

# IncP-1 $\beta$ Plasmid pGNB1 Isolated from a Bacterial Community from a Wastewater Treatment Plant Mediates Decolorization of Triphenylmethane Dyes<sup>∇</sup>

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Plasmid pGNB1 was isolated from bacteria residing in the activated sludge compartment of a wastewater treatment plant by using a transformation-based approach. This 60-kb plasmid confers resistance to the triphenylmethane dye crystal violet and enables its host bacterium to decolorize crystal violet. Partial sequencing of pGNB1 revealed that its backbone is very similar to that of previously sequenced IncP-1 $\beta$  plasmids. The two accessory regions of the plasmid, one located downstream of the replication initiation gene *trfA* and the other located between the conjugative transfer modules *Tra* and *Trb*, were completely sequenced. Accessory region L1 contains a transposon related to Tn5501 and a gene encoding a Cupin 2 conserved barrel protein with an unknown function. The triphenylmethane reductase gene *tmr* and a truncated dihydroliipoamide dehydrogenase gene that is flanked by IS1071 and another putative insertion element were identified in accessory region L2. Subcloning of the pGNB1 *tmr* gene demonstrated that this gene is responsible for the observed crystal violet resistance phenotype and mediates decolorization of the triphenylmethane dyes crystal violet, malachite green, and basic fuchsin. Plasmid pGNB1 and the associated phenotype are transferable to the  $\alpha$ -proteobacterium *Sinorhizobium meliloti* and the  $\gamma$ -proteobacterium *Escherichia coli*. This is the first report of a promiscuous IncP-1 $\beta$  plasmid isolated from the bacterial community from a wastewater treatment plant that harbors a triphenylmethane reductase gene. The pGNB1-encoded enzyme activity is discussed with respect to bioremediation of sewage polluted with triphenylmethane dyes.

Bacteria living in wastewater habitats have to adapt rapidly to changing conditions depending on the pollutant composition of the sewage. The horizontally mobile gene pool of bacteria has been recognized to be very important for adaptive responses to selective pressures caused by diverse chemical compounds (6, 11, 43, 45). In particular, mobile broad-host-range plasmids significantly contribute to the dissemination of beneficial genetic traits among the population of a certain species and even beyond species boundaries. In recent years several promiscuous broad-host-range plasmids belonging to the IncP-1 group and carrying different accessory modules conferring resistance and degradation capabilities were identified in different hosts (7, 35). Among these, plasmids encoding resistance to nearly all clinically relevant antimicrobial drug classes were isolated from bacterial populations residing in wastewater treatment plants (15, 33, 34, 39, 41, 42). Other IncP-1 plasmids harbor genes for the degradation of different pollutants, such as the xenobiotics atrazine, 2-chlorobenzoate, 3-chlorobenzoate, 2,5-dichlorobenzoate, 2,4-dichlorophenoxyacetic acid, *p*-toluenesulfonate, and haloacetates (18, 20, 37, 46–48). Many more catabolic gene regions were identified on plasmids belonging to other Inc groups (7, 8, 28). Catabolic gene clusters on plasmids have frequently been found to be associated with mobile genetic elements, e.g., insertion se-

quences (IS elements) and transposons, which suggests that these elements play an important role in the distribution of catabolic capacities (27, 38, 40, 45).

Triphenylmethane dyes, such as malachite green, crystal violet, and basic fuchsin, are widely used in the textile, pharmaceutical, food, and cosmetic industries, in paper printing, and in photochemical processes (29). Considerable amounts of these toxic and mutagenic dyes are discharged into wastewater treatment facilities and thus impose a selective pressure on the microbial flora residing in wastewater habitats. In recent years several microorganisms capable of dye decolorization were identified. Among these, an *Aeromonas hydrophila* strain possessing a broad-spectrum dye decolorization activity was described (30). Likewise, different *Mycobacterium* species are able to tolerate high concentrations of triphenylmethane dyes (19). Jang et al. (17) described a biochemical analysis of a triphenylmethane reductase purified from *Citrobacter* sp. strain KCTC. This enzyme catalyzes the NADH-dependent reduction and thus decolorization of certain triphenylmethane dyes.

