RESEARCH ARTICLE

A Multiprotein DNA Translocation Complex Directs Intramycelial Plasmid Spreading during *Streptomyces* Conjugation

Lina Thoma,[a] Hyazinth Dobrowinski,[a] Constanze Finger,[a] Jamil Guezguez,[a] Dirk Linke,[b]* Edgardo Sepulveda,[a]* Günther Muth[a]

Interfakultaeres Institut für Mikrobiologie und Infektionsmedizin Tuebingen IMIT, Mikrobiologie, Biotechnologie, Eberhard Karls Universitaet Tuebingen, Tuebingen, Germany[a]; Max-Planck-Institut für Entwicklungsbiologie, Tuebingen, Germany[b]

* Present address: Dirk Linke, University of Oslo, Department of Biosciences, Blindernveien, Oslo, Norway; Edgardo Sepulveda, Max-Planck-Institut für Entwicklungsbiologie, Tuebingen, Germany.

ABSTRACT **Conjugative DNA transfer in mycelial *Streptomyces* is a unique process involving the transfer of a double-stranded plasmid from the donor into the recipient and the subsequent spreading of the transferred plasmid within the recipient mycelium. This process is associated with growth retardation of the recipient and manifested by the formation of circular inhibition zones, named pocks. To characterize the unique *Streptomyces* DNA transfer machinery, we replaced each gene of the conjugative 12.1-kbp *Streptomyces venezuelae* plasmid pSVH1, with the exception of the *rep* gene required for plasmid replication, with a hexanucleotide sequence. Only deletion of *traB*, encoding the FtsK-like DNA translocase, affected efficiency of the transfer dramatically and abolished pock formation. Deletion of *spdB3*, *spd79*, or *spdB2* had a minor effect on transfer but prevented pock formation and intramycelial plasmid spreading. Biochemical characterization of the encoded proteins revealed that the GntR-type regulator TraR recognizes a specific sequence upstream of *spdB3*, while Orf108, SpdB2, and TraR bind to peptidoglycan. SpdB2 promoted spheroplast formation by T7 lysozyme and formed pores in artificial membranes. Bacterial two-hybrid analyses and chemical cross-linking revealed that most of the pSVH1-encoded proteins interacted with each other, suggesting a multiprotein DNA translocation complex of TraB and Spd proteins which directs intramycelial plasmid spreading.**

IMPORTANCE **Mycelial soil bacteria of the genus *Streptomyces* evolved specific resistance genes as part of the biosynthetic gene clusters to protect themselves from their own antibiotic, making streptomycetes a huge natural reservoir of antibiotic resistance genes for dissemination by horizontal gene transfer. *Streptomyces* conjugation is a unique process, visible on agar plates with the mere eye by the formation of circular inhibition zones, called pocks. To understand the *Streptomyces* conjugative DNA transfer machinery, which does not involve a type IV secretion system (T4SS), we made a thorough investigation of almost all genes/proteins of the model plasmid pSVH1. We identified all genes involved in transfer and intramycelial plasmid spreading and showed that the FtsK-like DNA translocase TraB interacts with multiple plasmid-encoded proteins. Our results suggest the existence of a macromolecular DNA translocation complex that directs intramycelial plasmid spreading.**

Received 23 December 2014 **Accepted** 22 April 2015 **Published** 26 May 2015

Citation Thoma L, Dobrowinski H, Finger C, Guezguez J, Linke D, Sepulveda E, Muth G. 2015. A multiprotein DNA translocation complex directs intramycelial plasmid spreading during *Streptomyces* conjugation. mBio 6(3):e02559-14. doi:10.1128/mBio.02559-14.

Invited Editor Carton W. Chen, National Yang-Ming University **Editor** Stephen Carlyle Winans, Cornell University

Address correspondence to Günther Muth, gmuth@biotech.uni-tuebingen.de.

Streptomyces conjugation has been studied for nearly 60 years, but its molecular mechanism is poorly understood (1, 2). Like other conjugation systems, *Streptomyces* conjugation depends on the presence of conjugative plasmids (3). Nevertheless, its underlying mechanism fundamentally differs from known conjugation processes via type IV secretion systems (T4SS) (4–7). In contrast to T4SS, *Streptomyces* conjugation involves the transfer of a double-stranded DNA molecule (8). A single plasmid-encoded protein, TraB, is sufficient for conjugative DNA transfer. This has been demonstrated by integrating the *traB* homologue *kilA* of pIJ101 together with its corresponding regulatory gene *korA* (*traR*) into the chromosome of *Streptomyces lividans* (9). *traB-traR* of most plasmids constitute a Kil-Kor system, since inactivation of *traR* is not tolerated in the presence of *traB*, probably due to toxicity of unregulated *traB* expression (10–12). TraB directs plasmid transfer by binding to a specific plasmid region (13), the *cis*-acting locus of transfer, *clt* (9, 14). The *clt* regions of different plasmids contain direct 8-bp repeats, which are recognized by the corresponding TraB protein (15, 16). Characterization of the TraB domain architecture, its structure, its enzymatic activity, and its mode of DNA interaction revealed that TraB highly resembled the septal DNA translocator proteins FtsK/SpoIIIE, which are involved in the segregation of the chromosome during bacterial cell division and sporulation (16). This similarity suggested that *Streptomyces* adapted the intracellular FtsK/SpoIIIE chromosome segregation system for the DNA transfer across the envelopes of donor and recipient (4, 5).

A striking feature of *Streptomyces* conjugation is the formation of circular inhibition zones which have been named pocks (17–20). Pocks of up to 3 mm in size are formed when spores of a

plasmid-carrying donor are plated with an excess of plasmid-free recipient spores. These temporary growth retardation zones indicate areas within the mycelial lawn where a recipient has obtained a plasmid by conjugation (18). Although the molecular mechanism underlying the formation of pocks has not been elucidated, they have been interpreted as the consequence of intramycelial plasmid spreading via the septal cross walls of the recipient mycelium (6, 18). Pock formation requires the presence of 3 to 7 *spd* genes, which are organized in operon structures, often with overlapping stop/start codons. These genes are diverse, and from their sequence it is often not possible to assign homologues on other *Streptomyces* plasmids. The molecular function of most Spd proteins is unknown (21). SpdB2 of plasmid pSVH1 has been shown to be an oligomeric integral membrane protein that binds to DNA in a nonspecific manner (22). The role of SpdA in plasmid spreading is unclear. Whereas *spdA* of plasmid pSN22 has been shown to influence plasmid spreading, the *spdA* homologue *spdA2* of plasmid pIJ101 did not affect pock formation but was characterized as a stability function (23, 24). Purified SpdA2 bound to a highly conserved palindromic sequence motif associated with the *spdA* coding regions of *Streptomyces* plasmids (23).

Plasmid pSVH1, isolated from the chloramphenicol producer *Streptomyces venezuelae*, contains 12 open reading frames, probably involved in replication, conjugative transfer, and intramycelial plasmid spreading (10). Of these, only TraB and TraR have functionally characterized homologues in databases; thus, predictions concerning the molecular functions of the putative spread proteins are hampered. Since the proteins are thought to be involved in DNA transfer across the septal cross walls, one has to postulate that these proteins might interact with membranes, peptidoglycan (PG), and DNA. Here, we describe the mutational analysis of pSVH1 and the biochemical characterization of several pSVH1-encoded proteins. Our results show that *spdB3*, *spd79*, and *spdB2* are crucial for intramycelial plasmid spreading, that many pSVH1-encoded proteins bind to PG, and that SpdB2 permeabilizes membranes *in vivo* and *in vitro*. Moreover, our protein-protein interaction assays showed that most pSVH1-encoded proteins interact with each other, suggesting a large multiprotein complex involved in DNA translocation.

