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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

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Ten different antibiotic resistance plasmids conferring high-level erythromycin resistance were isolated from an activated sludge bacterial community of a wastewater treatment plant by applying a transformation-based approach. One of these plasmids, designated pRSB101, mediates resistance to tetracycline, erythromycin, roxythromycin, sulfonamides, cephalosporins, spectinomycin, streptomycin, trimethoprim, nalidixic acid and low concentrations of norfloxacin. Plasmid pRSB101 was completely sequenced and annotated. Its size is 47 829 bp. Conserved synteny exists between the pRSB101 replication/partition (*rep*/*par*) module and the pXAC33-replicon from the phytopathogen *Xanthomonas axonopodis* pv. *citri*. The second pRSB101 backbone module encodes a three-Mob-protein type mobilization (*mob*) system with homology to that of IncQ-like plasmids. Plasmid pRSB101 is mobilizable with the help of the IncP-1α plasmid RP4 providing transfer functions *in trans*. A 20 kb resistance region on pRSB101 is located within an integron-containing Tn*402*-like transposon. The variable region of the class 1 integron carries the genes *dhfr1* for a dihydrofolate reductase, *aadA2* for a spectinomycin/streptomycin adenylyltransferase and bla_{TLA-2} for a so far unknown Ambler class A extended spectrum β-lactamase. The integron-specific 3′-segment (*qacE*Δ*1-sul1-orf5*Δ) is connected to a macrolide resistance operon consisting of the genes *mph*(A) (macrolide 2′-phosphotransferase I), *mrx* (hydrophobic protein of unknown function) and *mphR*(A) (regulatory protein). Finally, a putative mobile element with the tetracycline resistance genes *tetA* (tetracycline efflux pump) and *tetR* was identified upstream of the Tn*402*-specific transposase gene *tniA*. The second 'genetic load' region on pRSB101 harbours four distinct mobile genetic elements, another integron belonging to a new class and footprints of two more transposable elements. A tripartite multidrug (MDR) transporter consisting of an ATP-binding-cassette (ABC)-type ATPase and permease, and an efflux membrane fusion protein (MFP) of the RND-family is encoded between the replication/partition and the mobilization module. Homologues of the macrolide resistance genes *mph*(A), *mrx* and *mphR*(A) were detected on eight other erythromycin resistance-plasmids isolated from activated sludge bacteria. Plasmid pRSB101-like *repA* amplicons were also obtained from plasmid-DNA preparations of the final effluents of the wastewater treatment plant indicating that pRSB101-like plasmids are released with the final effluents into the environment.

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Abbreviations: DR, direct repeat; Inc, incompatibility; MDR, multidrug resistance; MFP, membrane fusion protein; OMF, outer-membrane factor; RND, resistance nodulation division.

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INTRODUCTION

Antibiotics are efficiently utilized in the therapy of infectious diseases caused by pathogenic bacteria. In recent years clinical isolates, especially from intensive-care units, have been found that are resistant or multiresistant to frequently used antimicrobial drugs. This antibiotic resistance problem threatens progress in medicine. It was recognized very early that mobile genetic elements such as self-transmissible or mobilizable plasmids and conjugative transposons play an important role in the transfer and interspecies dissemination of resistance determinants (Davies, 1994; Tschäpe, 1994; Davison, 1999; Mazel & Davies, 1999). In addition, insertion sequence (IS) elements, transposons and integrons facilitate the assembly, rearrangement/recombination, supplementation and translocation of resistance gene regions (Hall & Collis, 1995; Bennett, 1999; Rowe-Magnus & Mazel, 1999; Smalla & Sobecky, 2002). Although several antibiotic resistance plasmids were isolated from clinical and environmental micro-organisms, our knowledge on the prevalence, diversity and evolution of plasmids is still very fragmentary, especially if bacteria which are difficult to access are involved. Likewise, little information is available on plasmids encoding cryptic functions which are not primarily linked to resistance. These kind of plasmids might contribute to the pool of mobile genetic elements serving as possible transport vehicles for resistance genes (Vivian *et al.*, 2001). It is generally believed that a cycle of resistance acquisition (Davies, 1994) exists in nature which involves transfer of resistance determinants from pathogenic bacteria to environmental bacteria and *vice versa*.

Macrolides with the prototype drug erythromycin are clinically important antibiotics (Blondeau, 2002; Blondeau *et al.*, 2002). Their structures consist of 14-, 15- and 16-membered lactone ring systems with amino and/or neutral sugars attached via glycosidic bonds (Roberts *et al.*, 1999). Macrolides are applied in the treatment of upper and lower respiratory tract infections (including e.g. pneumonia and bronchitis), infections of the skin, the genito-urinary and intestinal tract caused by certain bacterial species belonging to the genera *Pneumococcus*, *Legionella*, *Bordetella*, *Mycobacterium*, *Mycoplasma*, *Chlamydia* and *Corynebacterium* (Stratton, 1998; Roberts *et al.*, 1999; Zhanel *et al.*, 2001). Macrolides act by inhibition of protein biosynthesis at the large subunit (50S) of ribosomes. They dissociate the peptidyl-tRNA molecule from the ribosome resulting in termination of the growing peptide chain (Gaynor & Mankin, 2003). Resistance to this class of antibiotics is mainly conferred by three different mechanisms: (i) modification of the 23S rRNA target site by different methyltransferases; (ii) efflux of the drug mediated by ATP-binding cassette (ABC)-type transport systems or by exporters belonging to the major facilitator superfamily (MFS); (iii) inactivation of the molecule by different enzymes such as esterases, hydrolases, transferases and phosphorylases (Sutcliffe *et al.*, 1996; Vester & Douthwaite, 2001). Macrolide resistance genes were frequently found to be located on mobile plasmids which, to some extent, contribute to increased exchange of resistance to macrolide antibiotics (Matsuoka *et al.*, 1998; Lin & Chung, 1999; Liebl *et al.*, 2002; Tauch *et al.*, 2003).

Several environmental hot-spots for the horizontal exchange of genetic material were discovered (Sèveno *et al.*, 2002). Wastewater purification facilities are considered to play an important role for recombination, exchange and distribution of resistance determinants because they receive bacteria which were previously exposed to antimicrobial compounds with the inflow sewage water originating from hospitals, private households, industry and agriculture. Selective pressure might actually persist in sewage treatment plants since low concentrations of several antimicrobial drugs have been detected in sewage water (Ohlsen *et al.*, 2003). Wastewater treatment plants were found to be reservoirs for antibiotic-resistant bacteria and resistance plasmids (Mach & Grimes, 1982; Blázquez *et al.*, 1996; Smalla & Sobecky, 2002). Self-transmissible, broad-host-range plasmids belonging to the IncP-1 group and mediating diverse resistance spectra were frequently isolated from activated sludge bacteria of municipal wastewater treatment plants (Dröge *et al.*, 2000; Tennstedt *et al.*, 2003). Two such plasmids, namely the conjugative IncP-1β plasmids pB4 and pB10, were completely analysed at the DNA sequence level (Schlüter *et al.*, 2003; Tauch *et al.*, 2003). Plasmid pB4 possesses IncP-1β-specific backbone modules and is loaded with different mobile genetic elements carrying resistance genes. Among other resistance determinants pB4 encodes a tripartite antibiotic efflux system composed of a resistance-nodulation-division (RND) type transporter, a periplasmic membrane fusion protein (MFP) and an outer-membrane factor (OMF). Functional analysis showed that this efflux system conferred high-level resistance to the macrolide antibiotics erythromycin and roxythromycin on the host bacterium *Pseudomonas* sp. B13 (Tauch *et al.*, 2003). Since this was the first example of a macrolide resistance determinant of the RND-MFP-OMF-type to be found on a plasmid genome, the objective of the work described here was the isolation of other erythromycin-resistance plasmids from activated sludge bacteria of a wastewater treatment plant. Different erythromycin-resistance plasmids were characterized with respect to their replicon and erythromycin-resistance genes. Plasmid pRSB101 was selected for complete sequencing to learn more about the nature and organization of its replicon and resistance genes. In addition, we addressed the question of whether bacteria carrying erythromycin-resistance plasmids were released into the environment with the final effluents of the wastewater treatment plant.

METHODS

Bacterial strains, growth conditions and genetic techniques. *Escherichia coli* DH5α *mcr* (Grant *et al.*, 1990) containing the multiresistance plasmid pRSB101 was grown at 37 °C in Luria–Bertani (LB) medium supplemented as needed with antibiotics at the following final concentrations: 100 μg streptomycin ml^{-1}, 100 μg

spectinomycin ml^{-1} or 5 μg tetracycline ml^{-1}. *E. coli* DH5α *mcr* containing the plasmids pRSB102, pRSB103, pRSB104, pRSB105, pRSB106, pRSB107, pRSB108 and pRSB110 [formerly designated pCEm1 by Tennstedt *et al.* (2003)], were grown at 37 °C in LB medium supplemented as needed with 300 μg erythromycin ml^{-1}. *Pseudomonas* sp. B13 GFP1 containing the multiresistance plasmid pRSB101 was grown at 30 °C in LB medium supplemented as needed with antibiotics at the following final concentrations: 40 μg streptomycin ml^{-1}, 100 μg spectinomycin ml^{-1} or 5 μg tetracycline ml^{-1}. *Pseudomonas* sp. B13 GFP1 containing the resistance plasmid pRSB109 was grown at 30 °C in LB medium supplemented as needed with 300 μg erythromycin ml^{-1}. For solid media 15 g agar $(l\ medium)^{-1}$ were added. Indicator medium for strains expressing an active β-galactosidase was supplemented with 40 μg 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) ml^{-1} (final concentration).

Antimicrobial susceptibility testing was carried out as described by Tennstedt *et al.* (2003). MIC tests were carried out as described by Dröge *et al.* (2000) with minor modifications: *E. coli* DH5α with corresponding cloning vector without insert was used as negative control.

