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Erythromycin Resistance-Conferring Plasmid pRSB105, Isolated from a Sewage Treatment Plant, Harbors a New Macrolide Resistance Determinant, an Integron-Containing Tn*402*-Like Element, and a Large Region of Unknown Function▽

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The erythromycin resistance plasmid pRSB105 was previously isolated from an activated sludge bacterial community of a municipal wastewater treatment plant. Compilation of the complete pRSB105 nucleotide sequence revealed that the plasmid is 57,137 bp in size and has a mean G+C content of 56.66 mol%. The pRSB105 backbone is composed of two different replication and/or partitioning modules and a functional mobilization region encoding the mobilization genes *mobCDE* and *mobBA*. The first replicon (Rep1) is nearly identical to the corresponding replication module of the multiresistance plasmid pRSB101 isolated from an unknown activated sludge bacterium. Accordingly, pRSB101 and pRSB105 are sister plasmids belonging to a new plasmid family. The second replicon (Rep2) of pRSB105 was classified as a member of the IncP-6 group. While Rep1 confers replication ability only in γ-proteobacteria, Rep2 extents the host range of the plasmid since it is also functional in the β-proteobacterium *Ralstonia eutropha*. Plasmid pRSB105 harbors the macrolide resistance genes *mel* and *mph*, encoding, respectively, a predicted ABC-type efflux permease and a macrolide-2′-phosphotransferase. Erythromycin resistance is mainly attributed to *mel*, whereas *mph* contributes to erythromycin resistance to a lesser extent. The second resistance region, represented by an integron-containing Tn*402*-like element, includes a β-lactam (*oxa10*) and a trimethoprim (*dfrB2*) resistance gene cassette. In addition to antibiotic resistance modules, pRSB105 encodes a functional restriction/modification system and two nonresistance regions of unknown function. The presence of different mobile genetic elements that flank resistance and nonresistance modules on pRSB105 indicates that these elements were involved in acquisition of accessory plasmid modules. Comparative genomics of pRSB105 and related plasmids elucidated that pRSB105 evolved by integration of distinct modules from different plasmid sources, including *Pseudomonas aeruginosa* plasmids, and thus represents a mosaic plasmid.

Wastewater treatment plants (WWTP) seem to be hotspots for dissemination of antibiotic resistance determinants since resistant bacteria, resistance genes, and mobile genetic elements carrying resistance determinants were frequently isolated from bacterial communities residing in WWTPs. Several multiresistance plasmids could be isolated from activated sludge bacteria by applying the so-called exogenous plasmid isolation method (9, 21, 42, 43, 47, 48, 50). Some of these plasmids were analyzed in-depth at the genomic level. In addition to exogenously isolated plasmids, 10 different erythromycin resistance plasmids, termed pRSB101 to pRSB110, were previously isolated from WWTP bacteria by applying a transformation-based approach (47). Erythromycin belongs to the macrolide antibiotics that are among the most often administered antimicrobial drugs. Resistances to these compounds often cause severe clinical complications. Complete sequencing of plasmids pRSB101 and pRSB107 revealed that both plasmids encode a *mph*(A)-*mrx*-*mphR*(A) macrolide resistance operon conferring high-level erythromycin resistance (46, 47). The *mph*(A) gene product is a macrolide-2′-phosphotransferase that modifies and thereby inactivates macrolide antibiotics (35). PCR analysis indicated that the genes *mph*(A), *mrx*, and *mphR*(A) are present on seven other plasmids of the pRSB series. Only pRSB105 seems to encode a different erythromycin resistance determinant since *mph*(A)-, *mrx*-, or *mphR*(A)-specific amplicons could not be detected with this plasmid as template DNA. The nature of the pRSB105 erythromycin resistance determinant also could not be elucidated by PCR using specific primers for other prevalent erythromycin resistance genes (47). Plasmid pRSB105 is approximately 58 kb in size and, in addition to the aforementioned resistance to erythromycin, confers resistance to ampicillin and trimethoprim. Sequencing of a pRSB105 *repA*-specific amplicon revealed that it differs only slightly compared to the homologous pRSB101 *repA* fragment, indicating that pRSB101 and pRSB105 are sister plasmids sharing similar replicon types (47). Both plasmids are thus members of a new replicon family that has not been described before. To learn more about the origin and evolution of plasmids belonging to this family and their resistance elements, we decided to determine the complete nucleotide sequence for plasmid pRSB105. Due to its size of 58 kb it was expected that pRSB105 also harbors nonresistance modules since only three antibiotic resistances are phenotypically apparent. The pRSB105 composition seems to be reminiscent of the IncF plasmid pRSB107 which, in addition to antibiotic resistances, encodes putative virulence-associated functions

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(46). A study of the comparative genomics of the pRSB105 nucleotide sequence was supplemented by functional analyses.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* DH5α (19), *E. coli* XL1-Blue (4), and the *E. coli* mobilisator strain S17-1 (44), containing the resistance plasmid pRSB105, were grown at 37°C in Luria broth (LB) medium supplemented as needed with antibiotics at the following final concentrations: 100 μg of ampicillin ml^{-1} or 300 μg of erythromycin ml^{-1}. *Xanthomonas campestris* pv. campestris B100 (22), *Ralstonia eutropha* GFP3 (50), *Sinorhizobium meliloti* 1021 (33), and *Agrobacterium tumefaciens* UBAPF2 (24) were grown in TY medium. Indicator medium for strains expressing an active β-galactosidase was supplemented with 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside ml^{-1} (final concentration).

Standard DNA techniques. Plasmid pRSB105 was isolated from *E. coli* strains containing the plasmid by using the Nucleobond kit PC100 and AX 100 columns (Macherey-Nagel, Düren, Germany) in accordance with the manufacturer's protocol. The plasmid content of recipient strains after, respectively, transformation and conjugative transfer was checked in Eckhardt gels as described previously (24). Isolation of small multicopy plasmids was done with the Qiaprep Spin Miniprep kit (QIAGEN, Hilden, Germany). Digestion of plasmid-DNA with restriction enzymes, agarose gel electrophoresis, and transformation of *E. coli* strains were carried out as described previously (40).

Library construction, DNA sequencing of pRSB105, and sequence analysis. Plasmid DNA of pRSB105 was partially digested with the restriction endonuclease Sau3A. The resulting restriction fragments were size fractionated, and the 1- to 3-kb fraction was extracted from an 1% agarose gel by using the Sephaglas BandPrep kit (Amersham Pharmacia Biotech, Inc.) and then cloned into the BamHI-digested sequencing vector pUC19 (54). A second library was generated by cloning PstI, SphI, SalI, BssHII, SacII, and ClaI restriction fragments into the sequencing vectors pUC19 (54), pGEM-T Easy (Promega GmbH, Mannheim, Germany), pBluescript KS (Stratagene, La Jolla, CA), and pBCKS (Stratagene).

Templates for DNA sequencing were prepared by the IIT Biotech GmbH (Bielefeld, Germany) applying the TempliPhi DNA sequencing template amplification kit (GE Healthcare). Template DNA was sequenced by the dideoxy chain termination method using PCR based sequencing with a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). Standard shotgun sequencing reactions were separated on an ABI 3730 (Applied Biosystems) DNA sequencer, resulting in 902 sequencing reads with a 496-base mean sequence length. Base calling and quality control were performed by using the in-house software tool SAMS (Bielefeld University, unpublished data) in which a normalization step applying PHRED (11, 12) was implemented. Sequencing reads were assembled with the CONSED/AUTOFINISH software tool (17, 18). Gap closure and polishing of the sequence was done by primer walking on pRSB105 plasmid-DNA as a template, using the dye terminator chemistry on an ABI 377 sequencer (Applied Biosystems). We decided to rely on Phred40 quality in the consensus sequence. Final assembly and editing of the DNA sequence data resulted in a single circular molecule with a total length of 57,137 bp. The finished pRSB105 sequence was annotated by using the GenDB annotation tool (34).