RESULTS

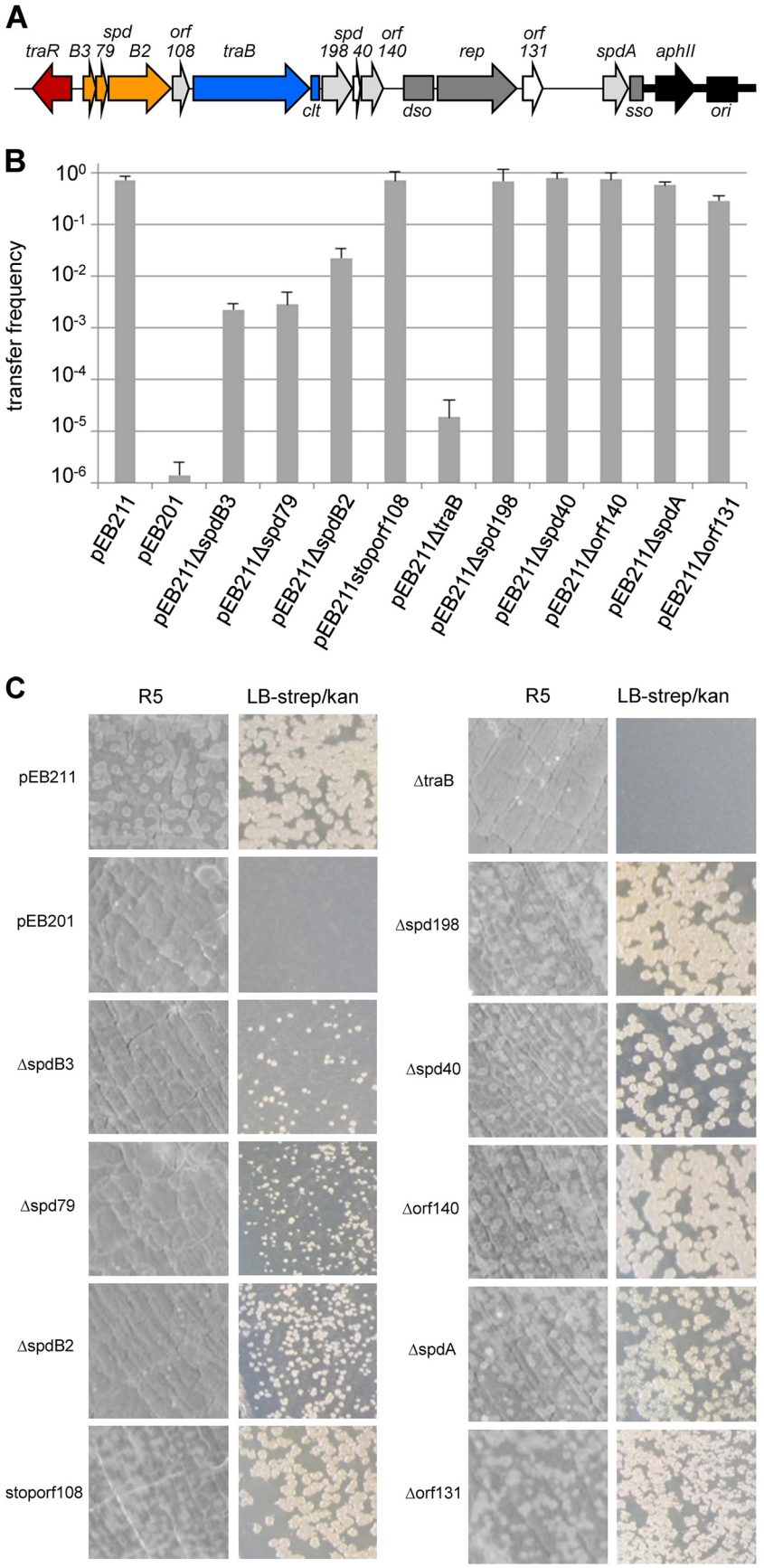
Identification of genes involved in conjugative transfer of pSVH1. Plasmid pEB211 is a conjugative pSVH1 derivative (Fig. 1A) which promotes the formation of pocks with a diameter of ~1 mm, slightly smaller in size than those reported for other plasmids (10, 18, 19). pEB211 was used to delete the pSVH1-carried genes by λ-Red-mediated homologous recombination (25–27), preserving the start and stop codons. In the final constructs, the coding region of each gene was replaced by the CA ATTG sequence, thereby making polar effects on the expression of the downstream genes unlikely. Since *orf108* is located upstream of *traB* and therefore could contain or overlap sequences involved in controlling *traB* expression, *orf108* was not completely deleted but inactivated by inserting a translational stop codon 3 bp after the predicted *orf108* start codon.

Respective plasmids were introduced into *S. lividans* TK54 by polyethylene glycol (PEG)-mediated protoplast transformation. Only plasmid pEB211ΔtraR did not yield any transformants, most likely due to the killing function of unregulated *traB* expression. Transfer properties were characterized in mating experiments with *S. lividans* TK64 as a recipient (Fig. 1B; see also Table S5 in the supplemental material). Although the negative-control plasmid pEB201 lacks all transfer genes, a transfer rate of 1.4×10^{-6} ($\pm 1.1 \times 10^{-6}$) was calculated. This value probably represents the background of our selection system. Plasmids pEB211Δorf131, pEB211ΔspdA, pEB211Δorf140, pEB211Δspd40, pEB211Δspd198, and pEB211stoporf108 were transferred with a rate of 3.0×10^{-1} to 7.9×10^{-1} (Fig. 1A; see also Table S5), similar to the transfer rate of the parent plasmid pEB211 ($7.3 \times 10^{-1} \pm 1.6 \times 10^{-1}$). In contrast, conjugative transfer of pEB211ΔtraB was reduced by five orders of magnitude. pEB211ΔspdB3, pEB211Δspd79, and pEB211ΔspdB2 showed a 30- to 300-fold reduction in transfer efficiency.

When plating the donor strains with an excess of recipient spores, pEB211Δorf131, pEB211ΔspdA, pEB211Δorf140, pEB211Δspd40, pEB211Δspd198, and pEB211stoporf108 formed pocks resembling those caused by pEB211 (Fig. 1C). Also, after replica plating on selective agar, the sizes of the transconjugant zones, the areas where the recipient mycelium obtained a plasmid by conjugation and intramycelial plasmid spreading, did not apparently differ. In contrast, no clearly visible pocks were formed when pEB211ΔspdB3, pEB211Δspd79, pEB211ΔspdB2, pEB211ΔtraB, or pEB201 were plated. Whereas no colonies grew on the replica plates of pEB201 or pEB211ΔtraB, being consistent with the defect in plasmid transfer, tiny transconjugant zones developed when the pEB211ΔspdB3, pEB211Δspd79, and pEB211ΔspdB2 crosses were replica plated. This observation suggested an essential role of these three genes in plasmid spreading and pock formation.

Heterologous expression of pSVH1-encoded proteins. The SpdA homologue SpdA2 of pIJ101 has been recently shown to affect segregational stability (23), and the biochemical characterization of SpdB2 and TraB has been reported previously (16, 22). To elucidate their molecular function, we tried to purify the remaining pSVH1-encoded proteins. *spdB3*, *spd198*, *orf140*, *orf108*, and *traR* genes were amplified by PCR (primers listed in Table S4 in the supplemental material) and cloned into the expression vectors pJOE2775 or pRSETB, creating fusions to a His tag-encoding sequence. Induction of *spdB3* expression turned out to be highly toxic to *Escherichia coli*, preventing production of SpdB3 protein. Although Spd198 could be expressed in reasonable amounts, Spd198-His was completely insoluble and could not be purified, not even under denaturing conditions (data not shown). In contrast, TraR, Orf108, and Orf140 could be purified in sufficient quality (see Fig. S1 in the supplemental material) for further studies.