To test the transfer properties of pRSB101, this plasmid was transferred into the mobilizer strain *E. coli* S17-1 and mated with the recipients *E. coli* CV60 GFP, *Pseudomonas* sp. B13 GFP1 and *Ralstonia eutropha* GFP3 on cellulose acetate filters. Putative transconjugants were selected on LB media containing 30 μg rifampicin ml^{-1} and 5 μg tetracycline ml^{-1} for *E. coli* CV60 and 50 μg kanamycin ml^{-1}, 30 μg gentamicin ml^{-1} and 5 μg tetracycline ml^{-1} for *Pseudomonas* sp. B13 GFP1 and *R. eutropha* GFP3, respectively.

Standard DNA techniques. Plasmid DNAs from the plasmid-containing *E. coli* DH5α *mcr* and *Pseudomonas* sp. B13 GFP1 derivatives were isolated with the Nucleobond PC100 kit on AX 100 columns (Macherey-Nagel) according to the protocol supplied by the manufacturer.

The pRSB101 plasmid DNA for the generation of a shotgun library was isolated with the Large-Construct kit (Qiagen) according to the manufacturers' instructions.

Total-plasmid-DNAs from bacteria of activated sludge and the final effluents were isolated with the Nucleobond PC100 kit on AX 100 columns according to the protocol supplied by the manufacturer. These total plasmid-DNAs were used to transform *E. coli* DH5α *mcr* by the $CaCl_2$ method as described by Tennstedt *et al.* (2003) or to transform *Pseudomonas* sp. B13 GFP1 by electroporation. Preparation of *Pseudomonas* sp. B13 GFP1 cells for electroporation was carried out as described by Artiguenave *et al.* (1997). Electroporation of *Pseudomonas* sp. B13 GFP1 cells was done according to the Gene Pulser (Bio-Rad) Electroprotocol as for *Pseudomonas putida* ATCC 12633^T. Determination of the plasmid content of *E. coli* DH5α and *Pseudomonas* sp. B13 GFP1 transformants was done by Eckhardt-gel analysis as described by Hynes *et al.* (1985).

Recombinant pGEM-T-Easy (T-cloning vector; Promega), pBCKS (chloramphenicol resistant derivative of pBluescript), pUC18 (Yanisch-Perron *et al.*, 1985), pK18mob (Schäfer *et al.*, 1994) and pBluescript (Stratagene) derivatives were isolated using the QIAprep Spin Miniprep kit according to the manufacturers' instructions. Restriction enzyme digestion, agarose gel electrophoresis, DNA cloning and transformation of *E. coli* DH5α was carried out according to Sambrook *et al.* (1989).

Construction of a shotgun library and DNA sequencing of pRSB101. Purified pRSB101 plasmid DNA was partially restricted with the restriction enzyme *Sau*3A. Restriction fragments with sizes ranging from 1 to 3 kb were extracted from an agarose gel with the Sephaglas BandPrep kit (Amersham Pharmacia Biotech) according to the manufacturers' instructions and cloned into the *Bam*HI-digested vector pZErO-2 (Invitrogen). Plasmid DNA was prepared from *E. coli* shotgun clones by an automated alkaline lysis with the RoboPrep 2500 (MWG) and BioRobot 9600 (Qiagen).

Sequencing reactions using dye-terminator chemistry were separated on MegaBACE 1000 capillar-sequencer (Amersham Biosciences) and ABI 377 (Applera; Applied Biosystems) DNA sequencers.

Sequencing reads were assembled using the Staden (GAP4) software package (Staden, 1996). Gap closure and polishing of the sequence was achieved by primer walking with walking primers designed on contig-DNA-sequences. This approach resulted in a single, circular molecule with a total length of 47 829 bp.

DNA sequence analysis and annotation. Annotation of the finished pRSB101 sequence was done by using the GenDB (version 2.0) Annotation Tool (Meyer *et al.*, 2003) as recently described by Tauch *et al.* (2003). Repeat regions within the pRSB101 sequence were identified and analysed by using the REPuter software (Kurtz *et al.*, 2001). Global amino acid sequence similarities were determined by using the Align Plus 4 (version 4.10) software package incorporated in the Clone Manager Professional Suite (Scientific & Educational Software) with the scoring matrix 'Standard Linear'. The annotated sequence of pRSB101 is available under the GenBank/EMBL accession number AJ698325.

Replicon typing by PCR. Incompatibility (Inc)-group typing of resistance plasmids was done by a PCR-based approach with replicon-specific primers for the Inc-groups P, W, Q, N and A/C (Götz *et al.*, 1996; Llanes *et al.*, 1996) and with pRSB101-*repA* specific primers (this work), repA-L409 (5′-GACGCTGACACAGACTTCCA) and repA-R854 (5′-GGCAAGTCCTTATCGAGCTG). The following Inc-group specific primer pairs were used: *trf*A2-1/-2 for IncP, *oriV*-1/-2 for IncQ, *oriV*-1/-2 for IncW, *kikA*-1/-2 for IncN and *rep*-1/-2 for IncA/C (Götz *et al.*, 1996; Llanes *et al.*, 1996).

Detection of the macrolide resistance operon genes *mph*(A), *mrx* and *mphR*(A) and the IS*26*- and IS*6100*-specific transposase genes (*tnpA*) by PCR. Macrolide resistance genes on plasmids were detected by PCR using primer pairs specific for *mph*(A) (mphA-L 5′-CTTGGGCTCGACTATAGGAT and mphA-R 5′-CTCCGTGTTGTCGATGAG), *mrx* (mrx-L 5′-GCTGTTTGTAGATGCAGGAC and mrx-R 5′-GCCATTGTAGCAAATTGAAG) and *mphR*(A) (mphR-L 5′-AAGTCCGATGACGAGGTACT and mphR-R 5′-TCGGGAAACATTAAACACAG).

The IS*26*- and IS*6100*-specific transposase genes were detected by PCR with the following primer pairs: IS26-L (5′-TTGCAAATAGTCGGTGGTGA) and IS26-R (5′-CGTAAGCCGTCTTCATGGAT) for $tnpA_{IS26}$ and IS6100-L (5′-CGCTGGTATTGTCGCTATCC) and IS6100-R (5′-CCAATGCCAAAAGCTCTCTC) for $tnpA_{IS6100}$.

Subcloning of DNA fragments generated by PCR. Amplification of *repA*-specific fragments on pRSB101, pRSB105 and total plasmid DNA isolated from bacteria of the final effluents as template DNAs was done by using the primers repA-L409 (see above) and repA-R854 (see above). The *repA*-specific amplicons were cloned into the vector pGEM-T-Easy (T-cloning vector; Promega) according to the pGEM-T-Easy Vector Systems protocol supplied by the manufacturer. Prior to cloning the PCR-products were purified on Sephacryl Microspin S-200 HR columns (Amersham Pharmacia Biotech). Recombinant pGEM-T-Easy derivatives were characterized by restriction analysis and by sequencing with standard sequencing primers.

RESULTS AND DISCUSSION

Plasmids conferring erythromycin resistance were isolated from activated sludge bacteria of a wastewater treatment plant

The main objective of this work was the analysis of bacterial populations residing in the activated sludge compartment of a wastewater treatment plant for the presence of plasmids conferring erythromycin resistance to the host bacterium. These kind of plasmids were isolated from activated sludge bacteria by a transformation-based approach. Bacteria from activated sludge samples were cultivated on LB agar containing 300 μg erythromycin ml^{-1}. Bacterial colonies growing on these agar plates were collected and subsequently used for total plasmid DNA preparations. The isolated plasmid DNA was then transformed into competent *E. coli* DH5α or *Pseudomonas* sp. GFP1 cells by the $CaCl_2$ method or electroporation, respectively. Approximately 200 putative transformants were tested by Eckhardt lysis for their plasmid content. *E. coli* DH5α and *Pseudomonas* sp. GFP1 transformants able to grow on LB agar with 300 μg erythromycin ml^{-1} contained plasmids ranging in size from 7 kb to 120 kb as shown by Eckhardt-gel analysis. The main characteristics for a collection of erythromycin-resistance plasmids which could be distinguished by their resistance profiles and restriction patterns are listed in Table 1. The selected plasmids confer resistance to up to eight different classes of antimicrobial compounds. Inc typing of the plasmids was carried out by PCR with primer pairs specific for the Inc groups IncP, IncQ, IncN, IncW and IncA/C and it was found that none of the plasmids could be assigned to any of these groups.

pRSB101 possesses two new type backbone modules for replication and mobilization and is loaded with two resistance gene regions and other accessory genes

Plasmid pRSB101 confers resistance to nine different antimicrobial compounds including erythromycin (see Table 1) and could not be assigned to any known incompatibility group of broad-host-range plasmids. It was therefore chosen for complete sequencing to characterize its replicon and the nature and genetic organization of the resistance genes located on the plasmid.

Determination of the complete pRSB101 sequence was achieved by applying a shotgun sequencing approach. Gap closure and polishing by primer-walking resulted in a circularly closed DNA sequence of 47 829 bp (Fig. 1) with a mean G+C content of 56·4 %. Subsequent annotation of the finished pRSB101 DNA sequence revealed that the plasmid contains 42 complete and nine disrupted or partially deleted coding sequences (cds), respectively (see Table 2). An inventory of the intact coding sequences showed that six have predicted functions in plasmid replication, maintenance and stable inheritance, three in plasmid mobilization, 11 in resistance and regulation of resistance, nine in transposition, recombination/inversion and DNA integration, three in transport processes and four in regulation. No function could be assigned to seven coding sequences. It appeared that approximately 18 % of

Table 1. Collection of erythromycin-resistance plasmids isolated from activated sludge bacteria

Plasmid	Recipient	Resistance spectrum*	Size (kb)†	Inc group	Integron‡
pRSB101	*Pseudomonas* sp. GFP1	Cpo, Ctx, Cxm, Em, Sm, Sp, Tc, Tp, Su	48	pXAC33-like§	+
pRSB102	*E. coli* DH5α	Ap, Em, Pen-G	8	−	−
pRSB103	*E. coli* DH5α	Ap, Em, Km, Pen-G	14	−	−
pRSB104	*E. coli* DH5α	Ap, Em, Pen-G, Sm, Su, Tc	75	−	−
pRSB105	*E. coli* DH5α	Ap, Em, Pen-G	58	pRSB101-like‖	−
pRSB106	*E. coli* DH5α	Ap, Em, Pen-G	28	−	−
pRSB107	*E. coli* DH5α	Ap, Cm, Em, Km, Nx, Pen-G, Sm, Su, Tc, Tp	120	F-like¶	−
pRSB108	*E. coli* DH5α	Ap, Em, Pen-G, Tc	76	−	+
pRSB109	*Pseudomonas* sp. GFP1	Cpo, Em, Tc	25	−	−
pRSB110	*E. coli* DH5α	Ap, Em, Pen-G	74	−	+

*Resistance patterns were determined by the disc-diffusion method (see Methods). Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Cpo, cefpirome; Ctx, cefotaxime; Cxm, cefuroxime; Em, erythromycin; Km, kanamycin; Nx, nalidixic acid; Pen-G, penicillin G; Sm, streptomycin; Sp, spectinomycin; Su, sulfonamides; Tc, tetracycline; Tp, trimethoprim.

