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 agalакticae has a very broad host range for conjugative plasmid transfer and mobilization. Its host range includes virtually all tested Gram-positive bacteria, including the multilocular 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 ICEBs1 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 (NICE) and replicates in both Gram-positive and Gram-negative bacteria.

Plasmid construction. The oriT region from the broad-host-range plasmid pIP501 (oriT pIP501) was subcoloned with primer pair oriT-HindIII-fw (fw stands for forward) and oriT-HindIII-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 pMSP355VA (6) via XmaI/XbaI with primer pair Prel-gfp-XmaI-fw and oriT-XbaI-re under the control of a nisin-inducible nisA promoter. Then, the λ phage λs terminator was cloned downstream of the oriT region via XbaI and XhoI sites with primer pair λs-term-XbaI-fw and λs-term-XhoI-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 (OD₆₀₀) 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...
agar with kanamycin (400 μg·ml⁻¹) and/or fusidic acid (50 μg·ml⁻¹) to enumerate transconjugants and recipients, respectively. The mean mobilization rate from three independent experiments was 1.42 · 10⁻⁵ ± 3.11 · 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 Tn1549 (32, 40).

### TABLE 1 Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
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</tr>
<tr>
<td>OG1RF</td>
<td>Rif² Fus²</td>
<td>10</td>
</tr>
<tr>
<td>OG1X</td>
<td>Sm⁰</td>
<td>22</td>
</tr>
<tr>
<td>T9</td>
<td>Tet⁰</td>
<td>21</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> subsp. natto DSM 4451</td>
<td>Sm⁰</td>
<td>DSMZ⁶</td>
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<table>
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<th>Plasmids</th>
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</tr>
<tr>
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<td>pVA380-1 and ColE1 replicons nisRK P_mRNA Km⁰</td>
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<td>This work</td>
</tr>
<tr>
<td>pVA-gfp-oriT</td>
<td>pMSP3535VA gfp oriT₉₋₉₋ t₀-term₉₋₉₋</td>
<td>This work</td>
</tr>
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*MLS⁰, macrolide-lincosamide-streptogramin B resistance; Hte, high transformation efficiency; Amy, amylase.

⁶ DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures).

**FIG 1** The pIP501 oriT region was subcloned into plasmid pJP₉₋₉₋GFP via HindIII. The gfp-oriT cassette was then cloned into plasmid pMSP3535VA via Xmal and XbaI sites, followed by insertion of the t₀ 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 μg ⋅ ml⁻¹), fusidic acid (50 μg ⋅ ml⁻¹), and nisin (100 ng ⋅ ml⁻¹) and amplification of the gfp gene by PCR with primer pair P₁₅gfp-Xmal-fw and oriT-Xbal-re (data not shown).

Since pLP501 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 pLP501: E. faecalis JH2-2(pVA-gfp-oriT), E. faecalis OG1RF(pLP501), and B. subtilis subsp. natto DSM 4451 were grown to an OD₆₀₀ 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 μg ⋅ ml⁻¹) and/or streptomycin (1,000 μg ⋅ ml⁻¹) to enumerate transconjugants and recipients, respectively. The mean mobilization rate obtained from three independent experiments was 4.10⁻⁶ ± 4.74 ⋅ 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 pLP501 (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 μg ⋅ ml⁻¹), streptomycin (1,000 μg ⋅ ml⁻¹), and nisin (100 μg ⋅ 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 ⋅ 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 gene in an E. coli cell that is necessary for conjugative element present in Bacillus species. Thus, GFP expression levels in our case might not have reached a threshold in most B. subtilis cells that is necessary for developing a green fluorescent phenotype (15).

pLP501-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(pLP501), and the recipient E. coli XL10 were grown to an OD₆₀₀ 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 (500 μg ⋅ ml⁻¹) and/or tetracycline (10 μg ⋅ ml⁻¹) to enumerate transconjugants and recipients, respectively. pLP501-mediated mobilization of pVA-gfp-oriT from E. faecalis OG1X to E. coli XL10 occurred with a rather low frequency of 2.30 ⋅ 10⁻⁸ ± 2.07 ⋅ 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.

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, 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 OD₆₀₀ 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 μg ⋅ ml⁻¹) and/or tetracycline (10 μg ⋅ ml⁻¹) to enumerate transconjugants and recipients, respectively. Retromobilization of pVA-gfp-oriT into E. faecalis T9 occurred with a frequency of 1.62 ⋅ 10⁻⁵ ± 1.04 ⋅ 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 μg ⋅ ml⁻¹) and/or tetracycline (10 μg ⋅ ml⁻¹), and nisin (100 ng ⋅ 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 OD₆₀₀ of 0.5 and mated overnight at 30°C. Serial dilutions were plated on LB agar supplemented with kanamycin (20 μg ⋅ ml⁻¹) and/or streptomycin (1,000 μg ⋅ ml⁻¹) to enumerate transconjugants and recipients, respectively. Green fluorescent B. subtilis transconjugants were obtained with a mean mobilization rate of 3.10⁻⁷ ± 3.32 ⋅ 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 pLP501 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 (M0B) 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 Tn916, Tn1545, and Tn1549 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 IncC18 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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**REFERENCES**