DNA binding activity of TraR. All conjugative *Streptomyces* plasmids encode a GntR-type transcriptional repressor TraR (28). TraR was shown to override the toxic effects of unregulated TraB expression by binding to so-called *tre* repeats in the promoter region of *traB* (29). To identify the binding sites of TraR of plasmid pSVH1, fragments covering the whole pSVH1 sequence were analyzed by electrophoretic mobility shift assays (EMSAs) with purified TraR-His (data not shown). A single pSVH1 fragment, comprising *traR* and the *traR-spdB3* intergenic region, was retarded (Fig. 2A). This fragment contained two direct 14-bp repeats, **TTTGGTACACAACT,** separated by 31 bp, and an incompletely conserved inverted repeat, **TTTGGTAC**CT**AAGT** (bold letters indicate identical residues). In addition, a 12-bp inverted repeat is located close to the *spdB3* start codon (Fig. 2C). In a more


A
traR
B3
spd 79
B2
orf 108
traB
clt
spd 198
40
orf 140
dso
rep
orf 131
spdA
sso
aphII
ori
B
transfer frequency
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
pEB211
pEB201
pEB211ΔspdB3
pEB211Δspd79
pEB211ΔspdB2
pEB211stoporf108
pEB211ΔtraB
pEB211Δspd198
pEB211Δspd40
pEB211Δorf140
pEB211ΔspdA
pEB211Δorf131
C
R5
LB-strep/kan
pEB211
pEB201
ΔspdB3
Δspd79
ΔspdB2
stoporf108
ΔtraB
Δspd198
Δspd40
Δorf140
ΔspdA
Δorf131

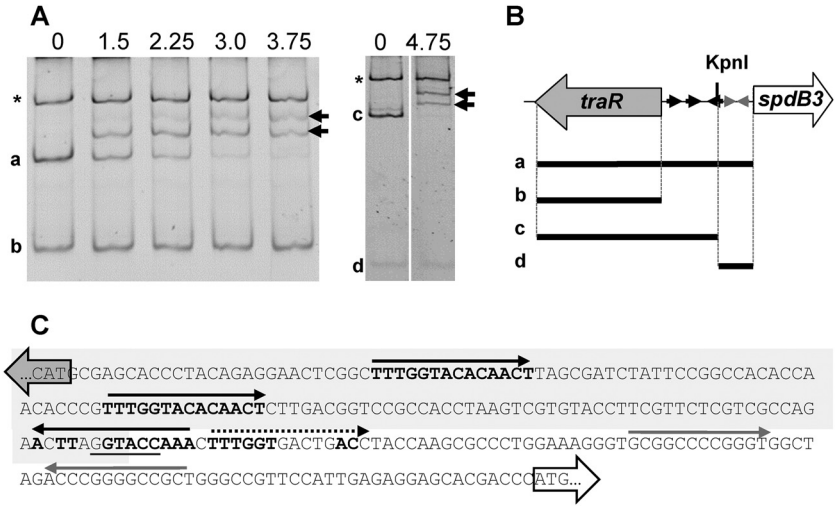


FIG 2 TraR specifically binds to the *traR-spdB3* intergenic region. (A) Electrophoretic mobility shift assays demonstrate binding of TraR-His to DNA fragments comprising the *traR-spdB3* intergenic region. With increasing TraR-His concentration (0 to 3.75 pmol), two retarded bands appeared (arrows). The negative-control fragment, marked by an asterisk, is not bound by TraR-His. (B) A schematic drawing of the DNA fragments (a to d) analyzed for TraR binding is given. The positions of the 14-bp TTTGGTACACAACT repeats (black arrows) and the 12-bp inverted repeat (grey arrows) are indicated. (C) Nucleotide sequence of the *traR-spdB3* intergenic region. The start codons of *traR* and *spdB3*, the 14-bp perfect repeats (black arrows), the imperfect repeat (dotted arrow), and the 12-bp inverted repeat (grey arrows) are marked. The KpnI site is underlined. DNA fragment "c," which is bound by TraR, is highlighted by shading.

detailed analysis with various DNA fragments, the TraR binding site could be narrowed down to a 139-bp fragment bordered by the ATG start codon of *traR* and the KpnI site (Fig. 2). This fragment contained only the two perfectly conserved 14-bp repeats. Interestingly, at higher protein concentrations, the EMSAs revealed two shifted bands (Fig. 2A), indicating either the presence of two TraR binding sites or the binding of TraR in its monomeric or dimeric form. The ability of TraR to form dimers was confirmed by chemical cross-linking, showing a higher-molecular-weight band corresponding to a TraR dimer (see Fig. S2A in the supplemental material).

Peptidoglycan binding activity of TraR, SpdB2, and Orf108. DNA transfer across the cell envelopes should involve proteins able to interact with PG. For TraB of pSVH1, a PG binding activity has already been reported (16). To study whether some of the other proteins might also interact with PG, TraR, SpdB2, Orf108, and Orf140, proteins were incubated with PG sacculi of *S. lividans*. After spinning down the PG sacculi, supernatants and pellet fractions were analyzed by SDS-PAGE and immunoblotting for the presence of the proteins. In control experiments, the proteins were treated the same way, but without peptidoglycan. As an additional control, we performed the assay with bovine serum albumin (BSA). Orf140 was unable to interact with PG and was found mainly in the supernatant. In contrast, TraR, SpdB2, and Orf108 were detected mainly in the PG-pellet fraction (Fig. 3), suggesting a PG binding activity of these proteins. Surprisingly, part of Orf108 seemed to disappear upon PG binding. This can be explained by SDS treatment not being sufficient to fully release PG-bound Orf108 required for electrophoretic separation.

SpdB2 is a pore-forming protein. When SpdB2-His was expressed under the control of the T7 promoter in BL21(DE3)/pLysS, spheroplasts were formed upon induction of the T7 polymerase with isopropyl-β-D-thiogalactopyranoside (IPTG) (Fig. 4A). LIVE/DEAD staining of osmotically stabilized cells with a LIVE/DEAD BacLight bacterial viability kit (Life Technology) showed that propidium iodide could not enter the cell to stain the DNA, demonstrating that the spheroplasts possessed an intact membrane (data not shown). Spheroplasts were not formed in the absence of SpdB2-His (Fig. 4B) or during expression of SpdB2 in *E. coli* BL21(DE3) missing the T7 lysozyme (Fig. 4C) or in Lemo21(DE3) (data not shown), encoding an enzymatic inactive T7 lysozyme. Thus, we concluded that SpdB2 might form a pore structure in the inner membrane, allowing the T7 lysozyme to reach the peptidoglycan layer. Degradation of the BL21 cell wall by T7 lysozyme then resulted in spheroplast formation.

To study the pore-forming ability of SpdB2 in artificial membranes, we performed single-channel recordings in planar lipid bilayers with SpdB2-His. To prevent contamination with outer membrane porins, the most frequent pore-forming contaminant, SpdB2-His was expressed in *S. lividans*. Following membrane isolation by ultracentrifugation and solubilization of SpdB2-His by β-dodecylmaltoside, SpdB2-His was purified by Ni-

FIG 1 Role of the distinct pSVH1 proteins on conjugative plasmid transfer. (A) Arrangement of genes in pEB211. The regulatory gene *traR* is drawn in red, *spd* genes in orange, *traB* in blue, putative *spd* genes in light grey, *rep* and *dso-sso* regions in dark grey. The *E. coli* part of pEB211 is highlighted in black. (B) Transfer frequencies of pEB211 derivatives. Approximately 10^7 spores of *S. lividans* TK54 containing pEB211 (Kan^r) and its mutated derivatives were plated with equal amounts of plasmid-free TK64 (Str^r). After 7 days of incubation, spores were harvested, and the transfer frequencies (ratio of transconjugants [Str^r, Kan^r] and recipients [Str^r]) were determined. Transfer frequencies are the mean values from three mating experiments (see Table S5). Error bars indicate standard deviations. (C) Pock formation, indicating intramycelial plasmid spreading. Approximately 10^2 spores of *S. lividans* TK54 containing derivatives of plasmid pEB211 (Kan^r) were streaked onto a lawn (~10^5 spores) of plasmid-free TK64 (Str^r). After 7 days of incubation at 30°C, pocks associated with the conjugative plasmid transfer were visible, and the fully sporulated plates were replica plated onto antibiotic-containing LB agar to select for transconjugants. Sizes of the pocks and the corresponding transconjugant patches indicate efficiency of plasmid transfer and intramycelial spreading.