†The sizes of the plasmids were estimated by restriction analysis.

‡Integron-specific variable regions were amplified by PCR with the primers 3′CS and 5′CS (Lèvesque *et al.*, 1995) binding to the 5′- and 3′-conserved segments of class 1 integrons.

§The basic replicon of pRSB101 is very similar to that of pXAC33 (accession no. NC_003921).

‖Partial sequencing of the pRSB105 *repA* gene showed that it is nearly identical to that of pRSB101.

¶pRSB107 belongs to the IncF-like group as revealed by partial sequencing of the pRSB107 replicon.

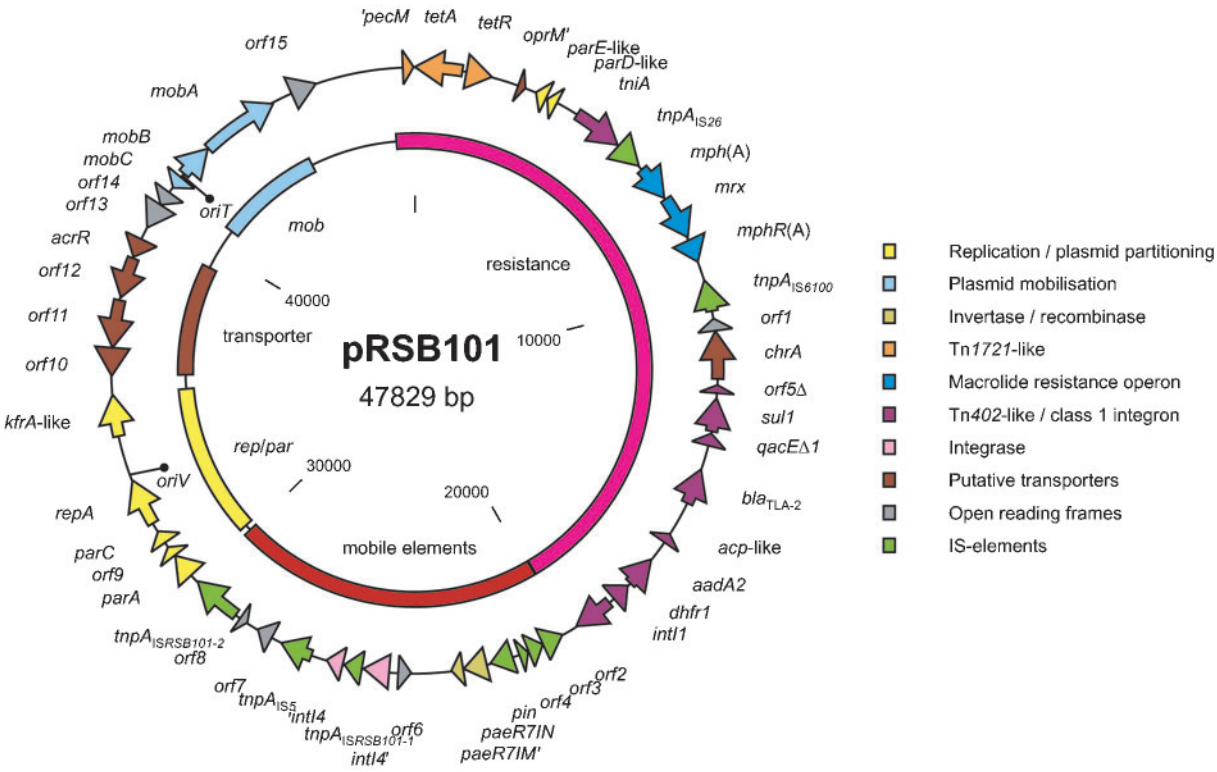

Fig. 1. Genetic map of the multiresistance plasmid pRSB101. Coding regions are shown by arrows indicating the direction of transcription. The positions of the origins of vegetative (oriV) and transfer replication (oriT) are marked by black circles. The different plasmid modules are indicated on the inner circle of the map. The backbone of the plasmid consists of a replication/partitioning module (*rep*/*par* – yellow) and a mobilization module (*mob* – blue). The 'genetic load' of the plasmid is composed of a 20 kb resistance region (magenta), another region containing different mobile genetic elements (red) and a region encoding a tripartite MDR transporter (brown). The colour code for the different gene categories present on pRSB101 is given on the right hand side of the map (see Table 2 for information on the encoded gene products).

the pRSB101 nucleotide sequence is occupied by genes for plasmid-specific functions whereas the rest (82 %) encodes accessory functions. The backbone region for plasmid replication (*rep*) and stable plasmid partitioning (*par*) during cell division is separated from a module for plasmid mobilization (*mob*) by insertion of a segment encoding a multidrug resistance (MDR) efflux system. A 20 kb antibiotic-resistance gene region is located downstream of the *mob*-module. This part of pRSB101 contains seven different resistance genes associated with mobile genetic elements. Another 'genetic load' region adjacent to the 20 kb resistance region consists of four complete transposable elements, footprints of two more transposable elements and a putative integron. The characteristics of the pRSB101 genes and a comparison of the deduced gene products to corresponding proteins encoded by other plasmids are listed in Table 2.

In summary, pRSB101 is a plasmid with a replicon that has not been categorized until now and which is loaded with several distinct mobile genetic elements some of which carry antibiotic resistance determinants. In addition, a new type MDR transport system and two integrons are specified by pRSB101. A detailed description of the different pRSB101 modules follows below.

The replication module of pRSB101 is very similar to that of plasmid pXAC33 from the phytopathogen *Xanthomonas axonopodis* pv. *citri*

The replication module of pRSB101 consists of the replication gene *repA* and the partition genes *parA* and *parC* with the same organization as compared to the corresponding genes present on plasmid pXAC33 of the phytopathogenic bacterium *Xanthomonas axonopodis* pv. *citri* (accession no. NC_003921). RepA of pRSB101 shows the highest degree of identity (77 %) to the replication protein A of the *Xanthomonas axonopodis* pv. *citri* plasmid pXAC33 and is a member of the replicase family (Pfam03090) which includes bacterial plasmid DNA replication initiator proteins. RepA of pRSB101 is also

Table 2. Predicted coding sequences (cds) of the multiresistance plasmid pRSB101 isolated from an unknown activated sludge bacterium

Coding sequence	Identity, similarity (%) of pRSB101 at the gene-product level to:*	Function	Pfam†	COG‡	Accession no.
tetA	98, 99; TetA from pTOJO2	Efflux of tetracyclines	–	COG2814	CAC42503
tetR	100, 100; TetR from RK2	Tetracycline repressor protein	Pfam02909	COG1309	AAD19682
oprM′§	OprM from *P. aeruginosa* PA01	Outer-membrane protein precursor	–	–	NP_249118
parE-like	62, 75; XAC2428 from *Xanthomonas axonopodis* pv. *citri*	Plasmid stabilization system protein	Pfam05016	COG3668	NP_642743
parD-like	61, 71; Xfas01411 from *Xylella fastidiosa* Ann-1	Transcriptional regulator	–	COG3905	ZP_00041599
tniA	97, 97; TniAΔ from pRMH760	Transposase	Pfam00665	–	AAM89410
$tnpA_{IS26}$	100, 100; TnpA from R751	Transposase	Pfam00665	COG3316	AAG45725
mph(A)	100, 100; Mph(A) from pTZ3509	Macrolide 3′-phosphotransferase I	Pfam01636	COG3173	BAB12239
mrx	100, 100; Mrx from pTZ3509	Unknown	–	–	BAB12240
mphR(A)	100, 100; MphR(A) from pTZ3509	Repressor protein	Pfam00440	COG1309	BAB12241
$tnpA_{IS6100}$	100, 100; TnpA from pRAS1	Transposase	Pfam00665	COG3316	CAD57187
orf1	45, 61; BH0575 from *Bacillus halodurans*	Transcriptional regulator	Pfam03551	COG1695	NP_241441
chrA	63, 74; PA4289 from *P. aeruginosa* PA01	Chromate transport protein	Pfam02417	COG2059	NP_252979
orf5′§	Orf5 from pVS1	–	–	–	D42646
sulI	100, 100; SulI from pB10	Dihydropteroate synthetase	Pfam00809	COG0294	CAD97505
qacEΔ1	100, 100; QacEΔ1 from pB10	Small multidrug resistance protein	Pfam00893	COG2076	NP_857995
bla_{TLA-2}	51, 64; TLA-1 from RZA92	β-Lactamase	Pfam00144	COG2367	AAD37403
acp-like	29, 54; Acp from *Thermosynechococcus elongatus* BP-1	Acyl carrier protein	–	–	NP_682662
aadA2	100, 100; AadA2 from Int22AM	Aminoglycoside adenylyltransferase	Pfam01909	–	AAG32132
dhfrI	99, 100; DhfrI from pTc1	Dihydrofolate reductase	Pfam00186	COG0262	AAM77080
intI1	100, 100; IntI1 from pVS1	Integrase	Pfam00589	COG4974	AAA25857
orf2	53, 56; XACa0010 from pXAC33	Hypothetical protein	–	–	NP_644696
orf3	100, 100; TNCP9 from pKLC102	Conserved hypothetical protein	–	COG5611	AAP22615
orf4	100, 100; TNCP10 from pKLC102	Conserved hypothetical protein	–	COG2002	AAP22616
pin	76, 79; TNCP11 from pKLC102	Putative invertase/recombinase	Pfam00239	COG1961	AAP22617
paeR7IN	100, 100; PaeR7IN from *P. aeruginosa*	DNA invertase-like protein	Pfam00239	COG1961	AAB34821
paeR7IM′§	PaeR7IM from *P. aeruginosa*	Modification methylase	–	–	P05103
orf6	26, 35; CAC0844 from *Clostridium acetobutylicum*	Barstar-like protein ribonuclease	Pfam01337	COG2732	NP_347480
intI′§	IntI4 from *V. cholerae* 01 biovar *eltor* str. N16961	Site-specific recombinase	Pfam00589	COG4974	NP_232687
$tnpA_{ISRSB101-1}$	69, 77; TnpA from *V. cholerae* 01 biovar *eltor* str. N16961	Transposase	Pfam01797	COG1943	NP_232602
′*intI*‖	IntI4 from *V. cholerae* 01 biovar *eltor* str. N16961	Site-specific recombinase	Pfam00589	COG4974	NP_232687
$tnpA_{IS5}$	90, 91; TnpA from *E. coli*	Transposase	Pfam01609	COG3039	NP_415888
orf7	95, 95; ORF2 from *K. pneumoniae*	Hypothetical protein	–	COG4273	CAA09861

Table 2. cont.