Here we report the isolation of a conjugative broad-host-range IncP-1 $\beta$  plasmid conferring resistance to and decolorization of triphenylmethane dyes from the bacterial community of a municipal wastewater treatment plant. This plasmid was characterized at the genomic and functional levels.

## MATERIALS AND METHODS

### Isolation of plasmid pGNB1 from an activated sludge bacterial community.

Activated sludge samples were taken from an activated sludge basin of the Bielefeld-Heepen wastewater treatment plant in Germany in October 2004. Plasmids were isolated from activated sludge bacteria as described previously (2).

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Briefly, activated sludge bacteria were cultivated on Luria broth (LB) agar containing crystal violet ( $10 \mu\text{g ml}^{-1}$ ) for 40 h at  $30^\circ\text{C}$ . Growing bacteria were collected and washed with 50 ml of an 0.85% NaCl solution, and total plasmid DNA was prepared using a Nucleobond PC100 kit and AX100 columns according to the protocol supplied by the manufacturer (Macherey-Nagel, Düren, Germany). Plasmid DNA preparations were subsequently used to transform  $\text{CaCl}_2$ -competent *Escherichia coli* KAM3 (25) cells. Transformants were selected on LB agar supplemented with crystal violet ( $10 \mu\text{g ml}^{-1}$ ).

**Bacterial strains and growth conditions.** *E. coli* strains KAM3 (25), DH5 $\alpha$  (14), XL1-Blue (4), and CSH52 (24) containing plasmid pGNB1 were grown in LB supplemented with crystal violet ( $10 \mu\text{g ml}^{-1}$ ) at  $37^\circ\text{C}$ .

**Standard DNA techniques.** Plasmid pGNB1 is a self-transmissible plasmid containing the complete genetic information for conjugal plasmid transfer and thus could be transferred to other *E. coli* strains and *Sinorhizobium meliloti* by conjugation. The plasmid contents of *E. coli* and *S. meliloti* strains harboring plasmid pGNB1 were visualized in an Eckhardt gel as described by Hynes et al. (16). Plasmid pGNB1 was isolated from *E. coli* strains with a Nucleobond PC100 kit (Macherey-Nagel, Düren, Germany), whereas recombinant plasmids containing subcloned pGNB1 restriction fragments were isolated by using a QIAprep Spin miniprep kit (QIAGEN, Hilden, Germany) according to the protocol supplied by the manufacturer. Restriction enzyme digestion, agarose gel electrophoresis, DNA cloning, and transformation of *E. coli* were carried out as described by Sambrook et al. (32).

**Restriction fragment library construction, sequencing, and sequence analysis of the pGNB1 accessory regions.** Purified pGNB1 plasmid DNA was restricted with restriction endonucleases BamHI, EcoRI, PstI, Sall, SphI, and NotI. The restriction fragments obtained were separately subcloned into the sequencing vectors pUC18 (50), pBluescript-II KS (Stratagene, La Jolla, CA), and pZErO-2 (Invitrogen). Sequencing of recombinant plasmids was done by IIT Biotech GmbH (Bielefeld, Germany) as previously described (39). Computer-assisted assembly and sequence quality control were carried out with the CONSED/AUTOFINISH software tool (12, 13). For annotation of the finished sequence, the annotation tool GenDB (version 2.2) was used (23).

**Decolorization of triphenylmethane dyes mediated by plasmid pGNB1.** To test for decolorization of triphenylmethane dyes mediated by plasmid pGNB1,  $10\text{-}\mu\text{l}$  portions of *E. coli* strains KAM3 and DH5 $\alpha$  with and without plasmid pGNB1 grown to the logarithmic phase (optical density at 580 nm, about 0.5) were applied to LB agar plates containing  $15 \mu\text{g ml}^{-1}$  crystal violet, malachite green, or basic fuchsin and grown overnight at  $37^\circ\text{C}$ . Likewise,  $10\text{-}\mu\text{l}$  portions of *S. meliloti*(pGNB1) and wild-type *S. meliloti* were applied to TY agar plates (1) containing  $15 \mu\text{g ml}^{-1}$  crystal violet, malachite green, or basic fuchsin and grown overnight at  $30^\circ\text{C}$ . Decolorization of the dyes was visible as a clear halo surrounding a colony. MICs of crystal violet were determined by growing strains in LB in the presence of 5, 10, 15, 20, 30, 50, 65, 70, 80, 90, 100, 110, and 120  $\mu\text{g ml}^{-1}$  crystal violet.

