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Green Fluorescent Protein-Labeled Monitoring Tool To Quantify Conjugative Plasmid Transfer between Gram-Positive and Gram-Negative Bacteria

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On the basis of pIP501, a green fluorescent protein (GFP)-tagged monitoring tool was constructed for quantifying plasmid mobilization among Gram-positive bacteria and between Gram-positive *Enterococcus faecalis* and Gram-negative *Escherichia coli*. Furthermore, retromobilization of the GFP-tagged monitoring tool was shown from *E. faecalis* OG1X into the clinical isolate *E. faecalis* T9.

The mechanisms of conjugative transfer in Gram-negative bacteria are fairly well understood (e.g., see references 2, 9, 18, 33, 36, and 41), whereas conjugation in Gram-positive bacteria has been studied in greater detail for only the last decade leading to the first model of a type IV secretion-like system in Gram-positive bacteria (1, 2, 16, 17, 41). The antibiotic resistance plasmid pIP501 from *Streptococcus agalactiae* has a very broad host range for conjugative plasmid transfer and mobilization. Its host range includes virtually all tested Gram-positive bacteria, including the multicellular filamentous streptomycetes and Gram-negative *Escherichia coli* (17, 24).

For Gram-positive systems, molecular tools for *in situ* detection of horizontal gene transfer by conjugation are still very limited in contrast to Gram-negative systems (4, 7, 8, 29, 37, 38). Nieto and Espinosa (30) have constructed a green fluorescent protein (GFP)-tagged derivative of plasmid pMV158 from *Streptococcus pneumoniae*, pMV158GFP, that was shown to be mobilizable to different low-GC Gram-positive bacteria like *Enterococcus faecalis* and *Lactococcus lactis*. Lorenzo-Díaz and Espinosa applied pMV158GFP to intra- and interspecies mobilization between different Gram-positive bacteria in large-scale filter mating assays (26).

Recently, Babic and coworkers (3) demonstrated conjugative transfer of the integrative and conjugative element ICE*Bs1* from *Bacillus subtilis* donor cells to *B. subtilis* recipient cells in real time using a *lacO* or LacI-GFP system for visualization of transfer events.

Here, we report the construction and mobilization of a GFP-tagged mobilizable plasmid based on the pIP501 *tra* region to monitor horizontal gene transfer between Gram-positive bacteria and between Gram-positive and Gram-negative bacteria by the formation of a green fluorescent phenotype in transconjugants. The mobilizable plasmid is based on a nisin-inducible expression system (NICE) and replicates in both Gram-positive and Gram-negative bacteria.

Plasmid construction. The *oriT* region from the broad-host-range plasmid pIP501 ($oriT_{pIP50}$) was subcloned with primer pair *oriT-Hin*dIII-fw (fw stands for forward) and *oriT-Hin*dIII-re (re stands for reverse) (see Table S1 in supplemental material) via HindIII into plasmid pJP$_{rel}$GFP encoding a *gfp* gene improved for expression in prokaryotes (28, 31). All bacterial strains and plasmids used in this work are described in Table 1. The *gfp-oriT*$_{pIP501}$ cassette was inserted into the *E. coli* shuttle plasmid pMSP3535VA (6) via XmaI/XbaI with primer pair P$_{rel}$-*gfp-Xma*I-fw and *oriT-Xba*I-re under the control of a nisin-inducible *nisA* promoter. Then, the λ phage t_0 terminator was cloned downstream of the *oriT* region via XbaI and XhoI sites with primer pair t_0-term-*Xba*I-fw and t_0-term-*Xba*I-re using the expression vector pQTEV (35) as the template, thus generating plasmid pVA-*gfp-oriT* (Fig. 1).

