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Comparative Biology of Two Natural Variants of the IncQ-2 Family Plasmids, pRAS3.1 and pRAS3.2[▽]

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Plasmids pRAS3.1 and pRAS3.2 are two closely related, natural variants of the IncQ-2 plasmid family that have identical plasmid backbones except for two differences. Plasmid pRAS3.1 has five 6-bp repeat sequences in the promoter region of the *mobB* gene and four 22-bp iterons in its *oriV* region, whereas pRAS3.2 has only four 6-bp repeats and three 22-bp iterons. Plasmid pRAS3.1 was found to have a higher copy number than pRAS3.2, and we show that the extra 6-bp repeat results in an increase in *mobB* and downstream *mobA*/*repB* expression. Placement of *repB* (primase) behind an arabinose-inducible promoter in *trans* resulted in an increase in *repB* expression and an approximately twofold increase in the copy number of plasmids with identical numbers of 22-bp iterons. The pRAS3 plasmids were shown to have a previously unrecognized toxin-antitoxin plasmid stability module within their replicons. The ability of the pRAS3 plasmids to mobilize the *oriT* regions of two other plasmids of the IncQ-2 family, pTF-FC2 and pTC-F14, suggested that the mobilization proteins pRAS3 are relaxed and can mobilize *oriT* regions with substantially different sequences. Plasmids pRAS3.1 and pRAS3.2 were highly incompatible with plasmids pTF-FC2 and pTC-F14, and this incompatibility was removed on inactivation of an open reading frame situated downstream of the *mobCDE* mobilization genes rather than being due to the 22-bp *oriV*-associated iterons. We propose that the pRAS3 plasmids represent a third, γ incompatibility group within the IncQ-2 family plasmids.

Plasmids of the IncQ family are small (<20 kb), have a broad host range, and are highly promiscuous due to their ability to be mobilized very efficiently by self-transmissible plasmids such as the IncP plasmids. They have been divided into two families, IncQ-1 and IncQ-2, based on the amino acid sequence relatedness of their RepA (helicase), RepB (primase), and RepC (DNA-binding) replication proteins and because the mobilization proteins of the two families are unrelated, consisting of three or five genes, respectively (31). IncQ-1 group plasmids include RSF1010 and the near-identical R1162, pDN1, pIE1107, pIE1115, and pIE1130, while IncQ-2 plasmids include pTF-FC2, pTC-F14, and pRAS3.

IncQ-2 plasmids pRAS3.1 and pRAS3.2 were isolated in Norway from the fish pathogens *Aeromonas salmonicida* subsp. *salmonicida* and atypical *A. salmonicida*, respectively, while investigating plasmids that conferred resistance to tetracycline (21). The two plasmids encode identical replication and mobilization proteins, with the most important differences in the plasmid backbone being that pRAS3.1 has four 22-bp iterons in its *oriV* region and five 6-bp repeat sequences upstream of its *mobB* gene, whereas pRAS3.2 has only three iterons and four 6-bp repeat sequences. No biological studies were carried out in the initial report of the pRAS3 plasmids. As a contribution to our studies on the evolution of IncQ plasmids, our longer-term aim is to address the question of why two natural versions of the plasmid exist. Here we report on the major differences in the biology of the two plasmids. In addition, we discovered the presence of *repC* and *mobB* genes that were not detected when the sequence of pRAS3 plasmids was previously reported. We also discovered a putative toxin-antitoxin (TA) postsegregational system different from that found in other members of the IncQ plasmids and tested it for functionality.

The IncQ-1 plasmids are subdivided into incompatibility groups α, β, and γ, (31), whereas the IncQ-2 plasmids are subdivided into two incompatibility groups, α and β (14). In this work we also report on the incompatibility between the pRAS3 plasmids and other members of the IncQ-2 plasmid family as well as the IncQ-1 family plasmids. Furthermore, we compare the functional relatedness of the pRAS3 mobilization system with that of previously studied IncQ-2 plasmids.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. *Escherichia coli* strains, cloning vectors, and plasmid constructs are shown in Table 1. Cultures of *E. coli* were grown in either Luria-Bertani broth (LB) or on Luria-Bertani agar (LA) plates. The growth medium was supplemented with antibiotics as required at the following concentrations: ampicillin (100 μg ml^{-1}), chloramphenicol (20 μg ml^{-1}), kanamycin (30 μg ml^{-1}), nalidixic acid (35 μg ml^{-1}), streptomycin (35 μg ml^{-1}), tetracycline (10 μg ml^{-1}).

General DNA techniques. Plasmid preparation, restriction endonuclease digestions, gel electrophoresis, and cloning were carried out using standard methods (2, 33). Where no suitable restriction sites were present, single-strand DNA primers were designed and DNA fragments to be cloned were amplified by PCR. An initial denaturation step of 90 s at 94°C was followed by 30 cycles of denaturation (30 s at 94°C), a variable annealing step, and a standard elongation step (240 s at 72°C). Annealing temperatures were based on the average primer annealing temperature, and extension times were altered as required based on primer sequence (Table 2). PCR was performed in a Sprint temperature cycling system (Hybaid) using the Expand high-fidelity PCR system DNA polymerase (Roche Molecular Biochemicals). The sequences of all constructs that required a PCR step were confirmed by DNA sequencing using the dideoxy chain termination method and an ABI Prism 3100 genetic analyzer.

Reverse transcription-PCR (RT-PCR). RNA was isolated using the RiboPure RNA isolation kit (Ambion) from mid-logarithmic *E. coli* DH5α cultures carrying the respective plasmids. The quality of the RNA was assayed on a 1%