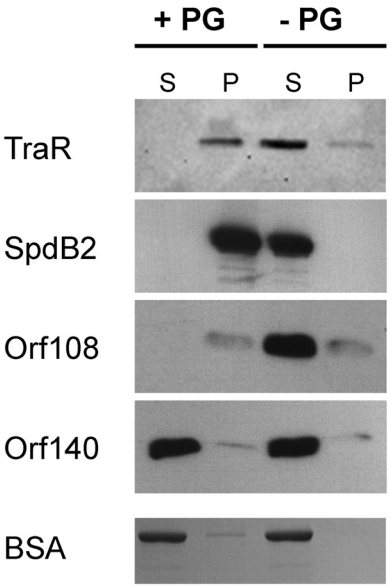


FIG 3 Peptidoglycan binding activity of pSVH1-encoded proteins. Purified soluble proteins were incubated with *S. lividans* PG sacculi (+) or buffer (−). Following precipitation of the PG sacculi by centrifugation, the supernatant (S) and the pellet (P) fractions were analyzed by immunoblotting with anti-His tag-specific antibodies for the presence of the respective proteins. Detection of the protein in the pellet fraction indicated PG binding activity.

nitrilotriacetic acid (NTA) and ion exchange chromatography (see Fig. S1B in the supplemental material). Single-channel recordings revealed that SpdB2-His inserted spontaneously in the membrane when added to the *cis*-side of the bilayer. The SpdB2 pores were consistently obtained with different samples of purified SpdB2-His protein but were very flickering and showed no discrete conductance steps (Fig. 4D and E). In contrast, when Ni-NTA-purified membrane extracts from cells carrying the empty expression plasmid were added, no changes in current flow were detected (data not shown), confirming that the channel recordings were caused by SpdB2-His and not by copurified contaminating proteins.

Complex interaction pattern of pSVH1-encoded proteins. To study whether the pSVH1-encoded proteins interact with each other, possibly forming a macromolecular DNA translocation complex, we analyzed interactions of the pSVH1 proteins using the bacterial two-hybrid system based on the catalytic domains T25 and T18 of the *Bordetella pertussis* adenylate cyclase (30). Translational fusions of all pSVH1 genes, with the exception of *rep*, encoding the replication initiator protein, and *traR*, encoding the transcriptional repressor, were made. For SpdB2 and TraB, not only the full-length coding regions but also subfragments were fused, to allow a more detailed study of the interacting domains. Protein-protein interactions were analyzed in cotransformation experiments of the *E. coli* cya mutant BTH101 (see Table S6).

With the exception of Orf131 and Spd40, we observed interactions for all other tested pSVH1 proteins. Interestingly, several proteins showed multiple interactions (Fig. 5A and B). Surprisingly, even proteins encoded by genes that had no clear phenotype in mating experiments, like Spd198, Orf140, and SpdA, interacted with TraB and/or Spd proteins, suggesting that also these proteins are somehow involved in conjugative DNA transfer or couple the conjugative transfer with other cellular processes. Spd198 interacted with the C-terminal DNA recognition domain of TraB (amino acids [aa] 717 to 772), whereas Orf140 interacted with the N-terminal part of TraB. SpdA even interacted with all proteins (SpdB3, Spd79, SpdB2, TraB) that were essential for pock formation and plasmid spreading. Furthermore, the C-terminal domain of SpdB2 interacted with the C-terminal part of TraB (aa 717 to 772) and with Spd79, which itself interacted with the N-terminal domain of TraB and with Spd198 (Fig. 5B; see also Table S6). The interaction network of TraB and Spd proteins indicates that TraB not only directs primary transfer but is also involved in the subsequent intramycelial plasmid spreading.

SpdB3, Spd79, SpdB2, Orf108, TraB, Spd198, and Orf140 showed self-interaction, suggesting that these proteins act as dimers or oligomers. Self-interaction of Orf140 could be confirmed by chemical cross-linking of purified protein, revealing even higher oligomeric states (see Fig. S2B). For SpdB2 and TraB, oligomerization was reported previously (16, 22). Interestingly, both the N-terminal 130 aa of TraB and the C-terminal part of TraB, comprising the DNA translocase and *clt* recognition domain, interacted with each other. Also, in case of SpdB2, both the N-terminal coiled-coil domain (aa 1 to 99) and the C-terminal domain (aa 206 to 409) showed self-interaction, suggesting that both parts of the protein contribute to oligomerization (Fig. 5B; see also Table S6).

In summary, the protein-protein interaction pattern of pSVH1-encoded proteins is in agreement with a model that the Spd proteins form a multiprotein DNA translocation apparatus.

DISCUSSION

During *Streptomyces* conjugation, even small plasmids encoding fewer than 10 genes are transferred to a recipient with high efficiency (19, 31). Despite the manageable number of plasmid-encoded proteins involved in conjugative plasmid transfer, the molecular function of most proteins is still widely unknown. Subcloning and deletion analyses revealed genes involved in replication (*rep*), regulation (*traR* [*korA*], *korB*), transfer (*traA*, *traB* [*kilA*]), and pock formation (*spd* genes), indicating a role in intramycelial plasmid spreading (6, 19, 32). These investigations were complicated by the genetic organization of the plasmids. Few transcriptional units containing several genes, in part very short ones, with overlapping stop and start codons hamper gene inactivation without polar effects on the downstream genes. Such difficulties were overcome in this study by replacing each open reading frame (ORF) of the bifunctional pSVH1 derivative pEB211 by a hexanucleotide sequence encoding only the 2 amino acids Gln and Leu.

Only the pEB211ΔtraR plasmid could not be introduced into *S. lividans*. This is in agreement with previous findings that the transcriptional repressor TraR is a Kor function, necessary to override the toxic effects of unregulated TraB expression (10, 12, 29). In conjunction with binding of TraR only to the upstream region of *spdB3*, it is plausible to conclude that TraR controls a large transcript, at least comprising *spdB3*, *spd79*, *spdB2*, *orf108*, and *traB*. PG binding activity of TraR, predicted to be a cytoplasmic protein, is unexpected. Although one cannot exclude that the interaction was caused by nonspecific charge-mediated PG binding, it could be possible that under mating conditions, TraR binds a PG-derived signaling molecule as a ligand to relieve repression of