Coding sequence	Identity, similarity (%) of pRSB101 at the gene-product level to:*	Function	Pfam†	COG‡	Accession no.
′orf8‖	27, 36; InsG from *E. coli*	Transposase	–	–	CAD66180
$tnpA_{ISRSB101-2}$	38, 53; Tnp from marine psychrophilic bacterium Mst37	Transposase	Pfam01609	COG3385	CAC84124
parA	90, 92; ParA from pXAC33	Partition protein A	Pfam00991	COG1192	NP_644704
orf9	35, 45; Xaca0019 from pXAC33	Hypothetical protein	–	–	NP_644705
parC	33, 47; ParC from pXAC33	Partition protein C	–	–	NP_644706
repA	77, 84; RepA from pXAC33	Replicase	Pfam03090	–	NP_644707
kfrA-like	41, 56 TlpA from *Salmonella typhimurium*	α-Helical coiled-coil protein	–	–	A44122
orf10	45, 61; GSU0947 from *G. sulfurreducens* PCA	ATP-binding protein	Pfam0 0005	COG1136	NP_952001
orf11	43, 59; GSU0948 from *G. sulfurreducens* PCA	Putative permease protein	Pfam02687	COG4591	NP_952002
orf12	28, 45; GSU0949 from *G. sulfurreducens* PCA	Membrane-fusion protein	–	COG0845	NP_952003
acrR	27, 46; Gmet1102 from *Geobacter metallireducens*	Transcriptional regulator	Pfam00440	COG1309	ZP_00299734
orf13	38, 46; Orf291 from pRA2	Hypothetical protein	–	–	AAD40349
orf14	31, 35; Orf291 from pRA2	Hypothetical protein	–	–	AAD40349
mobC	40, 57; MobC from pRAS3·2	Mobilization protein	–	–	NP_387458
mobB	16, 26; MobB from pTC-F14	oriT recognition protein	–	–	NP_835375
mobA	30, 37; MobA from pTC-F14	Relaxase/primase-like fusion protein	Pfam03432	–	NP_835376
orf15	26, 33; XF2427 from *Xylella fascidiosa* 9a5c	Hypothetical protein	–	–	NP_299706
′pecM‖	PecM-like from pB10	Hypothetical transmembrane protein	Pfam00892	COG5006	NP_858027

*Complete amino acid sequences were compared.

†Pfam is a large collection of multiple sequence alignments and hidden Markov models covering many common protein domains and families (Bateman *et al.*, 2004).

‡The database of Clusters of Orthologous Groups of proteins (COG) is an attempt on phylogenetic classification of proteins (Tatusov *et al.*, 2001).

§5′ part of a truncated gene.

‖3′ part of a truncated gene.

closely related to RepA of pPT23A-like plasmids from the phytopathogen *Pseudomonas syringae* (Sesma *et al.*, 1998, 2000). The partition genes *parC* and *parA* were identified upstream of *repA* on pRSB101. The deduced gene products of both genes are also homologous to the partition proteins C and A of pXAC33, respectively. ParA, belonging to the ParA family of ATPases (Pfam00991), most probably is involved in stable partitioning of plasmid replicates into daughter cells during cell division. The origin of replication (oriV) seems to be located downstream of *repA* since two different types of repeat sequences, a putative DnaA box and an A/T-rich region were found in this region on pRSB101. The first repeat type has the consensus sequence NNNTAGCC and occurs four times on the forward strand and four times on the reverse strand. Three copies of the second repeat type were identified (two on the forward and one on the reverse strand), which have the conserved sequence CCGCAGG. The DnaA box has the sequence TTATCCAC and is located on the reverse strand. Finally, the A/T-rich region is 53 bp long and has an A + T content of approximately 79 %.

pXAC33 encodes the avirulence (*avr*) genes *pthA1* and *pthA2* which are not present on pRSB101. No other significant functions could be assigned to pXAC33 (da Silva *et al.*, 2002). Therefore it has to be concluded that pRSB101 only shares its basic replicon-type with pXAC33.

The product of the adjacent gene downstream of *repA* is homologous (41 % identity) to the α-helical coiled-coil protein TlpA of the *Salmonella typhimurium* virulence plasmid pEX102 and the *Salmonella enterica* subsp. *enterica* serovar Choleraesuis virulence plasmid pKDSC50. It has been suggested that TlpA has a possible virulence-associated

regulatory function (Koski *et al.*, 1992; Haneda *et al.*, 2001). The TlpA-like protein of pRSB101 also shows significant similarity (42 %) to the regulatory protein KfrA of IncP-1β plasmid pADP-1 (accession no. NC_004956). KfrA was predicted to have a long α-helical tail which might form coiled-coil structures. It was hypothesized that KfrA has a function in the plasmid partitioning process during cell division (Adamczyk & Jagura-Burdzy, 2003). In addition, KfrA of IncP-1 plasmids negatively autoregulates its own expression. A *kfrA*-like gene is also present downstream of *repA* on the endogenous plasmid pRA2 of *Pseudomonas alcaligenes* NCIB 9867 (Kwong *et al.*, 2000) which resembles the organization of *repA* and the *kfrA*-like gene on pRSB101. Several amino acid residues conserved between KfrA of pRA2 and IncP-1 plasmids are also conserved in the pRSB101 KfrA-like protein (Kwong *et al.*, 1998). Taking together the findings described above, it is very likely that the KfrA-homologue of pRSB101 and related plasmids plays a role in stable plasmid inheritance and/or plasmid-regulation. In summary, the replication region present on pRSB101 is composed of at least four genes having functions in replication and stable plasmid inheritance (partitioning) and is related to replicons from phytopathogenic bacteria.

pRSB101 has a three-Mob-protein type mobilization system and can be mobilized by a self-transmissible helper plasmid

Plasmid pRSB101 contains a mobilization (*mob*) module encoding proteins homologous to MobA, MobB and MobC of IncQ-like broad-host-range plasmids. The pRSB101 MobA shows the highest degree of similarity (37 %) to the MobA relaxase/mobilization nuclease domain (Pfam03442) of the *Acidithiobacillus caldus* IncQ-like cryptic plasmid pTC-F14. This domain is predicted to function in nicking the plasmid DNA at the origin of transfer (oriT). The pRSB101 *mobB* and *mobC* gene products are homologous to the corresponding proteins of pTC-F14, the *Acidithiobacillus ferrooxidans* IncQ-like plasmid pTF-FC2 and the *Aeromonas salmonicida* tetracycline resistance plasmid pRAS3, respectively. Interestingly, pRSB101 MobB is more than twofold larger as compared to other MobB proteins and seems to possess a two-domain structure. It should be noted that the amino acid sequences of the two domains of pRSB101 MobB are not identical. MobB and MobC were proposed to be accessory mobilization proteins with possible functions in enhancement of nic-site cleavage and relaxosome stability. MobB might also help to recognize the origin of transfer (oriT) which is localized in the *mobC–mobB* intergenic region (Rawlings & Tietze, 2001). A possible nic-site motif (TCCTG$\downarrow$) was found upstream of the pRSB101 *mobC* gene which is divergently transcribed as compared to *mobB–mobA*. Since the pRSB101 mobilization module is most similar to corresponding systems encoded by the IncQ-like plasmids pTC-F14, pTF-FC2 and pRAS3 of *Acidithiobacillus caldus*, *Acidithiobacillus ferrooxidans* and *Aeromonas salmonicida*, respectively (Rohrer & Rawlings, 1992; Gardner *et al.*, 2001; L'Abée-Lund & Sørum, 2002), it is tentatively assumed that the pRSB101 *mob*-module originates from a plasmid related to IncQ-like plasmids.

Plasmids of the IncQ-family can be mobilized in the presence of self-transmissible helper plasmids. Conjugative IncP-1 type plasmids were shown to be particularly efficient in the mobilization of IncQ plasmids (Rawlings & Tietze, 2001). To test mobilization, pRSB101 was transformed into the *E. coli* mobilizer strain S17-1 carrying a derivative of the IncP-1α plasmid RP4 integrated in its chromosome. *E. coli* S17-1 (pRSB101) was mated with the recipients *E. coli* CV60 GFP, *Pseudomonas* sp. B13 GFP1 and *R. eutropha* GFP3 and transconjugants were selected on media containing the following antibiotic concentrations: 30 μg rifampicin ml^{-1}, 50 μg kanamycin ml^{-1} and 5 μg tetracycline ml^{-1} or 100 μg spectinomycin ml^{-1} (for *E. coli* CV60 GFP) and 30 μg gentamicin ml^{-1}, 50 μg kanamycin ml^{-1} and 5 μg tetracycline ml^{-1} or 100 μg spectinomycin ml^{-1} (for *Pseudomonas* sp. B13 GFP1 and *R. eutropha* GFP3). Plasmid pRSB101 could be mobilized from *E. coli* S17-1 to *E. coli* CV60 GFP.