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[b]	Reference or source
Strains		
DH5α	ϕ80Δ*lacZ*ΔM15 *endA1 recA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *relA1 supE44 deoR* Δ(*lacZYA-argF*)*U196*	Promega Corp., Madison, WI
S17-1	*recA pro hsdR* (RP4-2 Tc::Mu Km::Tn*7*)	34
CSH56	F$^-$ *ara* Δ(*lac pro*) *supD nalA thi*	Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
EC100D *pir*$^+$	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80Δ*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK* λ$^-$ *rpsL nupG pir*$^+$(*DHFR*)	Epicentre Biotechnologies
Plasmid vectors		
EZ-Tn*5*	Km^r, R6K γ-*ori*	Epicentre Biotechnologies
pACYC177	Ap^r Km^r, p15A replicon, cloning vector	6
pBAD28	Ap^r Cm^r, arabinose-inducible expression vector, pACYC184 replicon	17
pBR322	Ap^r Tc^r, ColE1 replicon, cloning vector	4
pGEM-T	Ap^r, T-tailed PCR product cloning vector	Promega Corp., Madison, WI
pOU82	Ap^r, *lacZYA*, R1 replicon	15
pUC19	Ap^r, *lacZ′*, ColE1 replicon, cloning vector	41
Plasmid constructs		
pBAD28-mobCDEorf3	Ap^r Cm^r, 2.7-kb ApaLI-ScaI fragment containing pRAS3.1 *mobCDE* and *orf3* cloned behind P_{BAD} promoter	This study
pBAD28-mobDEorf3	Ap^r Cm^r, 2.7-kb HindIII-ScaI fragment from pRAS3.1::mobC containing *mobDE* and *orf3* cloned behind P_{BAD} promoter	This study
pBAD28-orf3	Ap^r Cm^r, 1.25-kb PstI-ScaI fragment from pRAS3.1::mobE containing *orf3* cloned behind P_{BAD} promoter	This study
pBAD28-repAC	Ap^r Cm^r, 2.7-kb SalI-StuI fragment containing pRAS3.1 *repAC* cloned behind P_{BAD} promoter	This study
pBAD28-repB	Ap^r Cm^r, 1,212-bp PCR fragment containing pRAS3.1 *repB* (nt position 9891 to 8688)[a] cloned behind P_{BAD} promoter	This study
pBAD28-repBAC	Cm^r, 3.5-kb PvuI-SphI fragment containing pRAS3.1 *pemIK*-like and *repAC* genes cloned into pBAD28-repB after inactivation of the pBAD28 PvuI site	This study
pBAD28-repC	Ap^r Cm^r, 1,015-bp PCR fragment containing the pRAS3.1 *repC* (nt position 6903 to 5889)[a] cloned behind P_{BAD} promoter	This study
pGEM-OriV3.1	Ap^r, 742-bp PCR fragment containing pRAS3.1 *oriV* (nt position 3123 to 2387)[a] cloned into pGEM-T	This study
pIE1108Cm	Cm^r, pIE1107 replicon with nonessential *oriVa* deleted and St^r and Km^r genes replaced by Cm^r gene	13
pIE1130	Cm^r Km^r Sm^r Su^r, natural 10,687-bp IncQ-like plasmid isolated from piggery manure	35
pOriTF14	Ap^r, 203-bp HindIII-NcoI fragment containing pTC-F14 *oriT* cloned into pUC19	40
pOriTFC2	Ap^r, 208-bp HhaI-HhaI fragment containing pTF-FC2 *oriT* cloned into pUC19	40
pOriT-RAS3	Ap^r, 196-bp PCR fragment containing pRAS3.1 *oriT* (nt position 11,820 through 0 to 176)[a] cloned into pGEM-T	This study
pOU82-TA	Ap^r, 731-bp PCR fragment containing pRAS3.1 *pemIK*-like genes (nt position 8819 to 8088)[a] cloned into pOU82	This study
pR6K.3.1.repC$^\Delta$	Km^r, pRAS3.1::tet with *repC* and *tetR* truncated by NheI-NheI deletion	This study
pR6K.3.2.repC$^\Delta$	Km^r, pRAS3.2::tet with *repC* and *tetR* truncated by NheI-NheI deletion	This study
pRAS3.1	Tc^r, natural 11,851-bp plasmid isolated from *Aeromonas salmonicida* subsp. *salmonicida* with four iterons and five 6-bp repeats	21
pRAS3.1.34	Tc^r, pRAS3.1 derivative with three iterons obtained by random ligation of short iteron fragments after BstEII digestion and four 6-bp repeats from pRAS3.2 by exchange of a 2.9-kb HindIII-PvuI region	This study
pRAS3.1.35	Tc^r, pRAS3.1 derivative with three iterons obtained by random ligation of short iteron fragments after BstEII digestion	This study
pRAS3.1Km	Km^r, pRAS3.1 with Tc^r replaced by Km^r from pSKm2 at the BamHI-EcoRV sites	This study
pRAS3.1.44	Tc^r, pRAS3.1 derivative with four 6-bp repeats from pRAS3.2 by exchange of 2.9-kb HindIII-PvuI region	This study
pRAS3.1::mobC	Km^r Tc^r, pRAS3.1 with *mobC* interrupted by EZ-Tn*5* at position 296	This study
pRAS3.1::mobD	Km^r Tc^r, pRAS3.1 with *mobD* interrupted by EZ-Tn*5* at position 1082	This study
pRAS3.1::mobE1	Km^r Tc^r, pRAS3.1 with *mobE* interrupted by EZ-Tn*5* at position 1586	This study
pRAS3.1::mobE2	Km^r Tc^r, pRAS3.1 with *mobE* interrupted by EZ-Tn*5* at position 1614	This study
pRAS3.1::orf3	Km^r Tc^r, pRAS3.1 with *orf3* interrupted by EZ-Tn*5* at position 2089	This study
pRAS3.1::repB	Km^r Tc^r, pRAS3.1 with *repB* interrupted by EZ-Tn*5* at the PvuI site	This study
pRAS3.1::tetAR	Km^r, pRAS3.1 with *tetAR* interrupted by EZ-Tn*5* at the SphI-SphI sites	This study
pRAS3.2	Tc^r, natural 11,823-bp plasmid isolated from atypical *Aeromonas salmonicida* with three iterons and four 6-bp repeats	21
pRAS3.2Km	Km^r, pRAS3.2 with Tc^r replaced by Km^r from pSKm2 at the BamHI-EcoRV sites	This study
pRAS3.2::tetAR	Km^r, pRAS3.2 *tetAR* interrupted by EZ-Tn*5* at the SphI-SphI sites	This study
pTF-FC2Cm	Cm^r, natural pTF-FC2 plasmid with chloramphenicol resistance gene cloned into Tn*5467* (called pDR412 in previous manuscripts)	32
pTF-FC2Tet	Tc^r, Cm^r of pDR412 replaced by Tc^r of pACYC184 at the XbaI and EcoRV sites	G. Matcher
pTC-F14Cm	Cm^r, natural pTC-F14 plasmid with Cm^r inserted at the BamHI site	13
pTC-F14Km	Km^r, pTC-F14Cm with Cm^r replaced by Km^r from Tn*5*	40
R6K-OriV3.1	Km^r, pRAS3.1 *oriV* from pGEM-OriV3.1 transferred to EZ-Tn*5*	This study
RSF1010K	Km^r, 1,1704 bp of RSF1010 replaced by Tn*903*	G. Ziegelin

[a] The nucleotide (nt) positions refer to the positions on pRAS3.1 to which the PCR fragments correspond.
[b] Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline.

morpholinepropanesulfonic acid–EDTA agarose gel and quantified using a NanoDrop spectrophotometer. RNA (1 μg) was converted to cDNA using the Transcriptor first-strand cDNA synthesis kit (Roche Diagnostics).

Qualitative gene expression to verify whether *orf3* is expressed as part of the *mobCDE* operon was assayed using the FastStart *Taq* DNA polymerase (Roche Diagnostics) according to the manufacturer's protocol. Two microliters, or ~100 ng, of the cDNA was used in each reaction mixture with a final $MgCl_2$ concentration of 2.3 mM. The GC-rich solution was added to a 1× final concentration

TABLE 2. Primers used for cloning, quantitative gene expression, and plasmid copy number assays

Primer	Sequence
E.coli GAPA Fwd	5′-TGTTAGACGCTGATTACATGG-3′
E.coli GAPA Rev	5′-CTTTAACGAACATCGGAGTGT-3′
pRAS3A Fwd	5′-GGAGCCACTATCGACTACG-3′
pRAS3A Rev	5′-GAAGCAGCCCAGTAGTAGG-3′
pRAS3MobC Fwd	5′-ACACAACAGAGCAGCTAGA-3′
pRAS3MobC Rev	5′-TCTGGTCAAGCGTGTATCC-3′
pRAS3MobE Fwd	5′-GCATCAGCGGAAGCAGCC-3′
pRAS3MobE Rev	5′-GCCTATCGCACTTCGCC-3′
pRAS3ORF3 Fwd	5′-CCGTTCGATCTGGTAGACC-3′
pRAS3ORF3 Rev	5′-GTTCTTCCATGTCTCGACG-3′
pRAS3OriT Fwd	5′-CTTGCAGGATGAGCCAGAC-3′
pRAS3OriT Rev	5′-TGGTTGCGGAGTTGACAG-3′
pRAS3OriV Fwd	5′-GTCGAATTCGTACATTATGTTTCG-3′
pRAS3OriV Rev	5′-ATAGGTACCAGTCTTTTCCATCC-3′
pRAS3REPB Fwd[a]	5′-ACGAATTCATGTGCGGGAAG-3′
pRAS3REPB Rev[a]	5′-TCACTGCAGTGCAACATTGTA-3′
pRAS3REPB2 Fwd	5′-GCAACTATCAGGCCATCAT-3′
pRAS3REPB2 Rev	5′-TTGGGCTTGCGGTTCTC-3′
pRAS3REPC2 Rev[a]	5′-TATCTGCAGCTTGAACAGGTG-3′
pRAS3REPC3 Fwd[a,b]	5′-TAGAATTC***AGGAGG***AGGGCT**ATG**ACTCAGCAGC-3′
pRAS3SS2 Fwd[a]	5′-GAATTCAGTGGGAGAAGCTGGAAG-3′
pRAS3SS2 Rev[a]	5′-GGATCCGGAATGGTGTAGATCGTT-3′
R6KKANR Fwd	5′-CCATTCTCACCGGATTCAG-3′
R6KKANR Rev	5′-TCACCGAGGCAGTTCCATA-3′

[a] The primer includes an endonuclease restriction site (underlined).
[b] The primer overlaps the start codon (shown in bold) and includes an artificial ribosome binding site (in bold italics).

whenever the pRAS3MOBE forward and reverse primers (Table 1) were used, and 2.5% dimethyl sulfoxide was added to each PCR mixture when the pRAS3MOBC forward or reverse primers were used. The reaction mixtures were subjected to 35 cycles of denaturation, elongation, and extension as described above. The RNA samples were assayed for the presence of contaminating DNA by using ~400 ng of RNA in control reaction mixtures. PCR products were analyzed on a 1% agarose gel.