**Antimicrobial susceptibility testing.** Disk diffusion tests to determine antimicrobial susceptibility were carried out using the general experimental conditions recommended in the guidelines of the Clinical and Laboratory Standards Institute (5) and by the manufacturer of the disks (Oxoid GmbH, Wesel, Germany). The following disks purchased from Oxoid were used: amikacin (AK30), ampicillin (AMP25), azithromycin (AZM15), aztreonam (ATM30), bacitracin (B10), ceftazidim (CEC30), cefepim (FEP30), cefotaxim (CTX30), ceftiofur (CPO30), ceftazidim (CAZ30), ceftibuten (CFT30), cefuroxim (CXM30), chloramphenicol (C10), ciprofloxacin (CIP5), clarithromycin (CLR15), clindamycin (DA10), colistin sulfate (CT10), erythromycin (E30), gentamicin (CN10), kanamycin (K30), lincomycin (MY15), neomycin (N30), norfloxacin (NOR5), novobiocin (NV30), piperacillin (PRL30), polymyxin B (PB300), rifampin (RD30), spectinomycin (SH10), streptomycin (S10, S25), sulfonamide (S3 300), tetracycline (TE10), tobramycin (TOB10), and vancomycin (VA5). Roxithromycin (ROX15) and tylosin (TY30) disks were purchased from MAST Diagnostica (Reinfeld, Germany).

**Conjugative transfer of plasmid pGNB1.** Conjugative transfer tests with plasmid pGNB1 were done as described previously (39). *E. coli* CSH52 (a  $\gamma$ -proteobacterium) and *S. meliloti* (*fdxN::tet*; an  $\alpha$ -proteobacterium) (21) were used as recipients in mating experiments. *E. coli* CSH52 transconjugants were selected on LB containing streptomycin ( $600 \mu\text{g ml}^{-1}$ ) and crystal violet ( $15 \mu\text{g ml}^{-1}$ ), whereas *S. meliloti* transconjugants were selected on TY medium containing tetracycline ( $10 \mu\text{g ml}^{-1}$ ) and crystal violet ( $15 \mu\text{g ml}^{-1}$ ). Transfer frequencies were calculated per recipient.

**Amplification of 16S rRNA gene and internal *tmr* gene fragments.** Crude total DNA preparations from activated sludge isolates were obtained as described by Weidner et al. (49). Amplification of the 16S rRNA gene was performed as described previously (49) by using primers 1385r ( $5\text{'-CGGTGTGTRCAAGGC}$

CC-3', where R is A or G) and 27f ( $5\text{'-GAGTTTGTATCTGGCTCA-3'}$ ). The numbers in the primer designations refer to the binding positions in the *E. coli* 16S rRNA gene. Primers specific for the pGNB1 *tmr* gene were designed by using the Primer 3 program. The primer combination consisting of *tmr*-L1 ( $5\text{'-GTCA AATCATTGCCATCGTG-3'}$ ) and *tmr*-R1 ( $5\text{'-TCGAATGAGACAGGCTGAT G-3'}$ ) was used to amplify *tmr*-specific amplicons from total DNA preparations from activated sludge bacteria by PCR. PCRs were done under standard conditions using the BIO-Taq DNA polymerase (BIOLINE). The amplicons obtained were verified by sequencing.

**Nucleotide sequence accession numbers.** The annotated nucleotide sequences of pGNB1 accessory gene regions L1 and L2 have been deposited in the GenBank database under accession numbers EF628291 and EF628292, respectively.