Incorporation of a mobilization module into the pRSB101 genome arguably was a prerequisite for acquisition of most of its accessory genes, which are of diverse origins (see below).

pRSB101 contains a tetracycline resistance module next to the Tn*402*-specific inverted repeat motif

A derivative of the transposon Tn*402* constitutes the core element of a 20 kb resistance region present on plasmid pRSB101 (see Fig. 2a). A tetracycline resistance (*tet*) module was found at one end of this resistance region. The 25 bp inverted repeat (IR)-element of a Tn*402*-like transposon is conserved at the outermost end of the resistance region, but the Tn*402* transposition gene *tniA* was separated from the IR by insertion of the *tet* module. A 2792 bp DNA segment containing the 3′-end of a *pecM* gene, *tetA* encoding a tetracycline efflux pump and *tetR* for a regulatory protein is 99·9 % identical to the corresponding region present on the 'Birmingham' IncP-1α plasmids (Pansegrau *et al.*, 1994). Tetracycline resistance genes were frequently found in plant-associated and phytopathogenic bacteria since oxytetracycline is applied to protect plants from pathogens (McManus *et al.*, 2002). Schnabel & Jones (1999) reported on the detection of the *tetA* determinant in fluorescent and non-fluorescent *Pseudomonas* spp. isolated from apple orchards which were treated with oxytetracycline. Likewise, derivatives of the transposon Tn*1721*, which usually carries *tetA–tetR*, were found in *Xylella fastidiosa*, the causative agent of citrus variegated chlorosis (Ferreira *et al.*, 2002). The origin of the pRSB101 *tetAR* genes cannot be easily determined due to the wide dissemination of these genes among environmental and

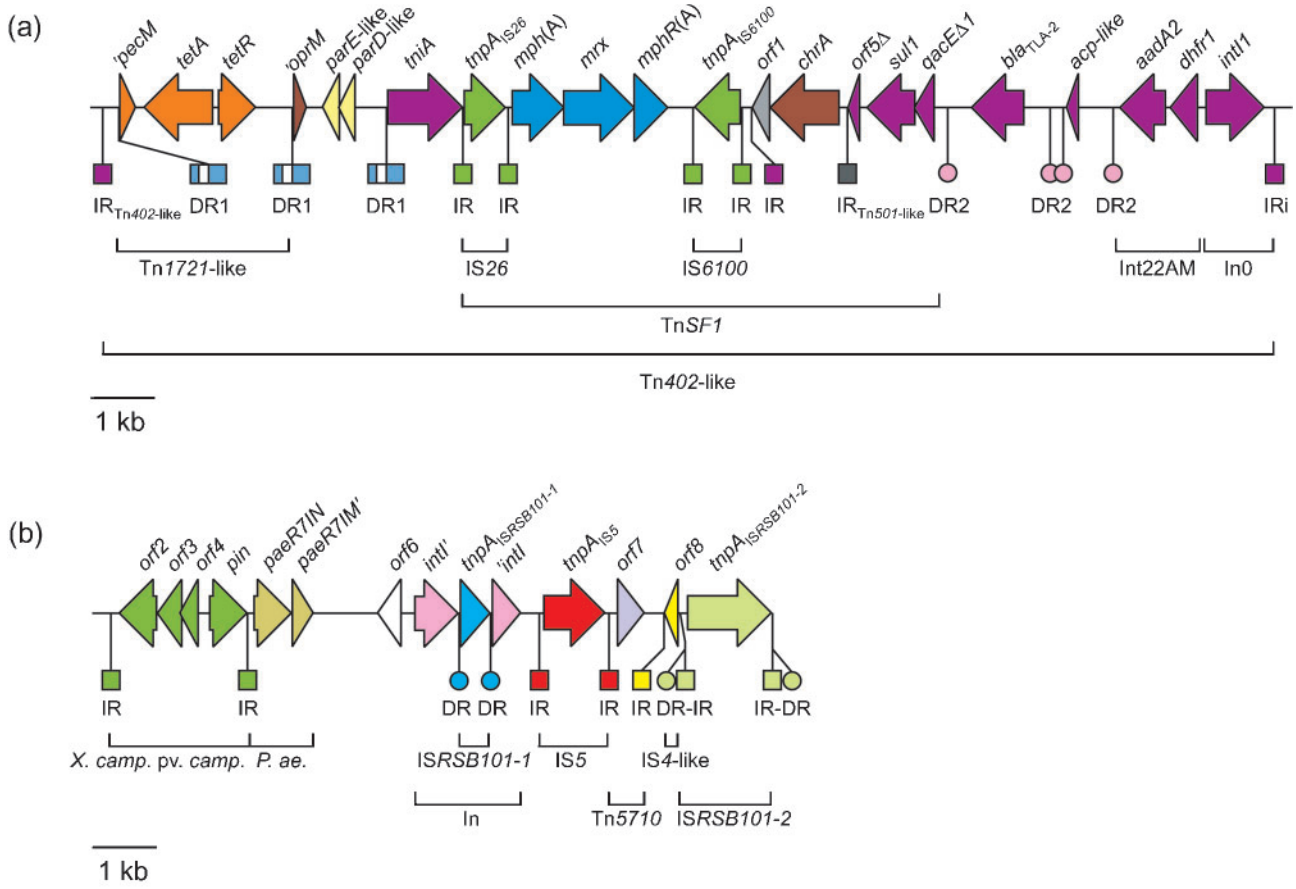

Fig. 2. Detailed map of the pRSB101 20 kb resistance region and the adjacent region containing mobile genetic elements. The 'genetic load' of pRSB101 consists of a 20 kb resistance region (a) and another region containing different mobile genetic elements (b). (a) The core element of the 20 kb region is a derivative of transposon Tn*402* which carries a class 1 integron (lilac). The 5′-conserved segment of the integron is identical to In0 of plasmid pVS1 from *P. aeruginosa*. The two resistance gene cassettes *dhfr1* and *aadA2* were also found in the integron Int22AM of *K. pneumoniae*. The 3′-conserved segment of the integron (*qacEΔ1–sul1–orf5Δ*) is connected to a macrolide-resistance operon [*mph*(A)–*mrx–mphR*], (blue) which is bordered by copies of IS*26* (green) and IS*6100* (green). The DNA segment which is nearly identical to Tn*SF1* of *Shigella flexneri* (accession no. AF188331) is marked below the map. The *parDE*-homologous genes (yellow) are predicted to constitute a plasmid-stabilization system. The DNA segment with the tetracycline resistance genes *tetA* and *tetR* (orange) corresponds to the central part of Tn*1721*. Terminal IRs are shown in the same colour as the element to which they belong. DRs (DR1 and DR2) are represented by blue-white boxes or lilac circles, respectively. More details on DR1 and DR2 are given in the text. The encoded gene products of the different genes are listed in Table 2. (b) The 'genetic load' region downstream of the integrase gene *intI1* is composed of four distinct mobile elements (green, blue, red, light green), another integron (pink) and footprints of two more transposable elements (light blue, yellow). These elements are nearly identical or homologous to corresponding elements found on TNCP9 of *P. aeruginosa*, IS*1004* of *V. cholerae*, IS*5* of *E. coli*, Tn*5710* of *K. pneumoniae* and IS*4* of *E. coli*, respectively. The IS element, IS*RSB101-2*, downstream of the IS*4*-footprint has not been described before. Terminal IRs and DRs are shown in the same colour as the element to which they belong.

pathogenic bacteria (Aminov *et al.*, 2002; Chopra & Roberts, 2001; Rhodes *et al.*, 2000).

To determine the tetracycline resistance level mediated by pRSB101, a 8087 bp *Xho*I–*Cla*I fragment containing the *tetAR* genes was cloned into the vector pBCKS. The recombinant plasmid conferred a tenfold higher resistance level (100 μg tetracycline ml^{-1}) to *E. coli* DH5α as compared to the basic vector.

The pRSB101 *pecM* gene was truncated by insertion of a 168 bp DNA element (designated DR1 in Fig. 2a) containing the 49 bp IR of IS*3000* at one end and the outer part of this IR (26 bp) at the other end. This DR1-element might represent a deletion derivative of IS*3000* or a distinct genetic element which possibly can be moved by a specific transposase provided *in trans*. Three identical copies of the DR1-element were found in the vicinity of the *tet* module. The second DR1 copy borders the *tet* module 532 bp

downstream of *tetR* and the third copy is located at the 5′-end of *tniA* (see Fig. 2a). The DNA segment between the second and third DR1 copy encodes two ORFs, the deduced gene products of which show the highest degree of identity to, respectively, a predicted transcriptional regulator (COG3905) of *Xylella fastidiosa* (accession no. ZP_00041599) (61 % identity) and a conserved hypothetical protein belonging to the group of plasmid stabilization proteins (ParE: COG3668, Pfam05016) of *Xanthomonas axonopodis* pv. *citri* (accession no. NP_642743) (62 % identity). On the resistance plasmid RK2 the *parD* gene is located upstream of *parE*. ParD belongs to a family of transcriptional repressors and has been shown to bind to DNA in the *parD* promoter region (Roberts & Helinski, 1992). The predicted transcriptional regulator encoded upstream of the *parE*-like gene on pRSB101 seems to be the orthologue of ParD, although it exhibits only weak similarity (36 %) as compared to ParD of RK2. The gene products of the *parD*-like and *parE*-like genes are predicted to constitute a second system for plasmid stabilization involved in post-segregational killing (*psk*) of plasmid-free segregants (Roberts & Helinski, 1992). In addition to the ParD/ParE system, pRSB101 also possesses the partitioning genes *parA* (ATPase), *parC* and *kfrA* (putative filamentous α-helical coiled-coil protein). These systems have predicted functions in active plasmid partitioning during cell division and in post-segregational killing (*psk*) of plasmid-free segregants (Roberts & Helinski, 1992; Adamczyk & Jagura-Burdzy, 2003). Presence of at least five putative stability genes could be the reason why we never observed loss of pRSB101, even under non-selective conditions, during our work with this plasmid.