Assessment of green fluorescence by FACS. The fluorescence of *E. faecalis* OG1X(pVA-*gfp-oriT*) was quantified by fluorescence-activated cell sorting (FACS) (FACScan flow cytometer; BD Biosciences, Heidelberg, Germany) after induction of the *nisA* promoter with 100 ng · ml^{-1} nisin on brain heart infusion (BHI) (Condalab, Madrid, Spain) agar plates supplemented with streptomycin (1,000 μg · ml^{-1}) and kanamycin (2,000 μg · ml^{-1}). More than 95% of the analyzed cells were shifted to a green fluorescent phenotype, indicating efficient induction of the *nisA* promoter and expression of GFP with the plasmid construct (data not shown).

pIP501-mediated mobilization of the GFP-tagged plasmid to *E. faecalis* and *B. subtilis*. pVA-*gfp-oriT* was tested for its ability to be mobilized by pIP501 in filter matings. The donor *E. faecalis* OG1X harboring pIP501 and pVA-*gfp-oriT* and the recipient *E. faecalis* JH2-2 were grown to an optical density at 600 nm (OD_{600}) of 0.5, mixed in a 1:10 ratio, and passed through a sterile nitrocellulose membrane filter (0.45 μm) (Millipore, Schwalbach, Germany). After overnight incubation on BHI agar at 37°C, the cells were recovered in 1 ml phosphate-buffered saline (PBS), and serial dilutions were plated on BHI

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TABLE 1 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant genotype or phenotype[a]	Source or reference
Bacterial strains		
Escherichia coli XL10	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lacI*^{q}*Z*ΔM15 Tn*10*(Tet^r) Amy Cm^r]	Stratagene
Enterococcus faecalis		
JH2-2	Rif^r Fus^r	23
OG1RF	Rif^r Fus^r	10
OG1X	Sm^r	22
T9	Tet^r	21
Bacillus subtilis subsp. *natto* DSM 4451	Sm^r	DSMZ[b]
Plasmids		
pIP501	Tra Cm^r MLS^r	12
pMSP3535VA	pVA380-1 and ColE1 replicons *nisRK* P_{nisA} Km^r	6
pJP$_{rel}$GFP	P_{rel} *gfp*	31
pQTEV	P_{t4} *lacI*^{q} His_7 Amp^r	35
pJP$_{rel}$GFP-*oriT*	pJP$_{rel}$GFP $oriT_{pIP501}$	This work
pVA-*gfp-oriT*	pMSP3535VA *gfp* $oriT_{pIP501}$ t_0-term$_{pQTEV}$	This work

[a] MLS^r, macrolide-lincosamide-streptogramin B resistance; Hte, high transformation efficiency; Amy, amylase.
[b] DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures).

agar with kanamycin (400 $\mu g \cdot ml^{-1}$) and/or fusidic acid (50 $\mu g \cdot ml^{-1}$) to enumerate transconjugants and recipients, respectively. The mean mobilization rate from three independent experiments was $1.42 \cdot 10^{-5} \pm 3.11 \cdot 10^{-6}$ transconjugants per recipient cell. Mobilization rates were approximately three times lower than pIP501 transfer rates in experiments under the same conditions, suggesting a possible *cis*-acting preference of the Orf1 relaxase in agreement with observations made for the TraA relaxase of plasmid pRetCF2d and relaxase Orf28 of the conjugative transposon Tn*1549* (32, 40).