## RESULTS AND DISCUSSION

**Isolation of plasmid pGNB1 from an activated sludge bacterial community from a wastewater treatment plant.** Bacteria in an activated sludge sample from the Bielefeld-Heepen wastewater treatment plant (Bielefeld, Germany) were selected on LB agar containing the triphenylmethane dye crystal violet (final concentration,  $10 \mu\text{g ml}^{-1}$ ). Total plasmid DNA was isolated from growing colonies and subsequently used to transform *E. coli* KAM3 carrying a deletion in the multidrug efflux system genes *acrAB* (25). *E. coli* KAM3 transformants were selected on LB agar containing  $10 \mu\text{g ml}^{-1}$  crystal violet. Three of 12 transformants that were able to grow on this medium carried an approximately 60-kb plasmid as determined by Eckhardt gel analysis. Isolation of the plasmids and restriction with endonucleases BamHI, HindIII, and PstI revealed that the three plasmids had identical restriction patterns. One of the plasmids conferring crystal violet resistance was designated pGNB1 and chosen for further analysis. Interestingly, colonies of *E. coli* KAM3 carrying pGNB1 were able to decolorize crystal violet. Since *E. coli* KAM3 also harbors an F' plasmid, it was necessary to separate pGNB1 from F', which was achieved by transformation of *E. coli* DH5 $\alpha$  with plasmid DNA isolated from *E. coli* KAM3(pGNB1). *E. coli* DH5 $\alpha$  transformants carrying pGNB1 were selected on LB agar supplemented with crystal violet ( $10 \mu\text{g ml}^{-1}$ ), and their plasmid content was checked using Eckhardt gels.

**Partial sequencing of plasmid pGNB1 revealed that it is an IncP-1 $\beta$  plasmid.** To determine the replicon type of pGNB1, a restriction fragment library of the plasmid was constructed. Restriction fragments generated by the enzymes EcoRI, PstI, Sall, and SphI were cloned into appropriate sequencing vectors. Terminal end sequencing of 17 different recombinant plasmids revealed that pGNB1 is an IncP-1 $\beta$  plasmid closely related to the IncP-1 $\beta$  plasmids pTP6 (36), R751 (44), pB8 (34), pB10 (33), pADP-1 (20), pJP4 (47), and pUO1 (37). The IncP-1 $\beta$ -specific genes *kfrC*, *kleA*, *klcC*, *klcB*, *kluB*, *trfA*, *trbB*, *trbN*, *trbO*, *traC*, *traG*, and *traI* could be identified on the subcloned restriction fragments. Since the backbone regions of IncP-1 $\beta$  plasmids are highly conserved and very well known, we decided to limit our sequencing approach to the accessory regions of the plasmid. Analysis of sequencing data in the restriction fragment library showed that pGNB1 contains two accessory regions, one located downstream of the replication initiation gene *trfA1* and the other between the *tra* and *trb* conjugative transfer modules. The restriction fragment clones representing accessory regions were completely sequenced by using a primer walking strategy. Gaps between restriction fragments were covered by walking reads on pGNB1 plasmid