In summary, the described region can formally be interpreted as two DR1-bounded 'cassettes' carrying *tetA–tetR* and *parDE*-like genes, respectively, which were tandemly inserted into the terminal part of a Tn*402*-like transposon.

The macrolide resistance operon present on pRSB101 confers high-level erythromycin resistance

Plasmid pRSB101 contains a macrolide resistance region flanked by copies of the insertion sequence elements IS*26* and IS*6100* (see Fig. 2a). A 5055 bp DNA segment containing the complete macrolide resistance operon, IS*26* and IS*6100* is 99·8 % identical to a corresponding segment of the *Shigella flexneri* Tn*21*-like transposon Tn*SF1* (accession no. AF188331). The macrolide resistance operon is composed of the genes *mph*(A), *mrx* and *mphR*(A) which encode a macrolide-2′-phosphotransferase I, a hydrophobic protein of unknown function and a negative transcriptional regulator, respectively. An identical macrolide resistance region was first cloned and sequenced from the clinical *E. coli* isolate Tf481A which exhibits high-level erythromycin resistance (Noguchi *et al.*, 1995, 2000). Sequences upstream of *E. coli mph*(A) and downstream of *mphR*(A) suggest that this region is also flanked by IS*26* (synonym: IS*176*) and IS*6100*. IS*26* and IS*6100* both belong to the insertion sequence family IS*6* and possess almost identical 14 bp IR-elements. Therefore it is possible that these IS-elements, framing the macrolide resistance operon, constitute a composite transposon. The sequence upstream of the IS*6100 tnpA* gene on pRSB101 is nearly identical for 1309 bp to the corresponding region of *Shigella flexneri* Tn*SF1*. The first 123 bp next to the insertion site of IS*6100* are identical to one end of Tn*402* and Tn*1696* (IRt-end) including the 25 bp inverted repeat sequence (IRt) terminating these transposons (Partridge *et al.*, 2001). An IRt element is missing at the other end of IS*6100*. The DNA region between the IS*6100* element and the 3′-conserved segment of a class 1 integron (see below) on pRSB101 contains two ORFs, *orf1* and *chrA*, the first of which encodes a hypothetical protein that is conserved in various bacterial species (see Table 2). It possesses the Pfam PadR-specific motif (Pfam03551) which is characteristic for transcriptional regulators belonging to the PadR-like family. The gene product of *chrA* shows 63 % identity to a probable chromate ion transporter (accession no. NP_252979) designated ChrA in other bacteria. *Pseudomonas* sp. B13 GFP1 and *E. coli* DH5α harbouring pRSB101 were checked for their growth properties in media containing potassium chromate (50–800 μg ml^{-1}) and it was found that pRSB101 does not mediate chromate resistance to these host bacteria.

To determine the resistance level mediated by the pRSB101 macrolide resistance operon an appropriate restriction fragment was subcloned into the vector pUC18. The recombinant plasmid was shown to confer high level erythromycin resistance (up to 4500 μg ml^{-1}) to the host bacterium *E. coli* DH5α as compared to the basic vector (400 μg ml^{-1}). To our knowledge pRSB101 is the first example for a plasmid-borne *mph*(A)–*mrx*–*mphR*(A) macrolide resistance operon. Since the pRSB101 macrolide resistance operon and adjacent sequences are very closely related to a corresponding segment on the transposon Tn*SF1* (accession no. AF188331) of the human pathogen *Shigella flexneri* (strain SH595), which is the causative agent of bacillary dysentery and diarrhoea, we assume that pRSB101 captured part of a Tn*SF1*-like transposon from an enteric bacterium sharing its mobile genetic element pool with *Shigella flexneri*.

Nine different erythromycin resistance plasmids were isolated in parallel with pRSB101. To determine whether these plasmids also contain the macrolide resistance operon *mph*(A)–*mrx*–*mphR*(A), specific primer pairs were designed for each gene. These primers were used to detect the respective genes by PCR on isolated plasmid DNAs as template. Plasmids pRSB101, pRSB102, pRSB103, pRSB104, pRSB106, pRSB107, pRSB108, pRSB109 and pRSB110 gave amplification products of the expected size for *mph*(A), *mrx* and *mphR*(A). In addition, internal fragments of IS*6100* and IS*26* which flank the *mph*(A)–*mrx*–*mphR*(A) operon of pRSB101 were also detected on the plasmids listed above. Plasmid pRSB105 does not

contain a macrolide resistance operon and gave no PCR products with primers specific for the erythromycin esterification genes *ereA*, *ereB* and for the macrolide efflux genes *mefA*/*E* (Sutcliffe *et al.*, 1996).

In summary, the *mph*(A)–*mrx*–*mphR*(A) operon seems to be the most abundant erythromycin resistance determinant on erythromycin-resistance plasmids isolated from activated sludge bacteria of the wastewater treatment plant under study.

pRSB101 contains a class 1 integron with an unusual composition of genes including a new class A β-lactamase gene

An intact class 1 integron, potentially capable of integrating and disseminating resistance gene cassettes, was identified upstream of the putative chromate transporter gene *chrA* on pRSB101 (Fig. 2a). The variable region of this integron contains an unusual arrangement of gene cassettes and genes.

The integron-specific 5′-conserved segment on pRSB101, including the terminal inverted repeat (IRi), the *intI1* integrase gene and the first recombination site (attI), is identical to the corresponding part of In0 on the *Pseudomonas aeruginosa* plasmid pVS1 (Bissonnette & Roy, 1992). Even the target site duplication (5 bp direct repeat, DR) next to the IRi-element is conserved in these integrons. The pRSB101 integron contains two resistance gene cassettes, namely *dhfr1* for a dihydrofolate reductase conferring trimethoprim resistance and *aadA2* encoding an aminoglycoside adenylyltransferase mediating streptomycin/spectinomycin resistance. The arrangement of these gene cassettes, including the 59-base elements, is essentially the same as described for the *Klebsiella pneumoniae* integron Int22AM (accession no. AY007807). Streptomycin resistance genes were frequently found in plant-associated and phytopathogenic bacteria since streptomycin has been widely used to control bacterial diseases in plants, especially in fruit orchards (Sundin & Bender, 1996; Vivian *et al.*, 2001; McManus *et al.*, 2002; Sundin, 2002). Some pPT23A-like plasmids of the phytopathogenic bacterium *P. syringae* as well as pRSB101 confer resistance to trimethoprim and streptomycin (Cooksey, 1990). As outlined above the pPT23A RepA replication protein is related to RepA of pRSB101. Recently, the integron-specific streptomycin resistance gene cassette *aadA2* which is present on pRSB101 was identified on the transposon Tn*1404* from *Pseudomonas* sp. R9 which was isolated from a streptomycin-treated apple orchard (Schnabel & Jones, 1999). Thus, we speculate that pRSB101 acquired the streptomycin resistance determinant during its evolution in a bacterium which lived in a streptomycin-contaminated environment. To determine spectinomycin and streptomycin resistance levels conferred by the *aadA2* gene product, a 2282 bp *Sph*I-fragment carrying *aadA2* was cloned into the vector pUC18. The recombinant vector conferred high-level spectinomycin resistance (>2000 μg ml^{-1}) and streptomycin resistance (300 μg ml^{-1}) to the host bacterium *E. coli* DH5α as compared to the basic vector (100 μg spectinomycin ml^{-1} and 6 μg streptomycin ml^{-1}, respectively).

Surprisingly, the composition of the DNA region downstream of the *aadA2* cassette is puzzling because the genes *acp*-like, for a putative acyl-carrier protein, and $bla_{\text{TLA-2}}$, encoding a new extended-spectrum class A β-lactamase, do not seem to be located on integron-specific gene cassettes. Sequence motifs resembling the integron-specific 59-base element could not be found in the vicinity of *acp* and $bla_{\text{TLA-2}}$, but it is noticeable that both genes are flanked by 145 bp direct repeats (termed DR2 in Fig. 2a). Since these DR2-elements contain the consensus core-motif (GTTAAA) of integron-specific recombination sites we speculate that these DR-elements played a role in the integration of *acp* and $bla_{\text{TLA-2}}$ into the variable region of the integron. The origin of these genes remains unknown. The deduced gene product of the *acp*-like gene is 29 % identical and 54 % similar to an acyl carrier protein of unknown function from *Thermosynechococcus elongatus* (accession no. NP_682662). The signature pattern for the acyl carrier protein phosphopantetheine domain (PROSITE no. PS50075) is conserved in the putative pRSB101 Acp protein.

The $bla_{\text{TLA-2}}$ gene encodes a β-lactamase of 304 amino acid residues. A putative ribosome-binding site (RBS) and possible −10/−35 promoter motifs were found upstream of the ATG start codon. The $bla_{\text{TLA-2}}$ gene was integrated into the variable region of the integron quite recently, since its G+C content of 38 % differs considerably from the mean G+C content of the whole plasmid (56 %). The deduced TLA-2 protein shows the highest degree of similarity to TLA-1 encoded by the conjugative plasmid RZA92 of the clinical *E. coli* isolate R170 (51 % identity and 64 % similarity) (Silva *et al.*, 2000). The motifs S_{70}xxK, S_{130}DN and K_{234}TG, common to Ambler class A β-lactamases are conserved.

A 1771 bp *Sph*I-fragment containing the complete $bla_{\text{TLA-2}}$ gene and 359 bp of the upstream region was cloned into pZErO-2 and it was found that the recombinant plasmid pZErO-2-$bla_{\text{TLA-2}}$ confers resistance to cefotaxime (30 μg ml^{-1}), cefuroxime (550 μg ml^{-1}) and cefpirome (30 μg ml^{-1}) to the host bacterium *E. coli* DH5α. These resistance levels are, respectively, sixfold, 22-fold and sixfold above the intrinsic resistance of *E. coli* DH5α harbouring the basic vector. These results justify the classification of the pRSB101 TLA-2 β-lactamase as an extended spectrum β-lactamase.

In summary, the $bla_{\text{TLA-2}}$ gene of pRSB101 encodes a new β-lactamase belonging to Ambler class A and most probably originates from a clinical bacterium which was exposed to expanded spectrum cephalosporins. The mechanism by which the gene has integrated into the pRSB101 integron structure remains unknown.