Functional analysis of the pRSB105 replicon modules. A 4,673-bp SacII restriction fragment containing the pRSB105 Rep1 module was converted to a SphI/PstI fragment by ligation with appropriate linker fragments and subsequently cloned into the mobilizable suicide plasmid pK18-mob (41). The resulting construct was designated pNK100-Rep1. In addition, the pRSB105 Rep2 module was cloned on a 8,782-bp PstI restriction fragment into the mobilizable suicide vector pSUP202 (44), resulting in construct pIK100-Rep2. To test the replication ability of Rep1 and Rep2 in different recipients, the constructs pK18-mob-Rep1 and pSUP202-Rep2 were transferred from the mobilisator strain S17-1 to *Xanthomonas campestris* pv. campestris B100, *Ralstonia eutropha* GFP3, *Sinorhizobium meliloti* 1021, and *Agrobacterium tumefaciens* UBAPF2. Matings were done as described previously (9). *X. campestris* pv. campestris, *S. meliloti*, or *A. tumefaciens* transconjugants were selected on TY medium containing 200 μg of streptomycin ml^{-1}, 10 μg of tetracycline ml^{-1}, or 120 μg of neomycin ml^{-1}. Putative *R. eutropha* transconjugants were selected on LB medium containing 50 μg of kanamycin ml^{-1} and 5 μg of tetracycline ml^{-1}. The plasmid content of transconjugants was checked in Eckhardt gels.

Functional analysis of the pRSB105 mobilization module. To test the functionality of the pRSB105 mobilization module, the plasmid was transferred into the mobilisator strain S17-1, which carries an chromosomally integrated derivative of the IncP-1α plasmid RP4 providing transfer functions in *trans*. *E. coli* S17-1(pRSB105) was mated with the recipient strain *E. coli* XL1-Blue as described previously (9). *E. coli* transconjugants were selected on LB medium containing 100 μg of ampicillin ml^{-1} and 10 μg of tetracycline ml^{-1}.

Antimicrobial susceptibility testing. Disk diffusion tests for antimicrobial susceptibility testing were carried out under the experimental conditions recommended in general in the guidelines of the Clinical and Laboratory Standards Institute (6) and 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), cefaclor (CEC30), cefepime (FEP30), cefotaxime (CTX30), cefpirom (CPO30), ceftazidime (CAZ30), ceftibuten (CFT30), cefuroxime (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) were purchased from MAST Diagnostica (Reinfeld, Germany). MICs were determined as described by Dröge et al. (9).

The complete pRSB105 macrolide resistance gene region was cloned on a 5,061-bp SalI restriction fragment into the vector pBCSK. A 2,036-bp PstI/DraI fragment containing only the *mel* gene was subcloned into pUC19, resulting in the construct pNK-*mel*. Recombinant plasmid pNK-*mph* carries a 1,922-bp EcoRV/BamHI fragment with the complete *mph* gene in pBluescript II KS. These constructs were tested for conferring erythromycin and azithromycin resistance by determining the corresponding MICs.

Transcription of *mel* and *mph* from the constructed plasmids was verified by real-time reverse transcription-PCR experiments as described previously (23). For this purpose RNA was isolated from *E. coli* DH5α carrying, respectively, pNK-*mel*-*mph*, pNK-*mel*, pNK-*mph*, or the corresponding vector without insert by means of the RNeasy minikit (QIAGEN). The mRNA-levels of *mel* and *mph* were analyzed by RT-PCR using *mel*-specific (5′-GGTGATAACGGAGCAGG AAA-3′ and 5′-GCCACTCATTGTGTCGTTTT-3′) and *mph*-specific (5′-TGTT GCTTACGGACAAAATGA-3′ and 5′-TGAAATTCTCCAATCAGGAACC-3′) primer pairs.

Functional analysis of the pRSB105-encoded restriction and/or modification system. *E. coli* S17-1 carrying pRSB105 was infected by serial dilutions of phage Lambda λ-vir (strain collection Bielefeld University) on LBMM (maltose, 11.1 mM; $MgCl_2 \cdot 6H_2O$, 9.8 mM) soft agar medium. The efficiency of plating was determined in comparison to the control strain S17-1 without the plasmid. Phage lysates were prepared from rare plaques on S17-1(pRSB105) and used to infect S17-1(pRSB105) and the plasmid-free control strain. The efficiencies of plating were also determined for this second round of infection.

Nucleotide sequence accession number. The annotated nucleotide sequence of the resistance plasmid pRSB105 is accessible under the GenBank accession no. DQ839391.

RESULTS AND DISCUSSION

Complete nucleotide sequencing of plasmid pRSB105. The complete nucleotide sequence of the resistance plasmid pRSB105 was established by terminal sequencing of inserts from a Sau3A shotgun library and a restriction fragment library and subsequent primer walking strategies. The nucleotide sequence was determined with a mean coverage of 7.8, resulting in a circular plasmid sequence of 57,137 bp with an average G+C content of 56.66%. Correct assembly of the sequencing reads was verified by comparing the predicted pRSB105 restriction map on the basis of the nucleotide sequence to digests of plasmid DNA with the restriction enzymes BamHI, BssHII, EcoRI, EcoRV, HindIII, PstI, SacII, SalI, and SphI. Annotation of the plasmid nucleotide sequence revealed that it contains 67 predicted complete coding sequences (CDSs) which can be categorized as follows: replication, stable inheritance, and partitioning (9 CDSs); mobilization (5 CDSs); transposition and recombination (13 CDSs); resistance (6 CDSs); restriction and modification (2 CDSs); and unknown function (32 CDSs). The pRSB105 backbone is composed of two replication modules and a mobilization region. The plasmid car-