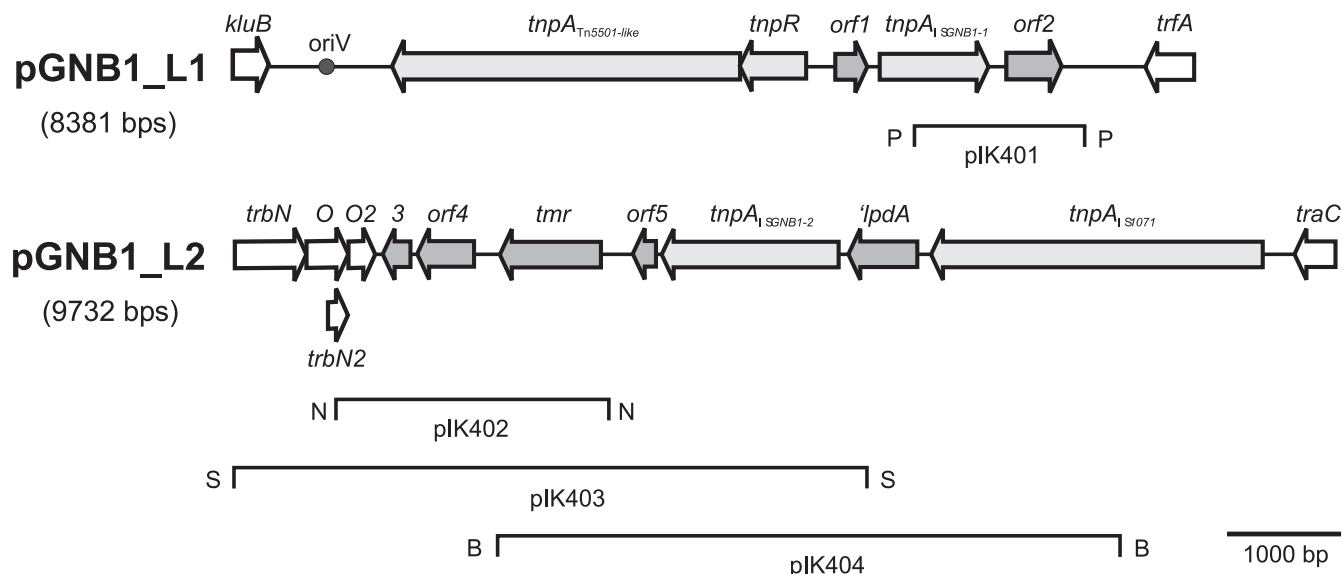


FIG. 1. Genetic map of pGNB1 accessory regions L1 and L2. Coding regions are indicated by arrows showing the direction of transcription. *kluB* (putative postsegregational killing gene), *trfA* (replication initiation gene), *trbN* and *trbO* (mating pair formation genes), and *traC* (conjugative transfer gene) are indicated by open arrows and represent plasmid backbone genes. Accessory region L1 contains a transposon related to Tn5501, a new insertion sequence element (*ISGNB1-1*), and a gene, *orf2*, encoding a conserved protein (Cupin 2 conserved barrel protein) having an unknown function. Recombinant plasmid pIK401 contains a PstI (P) restriction fragment with *orf2*. The origin of vegetative replication (*oriV*) is indicated by a filled circle. Accessory region L2 contains the triphenylmethane reductase gene (*tmr*) and a truncated dihydroliipoamide dehydrogenase gene (*'lpdA*) which is flanked by *IS1071* and *ISGNB1-2*. Subcloning of a 2.4-kb NotI (N) restriction fragment, a 5.8-kb SphI (S) restriction fragment, and a 5.4-kb BamHI (B) restriction fragment resulted in recombinant plasmids pIK402, pIK403, and pIK404, respectively. These plasmids were tested to determine whether they conferred resistance to antimicrobial drugs and triphenylmethane dyes.

DNA. This approach led to the establishment of the complete nucleotide sequences of the two pGNB1 accessory regions in Phred40 quality. Accessory region L1 is flanked by the *kluB* gene (putative postsegregational killing gene) and the *trfA1* backbone gene, whereas *trbNO* and *traC* flank accessory region L2 (Fig. 1).

**Accessory region L1 on plasmid pGNB1 contains a transposon related to Tn5501.** A transposon bordered by 38-bp inverted repeats and related to Tn5501 was inserted between the pGNB1 *kluAB* operon (putative postsegregational killing genes) and the replication initiation gene *trfA*. The insertion site of Tn5501, marked by 5-bp direct repeats (TTCTC), is located 289 bp downstream of *trfA* between TrfA-binding sites 9 and 10 (numbering of Smalla et al. [36]). A 4,230-bp segment of the transposon consisting of the transposition module genes *tnpA* (transposase) and *tnpR* (resolvase), *orf1*, and the 5' end of a *parE*-like gene is 99.5% identical to the Tn5501-like transposon previously identified on IncP-1 $\beta$  plasmid pB8 isolated from an unknown bacterium from an activated sludge community (34). The pGNB1 *orf1-parE'* module encoding a putative addiction system was truncated by insertion of a new 1,047-bp insertion sequence, designated *ISGNB1-1*, which is flanked by 15-bp inverted repeat motifs. The encoded transposase of *ISGNB1-1* belongs to the Transposase\_11 family (accession no. pfam01609) and is 73% identical to TnpA of *Burkholderia vietnamiensis* G4 IS4 (accession no. EAM29391). Adjacent to *ISGNB1-1* the *orf2* gene encoding a conserved protein which contains double-stranded  $\beta$ -helix domains was identified. The *orf2* gene product is identical to the Cupin 2 conserved barrel

protein of plasmid 2 residing in *Ralstonia metallidurans* CH34 (accession no. YP\_582148).