Quantitative gene expression of the *repB* and *orf3* genes using the pRAS3REPB2 and pRAS3ORF3 primer sets was assayed using a LightCycler as described above. The cDNA was diluted twofold and a total of 50 ng was used in each reaction mixture. The amplification efficiencies were determined as described above using serial dilutions of the cDNA, and the reaction parameters were set as described above for the plasmid copy number determinations. Relative gene expression was determined using the REST analysis tool (30). The R6KkanR primer set was used as a calibrator.

Copy number determinations. Total genomic DNA was prepared from *E. coli* DH5α cultures containing the respective plasmids during exponential growth. Cells were grown overnight (in the presence of antibiotics), reinoculated (1/100) into 50 ml of prewarmed LB medium (no antibiotics), and grown while shaking at 37°C to an optical density at 600 nm of ~0.8. Total genomic DNA was extracted from 1 ml of culture using the QIAamp DNA minikit (Qiagen). The genomic DNA was eluted in 60 μl elution buffer, and the concentration and purity were checked using a Nanodrop spectrophotometer.

Real-time quantitative PCR (qPCR) amplification was performed using a LightCycler (version 2.0) with the LightCycler FastStart DNA master SYBR green I kit (Roche Diagnostics). A total of 4 ng of total DNA was added to each amplification reaction mixture, and the thermal cycling protocol of Lee et al. (22) was followed, except that primer annealing was at 56°C for 4 s and DNA extension was at 72°C for 15 s.

To generate standard curves for plasmid copy number determinations, the *gapA* amplicon (Table 2) was cloned into pGEM-T(Easy). The pGEM-*gapA* and pRAS3.1 plasmids were extracted from *E. coli* DH5α using a Nucleobond AX plasmid DNA purification kit. The concentrations of both DNA samples were determined (six replicates) by using a NanoDrop spectrophotometer. A 10-fold dilution series (10^0 to 10^{-5}) was set up for each plasmid. Samples were amplified with thermal cycle parameters as specified above, and the threshold cycle (C_t) values were plotted against the number of DNA molecules in each sample. The R^2 value for both calibration standard curves was greater than 0.9995. Absolute plasmid copy number was determined by amplification of pRAS3.1 and pRAS3.2 in the same cycle as the calibration curves. The C_t values were used to extrapolate the total amount of chromosome and plasmid present in each sample from the standard curves, using the LightCycler software (version 3.5) according to the calculations of Lee et al. (22). Relative plasmid copy numbers were determined using the same conditions and cycle parameters as described above. All copy numbers were determined relative to pRAS3.1.35 by using the REST analysis tool (30). The same standard curves that were used for absolute copy number determinations were used to calculate the amplification efficiency.

Plasmid copy numbers in the presence of excess replication proteins which were expressed from the P_{BAD} arabinose-inducible promoter of pBAD28 were measured by means of qPCR relative to the same sample with only pBAD28 in *trans.* Addition of arabinose to Luria-Bertani broth resulted in slow cell growth and proved unnecessary, as *rep* genes cloned behind the P_{BAD} promoter were sufficiently expressed to be able to complement their respective *rep* deletion mutants.

Mobilization assay. *E. coli* S17.1 donor and *E. coli* CSH56 recipient cells were cultured separately overnight with appropriate antibiotic selection. Cells were washed three times in phosphate-buffered saline (PBS, pH 7.4) and mixed in a donor-to-recipient ratio of 1:10 or 1:100. One hundred microliters of this mixture was spotted onto an LA plate and incubated at 37°C for 60 min (unless specified otherwise). The agar plug was excised and suspended in 10 ml PBS (pH 7.4) and vigorously shaken, after which 8 ml was collected, pelleted, and resuspended in 1 ml PBS (pH 7.4). Serial dilutions were plated onto donor and transconjugant selective media, and the number of transconjugants per donor was calculated.

Plasmid stability assay. Stability assays using the pOU82-based test system were performed by growing plasmid-containing *E. coli* cells without selection in 5 ml LB at 30°C for 4 days, with transfer of ~1,000 cells to fresh LB at ~20-generation intervals. Samples taken at ~20-generation intervals were diluted in PBS (pH 7.4) and plated onto LA plates supplemented with 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and incubated at 37°C. The percentage plasmid loss was determined by calculating the ratio of plasmid-containing (blue) to plasmid-free (white) colonies (15).

Incompatibility assays. Plasmid-containing *E. coli* DH5α cells were transformed with a second plasmid and plated on LA plates with antibiotic selection for both plasmids. Single colonies were picked into LB containing appropriate antibiotics and incubated overnight at 30°C. Survival of the plasmids was then tested by removing selection for both plasmids and growing the cells in 5 ml LB for ~100 generations, with transfer of approximately ~1,000 cells to fresh medium at ~20-generation intervals. Finally, 50 colonies were replica plated to antibiotic-containing LA plates to score for plasmid retention. Cells containing individual plasmids were similarly grown and plated as a control to account for spontaneous plasmid loss.

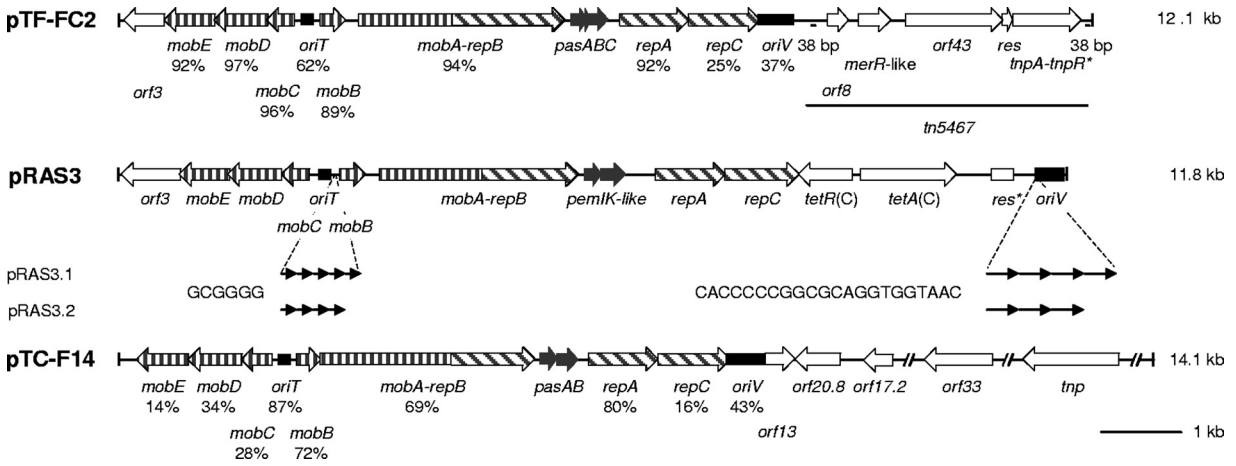

FIG. 1. Comparison of the genetic maps of the pRAS3 plasmids with pTF-FC2 and pTC-F14. Percentages below the plasmid backbone genes of pTF-FC2 and pTC-F14 indicate the percent amino acid sequence identity of the gene product with that of the pRAS3 plasmids. Percentages below the *oriT* and *oriV* regions indicate nucleotide sequence identity. Plasmids pRAS3.1 and pRAS3.2 have different numbers of 6-bp repeats and 22-bp iterons, while the nucleotide sequence of each repeat or iteron is identical, as indicated below pRAS3.

Displacement assays. Competent *E. coli* DH5α cells containing a resident plasmid were transformed with a second incoming plasmid and plated on antibiotic-containing medium that selected only for the incoming plasmid. Sixteen colonies were picked and plated onto three sets of solid media, two containing single antibiotics to separately test for the presence of the resident or incoming plasmid and one containing no antibiotics as a control for cell viability. Controls to check for spontaneous loss of the resident plasmids were carried out using the same procedure except that the initial competent *E. coli* cells containing the resident plasmids were taken through a cycle of growth on solid medium without antibiotic selection before testing for retention of the resident plasmid.

Random knockouts and screening for an incompatibility determinant. Random knockouts of pRAS3.1 were generated using the EZ-Tn*5* transposon system (Epicenter Biotechnologies). The transposon (0.025 pmol) mutagenesis was carried out in vitro using 0.05 pmol pRAS3.1 as per the manufacturer's protocol. The reaction mixture was transformed into electrocompetent *E. coli* EC100D, and all the colonies were scraped off the plates into 100 ml fresh LB medium and incubated for 1 h at 37°C. The plasmid DNA was purified using the Nucleobond AX plasmid purification kit (Macherey-Nagel).

