing in several aspects from the

paradigm conjugation system of uni-cellular bacteria: i. it does not require a plasmid-encoded specific mating pair formation system, like pili or aggregating proteins. ii. it does not depend on a conjugative relaxase and a type IV secretion system, but relies on a single plasmid-encoded protein, the FtsK-like DNA translocase TraB. iii. it transfers a double-stranded DNA molecule. iv. it includes two distinct DNA-translocation processes. The initial DNA-transfer proceeds via a tight connection between the lateral walls of donor and recipient hyphae, involving a membrane pore formed by TraB hexamers. TraB directs plasmid transfer by specifically recognizing the non-coding *clt* locus. Subsequently, a cross wall localized multi-protein assembly of TraB and plasmid-encoded Spd proteins directs invasion of neighboring mycelial compartments.

The efficiency of a conjugative plasmid to colonize the recipient mycelium is manifested by the size of pock structures, which are visible with the mere eye and which exactly match the transconjugant area. Conjugative plasmids adapted to the *Streptomyces* life style and use the combined activities of distinct processes to efficiently spread within the *Streptomyces* colony (Fig. 5). The plasmid propagates in the extending tip compartments and enters new hyphae at branching points, before the formation of cross walls. The initial conjugative transfer event is then multiplied by subsequent rounds of conjugation, since the transconjugant hyphae have been transformed into donors and can transfer the plasmid, whenever a recipient is met. In addition, the plasmid developed the Spd apparatus, allowing the plasmid to travel across preformed cross walls in older mycelial compartments. The ability of the plasmid to spread to older parts of the recipient colony seems to be the most significant process, as demonstrated by the dramatic reduction in pock size of a *spd* mutant. Putting efforts to reach non-growing mycelial compartments seems to be not a clever decision on the first view, since only extending hyphae have a chance to find a new mating partner. But the intra-mycelial travelling through “old” compartments is also a route for the plasmid to reach new tip compartments. Moreover, preferentially the old parts of a *Streptomyces* colony, suffering from nutrient depletion, start to differentiate into aerial hyphae, which eventually are converted into spore chains. Therefore, from a plasmids point of view it is an effective strategy to reach the “old” compartments of the mycelium before they start to differentiate. Plasmids arriving in the differentiating hyphae in time have a much better chance to enter the spores for further dissemination in the soil.

Declarations of interest

None.

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