The remaining part of the sequenced fragment (717 bp) contains the replication initiation gene *trfA* and is 100% identical to *Burkholderia cepacia* AMMD IncP-1 $\beta$  plasmid 1 (accession no. CP000443).

**Second accessory region on plasmid pGNB1, L2, contains a triphenylmethane reductase gene flanked by an insertion sequence element.** To establish the complete nucleotide sequence of pGNB1 accessory region L2, restriction fragments covering this region (Fig. 1) were subcloned and subsequently sequenced. Gaps were closed by using a primer walking strategy with pGNB1 plasmid DNA. The left part of the sequenced fragment contains the IncP-1-specific gene *trbN* encoding a lytic transglycosylase involved in mating pair formation and the 5' part of *trbO*. A 832-bp region spanning *trbN-trbO'* is 99.9% identical to the corresponding segments of IncP-1 $\beta$  plasmids pJP4, pTSA, pUO1, pB8, pB10, and pTP6, indicating that these plasmids originated from a common ancestor. A 368-bp region containing *'trbN-trbO'* is reiterated downstream of the truncated first copy of *trbO'*. The accessory module inserted next to *trbO'* encodes a predicted triphenylmethane reductase that is 99.7% identical to the corresponding enzyme previously identified in *Citrobacter* sp. strain KCTC 18061P (17). Triphenylmethane reductase was shown to catalyze the NADH-dependent reduction of triphenylmethane dyes, such as crystal violet, malachite green, and basic fuchsin. The dinucleotide-binding motif G-X<sub>2</sub>-G-X<sub>2</sub>-G conserved in NAD(P)H-dependent enzymes is also present in the N terminus of pGNB1

triphenylmethane reductase. The gene downstream of pGNB1 *tmr* encodes a conserved hypothetical protein (Orf4) that is identical to a corresponding protein of plasmid pLM80 identified in the gram-positive bacterium *Listeria monocytogenes* (26). Interestingly, the pLM80 gene for the hypothetical protein is also clustered with a *tmr*-like gene. There is no indication that the pGNB1 *tmr-orf4* module is part of a mobile genetic element. Thus, it is not known how the gene region was incorporated into the plasmid.

The module upstream of *tmr* on pGNB1 encodes a truncated dihydrolipoamide dehydrogenase (LpdA) possessing an intact pyridine nucleotide-disulfide oxidoreductase dimerization domain (accession no. pfam02852). Compared to other dihydrolipoamide dehydrogenase genes, only the 3' part (591 bp) of *lpdA* is retained on pGNB1. Interestingly, the truncated pGNB1 *lpdA* gene has a GTG start codon that is preceded by a convincing ribosome-binding site (AGAGGAG). Accordingly, the *lpdA* gene product might be produced. LpdA of pGNB1 is identical to the corresponding part of dihydrolipoamide dehydrogenase encoded in the genome of the nitroaromatic compound degrader *Acidovorax* sp. strain JS42 (accession no. ZP\_01383531). Complete *lpdA* genes were previously identified on plasmids pADP-1 and pAA1 of the atrazine-degrading bacteria *Pseudomonas* sp. strain ADP (20) and *Arthrobacter aurescens*, respectively (31). Truncation of *lpdA* on pGNB1 was caused by insertion of *IS1071* four bases upstream of *lpdA*. Transcription of the *IS1071 tnpA* gene is oriented towards *lpdA*, so that read-through transcription of *lpdA* starting from the *tnpA*<sub>*IS1071*</sub> promoter seems to be possible. *IS1071* elements nearly identical (99.9%) to the element on pGNB1 were previously identified on R906 of *Bordetella bronchiseptica* (accession no. DQ471307), pJP4 of *Ralstonia eutropha* (47), pAA1 of *A. aurescens* (31), pUO1 of *Delftia acidovorans* (37), and pADP-1 of *Pseudomonas* sp. strain ADP (20). *IS1071* was frequently found to be associated with degradative genes (9). On the other side *lpdA* of pGNB1 is flanked by a putative IS element (*ISGNB1-2*) closely related to *ISPps1* (*IS91* family) of *Herbaspirillum huttiensis*. The *tnpA* gene of this insertion sequence encodes a transposase belonging to the Transposase\_32 family (accession no. pfam04986) that is 97% identical to TnpA of *H. huttiensis* (10).