The bank of random knockouts was screened for a compatible phenotype by transforming 1 ng of the plasmid DNA into electrocompetent *E. coli* EC100D cells containing a resident pTF-FC2Cm (pTF-FC2 with Cm^r gene) plasmid. EZ-Tn*5* and pRAS3.1::Tet were used as controls for compatibility and incompatibility, respectively, on double-selective plates. The expression mix was spread on plates containing antibiotic selection for both plasmids, and colonies were replica plated for two rounds of growth on plates selecting for the pRAS3.1 knockouts only. This was followed by selection for cells that still retained pTF-FC2Cm. After restriction analysis of the extracted plasmid DNA, the random knockout plasmids from selected positive colonies were separated from the coresident pTF-FC2Cm plasmid by transforming the extracted DNA into *E. coli* DH5α and selecting for only the pRAS3.1 knockouts that had allowed pTF-FC2Cm to be retained.

Sequence analysis and bioinformatics. The DNA sequence previously deposited as pRAS3.1 (accession number AY043298) and pRAS3.2 (accession number AY043299) by L'Abée-Lund and Sørum (21) was analyzed using a variety of software programs but mainly a combination of the Glimmer 2 (www.tigr.org/softlab) (9) and DNAMAN (Lynnon BioSoft) programs. Comparison searches were performed using the gapped BLAST program at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) (1).

RESULTS

Reanalysis of the sequences of pRAS3.1 and pRAS3.2. An analysis of the sequences of the pRAS3 plasmids was previously reported by L'Abée-Lund and Sørum (21). Given the high similarity in gene organization and sequence with plasmid pTF-FC2, we were surprised by the apparent absence of *mobB* and *repC* genes in the pRAS3 plasmids. When the sequence data were reanalyzed, four genes were identified that were not detected previously. These were the *mobB* and *repC* genes, with the amino acid sequences of their products being 87% and 37% identical to the equivalent gene products of pTF-FC2, respectively. In addition two genes were identified for what appears to be a TA postsegregation killing system. The TA genes are distantly related to the previously published *pemIK* (*parDE*) (5, 38) and *mazEF* (*chpAI chpAK*) (24) systems. A comparison of the pRAS3 plasmids with pTF-FC2 and pTC-F14, the only other two plasmids of the IncQ-2 group identified to date, is shown in Fig. 1. In general, the nucleotide sequences of the backbones of the pRAS3 plasmids are more closely related to pTF-FC2, with the exceptions being the sequences of the *oriT* and *oriV* regions, whereas the pRAS3 plasmids were more closely related to pTC-F14. The putative TA system of the pRAS3 plasmids was unrelated to that of either pTF-FC2 or pTC-F14 (which are closely related to each other [8]). However, in all plasmids the TA system is situated in a similar position between the *repB* and *repA* genes.

Copy numbers of pRAS3.1 and pRAS3.2. The absolute and relative plasmid copy numbers (PCN) of pRAS3.1 and pRAS3.2 were determined in *E. coli* DH5α by quantitative real-time PCR using the chromosomal *gapA* gene as a calibration standard. The copy number of pRAS3.1 was found to be 45 ± 13 ($n = 11$) plasmids per chromosome and that for pRAS3.2 was 30 ± 5 ($n = 4$). This large difference in copy number was surprising, as pRAS3.2 has three *oriV*-associated 22-bp iterons while pRAS3.1 has four of these identical 22-bp iterons, and one would expect the plasmid with fewer iterons to have the higher copy number. Furthermore, other IncQ-2 family plasmids have been reported to have a considerably lower PCN of 12 to 16 plasmids per chromosome (10, 36), which is two- to threefold lower than determined for pRAS3.2 and pRAS3.1, respectively. The PCN of the well-known cloning vector pBR322 has been determined to be ~18 plasmids per chromosome based on both absolute and relative quantifica-

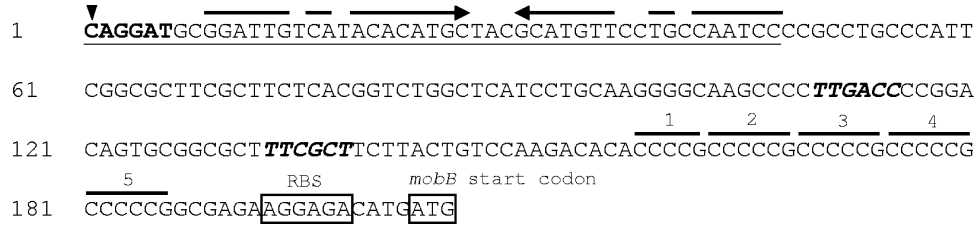

FIG. 2. The intergenic sequence between the *oriT* and *mobB* of pRAS3.1, showing the position of the 6-bp repeats. The *oriT* is underlined and the imperfect inverted repeat within the *oriT* is indicated by broken inverted arrows. The conserved hexameric nick site is indicated in bold with a vertical arrow indicating the putative nick position. The 6-bp CCCCCG repeats are labeled 1 to 5. The first repeat consists of only 5 bp, as it lacks a cytosine base. A putative promoter with a near-consensus −35 region and a weak −10 region is shown in bold italics and is separated by a 17-bp spacer.

tion real-time PCR methods (22). As the tetracycline resistance gene of pBR322 is identical to that of pRAS3.2, the PCN of pBR322 could be quantified using the same primer sets. It was therefore included in an additional set of real-time PCR assays as a copy number control. The relative PCN of pRAS3.1 and pRAS3.2 were 2.3- and 1.7-fold higher than that of pBR322, respectively. Based on a copy number of 18 for pBR322, this is equal to PCN values of approximately 41 for pRAS3.1 and 30 for pRAS3.2.

Reason for the difference in copy number between pRAS3.1 and pRAS3.2. The observation that pRAS3.1 with four 22-bp *oriV*-associated iterons had a higher copy number than pRAS3.2 with three 22-bp iterons was unexpected. We therefore investigated the effect on PCN of the additional 6-bp CCCCCG repeat upstream of the *mobB* gene of pRAS3.1. The repeats are located 6 bp upstream of a putative ribosomal binding site of the *mobB* gene (Fig. 2). Three derivatives of pRAS3.1 (five 6-bp repeats and four 22-bp iterons) were constructed to isolate the effects of the 6-bp repeats and 22-bp iterons on PCN (Table 1). When the number of 22-bp iterons in pRAS3.1 was decreased from four to three (pRAS3.1.35), the calculated PCN increased from approximately 41 to 59 copies (Table 3). The five 6-bp repeats in this construct were then exchanged for those in pRAS3.2 (Table 1) to give a construct still with three 22-bp iterons but now with only four 6-bp repeats (pRAS3.1.34). If the number of 6-bp repeats had an influence on PCN, the PCN of pRAS3.1.34 should fall to that of pRAS3.2, as these two plasmids have equal 6-bp repeat and 22-bp iteron copy numbers. This is what was found, as the relative PCN of pRAS3.1.34 decreased by approximately 47% to 31 copies, the same as that of pRAS3.2. When the number of 22-bp iterons in this lower-copy-number plasmid was increased from three to four (pRAS3.1.44) the PCN decreased further to approximately 23 copies. Therefore, the presence of a fifth 6-bp repeat resulted in the PCN of a plasmid with four 22-bp iterons to increase by 1.77-fold (pRAS3.1.44 compared with pRAS3.1) and a plasmid with three 22-bp iterons increased by 1.89-fold (pRAS3.1.34 compared with pRAS3.1.35). A decrease in the number of 22-bp iterons from four to three also resulted in an increase in PCN. This was 1.45-fold in a plasmid with five 6-bp repeats (pRAS3.1 compared with pRAS3.1.35) and 1.35-fold in a plasmid with four 6-bp repeats (pRAS3.1.44 compared with pRAS3.1.34).