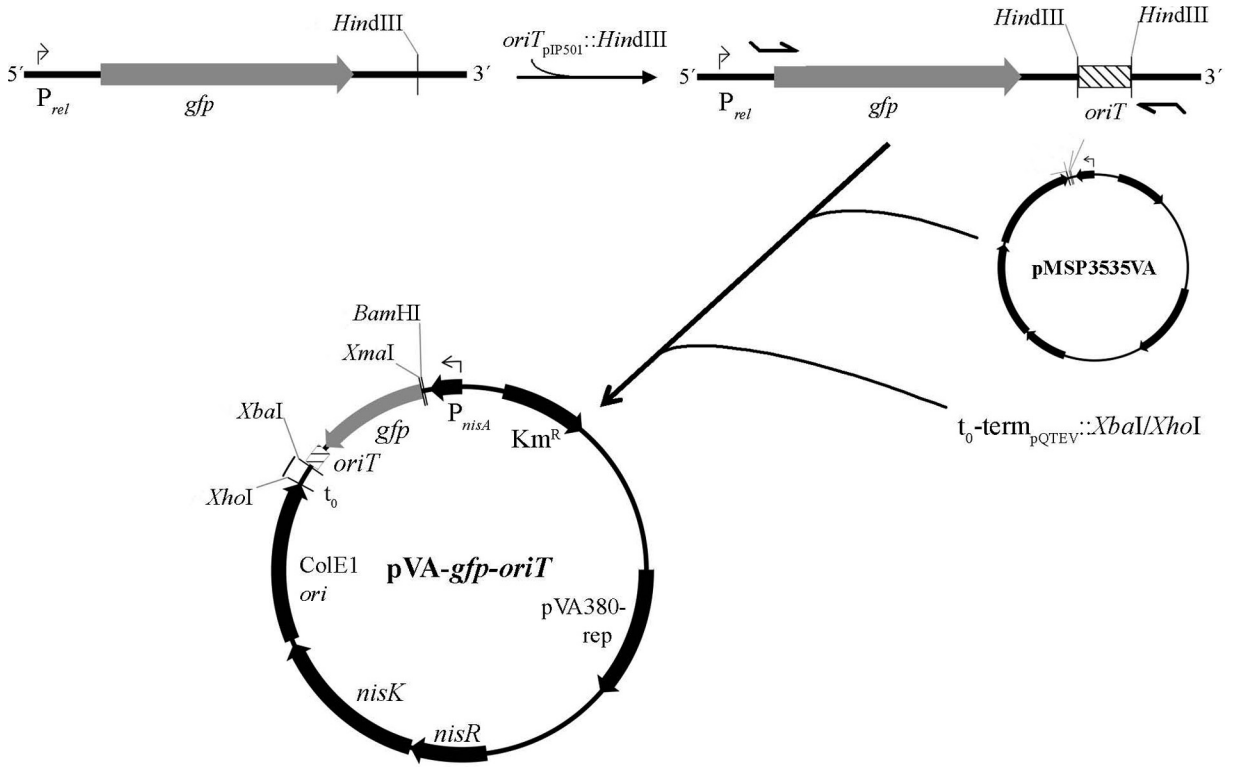

FIG 1 The pIP501 *oriT* region was subcloned into plasmid pJP$_{rel}$GFP via HindIII. The *gfp-oriT* cassette was then cloned into plasmid pMSP3535VA via XmaI and XbaI sites, followed by insertion of the t_0 termination sequence via XbaI and XhoI, generating pVA-*gfp-oriT*.

Transconjugants were verified by green fluorescent phenotype after induction of the *nisA* promoter on BHI agar supplemented with kanamycin (400 $\mu g \cdot ml^{-1}$), fusidic acid (50 $\mu g \cdot ml^{-1}$), and nisin (100 $ng \cdot ml^{-1}$) and amplification of the *gfp* gene by PCR with primer pair P_{rel}-*gfp*-*Xma*I-fw and *oriT*-*Xba*I-re (data not shown).

Since pIP501 can be transferred and stably maintained in various Gram-positive bacteria, we tried to mobilize pVA-*gfp*-*oriT* to *B. subtilis* in a triparental mating with the help of pIP501: *E. faecalis* JH2-2(pVA-*gfp*-*oriT*), *E. faecalis* OG1RF(pIP501), and *B. subtilis* subsp. *natto* DSM 4451 were grown to an OD_{600} of 0.5 and mated overnight at 30°C in a ratio of 1:1:10. Serial dilutions were plated on Luria-Bertani agar supplemented with kanamycin (20 $\mu g \cdot ml^{-1}$) and/or streptomycin (1,000 $\mu g \cdot ml^{-1}$) to enumerate transconjugants and recipients, respectively. The mean mobilization rate obtained from three independent experiments was $4.10 \cdot 10^{-6} \pm 4.74 \cdot 10^{-7}$ transconjugants per recipient. Thus, the mobilization rates are similar to those obtained for mobilization of pMV158 from *E. faecalis* OG1X to *B. subtilis* MB46 SL601 by pIP501 (24). Mobilization of pVA-*gfp*-*oriT* was verified by a green fluorescent phenotype for *B. subtilis* subsp. *natto* DSM 4451(pVA-*gfp*-*oriT*) (not shown) after induction of the *nisA* promoter on LB agar supplemented with kanamycin (20 $\mu g \cdot ml^{-1}$), streptomycin (1,000 $\mu g \cdot ml^{-1}$), and nisin (100 $\mu g \cdot ml^{-1}$) and amplification of the *gfp* gene by PCR (data not shown).