The DNA sequence of the integron-specific 3′-segment present on pRSB101, including *qacEΔ1*, *sul1* and *orf5Δ*, is essentially the same as compared to the integron on Tn*SF1* (99·7 % identity at the DNA sequence level). The only difference is that the core-site of the 59-base element which is normally present upstream of *qacEΔ1* is missing in the pRSB101 sequence. This sequence motif was replaced by a DR2 element (see Fig. 2a) which might have played a role in the integration of the $bla_{TLA\text{-}2}$ gene. Deletion of *orf5Δ* in Tn*SF1* and the pRSB101 integron most probably occurred by insertion of a Tn*501*-like transposon since a 38 bp IR element very similar to those of Tn*21*/Tn*501* transposons was found at the 3′-end of *orf5Δ* (see Fig. 2a). A 8384 bp fragment of pRSB101 containing IS*26*, the *mph*-operon, IS*6100*, *chrA* and the integron-specific 3′-conserved segment is also an integral part of the *Shigella flexneri* transposon Tn*SF1* (99·8 % identity at the DNA sequence level). Unfortunately, Tn*SF1* has not been published until now and the DNA-sequence of the database entry (accession no. AF188331) was only partially annotated.

pRSB101 encodes a putative MDR system of the ABC-type associated with a membrane fusion protein (MFP)

A putative MDR transport system consisting of three components and a regulatory protein is encoded between the replication and the mobilization module on pRSB101.

The first gene of the presumptive MDR region encodes a transcriptional regulator of the TetR/AcrR family (COG1309, Pfam00440). The *acrR* gene product shows 40 % similarity to the corresponding gene product of *Geobacter sulfurreducens* PCA (accession no. NP_952005) and 35 % similarity to the AcrR of *E. coli* K-12 (accession no. NP_414997). The repressor AcrR modulates the regulation of *acrAB* for a multidrug efflux pump in *E. coli* (Ma *et al.*, 1996). The pRSB101 AcrR regulator possesses a DNA-binding helix-turn-helix motif near the N terminus (residues 33 to 54) which is typical for repressor proteins belonging to the TetR/AcrR family. A 28 bp palindromic sequence motif which might serve as a binding site for AcrR was found 12 bp upstream of the ATG start-codon of the *acrR* gene on pRSB101. The *acrR* gene and the downstream gene are most probably translationally coupled since the stop-codon of the first gene overlaps with the start-codon of the second one. It is therefore very likely that the AcrR regulator controls transcription of the complete MDR-operon including the regulatory gene. The gene downstream of the *acrR* gene encodes a protein that shows 45 % similarity to the probable RND efflux MFP of *G. sulfurreducens* (accession no. NP_952003) and 34 % to the MFP of *E. coli* K-12 (accession no. NP_414996) (see Fig. 3). Classification of the pRSB101 MFP reveals that this protein clusters within the COG0845 group of putative membrane fusion proteins. The prototype of this group, AcrA, represents the accessory periplasmic protein of an RND-type multidrug efflux system which pumps out a wide variety of lipophilic and amphiphilic compounds (Nikaido & Zgurskaya, 2001). MFP of pRSB101 possesses a signal-peptide which could be involved in guidance of the protein into the periplasmic space. The possibility also exists that the hydrophobic N-terminal segment anchors the protein in the cytoplasmic membrane. It is thought that MFPs physically connect the cytoplasmic transporter component (see below) to an outer-membrane factor (OMF) facilitating transport across the outer membrane of Gram-negative bacteria (Zgurskaya, 2002). An OMF is not encoded in the pRSB101 MDR-region but the 3′-end of an *oprM* gene for an outer-membrane protein is located downstream of the tetracycline regulator gene *tetR* on pRSB101. It has been shown that OMFs function with more than one permease/MFP pair (Saier & Paulsen, 2001; Paulsen *et al.*, 1997), so that we speculate that a heterologous, host-encoded OMF interacts with the pRSB101 MDR efflux components. The gene for the MFP is also translationally coupled to the downstream gene of the MDR-operon since its stop-codon overlaps with the start-codon of the following gene (combined ATGA start/stop motif). The deduced gene product of the third gene of the MDR-operon is 59 % similar to a possible ABC-type transporter transmembrane subunit of *G. sulfurreducens* (accession no. NP_952002). Analysis of the membrane topology using the TopPred tool revealed that the pRSB101 protein contains six putative transmembrane helices with the N and C termini being localized at the cytoplasmic site of the membrane. Similar membrane topologies were predicted for most ABC-type permeases (van Veen & Konings, 1998; Higgins, 2001). Another interesting feature of the pRSB101-encoded permease is that its C-terminal part gives a hit to the Pfam FtsX-family (Pfam02687) which includes predicted permeases some of which were shown to transport lipophilic substrates targeted to the outer membrane across the inner membrane. The transport process of ABC-transport systems is normally coupled to the hydrolysis of ATP. A putative ATP-binding protein is encoded by the last gene of the MDR-region. The corresponding gene product is 45 % identical to an ATP-binding component of an ABC-transporter of *G. sulfurreducens* (accession no. NP_952001). The amino acid sequence of the pRSB101 protein possesses a conserved Walker A motif (G_{42}xxGxG**K**S), Q-loop (glutamine-loop, Q_{96}), Walker B motif (V_{168}ILA**D**) and a switch region (histidine-loop, H_{206}). These sequence motifs are involved in forming a nucleotide-binding site (Schneider & Hunke, 1998). In addition, a signature-conserved motif also known as linker peptide (L_{148}SGGEQQR) specific for the ABC ATP-binding component was found in the pRSB101 protein. The possible function of this ATP-binding protein is to energize the transport process via ATP-hydrolysis.

The MDR transporter genes seem to represent the most original load of pRSB101 since these genes are not located on a transposable element. The only indication that the MDR transporter region entered the plasmid by transposition is the relict of a 38 bp Tn*21*/Tn*501*-like IR upstream of the MDR-operon.

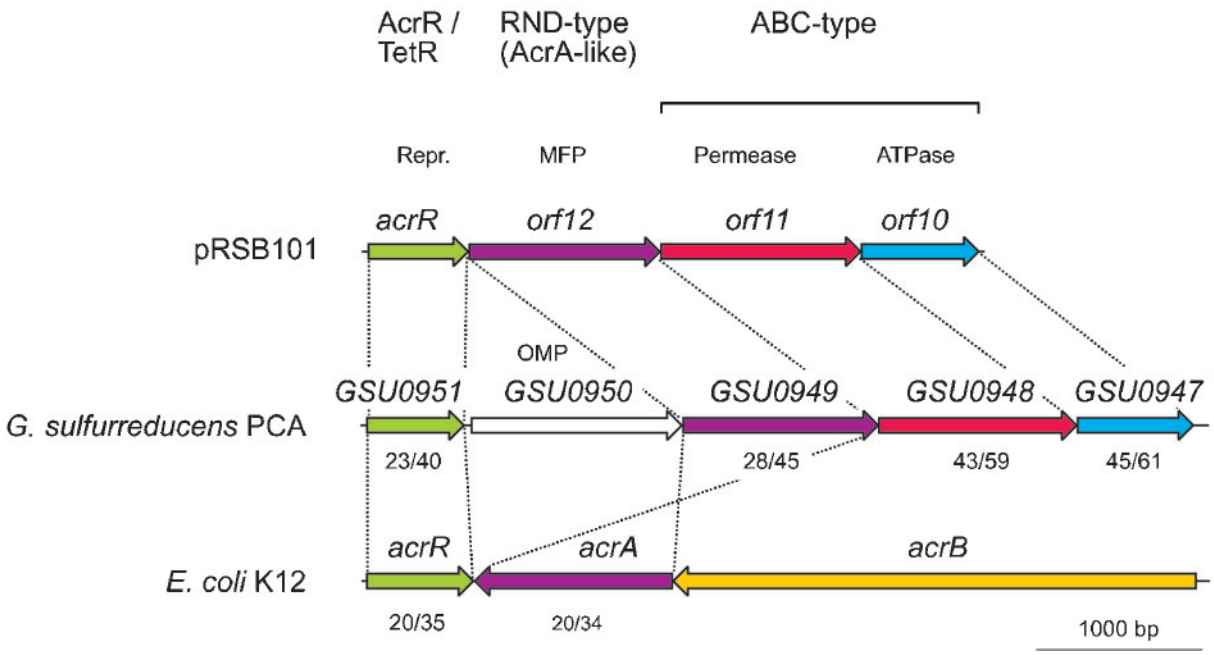

Fig. 3. Comparison of the pRSB101 MDR efflux gene region to related regions of *G. sulfurreducens* PCA and *E. coli* K-12. Coding sequences are shown by arrows indicating the direction of transcription. The protein sequences used for this comparison were taken from the following sources: GSU0951 (accession no. NP_952005), GSU0950 (accession no. NP_952004), GSU0949 (accession no. NP_952003), GSU0948 (accession no. NP_952002), GSU0947 (accession no. NP_952001) from *G. sulfurreducens* PCA and AcrA (accession no. NP_414996), AcrB (accession no. NP_414995) and AcrC (accession no. NP_414997) from *E. coli* K-12. The identities and similarities (in %), respectively, of the gene products which correspond to those encoded by pRSB101 are shown below the arrows. Abbreviations: AcrR, acridine resistance regulator; TetR, tetracycline resistance regulator; RND, resistance-nodulation-division; ABC, ATP-binding cassette; Repr, repressor; MFP, membrane-fusion protein; OMP, outer-membrane factor.