TABLE 3. Effects of the number of 6-bp repeats and 22-bp iterons on plasmid copy number

Plasmid construct	No. of 6-bp repeats	No. of 22-bp iterons	Relative plasmid copy no.[b]	Calculated plasmid copy no.[c]
pRAS3.2	4	3	0.51 ± 0.09	30 ± 5
pRAS3.1	5	4	0.69 ± 0.062	41 ± 4
pRAS3.1.35[a]	5	3	1.0	59
pRAS3.1.34	4	3	0.53 ± 0.002	31 ± 1
pRAS3.1.44	4	4	0.39 ± 0.037	23 ± 2

[a] pRAS3.1.35 served as the reference for the determination of relative copy numbers and standard deviations.
[b] The number of replicates for relative copy number determinations was four to six, and a *P* value of 0.001 was obtained for each qPCR experiment.
[c] Plasmid numbers were calculated to the nearest whole plasmid.

These results suggested that it was the presence of an additional 6-bp repeat upstream of the *mobB* gene that resulted in a higher PCN and raised the question of how the extra 6-bp repeat exerted this effect on PCN. The most obvious possibility was that the 6-bp repeat affected the level of expression of *mobB* as well as the downstream *mobA*/*repB* genes (Fig. 2). To test this, the levels of expression of *mobB*, *mobA*/*repB*, and the divergently transcribed *mobCDE* and *orf3* operon were determined for pRAS3.1 and compared with pRAS3.2 by using qPCR. To carry out the comparison, an R6K *oriV* and kanamycin resistance gene (EZ-Tn*5*) were cloned into the *tetAR* genes of both pRAS3.1 and pRAS3.2, whereafter the native replicons were inactivated through truncation of the *repC* and *tetR* genes. This ensured that both plasmids had the same copy number and allowed the relative levels of gene expression to be determined. The expression of *mobA*/*repB* in the case of a pRAS3.1 equivalent (five 6-bp repeats; pR6K.3.1.repC$^{\Delta}$) was approximately twofold higher (2.0 ± 0.9; $n = 12$; $P = 0.048$) relative to a pRAS3.2 equivalent (four 6-bp repeats; pR6K.3.2.repC$^{\Delta}$). In contrast, expression in the opposite direction (*mobCDE-orf3*) was the same (1.1 ± 0.5; $n = 4$; $P = 0.875$) for both pRAS3.1 and pRAS3.2 equivalents. These results suggested that the reason for the increase in PCN of pRAS3.1 compared to pRAS3.2 was that the additional 6-bp repeat resulted in an increase in *mobB-mobA*/*repB* expression and that increased expression of *repB* was the actual cause.

Effect of increased *repBAC* expression on plasmid copy number. To confirm that the additional *repB* expression resulted in an increased PCN as well as to determine whether the products of the *repA* and *repC* genes affected PCN, the *repC*, *repAC*, and *repB* genes were cloned behind the P_{BAD} promoter of the vector pBAD28. The arabinose-inducible *repB* construct was

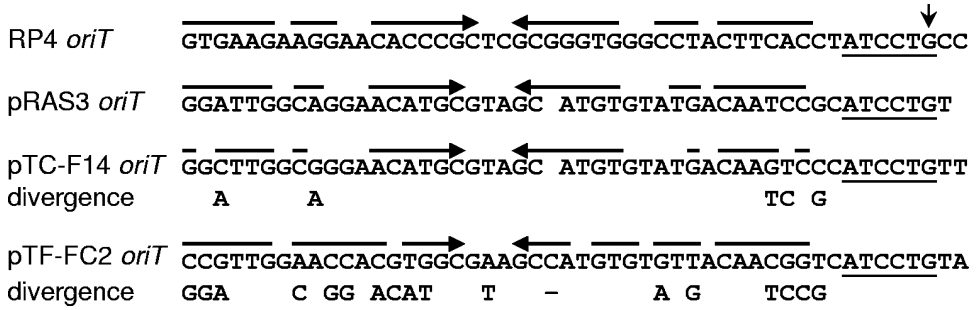

FIG. 3. Alignment of *oriT* regions of IncQ-2 plasmids and the IncPα plasmid RP4, showing the sequence divergence that could be tolerated by the Mob proteins of plasmid pRAS3 while they were still able to mobilize DNA from an *oriT*. A vertical arrow indicates the relaxase *nic* site at which single-stranded cleavage takes place as determined for plasmid RK2/RP4 (29).

placed in *E. coli*(pRAS3.1.34) cells and the copy number was compared with the same cells containing the pBAD28 vector only. With the *repB*-containing construct in *trans*, the PCN of pRAS3.1.34 was increased approximately 2.2-fold ± 0.53-fold ($n = 4$; $P = 0.001$) relative to the vector control. This is approximately equal to the 1.96-fold difference in PCN between plasmids differing only in their number of 6-bp repeats, such as pRAS3.2 (three iterons; four 6-bp repeats) and pRAS3.1.35 (three iterons; five 6-bp repeats), as shown in Table 3. Therefore, the additional *repB* expression raised the copy number of a plasmid which contained four 6-bp repeats to approximately the same as that of a plasmid that had five 6-bp repeats.

To examine the effects of additional *repC* or *repAC* gene products, plasmid pRAS3.1.35 was used, as this plasmid with five 6-bp repeats already had high levels of *repB* expression and it was possible that the *repC* or *repAC* gene products were limiting. Expression of *repC* in *trans* did not result in a change in PCN (1.02 ± 0.22; $n = 4$; $P = 0.874$), whereas expression of *repAC* (pBAD28-repAC) resulted in an approximately 30% reduction in PCN (0.67 ± 0.13; $n = 4$; $P = 0.008$). This reduction in PCN due to overexpression of *repAC* was consistent with that reported by Matcher and Rawlings (25).

Comparison of the mobilization frequencies of pRAS3.1 and pRAS3.2. As the mobilization frequencies of pRAS3.1 and pRAS3.2 were not reported in the study by L'Abée-Lund and Sørum (21), we determined the mobilization frequency between *E. coli* S17.1 donor and *E. coli* CSH56 recipient cells. This was found to be 0.032 ± 0.014 for pRAS3.1 and 0.021 ± 0.013 for pRAS3.2. Experiments using plasmids with different numbers of 6-bp repeats but identical 22-bp iteron numbers, as well as plasmids with identical 6-bp repeats but different 22-bp iteron numbers, showed that neither had any significant effect on the mobilization frequencies. Thus, although increased transcription in the direction *mobB-mobA/repB* compared with *mobCDEorf3* as a result of the extra 6-bp repeat had an effect on plasmid replication, it did not have a marked effect on plasmid mobilization.

Functional relatedness of mobilization regions within the IncQ-2 plasmids. Prior to plasmid conjugation a relaxase cleaves one of the DNA strands at the origin of transfer (*oriT*), forming a covalent protein-DNA complex in the donor cell that is transferred to the recipient. In the case of plasmid F, the relaxase-*oriT* recognition has a high degree of structural and sequence specificity (12, 26). In contrast, the *oriT* region of the IncQ-1 plasmid R1162 is small, structurally simple, and can accommodate base pair changes without a complete loss of function (3, 20, 27). Since the five-protein mobilization operon of the IncQ-2 plasmids has more in common with the IncP plasmids than with the three mobilization protein operons of IncQ-1 plasmids, we tested whether the relaxase-*oriT* recognition of the pRAS3 plasmids was relaxed like that of R1162 or specific like plasmid F and related plasmids. The amino acid sequences of the *mob* gene products of the pRAS3 plasmids were closely related to that of pTF-FC2, with an average amino acid sequence identity of over 90% for the five *mob* gene products (Fig. 1). This contrasted with pTC-F14, where the average amino acid sequence identity was approximately 25% for the MobCDE genes and 70% for the MobAB genes. However, when the nucleotide sequences of the *oriT* regions were compared (Fig. 3), the *oriT* of the pRAS3 plasmids was considerably more related to that of pTC-F14 (87% identity) than to pTF-FC2 (62% identity). We therefore tested whether the *oriT* of the pRAS3 plasmids was able to be mobilized by *E. coli* S17.1 cells containing the conjugative plasmid RP4 by the products of the *mob* genes of pTF-FC2 and pTC-F14. A 196-bp fragment from pRAS3.1 containing the *oriT* was PCR amplified and cloned into the nonmobilizable pUC19 vector to produce pOriT-RAS3, and the sequence was confirmed by DNA sequencing. Plasmid pOriT-RAS3 was mobilized at the saturation frequency within 60 min of mating by *E. coli* S17.1 when either pRAS3.1 or pRAS3.2 was coresident, while no transconjugants were obtained in the absence of coresident pRAS3 plasmids. When either pTF-FC2Cm or pTC-F14Cm was coresident instead of the pRAS3 plasmids, no transconjugants were obtained. However, in the reverse experiments, coresident pRAS3.1 plasmid was able to mobilize pUC19 vector containing the pTF-FC2 and pTC-F14 *oriT*s at frequencies of $1.20\ (\pm 0.44) \times 10^{-1}$ and $3.42\ (\pm 1.64) \times 10^{-4}$ transconjugants per donor in 60 min, respectively. The ability of Mob proteins of pRAS3 to mobilize the *oriT*s pTF-FC2 and pTC-F14 suggested that like R1162 these *oriT*s are also relaxed and that the Mob proteins pRAS3 can mobilize *oriT*s with very different sequences.