Interestingly, even after induction of the *nisA* promoter with different nisin concentrations (10, 100, and 1,000 $ng \cdot ml^{-1}$), only 5% of the *Bacillus* transconjugants expressed a green fluorescent phenotype. It has been previously demonstrated that the NICE system can be used for efficient inducible gene expression in Gram-positive bacteria like *Lactococcus*, *Bacillus*, and *Enterococcus* (6, 11, 27). However, Hirt and coworkers (20) demonstrated weak expression of the pCF10 encoded surface protein PrgB with plasmid pMSP3535 in *B. subtilis*. This finding together with our results leads to the hypothesis that the NICE vectors pMSP3535 and pMSP3535VA might not be suitable for efficient gene expression in *Bacillus* species. Thus, GFP expression levels in our case might not have reached a certain threshold in most *B. subtilis* cells that is necessary for developing a green fluorescent phenotype (15).

pIP501-mediated mobilization from the Gram-positive *E. faecalis* to the Gram-negative *E. coli*. pVA-*gfp*-*oriT* was shown to be suited to monitor the transfer of plasmid from the Gram-positive *E. faecalis* to the Gram-negative *E. coli* by formation of a green fluorescent phenotype in an *E. coli* recipient. The donor *E. faecalis* OG1X(pVA-*gfp*-*oriT*), the helper *E. faecalis* OG1RF(pIP501), and the recipient *E. coli* XL10 were grown to an OD_{600} of 0.5 and mated overnight at 37°C in a ratio of 1:1:10. Serial dilutions were plated on Luria-Bertani agar supplemented with kanamycin (50 $\mu g \cdot ml^{-1}$) and/or tetracycline (10 $\mu g \cdot ml^{-1}$) to enumerate transconjugants and recipients, respectively. pIP501-mediated mobilization of pVA-*gfp*-*oriT* from *E. faecalis* OG1X to *E. coli* XL10 occurred with a rather low frequency of $2.30 \cdot 10^{-8} \pm 2.07 \cdot 10^{-8}$ transconjugants per recipient cell. However, the *nisA* promoter in *E. coli* was functional, leading to a green fluorescent phenotype in approximately 95% of the induced *E. coli* cells (data not shown). Trieu-Cuot and coworkers (39) showed mobilization of shuttle vectors containing the RK2 *oriT* region from Gram-negative *E. coli* to Gram-positive *E. faecalis* with mobilization frequencies in the same range.

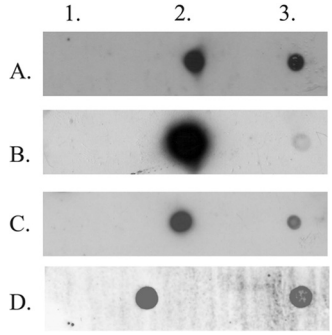

FIG 2 Dot blot hybridization of *nes* (A), *traE* (B), *traG* (C), and *traK* (D). PCR products were generated with digoxigenin-labeled pSK41-derived probes. Spot 1, negative control (no DNA template was applied to the PCR); spot 2, PCR product of *S. aureus*(pSK41) as a positive control, spot 3, PCR product of *E. faecalis* T9.

Retromobilization of pVA-*gfp*-*oriT* into the clinical strain *E. faecalis* T9. To investigate whether mobilization of pVA-*gfp*-*oriT* can also occur into pathogenic enterococcal strains, we tested the clinical *E. faecalis* T9 isolate (21). Molecular characterization of *E. faecalis* T9 revealed the presence of pSK41-like *nes* and *traE*, *traG*, and *traK* genes confirmed by dot blotting (Fig. 2; see Table S1 in the supplemental material for the primers used in dot blots), thus indicating the presence of a putative conjugative element in *E. faecalis* T9. However, plasmid isolation by the method of Woodford et al. (43) with the modifications of Werner et al. (42) and plasmid profiling by S1 nuclease macrorestriction (5, 13, 25) of *E. faecalis* T9 did not confirm the presence of plasmids (not shown).

