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Multiresistance in *Pasteurella multocida* Is Mediated by Coexistence of Small Plasmids▽

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In most gram-negative bacteria, acquired multiresistance is conferred by large plasmids compiling numerous antimicrobial resistance genes. Here, we show an evolutionary alternative strategy used by *Pasteurella multocida* to become resistant to multiple clinically relevant antibiotics. Thirteen β-lactam-resistant clinical isolates, concomitantly resistant to tetracyclines and/or streptomycin as well as to sulfonamides, were studied. Pulsed-field gel electrophoresis analysis revealed different profiles among the isolates, showing that clonal dissemination was not the sole event responsible for the spread of multiresistance. Each *P. multocida* strain carried two or three small plasmids between 4 and 6 kb in size. A direct association between resistance profile and plasmid content was found. Complete nucleotide sequencing of all plasmids revealed seven different replicons, six of them belonging to the ColE1 superfamily. All plasmids carried one, or a maximum of two, antimicrobial resistance determinants. Plasmids pB1000 and pB1002 bore bla_{ROB-1}, pB1001 carried *tet*(B), pB1003 and pB1005 carried *sul2* and *strA*, pB1006 harbored *tet*(O), and p9956 bore the *tet*(H) gene. All plasmids except pB1002 and pB1006 were successfully transformed into *Escherichia coli*. pB1000, also involved in β-lactam resistance in *Haemophilus parasuis* (A. San Millan et al., Antimicrob. Agents Chemother. 51:2260–2264, 2007), was mobilized in *E. coli* using the conjugation machinery of an IncP plasmid. Stability experiments proved that pB1000 was stable in *P. multocida* but highly unstable in *E. coli*. In conclusion, bla_{ROB-1} is responsible for β-lactam resistance in *P. multocida* in Spain. Coexistence and the spread of small plasmids are used by *P. multocida* to become multiresistant.

Pasteurella multocida is a worldwide-distributed pathogen responsible for a broad range of diseases in animals (14). In humans, this pathogen produces pneumonia, meningitis, urinary tract infections, sepsis, and peritonitis (13, 23). Transmission usually occurs through animal bites, scratches, and licks or is due to close contact with animals or animal products (13, 27). In recent years, there has been an increase in the number of reports of *P. multocida* human infections, associated mainly with immunocompromised patients (13, 23).

Human infections are treated primarily with penicillin, ampicillin, and cephalosporins (27). Although the frequency of resistance to β-lactams remains low (22), β-lactamase-producing strains have been described previously in this species (16, 23, 31). ROB-1 is the most frequent enzyme conferring β-lactam resistance, whereas TEM-1 has been reported from a human isolate in France (23) More recently, BlaP1 (Pse-1/CARB-2), conferring resistance to ampicillin and carbenicillin, has been described in a *P. multocida* strain of avian origin in Taiwan (31). Animal infections are commonly treated with tetracyclines. In Spain, a recent study showed a shift from 1.6% to 14.4% in oxytetracycline resistance between 1987 and 2004 in *P. multocida* strains isolated from pigs (22).

Antimicrobial resistance in *P. multocida* has been related to small, nonconjugative plasmids encoding determinants conferring resistance to ampicillin (24), tetracycline (18, 19), streptomycin (30, 31), or florfenicol (20). Several replicons harboring one or a maximum of two antimicrobial resistance genes have been completely sequenced (3, 20, 31). So far, nothing is known about the possibility of coexistence of multiple antimicrobial resistance plasmids in *P. multocida*. Here, we characterize the molecular basis of β-lactam resistance and multiresistance in *P. multocida* isolates recovered from diseased pigs in Spain between 2002 and 2005.