The toxin-antitoxin system on pRAS3 is functional. Careful analysis of the sequence of pRAS3.1 and pRAS3.2 revealed the presence of two small tandem ORFs encoding proteins of 74 and 108 amino acids (aa), respectively, situated between *repB*

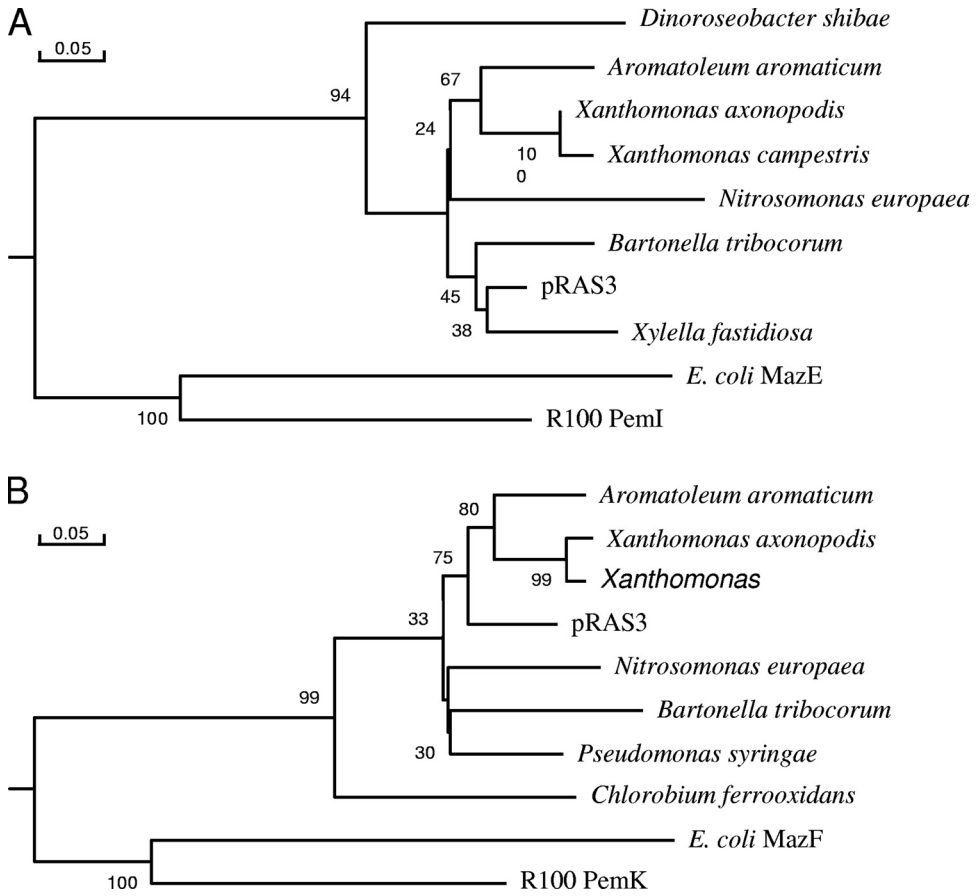

FIG. 4. Phylogenies of toxin-antitoxin proteins of pRAS3 and comparison with closely related proteins as well as the more distantly related PemIK and MazEF proteins. (A) Antitoxins were as follows: *Aromatoleum aromatium*, CAI08016; *Bartonella tribocorum*, CAK00897; *Dinoroseobacter shibae*, YP_001541878; *Nitrosomonas europaea* ATCC 19718, CAD85218; *Xanthomonas axonopolis* pv. *citri strain* 306, NP_644761; *Xanthomonas campestris* pv. *vesicatoria* strain 85-10, CAJ19793; *Xylella fastidiosa* Ann-1, ZP_00682677; *E. coli* MG1165 MazE, AAA69293; plasmid R100 PemI, P13975. (B) Toxins were as follows: *Aromatoleum aromatium*, CAI08015; *Bartonella tribocorum*, CAK00896; *Chlorobium ferrooxidans*, EAT59633; *Nitrosomonas europaea* ATCC 19718, CAD85217; *Pseudomonas syringae* pv. *phaseolicola*, AAZ37969; *Xanthomonas axonopolis* pv. *citri* strain 306, NP_644760; *Xanthomonas campestris* pv. *vesicatoria* strain 85-10, CAJ19792; *E. coli* MG1165 MazF, AAA69292; plasmid R100 PemK, P13976.

and *repA*. BLAST analysis of the proteins from these ORFs indicated that they were most closely related to pairs of proteins of similar size from adjacent ORFs detected in genome sequencing data obtained from a number of other bacteria, such as *Xanthomonas campestris*, *Xanthomonas axonopodis*, *Aeromatoleum aromaticum*, and *Nitrosomonas europaea* (Fig. 4A and B). These proteins are related to toxin-antitoxin post-segregational killing plasmid stability systems and are listed as being either PemIK-like or MazEF-like, although they group in a cluster well separated from either of these protein pairs. No members of the clusters shown in Fig. 4A and B, besides the distantly related PemIK and MazEF, have been tested for toxin-antitoxin activity. Using PCR we amplified the two ORFs from pRAS3.1 and cloned them into the segregationally unstable test plasmid pOU82, to give plasmid pOU82-TA. When grown without plasmid selection for 72 generations, approximately 98% of *E. coli* DH5α cells retained pOU82-TA, whereas only 35% of cells retained pOU82 (Fig. 5). This enhanced plasmid stability suggests that the other untested pro-

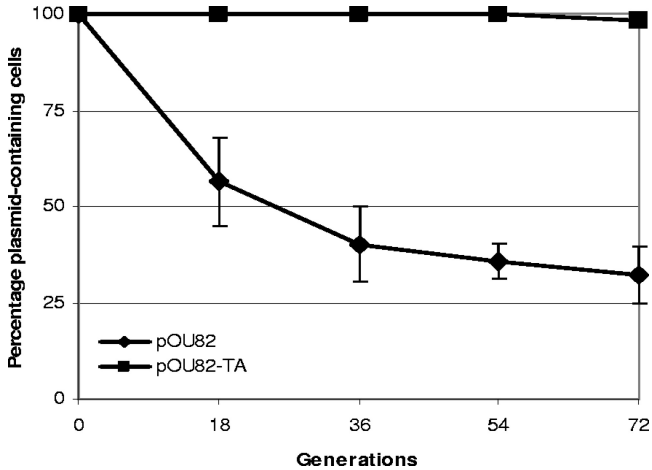

FIG. 5. Loss of the low-copy-number test plasmid, pOU82, with and without the PemIK-like TA genes from pRAS3 in the absence of plasmid selection.

teins shown in Fig. 4A and B are also likely to be toxin-antitoxin pairs.

Compatibility of pRAS3.1 and pRAS3.2 with other IncQ plasmids. We tested whether plasmids pRAS3.1 and pRAS3.2 were compatible with different IncQ-1 and IncQ-2 plasmids. Plasmids RSF1010 (16), pIE1108 (35), and pIE1130 (37) were used as representatives of the α, β, and γ incompatibility groups of InQ-1 plasmids, respectively. The incompatibility assay involved the placement of the two test plasmids into a single *E. coli* DH5α host cell and then testing for how many cells retained both plasmids after approximately 34 generations in the absence of selection. When present on their own, plasmids RSF1010K, pIE1108Cm, and pIE1130 were slightly less stable after 34 generations (94 to 100% retention) than pRAS3.1 or pRAS3.2 (100% retention). When either pRAS3.1 or pRAS3.2 was coresident with plasmid RSF1010K, pIE1108Cm, or pIE1130, the stability of each plasmid was indistinguishable from that when it was present in the *E. coli* host on its own.