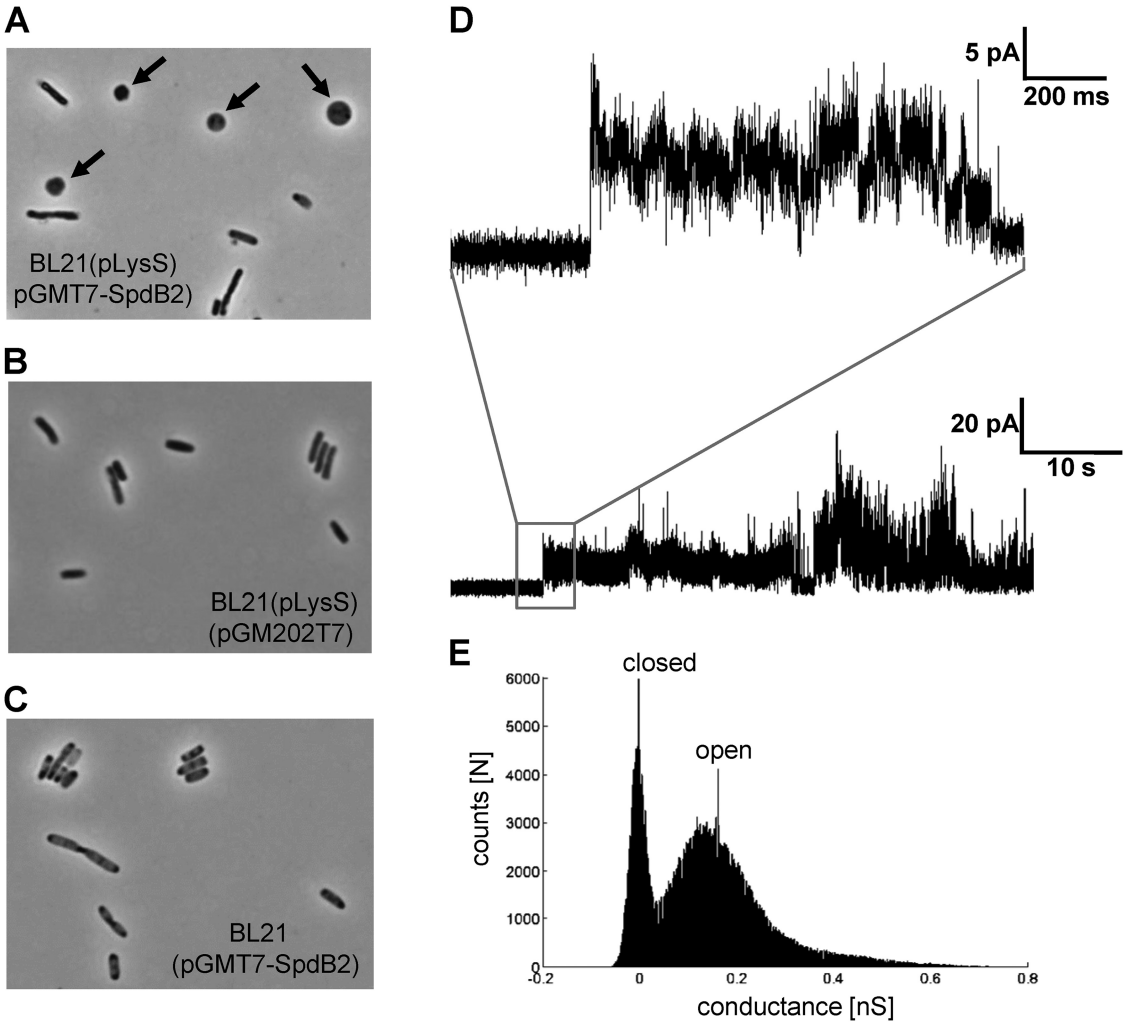


FIG 4 SpdB2-His promotes spheroplast formation by T7 lysozyme and forms unstable pores in planar lipid bilayers. (A) When expression of SpdB2-His was induced in BL21/pLysS, spheroplast (arrows) were formed. In contrast, spheroplasts were not observed in the absence of *spdB2* (B) or when expressing *spdB2* in BL21 lacking the T7 amidase (C). (D) When purified SpdB2-His was added to the *cis* side of a lipid bilayer, SpdB2-His inserted into the planar lipid bilayer, showing flickering pore structures. (E) The corresponding all-points conductance level histogram shows that there is no discrete conductance state of the open pore indicated by the long tail in the histogram.

the transfer genes. But this theory is highly speculative and has to be tested in further experiments.

The mating experiments singled out *traB* as the only gene crucial for conjugative transfer of pSVH1. This conforms to studies on plasmid pIJ101, where *traB* (*kilA*) alone was sufficient to promote conjugative DNA transfer (9). Analyses of pock formation revealed that although pSVH1 carries seven predicted *spd* genes (10), only three of them had a clear effect on pock formation. Inability to form pocks, but development of tiny transconjugant zones on the replica plates of pEB211ΔspdB3, pEB211Δspd79, and pEB211ΔspdB2, showed that inactivation of SpdB3, Spd79, or SpdB2 did not abolish the primary plasmid transfer into the recipient but specifically interfered with the subsequent intramycelial plasmid spreading. The observed reduction in the transfer rates of these mutants might be a side effect of the low transfer efficiency of pEB211 compared to transfer frequencies of other plasmids, which reach nearly 100% (12, 20, 31, 32). The contribution of intramycelial plasmid spreading to the overall transconjugant titer should be negligible if high donor and recipient concentrations (~10^7) are used, allowing each donor to immediately find a mating partner. However, if pEB211 transfer is less efficient compared to other conjugative *Streptomyces* plasmids (18), one can hypothesize that under such mating conditions, plasmid spreading affects the number of transconjugant colonies. Therefore, the reduced transfer rate (Fig. 1B) might not be caused by an effect on the primary transfer but rather reflects the defect in intramycelial plasmid spreading.

Pock morphology of pEB211Δspd198, pEB211Δspd40, pEB211Δorf140, pEB211ΔspdA, pEB211stoporf108, and pEB211Δorf131 did not apparently differ from pEB211 pocks. Therefore, Spd198, Spd40, Orf140, SpdA, Orf108, and Orf131 are either not involved in conjugative DNA transfer or they contribute to transfer only under certain environmental conditions, not reflected in the laboratory. Also, we might miss minor effects on plasmid spreading in this study due to the small size of pEB211 pocks, which prevents the detection of subtle changes in pock morphology.

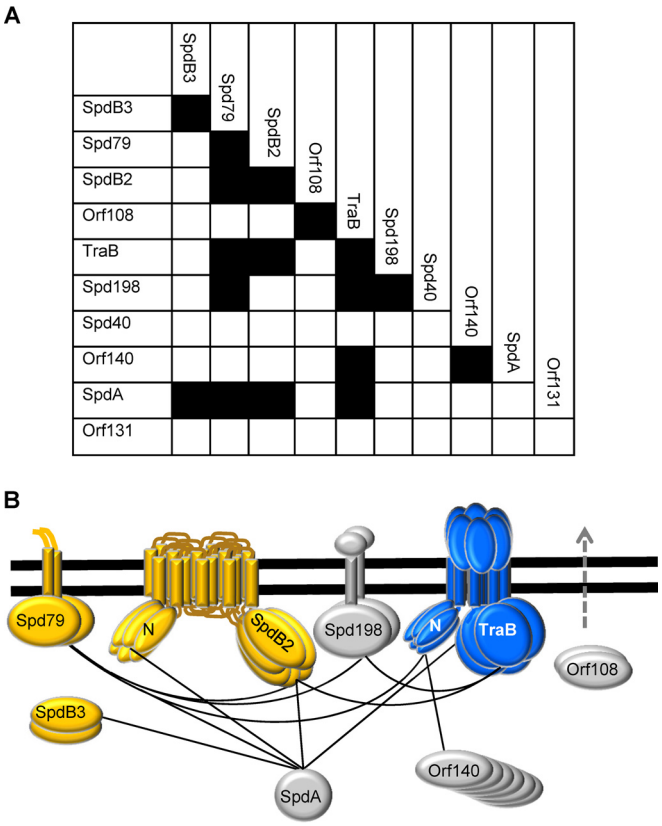


FIG 5 Complex interaction network of the pSVH1-encoded proteins. (A) Summary of the interactions of the pSVH1 proteins revealed by bacterial two-hybrid analyses. (B) Subcellular localization and the interaction pattern of pSVH1 proteins involved in conjugative DNA transfer and intramycelial plasmid spreading. The complex interaction network of TraB and various Spd proteins suggests a membrane-localized multiprotein DNA translocation apparatus involved in intramycelial plasmid spreading. Lines mark protein-protein interactions. Self-interactions are indicated by double ellipses. Transmembrane regions and protein orientations were predicted with TMpred (39). The dashed line indicates that Orf108 has to be secreted by an unknown route to be able to interact with PG.

The bacterial two-hybrid analyses of the pSVH1-encoded proteins revealed multiple protein-protein interactions, indicating a macromolecular DNA translocation complex involved in *Streptomyces* conjugation. In total, 36 positive interactions were found under 360 combinations tested. Although the 36 interactions should be confirmed by biochemical assays, several lines of evidence support the concept of the DNA translocation apparatus: (i) the *spd* genes are organized in an operon with translational coupling (10); (ii) hexamer formation of purified TraB has been demonstrated by chemical cross-linking, electron microscopy, and homology modeling (16); (iii) interaction of TraB with SpdB2 has been shown by copurification (22); (iv) oligomerization of SpdB2 has been revealed by chemical cross-linking (22), and blue native gel electrophoresis (see Fig. S2C in the supplemental material), oligomerization of Orf140 was demonstrated by chemical cross-linking (see Fig. S2B); (vi) subcellular localization of Spd79 was shown to depend on the presence of SpdB2 (22).

But how does this DNA transfer apparatus work? The key enzyme in DNA translocation is TraB. TraB hexamers were shown to build a central channel for DNA translocation in the membrane (16). The TraB C termini recognize the pSVH1 *clt* and the translocase domain pumps the plasmid, energized by ATP hydrolysis, to the other site (4). Interaction of TraB with the spread proteins indicates that TraB not only translocates the DNA across the envelopes of donor and recipient but is also involved in the subsequent plasmid spreading within the recipient mycelium. Evidence for the involvement of TraB in plasmid spreading has also been obtained by the characterization of distinct *tra* mutants of pIJ101, which were fully transfer proficient but specifically affected in plasmid spreading (33).