The remaining part of the sequenced fragment (495 bp) containing the 3' end of the conjugative transfer gene *traC* is identical to the corresponding regions of IncP-1 $\beta$  plasmids pTP6, R751, pB8, pB10, pADP-1, pJP4, and pUO1, again supporting the close relationship of these plasmids.

In summary, plasmid pGNB1 accessory region L2 contains a module encoding a triphenylmethane dye degradation gene and another element consisting of two insertion sequences which flank a truncated dihydrolipoamide dehydrogenase gene.

**pGNB1 *tmr* gene confers resistance to triphenylmethane dyes.** To further characterize the pGNB1 triphenylmethane reductase gene, the corresponding region was subcloned on a 5.6-kb SphI restriction fragment, on a 2.4-kb NotI fragment, and on a 5.4-kb BamHI fragment (Fig. 1). The resulting constructs, pIK402, pIK403, and pIK404, respectively, conferred resistance to 15  $\mu\text{g ml}^{-1}$  crystal violet and 15  $\mu\text{g ml}^{-1}$  malachite green to the host bacterium *E. coli* DH5 $\alpha$ , whereas DH5 $\alpha$  strains carrying the empty cloning vectors were not able to

grow in the presence of these dyes. *E. coli* DH5 $\alpha$  carrying either pIK402, pIK403, or pIK404 decolorized the dyes crystal violet, malachite green, and basic fuchsin in the vicinity of a colony, whereas the corresponding control strains carrying empty cloning vectors did not. The described decolorization phenotype was also observed for *E. coli* KAM3(pGNB1) and *E. coli* DH5 $\alpha$ (pGNB1). The plasmid-free strains KAM3 and DH5 $\alpha$  are sensitive to crystal violet and malachite green and could not decolorize these dyes. The MIC of crystal violet for *E. coli* strain DH5 $\alpha$  carrying pIK402 (*tmr*) is 120  $\mu\text{g ml}^{-1}$ , whereas the corresponding value for DH5 $\alpha$  without the plasmid is 2.5  $\mu\text{g ml}^{-1}$ . *E. coli* DH5 $\alpha$ (pGNB1) was able to decolorize LB medium contaminated with up to 110  $\mu\text{g ml}^{-1}$  crystal violet during overnight growth (15 h), which is a property that could have biotechnological relevance.

To summarize, the pGNB1 *tmr* gene confers the ability to degrade triphenylmethane dyes to the host bacterium.

**Plasmid pGNB1 does not confer resistance to commonly used antimicrobial compounds.** Annotation of the available pGNB1 nucleotide sequence revealed the presence of genes for which it was not possible to predict any function. These genes are *orf2*, *orf3*, *orf4*, *orf5*, and *lpdA*. To analyze whether one of these genes is involved in mediating antibiotic resistance, antimicrobial susceptibility tests using the disk diffusion method were carried out. *E. coli* DH5 $\alpha$  strains containing pGNB1 or recombinant plasmid pIK401, pIK402, pIK403, or pIK404, each of which harbors subcloned restriction fragments of pGNB1 accessory regions L1 and L2 (Fig. 1), were tested to determine whether they had reduced susceptibilities to 35 different antimicrobial compounds (see Materials and Methods). No reduced susceptibilities were detected for any of these strains. Thus, the function of pGNB1 accessory region L1 remains unknown.