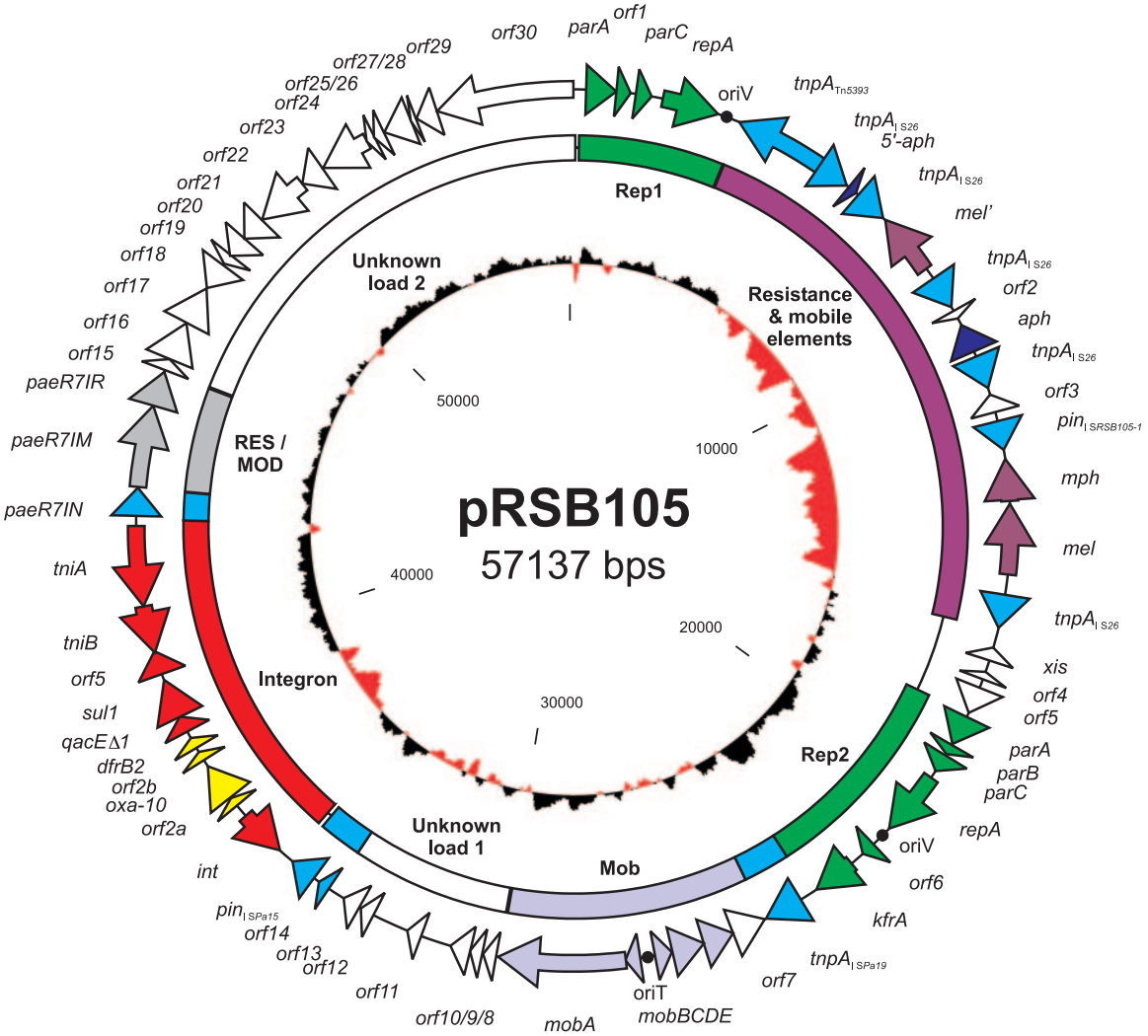

FIG. 1. Genetic map of the resistance plasmid pRSB105. Coding regions are indicated by arrows showing their orientation. The modular structure of the plasmid is shown on the median circle: plasmid replicons Rep1 and Rep2 (IncP-6) (green), the mobilization region Mob (light blue), the first resistance region including macrolide resistance genes and a truncated aminoglycoside-phosphotransferase gene (purple), the integron containing Tn*402*-like element (red), the modification/restriction region (gray), and two modules of unknown function (white). Insertion sequence (IS) and transposon (Tn) genes are shown in blue. Genes of integron gene cassettes are in yellow, whereas white arrows represent putative genes encoding unknown functions. Origins of vegetative (oriV) and transfer replication (oriT) are marked by black circles. The G+C plot is indicated on the inner circle, where a G+C content of <50% is shown in red and a G+C content of >50% is shown in black. The G+C plot was generated by using the GenDB (version 2.2) tool (34).

ries two antibiotic resistance regions, namely, a macrolide resistance determinant that is flanked by insertion sequences and an integron-containing Tn*402*-like element. In addition, ca. 26% of the plasmid is occupied by two gene regions of unknown function. The genetic map of pRSB105 is presented in Fig. 1, and the basic features of the plasmid nucleotide sequence are listed in Table 1.

Plasmid pRSB105 contains two replication modules. Plasmid pRSB105 harbors two different replication modules, which could mean that two formerly distinct plasmids fused to assemble plasmid pRSB105. The first replication module (Rep1) of pRSB105 extends from coordinates 89 to 3510 and comprises the partitioning genes *parA* and *parC*, an open reading frame (ORF) of unknown function and the replication gene *repA* (see Fig. 1). The deduced *parA* gene product represents an ATPase

TABLE 1. General features of plasmid pRSB105

Characteristic	Finding[a]
Length (bp)	57,137
%G+C content	56.66
Plasmid coding (%)	81.69
Maximum CDS length (bp)	2,883
Mean CDS length (bp)	672.57
Mean intergenic length (bp)	183.56
No. of CDSs on leading strand	43
No. of CDSs on lagging strand	27
Start codon usage (%)	
ATG	78.57
GTG	14.29
TTG	5.71
ATC	1.43

[a] Numbers were delivered by the GenDB_R-Plasmids-2.2 project statistics information.

TABLE 2. Replication functions of pRSB105 Rep1 and Rep2 in different proteobacteria

Construct	Subcloned pRSB105 replication module	Characteristics	Presence (+) or absence (−) of replication ability in:		
			S. meliloti, *A. tumefaciens* (α-proteobacteria)	*R. eutropha* (β-proteobacterium)	*X. campestris* pv. campestris (γ-proteobacterium)
pNK100-Rep1[a]	Rep1	Unknown Inc group, pRSB101-like[c]	−	−	+
pIK100-Rep2[b]	Rep2	IncP-6, Rms149-like[d]	−	+	+

[a] The pRSB105 Rep1 module was cloned on a 4,673-bp SacII fragment into the mobilizable suicide plasmid pK18-mob.
[b] The pRSB105 Rep2 module was cloned on a 8,782-bp PstI fragment into the mobilizable suicide plasmid pSUP202.
[c] The erythromycin resistance plasmid pRSB101 was isolated from an unknown activated sludge bacterium (47).
[d] The multiresistance plasmid Rms149 was isolated from a clinical *P. aeruginosa* strain (20).

of the ParA family predicted to be involved in plasmid partitioning (COG1192, pfam00991), whereas ParC could play an auxiliary function in the plasmid partitioning process. The gene product of *orf1* downstream of *parA* does not show convincing similarity to known ParB proteins but is nearly identical to the conserved hypothetical *orf9* gene product of plasmid pRSB101 isolated from an unknown activated sludge bacterium (47). ParA and ParC of the Rep1 partitioning module also show the highest degree of identity (99 and 83%, respectively) to corresponding proteins of the multiresistance plasmid pRSB101. A putative replication gene *repA* encoding a plasmid replication initiator protein (pfam03090) is located downstream of the Rep1 partitioning module. RepA is 98, 83, and 70% identical to the RepA of plasmids pRSB101, pXAC33, and pXAC64 of the phytopathogen *Xanthomonas axonopodis* pv. citri (7) and pPMA4326B of the radish and the *Arabidopsis* pathogen *Pseudomonas syringae* pv. maculicola (45). It might be speculated that the Rep1 replicon originates from a plant-associated bacterium. The very high similarity of Rep1 to the replication module of pRSB101 suggests that both plasmids evolved from a common ancestor and thus have to be considered as sister plasmids.

To test the replication ability of Rep1, the complete Rep1 module was cloned into the mobilizable suicide vector pK18-mob. The resulting construct pNK100-Rep1 could be mobilized from the *Escherichia coli* mobilisator strain S17-1 to the γ-proteobacterium *Xanthomonas campestris* pv. campestris B100, and Rep1 confers replication ability to the plasmid in this species (Table 2). Vector pK18-mob without Rep1 is a suicide plasmid for *Xanthomonas* species (41). Similar experiments with the α-proteobacteria *Sinorhizobium meliloti* and *Agrobacterium tumefaciens* and the β-proteobacterium *Ralstonia eutropha* as recipient strains were not successful, indicating that pK18-mob-Rep1 is not able to replicate in these species. Accordingly, Rep1 is a narrow-host-range replicon that is able to replicate in some γ-proteobacteria.