Plasmid spreading depends on three proteins encoded by the *spdB3-spd79-spdB2* operon. Bacterial two-hybrid analyses suggested a concerted action with SpdA. SpdB2 seems to have a key role in intramycelial plasmid spreading. It is the most conserved Spd protein, since putative SpdB2 homologues are encoded not only on all conjugative *Streptomyces* plasmids (28) but also on some actinophages (34, 35), where they might assist in the spreading of phage DNA. SpdB2 has been previously characterized as an oligomeric integral membrane protein with four predicted transmembrane helices (22). The ability to form pore structures *in vivo* and *in vitro* suggests that SpdB2 might build a channel for protein and/or DNA translocation. The nonspecific binding to double-stranded DNA (22) supports a direct interaction of SpdB2 with the transferred DNA. The multiple interactions with TraB and other Spd proteins suggest a multiprotein DNA transfer apparatus which might be anchored to the septal wall. Since in the PG binding assay part of Orf108 seemed to disappear upon PG binding, one could speculate that Orf108 binds very tightly, maybe covalently, to PG, preventing the subsequent solubilization of Orf108 from the PG sacculi by SDS. This speculation would point to a role of Orf108 in anchoring the DNA translocation apparatus to the PG.

Although most *Streptomyces* RCR plasmids contain an SpdA homologue, the function of *spdA* genes in *Streptomyces* plasmid biology is a matter of debate. Whereas the *spdA* homologue of pIJ101 seems to be a stability determinant rather than to affect plasmid spreading (23, 24), *spdA* of pSN22 has been shown to affect plasmid spreading (31). pEB211ΔspdA was transfer proficient and produced normal-sized pocks. However, the interaction of SpdA with all proteins encoded by the *spdB3-spd79-spdB2* operon and with TraB indicated a role in plasmid spreading. Since SpdA2 of plasmid pIJ101 was shown to bind the palindromic *spdA*-associated sequence *ssp* (23), it can be speculated that SpdA might represent the interface connecting the DNA substrate to the multiprotein DNA translocation apparatus.

MATERIALS AND METHODS

Bacterial strains and media. Cultivation of strains and procedures for DNA manipulation were performed as previously described (24, 36). Proteins were purified from *E. coli* Rosetta 2 (Merck), BL21(DE3), or BL21(DE3)/pLysS (Invitrogen) and *S. lividans* TK23 (24). *S. lividans* strains TK54 (24) and TK64 (24) were used for mating experiments. Strains and plasmids are listed in Table S1 in the supplemental material. Oligonucleotides used for PCR targeting, two-hybrid analyses, gene expression, and DNA binding studies are given in Tables S2 to S4.

Heterologous expression and purification of pSVH1 proteins. *spdB3-spd79* was amplified from template pSVH1 using primers Spd79upB/Spd79flaglow containing a BamHI site replacing the start codon of *spdB3* and a Flag tag-encoding sequence replacing the stop codon of *spd79*. The fragment was cloned as a BamHI/HindIII fragment into pRSETB (Life Technologies). All other genes were amplified using

primers containing an NdeI site overlapping with the start codon and a BamHI site replacing the stop codon. Primer sequences are listed in Table S4 in the supplemental material. Fragments were cloned under control of the rhamnose-inducible promoter in pJOE2775 (J. Altenbuchner, personal communication), generating a C-terminal fusion to a His tag-encoding sequence. For protein expression, overnight cultures (LB, 37°C) were induced with rhamnose at a final concentration of 0.2% or 1 mM IPTG (induction of T7 polymerase) for 6 h at 30°C.

Cells were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris [pH 8.0], 1 M NaCl, protease inhibitor mix [Roche], 5 $\mu g \cdot ml^{-1}$ DNase I, and 10 mM mercaptoethanol). Cells were broken by French pressing, and soluble proteins were separated by centrifugation at 21,000 × *g* for 10 min. His-tagged proteins were purified by Ni-NTA chromatography using a Superflow gravity flow column (1 ml) following the protocol of the supplier (IBA).

For SpdB2-His production, *Streptomyces lividans* TK23 harboring the expression plasmid pYT90 was grown for ~64 h in S medium (with 25 μg/ml kanamycin) at 27°C. Then, protein production was induced with thiostrepton at a final concentration of 12.5 μg/ml, and the cells were grown for a further 24 h at 18°C. Subsequently, the cells were harvested, resuspended in lysis buffer, and broken by French pressing. Membranes were spun down for 90 min at 48,000 × *g* and washed once with lysis buffer. Then, membranes were solubilized in lysis buffer supplemented with 1% (wt/vol) β-dodecylmaltoside for 1 h at 7°C. Nonsolubilized material was removed by centrifugation for 30 min at 20,000 × *g* at 4°C. The supernatant was used for subsequent purification by Ni-NTA affinity chromatography (Superflow Gravity flow column, 1 ml; IBA), and after being washed with elution buffer (50 mM Tris, 150 mM NaCl, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1% β-dodecylmaltoside [pH 7.6]) supplemented with increasing imidazole concentrations (20 mM, 70 mM), proteins were eluted with 250 mM imidazole. The eluted protein sample was diluted 1:20 with ion exchange buffer 1 (20 mM HEPES, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1% β-dodecylmaltoside [pH 7.9]) and further purified by ion exchange chromatography using an Äkta purifier with a 1-ml Hi-Trap SP-FF column (GE Healthcare). The proteins were eluted from the column with a gradient mixed of ion exchange buffer 1 and ion exchange buffer 2 (20 mM HEPES, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 M NaCl, 0.1% β-dodecylmaltoside [pH 7.9]). Subsequently, the buffer of the eluted protein sample was exchanged by dialysis to elution buffer supplemented with 10% (wt/vol) of glycerol, shock frozen in liquid nitrogen, and stored at −70°C.

Mutant construction. The apramycin cassette was amplified from plasmid pIJ773 (27) using primers containing 39-bp overhangs corresponding to 5′ sequences, including start codons, and 3′ sequences, including stop codons of the respective genes, followed by a MunI recognition sequence. Primer sequences are given in Table S2. The purified fragments were introduced by electroporation in *E. coli* BW25113 carrying plasmids pEB211 and pIJ790 in which expression of the λ-Red system (pIJ790) was induced by arabinose (0.2%). From apramycin/kanamycin-resistant transformants, plasmid DNA was isolated, and the correct replacement was confirmed by sequencing. Subsequently, the apramycin cassette was removed by MunI digestion and religation, leaving only the hexanucleotide sequence CAATTG.

Determination of transfer frequencies and pocking phenotypes. To determine transfer frequencies, 10^7 spores of the streptomycin resistant *S. lividans* TK64 were plated together with 10^7 spores of *S. lividans* TK54, carrying a pEB211-derivative, onto R5 agar containing 20 μM $CuCl_2$. After 1 week of growth at 30°C, spores were harvested and filtered through cotton, and spore titers were determined on LB-streptomycin (recipient/transconjugants) and on LB-streptomycin-kanamycin (transconjugants). Transfer frequency is given as the transconjugant titer divided by the recipient titer. Experiments were repeated three times.

To analyze pock formation, dilutions of *S. lividans* TK54 (Spt^r) containing pEB211 or its derivatives were streaked on a lawn (~10^5 spores) of plasmid-free TK64 (Str^r) on R5 plates containing 20 μM $CuCl_2$. Pocks associated with the conjugative plasmid transfer were visible after 2 days of incubation at 30°C. After 7 days of incubation, the fully sporulated plates were replica plated onto LB agar containing streptomycin and kanamycin to demonstrate transconjugant growth within the pock area.