When testing for the compatibility of pRAS3.1 and pRAS3.2 with pTF-FC2 and pTC-F14, members of the IncQ-2 α and β incompatibility groups, respectively, a different compatibility assay had to be used. The pRAS3.1 and pRAS3.2 plasmids were so highly incompatible with either pTF-FC2Cm or pTC-F14Cm that we were unable to isolate *E. coli* host cells containing two test plasmids. When attempts were made to transform competent *E. coli* pRAS3.1- or pRAS3.2-containing cells with either pTF-FC2Cm or pTC-F14Cm, no transformants were isolated, although the competent cells could be readily transformed with other plasmids, such as pUC19 and pACYC177. In the reciprocal experiments with either pTF-FC2Cm or pTC-F14Cm being resident in the competent cells prior to transformation with either pRAS3.1 or pRAS3.2, successful transformation by the incoming plasmid was achieved but the resident plasmid was immediately displaced.

Search for the source of strong plasmid incompatibility. Experiments were carried out in an attempt to identify the reason for this strong incompatibility. A functional 751-bp pRAS3.1 *oriV* region containing the four 22-bp iteron DNA was cloned into the *E. coli* pGEM-T vector to give pGEM-OriV3.1. However, this construct was fully compatible with either the pTF-FC2 or pTC-F14 replicons and therefore the iterons and associated DNA were not the cause of the strong incompatibility. The pRAS3.1 *repC*, *repAC*, and *repB* genes were cloned behind the P_{BAD} promoter of the pBAD28 expression vector and shown to be expressed by their ability to complement pRAS3.1 *repC* or *repB* mutants. Again, none of these clones displaced pTF-FC2 or pTC-F14. Next, the region containing the *repBAC* genes, including the genes for the *pemIK*-like TA system, was cloned behind the P_{BAD} promoterto give pBAD28-repBAC. This plasmid could support the replication of the 751-bp pRAS3.1 *oriV* region when cloned into a R6K vector (EZ-Tn*5*), which was unable to replicate in *E. coli* DH5α unless pBAD28-repBAC was present in *trans*. This showed that the *repBAC* fragment was functional, but this fragment also did not displace the pTF-FC2 or pTC-F14 replicons.

As plasmid incompatibility did not appear to be associated with the replicon region, we tested whether we could knock out whatever was responsible for incompatibility using the EZ-Tn*5* transposon mutagenesis system. A bank of random mutants of pRAS3.1 was generated and screened for mutants that did not displace *E. coli* containing a resident pTF-FC2Cm plasmid. Approximately 5% of mutants displayed a compatible phenotype by selection on plates for both plasmids. All 200 compatible pRAS3.1 mutant plasmids tested retained pTF-FC2Cm after two rounds of growth from a single cell to a colony when selecting for only the pRAS3.1 mutants. Restriction endonuclease analysis using BamHI and SalI for 48 of the 200 mutants indicated that the mutations fell into five groups, with all transposons located within a 1.75-kb region. Nucleotide sequencing of a representative of each group indicated that the five insertions were evenly spaced within the region containing the *mobCDE* genes and a previously unreported downstream ORF (called *orf3*), with one insertion in each gene and two in *mobE*. To determine whether *orf3* is expressed from the same transcript as *mobCDE*, mRNA was isolated from *E. coli* DH5α(pRAS3.1) cells and analyzed by RT-PCR. Primer sets to *mobC* and *orf3* or to *mobE* and *orf3* gave positive amplification products of the predicted sizes, while a lack of amplification products in experiments in which reverse transcriptase was omitted indicated that amplification products were not due to DNA contamination (data not shown). We concluded that *orf3* is expressed as part of the *mobCDE* operon. Since *orf3* is the last gene in the series of four genes, we predicted that insertion into the upstream *mob* genes presumably affected expression of *orf3* and that it was *orf3* that was probably responsible for plasmid incompatibility.

Role of *orf3*. *orf3* is 753 bp long and encodes a predicted protein of 250 aa that is preceded by a putative ribosome-binding site (GGAGG) 5 bp upstream of the ATG start. A BLAST analysis against the nonredundant NCBI protein database indicated two strong hits of 98 and 97% amino acid identity along the entire length of a hypothetical protein (240 aa) from an uncultured bacterium and to ORFX (163 aa) of plasmid Rms149 from *Pseudomonas aeruginosa* (18). Rms149 is a 57-kb IncP-6 plasmid that has a different replicon from IncQ plasmids but a 5.6-kb mobilization region that is very similar to that of the pRAS3 plasmids and pTF-FC2. Like the IncQ-2 plasmids this mobilization region includes a *mobB* followed by a *mobA*/*repB* gene fusion and the divergent *mobCDE* genes and has between 86 and 100% amino acid identity to the corresponding gene products of pRAS3 and pTF-FC2. The *orfX* of Rms149 is in the same location as *orf3*, immediately downstream of *mobE*, but appears to have been truncated by the insertion of Tn*1012*. Plasmid pTF-FC2 has an *orf4* encoding a 270-aa product in a similar location to *orf3* and *orfX*, but this ORF is unrelated in nucleotide or predicted amino acid sequence.

Despite the Tn*1012* insertion in *orfX*, the 5.6-kb mobilization region of Rms149 remained functional (18), but without an untruncated *orfX* the effect of *orfX* on mobilization frequency could not be tested. We therefore tested whether the insertion of EZ-Tn*5* into *orf3* of pRAS3.1, which was used to indentify *orf3* as the cause of incompatibility, affected the mobilization frequency. Construct pRAS3.1::tet was mobilized at a frequency of 12.44 ± 3.95 transconjugants per donor, while pRAS3::orf3 was mobilized twofold lower, at 6.04 ± 4.33 transconjugants per donor. We concluded that like *mobD* and *mobE*, *orf3* plays a minor role in mobilization frequency. To

determine whether the product of *orf3* was responsible for the strong plasmid incompatibility, *mobDE-orf3* and *orf3* were cloned behind the P_{BAD} promoter. The genes were shown to be expressed by using RT-PCR and primers specific to the *mobE* and *orf3* genes. However, when these constructs were placed in *trans* with pTF-FC2Tet (or pTC-F14Km) they did not cause plasmid incompatibility. Plasmid pTF-FC2Tet was, however, displaced when the entire operon (pBAD28-mobCDE-orf3) was placed in *trans*, and the reason for strong incompatibility requires further investigation.

DISCUSSION

The low nucleotide sequence identity between the *repC* genes of the pRAS3 plasmids with that of pTF-FC2 and pTC-F14, the other members of the IncQ-2 plasmid group, was unexpected. The iteron-binding protein, RepC, is essential for IncQ plasmid replication and is generally the most conserved Rep protein within the IncQ plasmid family (31). The other genes of the plasmid backbone (*mobEDCBA* and *repBA*) of pRAS3 and pTF-FC2 are highly conserved (Fig. 1), and it is unlikely that differences in the *repC* genes are due to a higher mutation rate. It is more likely that the *repC* gene of the pRAS3 plasmids has been acquired by gene swapping with some as-yet-unidentified IncQ-like plasmid that is different from any so far discovered.

In several other plasmids the total number of *oriV*-associated iterons in a cell has been shown to affect plasmid copy number (7, 23, 39), and our expectation was that pRAS3.1 with four 22-bp *oriV*-associated iterons would have a lower copy number than pRAS3.2, with three 22-bp iterons. Unexpectedly, we found that pRAS3.1 had a copy number considerably greater than pRAS3.2 despite of it having more iterons. This increase in copy number was due to the number of 6-bp repeats in the intergenic region between the *mobCBE-orf3* and the *mobB-mobA/repB* operons, with the higher-copy-number pRAS3.1 plasmid having five 6-bp repeats while plasmid pRAS3.2 had four 6-bp repeats. The additional 6-bp repeat resulted in an increase in expression of the *mobB-mobA/repB* operon and it was the increase in expression of the RepB primase that resulted in a higher plasmid copy number. The altered promoter could either increase the efficiency of initiation of *mobB-mobA/repB* transcription or decrease autorepression by RepB (14). Swapping the region with the repeats between pRAS3.1 and pRAS3.2 resulted in a corresponding change in copy number. We further demonstrated that the level of transcription of the *mobB-mobA/repB* operon was increased approximately twofold in the presence of five 6-bp repeats. An increase in PCN as a result of increased *repB* expression was confirmed by placement of a *repB* gene in *trans* under the control of an arabinose-inducible promoter. This resulted in a 2.2-fold increase in copy number of a plasmid containing four 6-bp repeats to approximately the level of a plasmid containing five 6-bp repeats. This provided strong evidence that the copy number of pRAS3.1 was affected by both the number of 22-bp *oriV*-associated iterons and the level of transcription of the *repB* gene.