To further analyse the transport function of the pRSB101 MDR system a 7214 bp *Sac*II/*Xho*I-fragment containing the complete region was subcloned into the *E. coli* vector pBluescript-II-KS. The recombinant plasmid confers resistance to nalidixic acid (550 μg ml^{-1}) and low-level resistance to norfloxacin (1·25 μg ml^{-1}) to the host bacterium *E. coli* DH5α. The basic vector confers resistance against 80 μg nalidixic acid ml^{-1} and 0·5 μg norfloxacin ml^{-1} to the host bacterium. Extrusion of antimicrobial drugs most probably is not the primary function of the pRSB107 efflux system. Very recently Burse *et al.* (2004) provided strong evidence that the RND-type AcrAB transport system of the phytopathogen *Erwinia amylovora* is responsible for resistance to hydrophobic and amphiphilic toxins and to phytoalexins. Likewise, ABC exporters were shown to reduce the toxic effects of phytoalexins in phytopathogenic fungi (Del Sorbo *et al.*, 2000; Schoonbeek *et al.*, 2001; Fleißner *et al.*, 2002). Although the pRSB101 transporter is not completely homologous to the *Erwinia amylovora* system, it remains to be determined whether the primary functions of these systems are similar.

In summary, pRSB101 encodes a tripartite MDR efflux system composed of an ABC-type ATP-binding protein, a corresponding transmembrane permease and an RND-type MFP. This efflux system contributes to the resistance phenotype mediated by pRSB101.

The third 'genetic load' region of pRSB101 contains four mobile genetic elements and a putative class 4 integron

Plasmid pRSB101 contains a third 'genetic load' region located between the 20 kb resistance region and the replication module. This segment harbours four complete mobile genetic elements, relicts of an insertion sequence element and a transposon, and a putative integron which was interrupted by insertion of the IS*RSB101-1* element (Fig. 2b).

The first putative mobile element possesses four genes and is bounded by 25 bp IRs, which show similarity to the IRi elements of class 3 and class 1 integrons. The deduced gene product of *orf2* is 53 % identical to a product which was annotated as IS*xac3* transposase on the *Xanthomonas axonopodis* pv. *citri* plasmid pXAC33 (accession no. NC_003921). The implied gene products are very short (110 and 90 amino acid residues, respectively) and are not homologous to any known transposases. Therefore the annotation of the IS*xac3* transposase remains questionable.

The products of *orf3*, *orf4* and *pin* located upstream of *orf2* show, respectively, 98, 81 and 89 % identity to conserved hypothetical proteins (designated XCC1632 and XCC1631) and an invertase/recombinase of *Xanthomonas campestris* pv. *campestris* (accession no. NC_003902). The organization of the corresponding genes is the same in *Xanthomonas campestris* pv. *campestris* and on pRSB101 and linkage of the region to an IS*xac3* transposase gene also exists in *Xanthomonas campestris* pv. *campestris*. Orf3 is a predicted nucleic-acid binding protein containing a PIN-domain (PilT N terminus, COG5611, Pfam01850) of about 100 amino acids with two conserved aspartate residues. The function of this domain is unknown but a role in signalling has been proposed. The reference protein for these PIN-domain proteins is PilT, a putative NTPase playing a role in pilus-dependent surface motility and other processes (Herdendorf *et al.*, 2002; Sakai & Komano, 2002). Orf4 belongs to the group of AbrB-homologues with a regulatory function during the transition state between vegetative growth and the onset of stationary phase (COG2002, Pfam04014). AbrB of *Bacillus subtilis* regulates diverse and unrelated genes during periods of suboptimal growth conditions (Vaughn *et al.*, 2001). Finally, the *pin* gene product is an invertase/recombinase-like protein possessing the signature motifs present in the N terminus of the resolvase family (Pfam00239) and in the site-specific recombinases/DNA-invertase Pin homologues having functions in DNA-replication, recombination and repair (COG1961). It should be pointed out that the closest relatives of the pRSB101 Pin protein were found to be encoded by the *Xanthomonas axonopodis* pv. *citri* plasmids pXAC33 and pXAC64, the *Acidithiobacillus ferrooxidans* plasmid pTF5 and the *Acidithiobacillus caldus* IncQ-like plasmid pTC-F14. It might be supposed that Orf2, Orf3 and Pin play a role in transposition or regulation/modulation of transposition of a mobile genetic element related to IS*xac3* of *Xanthomonas* species. Very recently, an element closely related to the one described above was identified on the large class I transposon TNCP23. This element is a composite of plasmid, integron and IS*6100* elements and constitutes a genomic island in *P. aeruginosa* (Klockgether *et al.*, 2004).

A 969 bp DNA segment downstream of the *pin* gene is 98 % identical to a corresponding region present in the genome of *P. aeruginosa* and encodes a DNA-invertase-like enzyme and the N-terminal 65 amino acids of a putative modification methylase. The deduced amino acid sequences are 100 and 95 % identical to *P. aeruginosa* PaeR7IN (invertase) and the N terminus of PaeR7IM (modification methylase), respectively (Theriault *et al.*, 1985; Vaisvila *et al.*, 1995). These findings indicate that the pRSB101 host bacterium and *P. aeruginosa* share a common gene pool.

Downstream of *paeR7IN–paeR7IM′* a genetic element was identified which might represent an integron. The integrase gene (*intI*) of the putative integron was interrupted by insertion of the IS*RSB101-1* element. The deduced gene product of the reconstructed *intI* open reading frame shows 57 % identity and 66 % similarity to the site-specific recombinase IntI4 of *Vibrio cholerae* and can be grouped into the XerC/XerD-family (COG4973, COG4974) of site-specific recombinases. Upstream of *intI* a putative site-specific recombination site (attI) with the inverse core-site motif -caaa**AAC**-, the core-site motif -**GTT**agcc- and imperfect 20 bp IRs in-between was detected. Therefore it might be speculated that *orf6* located upstream of *intI* represents an integron-specific gene cassette which entered the variable region of the integron by site-specific recombination via the putative recombination site described above. The N terminus of the *orf6* gene product contains the signature motifs COG2732 and Pfam01337 characteristic for barstar-like ribonuclease (barnase) inhibitors (Buckle *et al.*, 1994) but a function for Orf6 cannot be predicted.

The *intI* coding region was interrupted by insertion of a 621 bp transposable element designated IS*RSB101-1*. The encoded transposase is 69 % identical and 77 % similar to the IS*1004* transposase of *V. cholerae* O1 biovar eltor (accession no. NP_232602) which belongs to the transposase_17 family (Pfam01797, COG1943) of IS*200*-like elements. The IS present on pRSB101 represents a new member of the IS*200* family. Insertion of the element into the *intI* gene caused a 5 bp target site duplication (5 bp direct repeats). Although the integrase gene *intI* has been inactivated, the integron in its functional state might have contributed to the acquisition of genetic material in the course of pRSB101 evolution.

Downstream of the integrase gene *intI* another IS has integrated. This element is 1196 bp in length, possesses 16 bp terminal IRs and encodes a transposase of the transposase_11 family (IS*5*-family; Pfam1609, COG3039). The DNA sequence of this element is almost identical (97 %) to IS*5* present in the genome of *E. coli* K-12 (accession no. D90775).

The pRSB101 region adjacent to the IS*5* element contains relicts of transposon Tn*5710* originally found in *K. pneumoniae* subsp. *ozenae* (accession no. KPN011908) and an IS-element related to IS*4* of *E. coli* (accession no. NP_418698).

The IS*4*-like element was truncated by insertion of another transposable element, designated IS*RSB101-2*, which has not been described before. This element is 1388 bp in length, carries 19 bp imperfect terminal IRs flanked by 10 bp DRs and encodes a transposase which is homologous to a transposase (accession no. CAC84124) of the marine psychrophilic bacterium Mst37. The implied transposases belong to the transposase_11 family (Pfam01609, COG3385) and contain the DDE-domain with three conserved carboxylate residues essential for catalytic activity of the enzymes.

In summary, the pRSB101 region described in this section contains four complete IS-elements, footprints of another

IS-element and a transposon, and a new putative integron. This region might serve as 'matrix' for the incorporation of other 'genetic material' either by homologous recombination via IS-elements, site-specific recombination or transposition (illegitimate recombination).

Identification of pRSB101-like plasmids in different compartments of the wastewater treatment plant

Nine plasmids conferring erythromycin resistance were isolated in parallel with pRSB101 (see Table 1). To test whether these plasmids possess a similar replicon-type as compared to pRSB101, we tried to amplify internal *repA*-fragments by using primers (designated repA-L409 and repA-R854) specific for the pRSB101 *repA* replication gene.

An amplification product of the expected size (446 bp) was detected for plasmid pRSB105. Sequencing of this *repA*-specific amplicon revealed that it differs only in 9 bp as compared to the homologous pRSB101 *repA*-fragment.

To address the question of whether bacteria carrying pRSB101-like plasmids are released from the wastewater treatment plant into the environment, total plasmid-DNA preparations isolated from erythromycin-resistant bacteria of the final effluents were used as template DNAs in pRSB101 *repA*-specific PCR. This approach resulted in the amplification of an approximately 450 bp product which was cloned into the vector pGEM-T-Easy. Twenty-five insert-amplicons of the resulting *repA*-library were analysed for their RFLP-profiles by digestion with the restriction enzymes *Sau*3A and *Hha*I. It was found that 22 amplicons displayed identical restriction profiles as compared to the corresponding pRSB101 *repA*-fragment. The restriction patterns for the three other amplicons are identical to each other but differ to that of the pRSB101 *repA*-fragment. Sequencing of one of these amplicons revealed a difference in 7 bp as compared to pRSB101. These findings indicate that pRSB101-like plasmids are released with bacteria residing in the final effluents into the environment.

Conclusion

It is very likely that the pRSB101 progenitor plasmid accounted for an adaptive advantage to the host bacterium, for example low-level resistance to plant-borne or other toxic compounds. The presence of a multi-drug resistance determinant might have facilitated initial survival of the host bacterium and provided the opportunity to incorporate other resistance genes. Accordingly, plasmid pRSB101 might be regarded as a plasmid whose primary function was not related to antibiotic resistance. Later on in evolution, pRSB101 acquired antibiotic resistance determinants from environmental and/or pathogenic bacteria. The pRSB101 antibiotic resistance region was only recently extended by integration of genes which confer resistance to currently clinically important antimicrobial drugs such as cephalosporins and macrolides. Since pRSB101-like plasmids were also identified in the final effluents of the wastewater treatment plant it cannot be excluded that resistance determinants carried by these plasmids will be disseminated widely among environmental bacteria.

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