Bacterial two-hybrid interaction assays. To detect protein interactions, the different pSVH1 genes were amplified with primers listed in Table S3 containing XbaI and KpnI or MunI sites. Subsequently, PCR fragments were cloned as XbaI/KpnI or XbaI/MunI fragments into plasmids pKT25, pUT18, and pUT18c to generate translational fusions with the catalytic domains of the *B. pertussis* adenylate cyclase (30). The *E. coli* cya mutant BTH101 was cotransformed with pKT25 and pUT18 or pUT18c derivatives.

Overnight cultures in LB broth were washed with phosphate-buffered saline (PBS), and the resuspended cells were spotted on M63 minimal agar (0.4% lactose, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal] at 40 $\mu g \cdot ml^{-1}$) prepared in 48-well microtiter plates and incubated at 37°C (see Fig. S3). The ability of cotransformants to use lactose resulting in growth on minimal agar and blue color on X-Gal plates is based on a functional adenylate cyclase due to the interaction of the fusion proteins. As a positive control, BTH101 was transformed with plasmids pUT18c-Zip and pKT25-Zip (30). As a negative control, BTH101 carrying the empty vectors pUT18c and pKT25 was used. The experiments were repeated three times with independent cultures. Only those protein combinations which showed a positive reaction in all three experiments were regarded as interacting.

EMSA analyses. DNA regions were amplified by PCR using primer pairs listed in Table S4 in the supplemental material. Products were electrophoresed on a 1.5% agarose gel and purified by gel extraction using the Illustra GFX PCR DNA and gel band purification kit (GE Healthcare). Purified TraR was incubated with the desired fragments for 30 min at room temperature in a reaction volume of 10 μl in binding buffer (20 mM NaH_2PO_4, 300 mM NaCl, 50 mM KCl, 0.3 mM $MgCl_2$, 0.5 $mg \cdot ml^{-1}$ BSA [pH 8]). As a control for specificity, an unspecific DNA fragment (*traB* fragment; see Table S4) was included in the binding reaction mixture. Binding reaction mixtures were electrophoresed on a 6% Tris-acetate-polyacrylamide gel at 60 V for 1.5 h and stained with ethidium bromide for visualization with UV light.

PG binding assay. PG binding was analyzed according to Ursinus et al. (37) with some modifications. Approximately 100 μg of *S. lividans* peptidoglycan (PG) was incubated with ~3 μg of purified protein in 100 μl 0.1 M sodium acetate-acetic acid buffer (pH 5.4) for 30 min at room temperature. Subsequently, PG was pelleted (30 min at 21,000 × *g*, 4°C), and the supernatant (S) was loaded to an SDS-PA gel. The pellet was resuspended in 2% SDS and incubated for 1 h at 37°C on a shaker. After a further centrifugation step, the supernatant (P) was also loaded to the gel. The presence of the protein in the pellet fraction (P) suggests PG binding activity. As a negative control, the assay was performed using BSA.

Glutaraldehyde cross-linking. About 5 μg purified protein was cross-linked in a final volume of 30 μl of buffer (20 mM bicine [pH 7.2], 300 mM NaCl, 1 mM DTT) by the addition of glutaraldehyde to a final concentration of 0.01 to 0.1%. After incubation for 1 h on ice, the reaction was stopped by adding 1 M glycine to a final concentration of 100 mM. After boiling, samples were analyzed by SDS-PAGE and immunoblotting with anti-His tag antibodies.

Spheroplast formation by SpdB2-His. To express *spdB2* under control of the T7 polymerase, *spdB2-his* was cut out from pGB1 (22) by NdeI/HindIII digestion and ligated into pGM202T7. The resulting expression plasmid pGMT7-spdB2 was used to transform the different *E. coli* strains [BL21(DE3), BL21(DE3)/pLysS, Lemo21(DE3) (NEB)]. As a control, cells were also transformed with the empty expression plasmid pGM202T7. Transformants were inoculated in LB supplemented with 1% glucose and 50 μg/ml kanamycin, and cells were grown for about 6 h at 37°C. This preculture was used to inoculate 200 ml with an optical density at 600 nm (OD_{600}) of 0.05. When the culture with the empty expression plasmid reached an OD_{600} of 0.5, cells of all cultures were spun down,

resuspended in an equal volume of sucrose recovery medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20-mM glucose, 0.23 M sucrose [pH 7.0]) (38), and subsequently induced with 1 mM IPTG. After 3 to 4 h at 37°C, the cells were subjected to microscopy using an Olympus System Microscope BX60 equipped with appropriate filter sets, and pictures were taken with an Olympus F-view II camera.

LIVE/DEAD staining was carried out using the LIVE/DEAD BacLight bacterial viability kit (Life Technologies) according to the manufacturer's instructions.

Single-channel recordings in planar lipid bilayers. Planar lipid bilayer experiments were performed on an Ionovation Compact V02 system (Ionovation GmbH, Germany) at room temperature. Chambers consisting of two compartments separated by a Teflon septum with a 120-μm microhole were filled with approximately 1.3 ml of buffer (1 M KCl, 10 mM Tris [pH 7.6]) in each compartment. Lipid bilayers were made of a mixture of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) (Bilayer-Lipid II; Ionovation) in *n*-decane and were produced by pipetting the lipids next to the microhole and repetitively lowering and raising the buffer level. Bilayer formation was monitored optically and by checking the bilayer capacitance. Up to 3.5×10^{-11} mol SpdB2-His was added to the *cis* compartment, and a voltage of 50 mV was applied. Data analysis was performed with Patchmaster (HEKA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02559-14/-/DCSupplemental.

Figure S1, TIF file, 1.4 MB.
Figure S2, TIF file, 2.3 MB.
Figure S3, EPS file, 0.7 MB.
Table S1, DOCX file, 0.02 MB.
Table S2, DOCX file, 0.02 MB.
Table S3, DOCX file, 0.02 MB.
Table S4, DOCX file, 0.02 MB.
Table S5, DOCX file, 0.02 MB.
Table S6, DOCX file, 0.02 MB.

ACKNOWLEDGMENTS

This work was supported by DAAD scholarship A0876133 to E.S. and by the DFG (SFB766).

We thank D. Männle for his contribution to the bacterial two-hybrid studies.