The 30% reduction in pRAS3 copy number that occurred on overexpression of *repAC* from a P_{BAD} vector promoter is different from the result obtained by Haring et al. (19), who reported that the copy number of the IncQ-1 plasmid, RSF1010, was increased sixfold upon overexpression of *repAC*. However, the small reduction in copy number of pRAS3 is similar to the reduction in copy number that occurred on overexpression of *repAC* in the case of the related plasmid, pTF-FC2 (25). We observed that when *repAC* from either pRAS3 or pTF-FC2 was expressed from the P_{BAD} promoter, the *E. coli* host cells were slow growing and clearly stressed. Whether this might have affected the copy number is unknown.

Demonstration that the pRAS3 plasmids contain a TA system means that all of the IncQ-2 plasmids so far discovered contain a toxin-antitoxin plasmid stability module situated within the replicon in exactly the same position between the *repB* and *repA* genes. However, although the *pas* toxin-antitoxin systems of pTF-FC2 and pTC-F14 are related to each other and are deep-branching members of the *E. coli relBE* family, they are totally unrelated to the toxin-antitoxin system of the pRAS3 plasmids. As the *repB* and *repA* genes of pRAS3 plasmids are highly related to pTF-FC2 and pTC-F14 and flank the unrelated TA modules (Fig. 1), this implies that the TA modules of the pRAS3 plasmids were acquired independently of pTF-FC2 and pTC-F14. The observation that IncQ-2 plasmid replicons have acquired different TA systems located in the same position suggests that there may be a biological reason for their occurrence in that exact position. Matcher and Rawlings (25) have presented evidence that a strong, autoregulated promoter such as that provided by a TA system within the replicon of pTF-FC2 confers on the plasmid the ability to rapidly respond to a fall in copy number. This is because a transient burst of expression of the TA genes results in a related increase in expression of the downstream *repAC* genes. IncQ plasmids appear not to have an active partitioning system, and a fall in copy number might occur on cell division when one daughter cell could receive many more copies of a plasmid than the other. Similarly, a strongly expressed, autoregulated TA system would allow the rapid expression of *repAC* on arrival in a recipient cell following conjugation.

To date plasmids pRAS3.1, pRAS3.2, pTF-FC2, and pTC-F14 are the only reported representatives of the IncQ-2 plasmids. Previous work demonstrated that pTF-FC2 and pTC-F14 are fully compatible and therefore were placed in the IncQ-2 incompatibility groups α and β, respectively. When we tested the incompatibility of pRAS3.1 and pRAS3.2 against representatives of the IncQ-1 α, β, and γ incompatibility groups, they were fully compatible but were violently incompatible with other members of the IncQ-2 plasmids. Plasmid incompatibility is understood as being the inability of two plasmids to coexist in the same host in the absence of selective pressure and implies that they belong to the same incompatibility group (28). This is clearly not the case with pRAS3 plasmids and pTC-FC2 and pTC-F14, as the strong incompatibility observed would imply that they belong to the same incompatibility group. Strong incompatibility appeared to be due to a phenomenon associated with *orf3* that is still not fully understood. If, however, the region containing *orf3* was interrupted, then the replicon of pRAS3.1 was compatible with pTF-FC2 and pTC-F14. This suggests that the pRAS3 plasmids form a third, γ plasmid incompatibility grouping within the IncQ-2 plasmids. Support for this proposal is that the nucleotide sequence of the 22-bp *oriV*-associated iterons and the protein sequences of the

RepC iteron-binding proteins of the pRAS3 plasmids are very different from pTF-FC2 or pTC-F14.

In previous work, plasmid pTF-FC2 was mobilized at saturation frequency by the RP4 conjugative plasmid that had been integrated into the chromosome of *E. coli* S17.1, while pTC-F14 was mobilized at a frequency 3,500-fold lower (40). This difference in mobilization frequencies was due to differences in the MobD and MobE proteins, because when the genes for these proteins from pTF-FC2 were provided in *trans*, the mobilization frequency of pTC-F14 was raised to near saturation levels. The hypothesis for the differences in mobilization frequency was that pTF-FC2 and pTC-F14 had been adapted for efficient mobilization by two different conjugative plasmids and that the very different amino acid sequences of *mobCDE* gene products between the two plasmids were responsible for this. The pRAS3 plasmids have *mobCDE* genes that are closely related to those of pTF-FC2 (Fig. 1) and appeared to be more efficiently mobilized by RP4 than even pTF-FC2 was. Mating of the pRAS3 plasmids was so efficient that the mating time had to be shortened from 60 to 30 min and the donor-to-recipient ratio decreased from 1:10 to 1:100 to prevent mating from reaching saturation so that the mating frequency could be calculated. The finding that the pRAS3 plasmids could mobilize the *oriT*s of pTF-FC2 and pTC-F14 even though they have different sequences from the pRAS3 plasmids (Fig. 3) is interesting in the context of the work of Meyer (27). He showed that the IncQ-1 plasmid R1162 can initiate transfer from a 19-bp locus that is partly degenerate in sequence and that such sites are likely to occur by chance in a bacterial chromosome. R1162-dependent transfer of chromosomal DNA from such a potential *oriT* was demonstrated, and it was pointed out that this might indicate a previously unrecognized potential for the exchange of bacterial DNA. The relaxed nature of the relaxase and *oriT* interaction of the pRAS3 plasmids suggests that these plasmids also have the potential to mobilize chromosomal DNA from cryptic *oriT*-like sequences. The observations that the nucleotide sequence of the *oriT* of the pRAS3 plasmids is 25% more identical to pTC-F14 but that the pRAS3 plasmids do not complement the mobilization of pTC-F14 as well as pTF-FC2 suggest that conservation of specific base pairs within the *oriT* probably affects the mobilization frequency more than the general level of *oriT* sequence identity.

IncQ family plasmids are highly promiscuous, and related plasmids have been found in very different environments. An illustration of this was obtained while searching for genes with homology to those present on the pRAS3 plasmids. A genomic island containing tetracycline resistance genes has been isolated from seven isolates of the obligate intracellular human and animal pathogen *Chlamydia suis* (11). The tetracycline resistance genes were associated with a plasmid that is almost identical to pRAS3.2. Approximately 10.1 kb of plasmid-like DNA is present, the nucleotide sequence of which is 99% identical to pRAS3.2 and contains large stretches of perfect identity. The biggest difference is that a 1.7-kb fragment containing the *mobA*/*repB* gene fusion extending 64 bp into the antitoxin gene is missing. Although the toxin gene remains, this is not likely to be expressed, as its promoter lies upstream of the antitoxin gene and within the missing region. There is an 8-bp deletion in the region of the operator of the divergent *tetA* and *tetR* genes as well as a deletion in the *oriV* region, such that only one 22-bp iteron remains. The presence of pRAS3-like plasmids in an obligately intracellular parasite such as *Chlamydia suis* illustrates the remarkable promiscuity of IncQ family plasmids and their ability to participate in the horizontal gene pool.

We began this work as part of a study aimed at answering the question of why two versions of what are two highly similar plasmids existed. Although this question has not yet been fully addressed, we have established that the effect of the additional 6 bp upstream of the *mobB-mobA*/*repB* operon in pRAS3.1 was to raise plasmid copy number while the effect of the additional 22-bp *oriV*-associated iteron was to partly reduce the copy number (though this reduction was not down to the level of pRAS3.2). During evolution, plasmid variants would be expected to compete for space in the cytoplasm of the host but in such a way that they do not place an evolutionary significant additional metabolic burden on their host cells. For plasmids that do not have an active partitioning mechanism (such as the pRAS3 plasmids), one would predict that the ideal plasmid copy number would be a compromise between a copy number high enough to minimize plasmid loss that might occur on host cell division but not one so high that the plasmid-associated metabolic burden makes the host noncompetitive. In addition, there may also be selection for a plasmid to evolve in such a way that it is not easily displaced by its sister variant. Future studies will include the construction of a number of plasmid variants that will enable us to address this question.

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