The second replication module Rep2 is located in the vicinity of the macrolide resistance region and consists of the partitioning genes *parA*, *parB*, and *parC*, the replication gene *repA*, and a *kfrA*-like gene (Fig. 1). The *parA* gene product is an ATPase (NCBI conserved domain accession no. cd02042) involved in plasmid partitioning, whereas *parB* and *parC* encode predicted auxiliary partitioning proteins. The pRSB105 *parABC* module is 99.9% identical to the corresponding module of the IncP-6 multiresistance plasmid Rms149 isolated from a clinical *Pseudomonas aeruginosa* strain from Frankfurt, Germany, (20) and 98.7% identical to *parABC* of *Pseudomonas alcaligenes* pRA2 (27, 28). For pRA2 it was shown that ParB is a component of the partition nucleoprotein complex at the incompatibility determinant *parS* (28). The gene downstream of *parABC* on pRSB105 is nearly identical to *repA* of Rms149. The Rms149 *repA* gene confers replication ability to an *E. coli* cloning vector in *Pseudomonas putida* (20). Astonishingly, the C-terminal domains of pRSB105 and Rms149 RepA differ completely, suggesting that a recombination event occurred in *repA* either on pRSB105 or on Rms149. The second pRSB105 replication module is completed by a gene that is homologous to *kfrA* of IncP-1 plasmids. It contains an α-helical, coiled-coil tail and may act as a specific plasmid nucleoid organizer (1). The *kfrA* gene of Rms149 is separated from *repA* by insertion of the transposon Tn*5503*.

The function of Rep2 was analyzed by first cloning the complete replication module into the mobilizable suicide vector pSUP202 and subsequently transferring the construct to different proteobacteria. Plasmid pIK100-Rep2 confers replication ability only in the γ-proteobacterium *Xanthomonas campestris* pv. campestris and the β-proteobacterium *Ralstonia eutropha* (see Table 2). Thus, incorporation of Rep2 into pRSB105 extends the host range of the plasmid to the β-proteobacteria.

Plasmid pRSB105 encodes a five-Mob-protein mobilization module. Downstream of the IncP-6 replicon, plasmid pRSB105 contains a mobilization module that is composed of the *mob* genes *mobA*, *mobB*, *mobC*, *mobD*, and *mobE*. It could be classified as belonging to the MOB_P family of mobilization regions (15) since it shows the highest degree of similarity to corresponding regions of the *Aeromonas salmonicida* subsp. *salmonicida* antibiotic resistance plasmid pRAS3 (31), an integrated plasmid in the chromosome of the pig pathogen *Chlamydia suis* (10), the broad-host-range plasmid pTF-FC2 of *Acidithiobacillus ferrooxidans* (38), and the *Pseudomonas aeruginosa* archetype IncP-6 resistance plasmid Rms149 (20). The pRSB105 *mob* gene products also show limited similarity to, respectively, TraJ, TraI, TraK, TraL, and TraM of IncP-1 plasmids and have predicted functions in the recognition of and binding to the origin of transfer (oriT), relaxosome formation, and the initiation of transfer replication (36). MobA of pRSB105 represents a relaxase/primase fusion protein that is very similar to the corresponding protein encoded on pRAS3. The pRSB105 origin of transfer located in the intergenic region between *mobB* and *mobC* is identical to Rms149 oriT. The deduced gene products of *orf9* and *orf10* downstream of *mobA* are very similar to, respectively, Rms149 Ofn12 and Ofn11 of unknown function.

The functionality of the pRSB105 mobilization module was tested in mating experiments with the donor strain *E. coli* S17-1 which carries an integrated derivative of the IncP-1α plasmid RP4 providing transfer functions in *trans*. Plasmid pRSB105 could be transferred to *E. coli* XL1-Blue with a frequency of 5.3×10^{-1} per recipient cell and thus is mobilizable by a IncP-1 helper plasmid.

Interestingly, the sister plasmids pRSB101 and pRSB105 acquired different mobilization modules. A three-protein system is present on pRSB101 (47), whereas pRSB105 possesses the described module consisting of five *mob* genes. This demonstrates that plasmids represent mosaics incorporating different survival and mobility functions to evolve toward improved adaptability and competitiveness.

Plasmid pRSB105 carries a new macrolide resistance determinant. Plasmid pRSB105 contains a macrolide resistance region in the vicinity of the IncP-6 replicon module. The region consists of two genes, *mel* and *mph*, encoding, respectively, a predicted ABC-type efflux permease and a macrolide-2′-phosphotransferase. A 2,909-bp segment containing *mel* and *mph* is completely identical to corresponding regions on the self-transferable IncN plasmid pMUR050 from *E. coli* isolated from a diarrheic pig (16) and the transmissible plasmid pCTX-M3 from *Citrobacter freundii* (accession no. AF550415). Plasmid pMUR050 was described to confer resistance to aminoglycosides and sulfonamides and has an R46-like replicon. The deduced gene product of the pRSB105 *mel* gene possesses two nucleotide-binding domains (pfam00005) characteristic for transporters of the ATP-binding cassette family, whereas Mph is a member of the phosphotransferase enzyme family (pfam01636, COG3173). A gene homologous to *mel* is also present downstream of the macrolide efflux gene *mef*(E) located on a transposon of *Streptococcus pneumoniae*, and recent data indicated that both genes contribute to efflux-mediated macrolide resistance in *S. pneumoniae* (3, 8). The pRSB105 macrolide resistance region is flanked, respectively, by a copy of IS*26* and a putative new transposable element, designated IS*RSB105-1*. The latter element encodes a putative resolvase/site-specific recombinase (pfam00239, COG1961) that is related to a similar enzyme of *Psychrobacter cryohalolentis* plasmid 1 (NC_007968). A truncated duplication of *mel* is located further downstream of the intact macrolide resistance region. The G+C content of the whole macrolide resistance module, including surrounding IS elements, differs considerably from the rest of the plasmids (see Fig. 1), indicating that this segment was acquired only recently.

To test whether both pRSB105 macrolide resistance genes *mel* and *mph* are required for mediating macrolide resistance, both genes were cloned together and separately into appropriate cloning vectors. Construct pNK-*mel-mph*, which carries the complete *mel-mph* gene region confers resistance to 3,200 μg of erythromycin ml^{-1}, whereas a construct with the *mel* gene (pNK-*mel*) confers resistance to 2,500 μg ml^{-1}. The *mph* gene alone (pNK-*mph*) mediates resistance to 225 μg ml^{-1}. This low resistance level is not due to reduced transcription of *mph* on the construct since real-time PCR assays revealed that *mph* of plasmid pNK-*mph* is transcribed to a higher level (factor 1.7) compared to the original construct containing the complete *mel-mph* region. The MICs for the macrolide antibiotics erythromycin and azithromycin are given in Table 3. These results demonstrate that macrolide resistance is mainly attributed to *mel* on pRSB105 but *mph* also contributes to erythromycin and azithromycin resistance, although to a lesser extent.

TABLE 3. MICs of *E. coli* DH5α containing subcloned pRSB105 macrolide resistance genes

Construct in *E. coli* DH5α	Relevant gene(s)	MIC (μg ml^{-1})	
		Erythromycin	Azithromycin
pNK-*mel-mph*	*mel*, *mph*	3,400	150
pNK-*mel*	*mel*	2,700	150
pNK-*mph*	*mph*	250	17
pBluescript II KS		100	5

The integron-containing Tn*402*-like element of pRSB105 is bordered by IS*Pa15* and another putative insertion sequence. Plasmid pRSB105 harbors a second resistance region represented by an integron-containing Tn*402*-like element. The conserved segments of this element consisting of the integrase gene *intI1*, the downstream-end genes *qacEΔ1*, *sul1*, and *orf5*, and the transposition module genes *tniA* and *tniB* are identical or almost identical to corresponding segments of the Tn*402* element inserted in the multiresistance IncP-1β plasmid pB8 (43) that was previously isolated from an activated sludge bacterial community (9). The integron variable region contains the resistance gene cassettes *oxa10* and *dfrB2* encoding, respectively, a β-lactamase and a dihydrofolate reductase and two additional gene cassettes of unknown function (*orf2a* and *orf2b*). A construct containing the *oxa10* resistance cassette confers resistance to ampicillin and piperacillin to *E. coli* DH5α. The arrangement of gene cassettes in the pRSB105 integron is identical to that of plasmid pCEm5, isolated from an unknown bacterium of a wastewater treatment plant (49). Both variable regions only differ in two positions over a length of 1,943 bp and are unique for pCEm5 and pRSB105. The G+C content of the integron's variable region differs from the rest of the plasmid, which might indicate that the gene cassettes were integrated only recently.