To prove the presence of a conjugative element in *E. faecalis* T9, a biparental retromobilization experiment was performed: *E. faecalis* T9 as a helper strain and recipient and *E. faecalis* OG1X(pVA-*gfp*-*oriT*) as a donor were grown to an OD_{600} of 0.5 and mated overnight at 37°C in a ratio of 1:10 (donor/recipient [D/R]). Serial dilutions were plated on BHI agar supplemented with kanamycin (500 $\mu g \cdot ml^{-1}$) and/or tetracycline (10 $\mu g \cdot ml^{-1}$) to enumerate transconjugants and recipients, respectively. Retromobilization of pVA-*gfp*-*oriT* into *E. faecalis* T9 occurred with a frequency of $1.62 \cdot 10^{-5} \pm 1.04 \cdot 10^{-5}$, indicating a putative pSK41-like conjugative element in *E. faecalis* T9. Transconjugants were verified by PCR amplification of the *gfp* gene, plasmid isolation, and fluorescence microscopy after induction of the *nisA* promoter on BHI agar supplemented with kanamycin (500 $\mu g \cdot ml^{-1}$), tetracycline (10 $\mu g \cdot ml^{-1}$), and nisin (100 $ng \cdot ml^{-1}$) (data not shown). To the best of our knowledge, retromobilization into *E. faecalis* has been demonstrated for the first time.

To further investigate the conjugative element present in *E. faecalis* T9, *E. faecalis* T9 harboring pVA-*gfp*-*oriT* was used as a donor to mobilize the GPF-tagged plasmid in a biparental mating to *B. subtilis* subsp. *natto* DSM 4451. The strains were grown to an OD_{600} of 0.5 and mated overnight at 30°C. Serial dilutions were plated on LB agar supplemented with kanamycin (20 $\mu g \cdot ml^{-1}$) and/or streptomycin (1,000 $\mu g \cdot ml^{-1}$) to enumerate transconjugants and recipients, respectively. Green fluorescent *B. subtilis* transconjugants were obtained with a mean mobilization rate of $3.10 \cdot 10^{-7} \pm 3.32 \cdot 10^{-8}$ per recipient, indicating that mobilizable plasmids can be transferred to *B. subtilis* and *E. faecalis* by the conjugative element in *E. faecalis* T9. These results suggest that the pIP501 *oriT* region is recognized by the pSK41-like relaxase pres-

ent in *E. faecalis* T9. In favor of this hypothesis, Garcillan-Barcia et al. reported that pIP501 and pSK41 relaxases belong to the same mobilization protein (MOB) family (14).

To date, we have no information about traits that can be transferred by the conjugative element in *E. faecalis* T9. Most widely spread integrative and conjugative elements (ICEs) in *E. faecalis* such as Tn*916*, Tn*1545*, and Tn*1549* are genetically linked to genes conferring antibiotic resistance and have a broad host range (13). However, none of these transposons shows similarities to the pSK41 *tra* region originating from *Staphylococcus aureus*. Considering the clinical origin of *E. faecalis* T9, a pSK41-like plasmid might have been integrated into the chromosome of *E. faecalis* T9, thus allowing the capture of different traits from the clinical background.

It has been demonstrated that Inc18 plasmids like pIP501 might be involved in the spread of vancomycin resistance genes and the emergence of vancomycin-resistant *S. aureus* (VRSA) (44, 45). Recent studies indicate that the pIP501 replicon has a high prevalence in clinical *Enterococcus faecium* isolates and that it is often genetically linked with *vanA* resistance genes in enterococci (19, 34). Thus, the constructed mobilizable plasmid might be a powerful tool to screen for pIP501-like and pSK41-like conjugative elements in *Enterococcus* and *Staphylococcus* isolates.

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