**Plasmid pGNB1 is self-transmissible and can be transferred to  $\alpha$ - and  $\gamma$ -proteobacteria.** Restriction analysis and partial sequencing of plasmid pGNB1 showed that its backbone, including the conjugative transfer (*tra*) and mating pair formation (*trb*) modules, is conserved. IncP-1 plasmids generally are self-transmissible and possess a broad host range. To determine the transfer properties of plasmid pGNB1, mating experiments with the recipient bacteria *E. coli* (a  $\gamma$ -proteobacterium) and *S. meliloti* (an  $\alpha$ -proteobacterium) were carried out. Plasmid pGNB1 transfers at high frequencies (calculated per recipient cell) from *E. coli* DH5 $\alpha$  to the streptomycin-resistant *E. coli* strain CSH52 (transfer frequency,  $2.8 \times 10^{-1}$  per recipient) and from *E. coli* DH5 $\alpha$  to *S. meliloti* (transfer frequency,  $2.2 \times 10^{-3}$  per recipient). The transfer frequencies for pGNB1 are comparable to those for other IncP-1 $\beta$  plasmids isolated from wastewater treatment plant bacteria, and hence the classification of pGNB1 as a self-transmissible, broad-host-range plasmid is justified. *S. meliloti*(pGNB1) was tested for the ability to decolorize triphenylmethane dyes, and it was found that the plasmid-containing strain, in contrast to the plasmid-free parental strain, could reduce crystal violet, malachite green, and basic fuchsin. Thus, the decolorization phenotype was transferred by acquisition of plasmid pGNB1.

**Wastewater treatment plant bacteria related to *Delftia acidovorans* harbor a triphenylmethane reductase gene (*tmr*).** To determine the species affiliation of wastewater treatment plant isolates able to decolorize crystal violet, crude total DNA was

prepared from four randomly selected faint white colonies growing on LB agar plates supplemented with crystal violet (final concentration,  $10 \mu\text{g ml}^{-1}$ ). These DNA preparations were used as template DNAs to generate 16S rRNA gene-specific PCR amplicons by using primers 27f and 1385r. Sequencing of the obtained 16S rRNA gene amplicons of the four different colonies revealed that they were 99, 98, 98.5, and 97% identical to the 16S rRNA gene nucleotide sequence of the  $\beta$ -proteobacterium *Delftia acidovorans* (synonym, *Comamonas acidovorans*) (22). All four isolates also gave PCR amplicons with primers designed for the pGNB1 triphenylmethane reductase gene *tmr*. *D. acidovorans* and *Comamonas* sp. are known to be natural hosts for IncP-1 $\beta$  plasmids (3, 37, 46).

**Concluding remarks.** To our knowledge, this is the first report of an IncP-1 plasmid encoding a gene product that is able to confer resistance to and decolorize triphenylmethane dyes. Since IncP-1 plasmids are very well known for their high transfer activities and their extremely broad host ranges, it is very likely that pGNB1 facilitates dissemination of the encoded catabolic activity among a wide range of bacterial species.

Genomic analysis of plasmid pGNB1 revealed that it is very closely related to the IncP-1 $\beta$  antibiotic resistance plasmid pB8, which was also isolated from a bacterial community from a wastewater treatment plant. It thus appears that nearly identical plasmids can serve as vehicles for both antibiotic resistance determinants and catabolic traits, probably depending on the selective pressure to which the corresponding host bacterium has recently been exposed. Consequently, it is quite conceivable that catabolic plasmids can easily be rearranged by incorporation of antibiotic resistance modules, especially in environments contaminated with antibiotics and other pollutants, which is the case for sewage. Thus, catabolic plasmids should be considered vehicles for the emergence of new antibiotic resistance plasmids. Maintenance of this kind of plasmids in sewage bacteria is selected for by the presence of pollutants, such as triphenylmethane dyes.

Since pollution of water with triphenylmethanes may cause serious ecological damage, the possibility that plasmid pGNB1 might be useful for biotechnological processes aimed towards bioremediation of liquid wastes contaminated with triphenylmethane dyes should be considered.

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