The pRSB105 Tn*402*-like integron is flanked by two insertion sequences, namely, IS*Pa15* and another putative mobile element encoding the resolvase/site-specific recombinase PaeR7IN. This composition is reminiscent of composite transposons. Interestingly, the integrons present on the IncP-6 plasmid Rms149 (20) and the IncU tetracycline resistance plasmid pFBAOT6 (37) also are bordered by IS*Pa15* elements, and Haines et al. (20) suggested that these integrons entered the plasmids as composite transposons. Alternatively, the region adjacent to the Tn*402*-specific inverted repeat IRi might represent an insertion hotspot for elements related to IS*Pa15*. Plasmids pRSB105, pRSB101, and Rms149 acquired their Tn*402*-like elements independently since the associated target-sites are different.

Plasmid pRSB105 encodes a functional restriction/modification system. Next to the integron-containing Tn*402*-like element, pRSB105 contains genes for a restriction/modification system. A 3,280-bp region including the genes *paeR7IM*, *paeR7IR*, and *orf15* and the 5′ part of *orf16* is 99% identical to a corresponding segment of the transferable IncP-3 R-plasmid pMG7 isolated from a clinical *Pseudomonas aeruginosa* strain (51). This plasmid mediates resistance to gentamicin, kanamy-

cin, tobramycin, sulfonamides, and mercuric chloride and was shown to encode a new restriction and modification specificity (13, 25). PaeR7IM is a predicted modification methyltransferase (COG2890), whereas PaeR7IR specifies a type II restriction endonuclease of the XhoI family that recognizes the target sequence CTCGAG. The deduced gene products of *orf16* and *orf17* located downstream of *paeR7IR* are homologous to hypothetical proteins (PaerP_01002574 and PaerP_01002575) of *Pseudomonas aeruginosa* PA7 (ZP_01295446), again suggesting that the pRSB105 host bacterium had access to the *P. aeruginosa* gene pool. At the left-hand side the pRSB105 restriction/modification gene region is terminated by a putative mobile genetic element encoding the resolvase/site-specific recombinase PaeR7IN (pfam00239, COG1961) of the PinR family. A very similar element was also identified in exactly the same position on plasmid pMG7 (53). The pRSB105 sister plasmid pRSB101 only contains a truncated restriction/modification gene region consisting of *paeR7IN* and the 5′ part of *paeR7IM* (47).

Functionality of the pRSB105 restriction/modification system has been demonstrated by interference with Lambda phage propagation. Lambda-vir could be propagated in *E. coli* S17-1 carrying pRSB105 with an efficiency of plating of 3.2×10^{-2} compared to the plasmid-free S17-1 strain (efficiency of plating = 1) since Lambda-DNA is restricted by the endonuclease PaeR7IR in S17-1(pRSB105). In contrast, phages from the rare plaques on *E. coli* S17-1(pRSB105) propagate with comparable efficiencies on S17-1(pRSB105) and S17-1 without the plasmid, which can be explained by modification of the Lambda genome by the pRSB105-encoded PaeR7IM methylase.

Mobile genetic elements inserted in pRSB105. Plasmid pRSB105 harbors a series of different mobile genetic elements representing part of the accessory modules inserted in the plasmid. Downstream of the first replication module (Rep1), pRSB105 contains a truncated Tn*5393*-like transposon. A 1,869-bp region including the Tn*5393*-specific terminal inverted repeat motif and the 3′ part of the $tnpA_{\mathrm{Tn}5393}$ transposase gene is identical to corresponding parts of Tn*5393* inserted in the resistance plasmid pFL424 of a clinical *Alcaligenes faecalis* isolate (32), the IncP-1β plasmid pB4 isolated from an unknown activated sludge bacterium (48), the R plasmid pRAS2 from the fish pathogen *Aeromonas salmonicida* subsp. *salmonicida* (30), and the *Salmonella enterica* serovar Typhimurium plasmid R64 (AP005147). These observations demonstrate that elements related to the streptomycin resistance transposon Tn*5393* were disseminated among human and animal pathogens and environmental bacteria. Tn*5393* on pRSB105 was truncated by insertion of IS*26*. In all, five copies of IS*26* are present in the vicinity of the pRSB105 macrolide resistance determinant. Two IS*26* copies frame a remnant of the kanamycin resistance gene *aphA1* that is identical to *aphA1* identified in a 86-kb genomic resistance island of the epidemic, multidrug-resistant *Acinetobacter baumannii* strain AYE isolated in France (14). The *A. baumannii aphA1* gene is flanked by IS*26* elements. In contrast, *aphA1* on pRSB105 was disrupted by insertion of IS*26*, and consequently pRSB105 does not confer kanamycin resistance. Another IS*26* copy disrupted the reiterated macrolide efflux gene *mel*, whereas the fifth IS*26* element is located upstream of the functional *mel* gene. Obviously, IS*26* played an important role for the assembly of the pRSB105 macrolide resistance region. On the other side the pRSB105 macrolide resistance region is flanked by the new element IS*RSB105-1*, which encodes a resolvase/site-specific recombinase of the PinR family.

Plasmids pRSB105 and Rms149 both possess insertions of IS*Pa19* 175 bp downstream of the IncP-6 replicon gene *kfrA*. A copy of IS*Pa15* inserted downstream of pRSB105 *orf13*. This IS element was also identified on the IncP-6 plasmid Rms149 (20) in a position that is located 316 bp downstream of the integron integrase gene *intI1*, which is exactly the same target site compared to pRSB105. However, nucleotide sequences flanking the other hand sides of IS*Pa15* differ on both plasmids, indicating that a recombination event occurred on one of the plasmids. Another element encoding a *pinR*-like resolvase/site-specific recombinase gene (*paeR7IN*) is located upstream of the restriction/modification system genes.

Finally, pRSB105 harbors a Tn*402*-like element including a class 1 integron (see above). All mobile genetic elements identified on pRSB105 inserted in-between functional plasmid-backbone modules and never disrupted plasmid survival and mobility genes.

Plasmid pRSB105 harbors two regions of unknown function. Two regions of unknown function were identified on pRSB105. The first one is located between the mobilization module and the integron-containing Tn*402*-like element (see Fig. 1). It contains footprints and remnants of different mobile genetic elements such as inverted repeats of IS*Pa15* from *P. aeruginosa* (20), IS*Psy19* of *Pseudomonas syringae* pv. phaseolicola (26) and a transposon related to Tn*1721* (2), respectively. In addition, three putative ORFs (*orf11* to *orf13*) encoding hypothetical gene products of unknown function were predicted in that region.