REFERENCES

1. **Sermonti G, Spada-Sermonti I.** 1955. Genetic recombination in *Streptomyces*. Nature **176:**121. http://dx.doi.org/10.1038/176121a0.
2. **Hopwood DA.** 1959. Linkage and the mechanism of recombination in *Streptomyces coelicolor*. Ann N Y Acad Sci **81:**887–898. http://dx.doi.org/10.1111/j.1749-6632.1959.tb49374.x.
3. **Vivian A.** 1971. Genetic control of fertility in *Streptomyces coelicolor* A3(2): plasmid involvement in the interconversion of UF and IF strains. J Gen Microbiol **69:**353–364.
4. **Thoma L, Muth G.** 2012. Conjugative DNA transfer in *Streptomyces* by TraB: is one protein enough? FEMS Microbiol Lett **337:**81–88. http://dx.doi.org/10.1111/1574-6968.12031.
5. **Sepulveda E, Vogelmann J, Muth G.** 2011. A septal chromosome segregator protein evolved into a conjugative DNA-translocator protein. Mobile Genet Elem **1:**225–229. http://dx.doi.org/10.4161/mge.1.3.18066.
6. **Grohmann E, Muth G, Espinosa M.** 2003. Conjugative plasmid transfer in Gram-positive bacteria. Microbiol Mol Biol Rev **67:**277–301. http://dx.doi.org/10.1128/MMBR.67.2.277-301.2003.
7. **Alvarez-Martinez CE, Christie PJ.** 2009. Biological diversity of prokaryotic type IV secretion systems. Microbiol Mol Biol Rev **73:**775–808. http://dx.doi.org/10.1128/MMBR.00023-09.
8. **Possoz C, Ribard C, Gagnat J, Pernodet JL, Guérineau M.** 2001. The integrative element pSAM2 from *Streptomyces*: kinetics and mode of conjugal transfer. Mol Microbiol **42:**159–166. http://dx.doi.org/10.1046/j.1365-2958.2001.02618.x.
9. **Pettis GS, Cohen SN.** 1994. Transfer of the p1J101 plasmid in *Streptomyces lividans* requires a *cis*-acting function dispensable for chromosomal gene transfer. Mol Microbiol **13:**955–964. http://dx.doi.org/10.1111/j.1365-2958.1994.tb00487.x.
10. **Reuther J, Wohlleben W, Muth G.** 2006. Modular architecture of the conjugative plasmid pSVH1 from *Streptomyces venezuelae*. Plasmid **55:**201–209. http://dx.doi.org/10.1016/j.plasmid.2005.11.007.
11. **Kendall KJ, Cohen SN.** 1987. Plasmid transfer in *Streptomyces lividans*: identification of a *kil-kor* system associated with the transfer region of pIJ101. J Bacteriol **169:**4177–4183.
12. **Hagège J, Pernodet J-L, Sezonov G, Gerbaud C, Friedmann A, Guérineau M.** 1993. Transfer functions of the conjugative integrating element pSAM2 from *Streptomyces ambofaciens*: characterization of a *kil-kor* system associated with transfer. J Bacteriol **175:**5529–5538.
13. **Reuther J, Gekeler C, Tiffert Y, Wohlleben W, Muth G.** 2006. Unique conjugation mechanism in mycelial streptomycetes: a DNA-binding ATPase translocates unprocessed plasmid DNA at the hyphal tip. Mol Microbiol **61:**436–446. http://dx.doi.org/10.1111/j.1365-2958.2006.05258.x.
14. **Servín-González L.** 1996. Identification and properties of a novel *clt* locus in the *Streptomyces phaeochromogenes* plasmid pJV1. J Bacteriol **178:**4323–4326.
15. **Franco B, González-Cerón G, Servín-González L.** 2003. Direct repeat sequences are essential for function of the *cis*-acting locus of transfer (*clt*) of *Streptomyces phaeochromogenes* plasmid pJV1. Plasmid **50:**242–247. http://dx.doi.org/10.1016/S0147-619X(03)00063-5.
16. **Vogelmann J, Ammelburg M, Finger C, Guezguez J, Linke D, Flötenmeyer M, Stierhof YD, Wohlleben W, Muth G.** 2011. Conjugal plasmid transfer in *Streptomyces* resembles bacterial chromosome segregation by FtsK/SpoIIIE. EMBO J **30:**2246–2254. http://dx.doi.org/10.1038/emboj.2011.121.
17. **Bibb MJ, Hopwood DA.** 1981. Genetic studies of the fertility plasmid SCP2 and its SCP2* variants in *Streptomyces coelicolor* A3(2)-. J Gen Microbiol **126:**427–442. http://dx.doi.org/10.1099/00221287-126-2-427.
18. **Hopwood DA, Kieser T.** 1993. Conjugative plasmids of *Streptomyces*, p 293–311. In Clewell DB (ed), Bacterial conjugation. Plenum Press, New York, NY.
19. **Kieser T, Hopwood DA, Wright HM, Thompson CJ.** 1982. pIJ101, a multi-copy broad host-range *Streptomyces* plasmid: functional analysis and development of DNA cloning vectors. Mol Gen Genet **185:**223–238. http://dx.doi.org/10.1007/BF00330791.
20. **Bibb MJ, Ward JM, Kieser T, Cohen SN, Hopwood DA.** 1981. Excision of chromosomal DNA sequences from *Streptomyces coelicolor* forms a novel family of plasmids detectable in *Streptomyces lividans*. Mol Gen Genet **184:**230–240.
21. **Vogelmann J, Wohlleben W, Muth G.** 2011. *Streptomyces* conjugative elements, p 27–42. In Dyson P (ed), *Streptomyces*—molecular biology and biotechnology. Caister Academic Press, Norfolk, United Kingdom.
22. **Tiffert Y, Götz B, Reuther J, Wohlleben W, Muth G.** 2007. Conjugative DNA transfer in *Streptomyces*: SpdB2 involved in the intramycelial spreading of plasmid pSVH1 is an oligomeric integral membrane protein that binds to dsDNA. Microbiology **153:**2976–2983. http://dx.doi.org/10.1099/mic.0.2006/005413-0.
23. **Thoma L, Sepulveda E, Latus A, Muth G.** 2014. The stability region of the *Streptomyces lividans* plasmid pIJ101 encodes a DNA-binding protein recognizing a highly conserved short palindromic sequence motif. Front Microbiol **5:**499. http://dx.doi.org/10.3389/fmicb.2014.00499.
24. **Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA.** 2000. Practical *Streptomyces* genetics. John Innes Foundation, Norwich, United Kingdom.
25. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A **97:**6640–6645. http://dx.doi.org/10.1073/pnas.120163297.
26. **Sawitzke JA, Stahl FW.** 1992. Phage lambda has an analog of *Escherichia coli recO*, *recR* and *recF* genes. Genetics **130:**7–16.
27. **Gust B, Chandra G, Jakimowicz D, Yuqing T, Bruton CJ, Chater KF.** 2004. Lambda red-mediated genetic manipulation of antibiotic-producing *Streptomyces*. Adv Appl Microbiol **54:**107–128. http://dx.doi.org/10.1016/S0065-2164(04)54004-2.
28. **Thoma L, Muth G.** 2015. The conjugative DNA-transfer apparatus of *Streptomyces*. Int J Med Microbiol **305:**224–229. http://dx.doi.org/10.1016/j.ijmm.2014.12.020.

29. **Kataoka M, Kosono S, Seki T, Yoshida T**. 1994. Regulation of the transfer genes of *Streptomyces* plasmid pSN22: *in vivo* and *in vitro* study of the interaction of TraR with promoter regions. J Bacteriol **176:**7291–7298.
30. **Karimova G, Pidoux J, Ullmann A, Ladant D**. 1998. A bacterial two-hybrid system based on a reconstituted signal transduction pathway. Proc Natl Acad Sci U S A **95:**5752–5756. http://dx.doi.org/10.1073/pnas.95.10.5752.
31. **Kataoka M, Seki T, Yoshida T**. 1991. Five genes involved in self-transmission of pSN22, a *Streptomyces* plasmid. J Bacteriol **173:** 4220–4228.
32. **Servín-González L, Sampieri AI, Cabello J, Galván L, Juárez V, Castro C**. 1995. Sequence and functional analysis of the *Streptomyces phaeochromogenes* plasmid pJV1 reveals a modular organization of *Streptomyces* plasmids that replicate by rolling circle. Microbiology **141:**2499–2510. http://dx.doi.org/10.1099/13500872-141-10-2499.
33. **Pettis GS, Cohen SN**. 2000. Mutational analysis of the *tra* locus of the broad-host-range *Streptomyces* plasmid pIJ101. J Bacteriol **182:** 4500–4504. http://dx.doi.org/10.1128/JB.182.16.4500-4504.2000.
34. **Smith MC, Burns RN, Wilson SE, Gregory MA**. 1999. The complete genome sequence of the *Streptomyces* temperate phage straight phiC31: evolutionary relationships to other viruses. Nucleic Acids Res **27:** 2145–2155. http://dx.doi.org/10.1093/nar/27.10.2145.
35. **Van Dessel W, Van Mellaert L, Liesegang H, Raasch C, De Keersmaeker S, Geukens N, Lammertyn E, Streit W, Anné J**. 2005. Complete genomic nucleotide sequence and analysis of the temperate bacteriophage VWB. Virology **331:**325–337. http://dx.doi.org/10.1016/j.virol.2004.10.028.
36. **Sambrook JM, Russel DW**. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
37. **Ursinus A, van den Ent F, Brechtel S, de Pedro M, Höltje JV, Löwe J, Vollmer W**. 2004. Murein (peptidoglycan) binding property of the essential cell division protein FtsN from *Escherichia coli*. J Bacteriol **186:** 6728–6737. http://dx.doi.org/10.1128/JB.186.20.6728-6737.2004.
38. **Ranjit DK, Young KD**. 2013. The Rcs stress response and accessory envelope proteins are required for de novo generation of cell shape in *Escherichia coli*. J Bacteriol **195:**2452–2462. http://dx.doi.org/10.1128/JB.00160-13.
39. **Hofmann K, Stoffel W**. 1993. TMbase—a database of membrane spanning proteins segments. Biol Chem Hoppe Seyler **374:**166.