The second region (9 kb) extends between the Rep1 module and the restriction/modification gene region and includes *orf30* to *orf18*, which are all encoded on the complement DNA-strand (oriented counter-clock wise in Fig. 1) and closely follow on each other. All ORFs in this region are preceded by convincing ribosomal binding sites, but their deduced gene products could not be classified according to Pfam or COG categories, with the exception of Orf22, which belongs to the peptidase M23/M37 family (pfam01551). This family includes metallo-endopeptidases with a range of specificities. The gene products of *orf18*, *orf19*, and *orf21* are homologous to, respectively, a hypothetical protein (XCV1305) of *Xanthomonas campestris* pv. vesicatoria (52), a hypothetical protein (Orf111) of the endogenous plasmid pRA2 from *Pseudomonas alcaligenes* (29), and a hypothetical protein in the *mobE* 3′-region of the *Acidithiobacillus ferrooxidans* plasmid pTF-FC2 (38). Orf26, Orf27, and Orf29 are highly similar (72 to 84% identity) to hypothetical proteins of *Pseudomonas aeruginosa* PA7 (NZ_AAQE01000016), which is currently sequenced since it displays an unusual resistance pattern and is expected to contain variations in virulence and pathogenicity islands. Orf26 possesses two predicted transmembrane helices. Finally, Orf30 also is a predicted membrane protein that has eight putative transmembrane helices and shows similarity to a membrane protein (PSPT01097) of *Pseudomonas syringae* pv. tomato (AAO54626) and a TraY/DotA-like type IV secretion system protein of *Ralstonia metallidurans* (YP_584969). TraY and

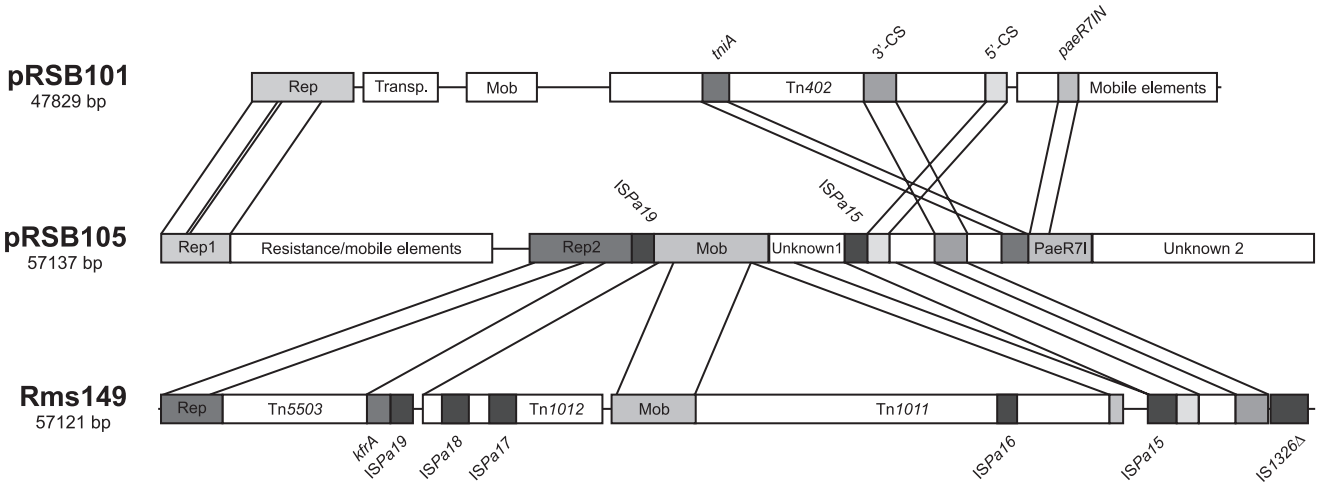

FIG. 2. Comparative analysis of the resistance plasmids pRSB105, pRSB101, and Rms149. The modular structures of the resistance plasmids pRSB105, pRSB101 (47), and Rms149 (20) are shown. Corresponding modules were given the same shading: Rep, replication; Transp., multidrug resistance efflux transporter; Mob, mobilization; Tni, Tn*402* transposition module genes; In-5′-CS, integron 5′-conserved module (*intI1*); In-3′-CS, integron 3′-conserved module (*qacEΔ1-sul1-orf5*); and PaeR7I, *Pseudomonas aeruginosa* restriction/modification module. Insertion sequences (IS) and transposons (Tn) are indicated.

DotA are components of bacterial type IV secretion systems that facilitate delivery of effector molecules to host cells (5). For example, DotA of the respiratory pathogen *Legionella pneumophila* is an inner membrane protein that was suggested to work in association with other proteins in the transduction of a signal to macrophages to direct trafficking of the *L. pneumophila* phagosome (39). The function of the putative membrane-associated protein complex encoded by pRSB105 *orf30* and adjacent ORFs currently remains unknown.

To analyze whether the gene regions of unknown functions encode any additional resistance determinants, disk diffusion tests with 35 different antimicrobial compounds (listed in Materials and Methods) were carried out. No additional reduced susceptibilities to the tested compounds were detected for *E. coli* DH5α (pRSB105) in comparison to the plasmid-free strain, suggesting that the implied gene regions are not involved in mediating antibiotic resistance.

Concluding remarks. Plasmid pRSB105 most probably represents a cointegrate that evolved by fusion of two formerly separate replicons, namely, Rep1 and the IncP-6 replicon. The presence of two replicons apparently enlarges the plasmid's host range since these replicons are optimized to facilitate replication in certain host bacteria. In addition, host range extension broadens access to genetic information. Indeed, the different accessory modules identified on pRSB105 seem to originate from different sources.

The archetype IncP-6 plasmid Rms149 from *Pseudomonas aeruginosa* is one of the nearest relatives of pRSB105. The Rms149 backbone modules for replication, stable inheritance, and mobilization were disconnected by multiple insertions of mobile genetic elements such as Tn*5533*, IS*Pa19*, Tn*1012*, and Tn*1011*, whereas pRSB105 only carries one insertion of IS*Pa19* downstream of the *kfrA*-like gene and hence represents the more original variant of the IncP-6 backbone modules. A comparative analysis of plasmids pRSB105 and Rms149 is shown in Fig. 2.

The integron-containing Tn*402*-like elements on Rms149 and pRSB105 are connected to insertions of IS*Pa15*, but the nucleotide sequences beyond IS*Pa15* differ on both plasmids. Since the terminal inverted repeats of IS*Pa15* are nearly identical to the integron repeats IRi and IRt, respectively, Haines et al. (20) suggested that IS*Pa15* could be capable of moving the complete integron as a composite transposon. At present, the mechanism by which the integron-containing element entered pRSB105 is not apparent.

It is very likely that pRSB105 Rep1 was connected to the large nonresistance gene region including the modification/restriction module (*paeR7IM-paeR7IR*) on the pRSB105 progenitor plasmid. This interpretation is supported by the fact that the sister plasmid of pRSB105, pRSB101, contains remnants of the PaeR7I modification/restriction gene region (see Fig. 2). The implied acquired segment seems to originate from *Pseudomonas aeruginosa*, suggesting that the pRSB105 host bacterium had access to the *P. aeruginosa* gene pool.

Acquisition of the pRSB105 macrolide resistance region also is not clear since the sister plasmid pRSB101 contains another erythromycin resistance determinant and Rms149 does not confer erythromycin resistance at all. Most probably, IS*26* elements played an important role for the integration of the erythromycin resistance module into pRSB105 by either transposition or homologous recombination. However, at least the origin of the pRSB105 macrolide resistance module is known, since identical genes were previously identified on plasmids residing in *Escherichia coli* and *Citrobacter freundii*.

In conclusion, the dual-replicon plasmid pRSB105 harbors two discrete and functional antibiotic resistance regions and an additional large module of hitherto-unknown function that can be further disseminated among diverse bacteria due to the mobility and extended host range of the plasmid.

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REFERENCES

1. **Adamczyk, M., P. Dolowy, M. Jonczyk, C. M. Thomas, and G. Jagura-Burdzy.** 2006. The *kfrA* gene is the first in a tricistronic operon required for survival of IncP-1 plasmid R751. Microbiology **152:**1621–1637.
2. **Allmeier, H., B. Cresnar, M. Greck, and R. Schmitt.** 1992. Complete nucleotide sequence of Tn*1721*: gene organization and a novel gene product with features of a chemotaxis protein. Gene **111:**11–20.
3. **Ambrose, K. D., R. Nisbet, and D. S. Stephens.** 2005. Macrolide efflux in *Streptococcus pneumoniae* is mediated by a dual efflux pump (*mel* and *mef*) and is erythromycin inducible. Antimicrob. Agents Chemother. **49:**4203–4209.
4. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. Xl1-Blue: a high-efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. BioTechniques **5:**376–378.
5. **Christie, P. J., and J. P. Vogel.** 2000. Bacterial type IV secretion: conjugation systems adapted to deliver effector molecules to host cells. Trends Microbiol. **8:**354–360.
6. **Clinical and Laboratory Standards Institute.** 2006. Performance standards for antimicrobial susceptibility testing; 15th informational supplement. M100–S16. Clinical and Laboratory Standards Institute, Wayne, PA.
7. **da Silva, A. C., J. A. Ferro, F. C. Reinach, C. S. Farah, L. R. Furlan, R. B. Quaggio, C. B. Monteiro-Vitorello, M. A. Van Sluys, N. F. Almeida, L. M. Alves, A. M. do Amaral, M. C. Bertolini, L. E. Camargo, G. Camarotte, F. Cannavan, J. Cardozo, F. Chambergo, L. P. Ciapina, R. M. Cicarelli, L. L. Coutinho, J. R. Cursino-Santos, H. El-Dorry, J. B. Faria, A. J. Ferreira, R. C. Ferreira, M. I. Ferro, E. F. Formighieri, M. C. Franco, C. C. Greggio, A. Gruber, A. M. Katsuyama, L. T. Kishi, R. P. Leite, E. G. Lemos, M. V. Lemos, E. C. Locali, M. A. Machado, A. M. Madeira, N. M. Martinez-Rossi, E. C. Martins, J. Meidanis, C. F. Menck, C. Y. Miyaki, D. H. Moon, L. M. Moreira, M. T. Novo, V. K. Okura, M. C. Oliveira, V. R. Oliveira, H. A. Pereira, A. Rossi, J. A. Sena, C. Silva, R. F. de Souza, L. A. Spinola, M. A. Takita, R. E. Tamura, E. C. Teixeira, R. I. Tezza, M. Trindade dos Santos, D. Truffi, S. M. Tsai, F. F. White, J. C. Setubal, and J. P. Kitajima.** 2002. Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. Nature **417:**459–463.
8. **Del Grosso, M., A. Scotto d'Abusco, F. Iannelli, G. Pozzi, and A. Pantosti.** 2004. Tn*2009*, a Tn*916*-like element containing *mef*(*E*) in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **48:**2037–2042.
9. **Dröge, M., A. Pühler, and W. Selbitschka.** 2000. Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. Mol. Gen. Genet. **263:**471–482.
10. **Dugan, J., D. D. Rockey, L. Jones, and A. A. Andersen.** 2004. Tetracycline resistance in *Chlamydia suis* mediated by genomic islands inserted into the chlamydial *inv*-like gene. Antimicrob. Agents Chemother. **48:**3989–3995.
11. **Ewing, B., and P. Green.** 1998. Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Res. **8:**186–194.
12. **Ewing, B., L. Hillier, M. C. Wendl, and P. Green.** 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res. **8:**175–185.
13. **Falkiner, F. R., C. T. Keane, M. Dalton, M. T. Clancy, and G. A. Jacoby.** 1977. Cross infection in a surgical ward caused by *Pseudomonas aeruginosa* with transferable resistance to gentamicin and tobramycin. J. Clin. Pathol. **30:**731–737.
14. **Fournier, P. E., D. Vallenet, V. Barbe, S. Audic, H. Ogata, L. Poirel, H. Richet, C. Robert, S. Mangenot, C. Abergel, P. Nordmann, J. Weissenbach, D. Raoult, and J. M. Claverie.** 2006. Comparative genomics of multidrug resistance in *Acinetobacter baumannii*. PLoS Genet. **2:**e7.
15. **Francia, M. V., A. Varsaki, M. P. Garcillan-Barcia, A. Latorre, C. Drainas, and F. de la Cruz.** 2004. A classification scheme for mobilization regions of bacterial plasmids. FEMS Microbiol. Rev. **28:**79–100.
16. **González-Zorn, B., T. Teshager, M. Casas, M. C. Porrero, M. A. Moreno, P. Courvalin, and L. Dominguez.** 2005. *armA* and aminoglycoside resistance in *Escherichia coli*. Emerg. Infect. Dis. **11:**954–956.
17. **Gordon, D., C. Abajian, and P. Green.** 1998. Consed: a graphical tool for sequence finishing. Genome Res. **8:**195–202.
18. **Gordon, D., C. Desmarais, and P. Green.** 2001. Automated finishing with autofinish. Genome Res. **11:**614–625.
19. **Grant, S. G., J. Jessee, F. R. Bloom, and D. Hanahan.** 1990. Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. Proc. Natl. Acad. Sci. USA **87:**4645–4649.
20. **Haines, A. S., K. Jones, M. Cheung, and C. M. Thomas.** 2005. The IncP-6 plasmid Rms149 consists of a small mobilizable backbone with multiple large insertions. J. Bacteriol. **187:**4728–4738.
21. **Heuer, H., R. Szczepanowski, S. Schneiker, A. Pühler, E. M. Top, and A. Schlüter.** 2004. The complete sequences of plasmids pB2 and pB3 provide evidence for a recent ancestor of the IncP-1β group without any accessory genes. Microbiology **150:**3591–3599.
22. **Hötte, B., I. Rath-Arnold, A. Pühler, and R. Simon.** 1990. Cloning and analysis of a 35.3-kilobase DNA region involved in exopolysaccharide production by *Xanthomonas campestris* pv. *campestris.* J. Bacteriol. **172:**2804–2807.
23. **Hüser, A. T., A. Becker, I. Brune, M. Dondrup, J. Kalinowski, J. Plassmeier, A. Pühler, I. Wiegrabe, and A. Tauch.** 2003. Development of a *Corynebacterium glutamicum* DNA microarray and validation by genome-wide expression profiling during growth with propionate as carbon source. J. Biotechnol. **106:**269–286.
24. **Hynes, M. F., R. Simon, and A. Pühler.** 1985. The development of plasmid-free strains of *Agrobacterium tumefaciens* by using incompatibility with a *Rhizobium meliloti* plasmid to eliminate pAtc58. Plasmid **13:**99–105.
25. **Jacoby, G. A., and L. Sutton.** 1977. Restriction and modification determined by a *Pseudomonas* R plasmid. Plasmid **1:**115–116.
26. **Joardar, V., M. Lindeberg, R. W. Jackson, J. Selengut, R. Dodson, L. M. Brinkac, S. C. Daugherty, R. Deboy, A. S. Durkin, M. G. Giglio, R. Madupu, W. C. Nelson, M. J. Rosovitz, S. Sullivan, J. Crabtree, T. Creasy, T. Davidsen, D. H. Haft, N. Zafar, L. Zhou, R. Halpin, T. Holley, H. Khouri, T. Feldblyum, O. White, C. M. Fraser, A. K. Chatterjee, S. Cartinhour, D. J. Schneider, J. Mansfield, A. Collmer, and C. R. Buell.** 2005. Whole-genome sequence analysis of *Pseudomonas syringae* pv. phaseolicola 1448A reveals divergence among pathovars in genes involved in virulence and transposition. J. Bacteriol. **187:**6488–6498.
27. **Kwong, S. M., C. C. Yeo, D. Chuah, and C. L. Poh.** 1998. Sequence analysis of plasmid pRA2 from *Pseudomonas alcaligenes* NCIB 9867 (P25X) reveals a novel replication region. FEMS Microbiol. Lett. **158:**159–165.
28. **Kwong, S. M., C. C. Yeo, and C. L. Poh.** 2001. Molecular analysis of the pRA2 partitioning region: ParB autoregulates *parAB* transcription and forms a nucleoprotein complex with the plasmid partition site, *parS*. Mol. Microbiol. **40:**621–633.
29. **Kwong, S. M., C. C. Yeo, A. Suwanto, and C. L. Poh.** 2000. Characterization of the endogenous plasmid from *Pseudomonas alcaligenes* NCIB 9867: DNA sequence and mechanism of transfer. J. Bacteriol. **182:**81–90.
30. **L'Abèe-Lund, T. M., and H. Sørum.** 2000. Functional Tn*5393*-like transposon in the R plasmid pRAS2 from the fish pathogen *Aeromonas salmonicida* subspecies *salmonicida* isolated in Norway. Appl. Environ. Microbiol. **66:**5533–5535.
31. **L'Abèe-Lund, T. M., and H. Sørum.** 2002. A global non-conjugative Tet C plasmid, pRAS3, from *Aeromonas salmonicida*. Plasmid **47:**172–181.
32. **Mantengoli, E., and G. M. Rossolini.** 2005. Tn*5393d*, a complex Tn*5393* derivative carrying the PER-1 extended-spectrum beta-lactamase gene and other resistance determinants. Antimicrob. Agents Chemother. **49:**3289–3296.
33. **Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. M. Ausubel.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. **149:**114–122.
34. **Meyer, F., A. Goesmann, A. C. McHardy, D. Bartels, T. Bekel, J. Clausen, J. Kalinowski, B. Linke, O. Rupp, R. Giegerich, and A. Pühler.** 2003. GenDB: an open source genome annotation system for prokaryote genomes. Nucleic Acids Res. **31:**2187–2195.
35. **Noguchi, N., A. Emura, H. Matsuyama, K. O'Hara, M. Sasatsu, and M. Kono.** 1995. Nucleotide sequence and characterization of erythromycin resistance determinant that encodes macrolide 2′-phosphotransferase I in *Escherichia coli*. Antimicrob. Agents Chemother. **39:**2359–2363.
36. **Rawlings, D. E., and E. Tietze.** 2001. Comparative biology of IncQ and IncQ-like plasmids. Microbiol. Mol. Biol. Rev. **65:**481–496.
37. **Rhodes, G., J. Parkhill, C. Bird, K. Ambrose, M. C. Jones, G. Huys, J. Swings, and R. W. Pickup.** 2004. Complete nucleotide sequence of the conjugative tetracycline resistance plasmid pFBAOT6, a member of a group of IncU plasmids with global ubiquity. Appl. Environ. Microbiol. **70:**7497–7510.
38. **Rohrer, J., and D. E. Rawlings.** 1992. Sequence analysis and characterization of the mobilization region of a broad-host-range plasmid, pTF-FC2, isolated from *Thiobacillus ferrooxidans*. J. Bacteriol. **174:**6230–6237.
39. **Roy, C. R., and R. R. Isberg.** 1997. Topology of *Legionella pneumophila* DotA: an inner membrane protein required for replication in macrophages. Infect. Immun. **65:**571–578.
40. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York, NY.
41. **Schäfer, A., A. Tauch, W. Jager, J. Kalinowski, G. Thierbach, and A. Pühler.** 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145:**69–73.
42. **Schlüter, A., H. Heuer, R. Szczepanowski, L. J. Forney, C. M. Thomas, A. Pühler, and E. M. Top.** 2003. The 64,508-bp IncP-1β antibiotic multiresistance plasmid pB10 isolated from a waste-water treatment plant provides evidence for recombination between members of different branches of the IncP-1β group. Microbiology **149:**3139–3153.
43. **Schlüter, A., H. Heuer, R. Szczepanowski, S. M. Poler, S. Schneiker, A. Pühler, and E. M. Top.** 2005. Plasmid pB8 is closely related to the prototype IncP-1β plasmid R751 but transfers poorly to *Escherichia coli* and carries a

new transposon encoding a small multidrug resistance efflux protein. Plasmid **54:**135–148.
44. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for in vivo genetic-engineering - transposon mutagenesis in gram-negative bacteria. BioTechnology **1:**784–791.
45. **Stavrinides, J., and D. S. Guttman.** 2004. Nucleotide sequence and evolution of the five-plasmid complement of the phytopathogen *Pseudomonas syringae* pv. maculicola ES4326. J. Bacteriol. **186:**5101–5115.
46. **Szczepanowski, R., S. Braun, V. Riedel, S. Schneiker, I. Krahn, A. Pühler, and A. Schlüter.** 2005. The 120,592-bp IncF plasmid pRSB107 isolated from a sewage-treatment plant encodes nine different antibiotic-resistance determinants, two iron-acquisition systems and other putative virulence-associated functions. Microbiology **151:**1095–1111.
47. **Szczepanowski, R., I. Krahn, B. Linke, A. Goesmann, A. Pühler, and A. Schlüter.** 2004. Antibiotic multiresistance plasmid pRSB101 isolated from a wastewater treatment plant is related to plasmids residing in phytopathogenic bacteria and carries eight different resistance determinants including a multidrug transport system. Microbiology **150:**3613–3630.
48. **Tauch, A., A. Schlüter, N. Bischoff, A. Goesmann, F. Meyer, and A. Pühler.** 2003. The 79,370-bp conjugative plasmid pB4 consists of an IncP-1β backbone loaded with a chromate resistance transposon, the *strA-strB* streptomycin resistance gene pair, the oxacillinase gene bla_{NPS-1}, and a tripartite antibiotic efflux system of the resistance-nodulation-division family. Mol. Genet. Genomics **268:**570–584.
49. **Tennstedt, T., R. Szczepanowski, S. Braun, A. Pühler, and A. Schlüter.** 2003. Occurrence of integron-associated resistance gene cassettes located on antibiotic resistance plasmids isolated from a wastewater treatment plant. FEMS Microbiol. Ecol. **45:**239–252.
50. **Tennstedt, T., R. Szczepanowski, I. Krahn, A. Pühler, and A. Schlüter.** 2005. Sequence of the 68,869-bp IncP-1α plasmid pTB11 from a waste-water treatment plant reveals a highly conserved backbone, a Tn*402*-like integron, and other transposable elements. Plasmid **53:**218–238.
51. **Theriault, G., P. H. Roy, K. A. Howard, J. S. Benner, J. E. Brooks, A. F. Waters, and T. R. Gingeras.** 1985. Nucleotide sequence of the PaeR7 restriction/modification system and partial characterization of its protein products. Nucleic Acids Res. **13:**8441–8461.
52. **Thieme, F., R. Koebnik, T. Bekel, C. Berger, J. Boch, D. Büttner, C. Caldana, L. Gaigalat, A. Goesmann, S. Kay, O. Kirchner, C. Lanz, B. Linke, A. C. McHardy, F. Meyer, G. Mittenhuber, D. H. Nies, U. Niesbach-Klösgen, T. Patschkowski, C. Rückert, O. Rupp, S. Schneiker, S. C. Schuster, F. J. Vorhölter, E. Weber, A. Pühler, U. Bonas, D. Bartels, and O. Kaiser.** 2005. Insights into genome plasticity and pathogenicity of the plant pathogenic bacterium *Xanthomonas campestris* pv. vesicatoria revealed by the complete genome sequence. J. Bacteriol. **187:**7254–7266.
53. **Vaisvila, R., G. Vilkaitis, and A. Janulaitis.** 1995. Identification of a gene encoding a DNA invertase-like enzyme adjacent to the PaeR7I restriction-modification system. Gene **157:**81–84.
54. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.