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MATERIALS AND METHODS

Bacterial strains, culture conditions, and identification of *P. multocida*. *P. multocida* ATCC 43137 served as the reference strain for pulsed-field gel electrophoresis (PFGE) and antimicrobial susceptibility analysis. All 13 strains characterized in this study were isolated in the Centro de Vigilancia Sanitaria Veterinaria laboratory (9) from diseased pigs belonging to five different farms in different geographic areas within Spain. Bacteria were cultured on Columbia agar plus 5% sheep blood plates (bioMérieux, France) and in brain heart infusion (BHI) broth at 37°C for 24 h. NovaBlue Singles *Escherichia coli* competent cells (Novagen, Merck Chemicals Ltd., United Kingdom) and $CaCl_2$-treated *E. coli* DH5α competent cells were used in transformation of the plasmids. *E. coli*

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TABLE 1. Primers used in this study

Primer	Sequence (5′→3′)	Amplicon size(s) (bp)	Plasmid(s)	Reference
MAP-1	GCTCTCTAATTCTTTCGATAA	1,087	pB1000/pB1002	25
MAP-2	TTTTGAAGAAAGCGACCTACC			25
MAP-3	TTCTGTGATGTCTGCTGAAAG	2,393	pB1000	25
rob-1D[a]	AATTGGTTGGACAATAACGCA			25
MAP-4	TAAAGCATTGGTATTAAAGGC	845	pB1000/pB1002	25
rob-1F	TGTTGCAATCGCTGCC			25
rob-1U[a]	ATCGTCATGCCTTTGCCAACG	1,602	pB1000/pB1002	25
MAP-5	GATTTTATCAACTCAACGTGG			25
MAP-6	CTACGCCTGTTTATTCTTCC	1,784 (rob-1D)	pB1002	This work
MAP-7	GCGAAGCGTCCCTACTACCAA	1,790 (MAP-3)	pB1002	This work
tet(B)invF	GGTTAGTTTTCCCTGTTTTA	4,500	pB1001	This work
tet(B)invR	ACCAACCGAACCACTTCACG			This work
tet(H)invF	GGGTCATCTTACCAGCATTA	5,166	p9956	This work
tet(H)invR	AGAAACCAAAATAGCGAGTT			This work
tet(O)invF	CATTGCATTTGAGGGATGTTA	5,500	pB1006	This work
tet(O)invR	AACAGTATACGGGTCTGTGCC			This work
strAinvF	GGTCTGATCGACCTTGGGCGG	4,680/3,860	pB1003/pB1005	This work
strAinvR	TGTTCCTCCTGCCAGTTGATC			This work
KMT1T7	ATCCGCTATTTACCCAGTGG	456		29
KMT1SP6	GCTGTAAACGAACTCGCCAC			29

[a] Primers rob-1D and rob-1U are used for the bla_{ROB-1} inverted PCR.

strain K-12, resistant to kanamycin and dibekacin and containing a chromosomal copy of the conjugation machinery from an IncP plasmid (4), was used for mobilization of pB1000. *E. coli* strain K802N was used as the recipient strain in mating experiments. Identification of *P. multocida* isolates was confirmed using species-specific PCR with primers KMT1T7 and KMT1SP6 (Table 1), as previously described (29).

Susceptibility testing. Antimicrobial susceptibility was determined by disk diffusion and microdilution methods by following CLSI guidelines (7). Commercially prepared, dehydrated Sensititre panels were used for MIC determination. Quality control of the panels was performed with collection strains ATCC 25922 (*E. coli*), ATCC 29213 (*Staphylococcus aureus*), and ATCC 29212 (*Enterococcus faecalis*).

DNA analysis and manipulation. Plasmid DNA extraction and purification of PCR fragments were performed using the plasmid midikit, QIAprep spin miniprep kit, Qiagen PCR purification kit, or gel extraction kit (Qiagen Inc., Chatsworth, CA). PCR and long-range PCR were performed using *Taq* polymerase and Certamp enzyme mix from Biotools (B & M Labs, Spain). Identification of pB1000 in resistant *P. multocida* strains was performed using PCR mapping by means of four PCRs with primers, as follows: MAP-1 and MAP-2 (PCR 1), MAP-3 and rob-1D (PCR 2), MAP-4 and rob-1F (PCR 3), and rob-1U and MAP-5 (PCR 4). For pB1002, two extra primers, MAP-6 and MAP-7, were used for mapping (Table 1). All primers used to perform inverted PCR of plasmids in this study are listed in Table 1. Plasmids were transformed into *E. coli* by heat shock at 42°C. Digestion with S1 nuclease (Promega, Madison, WI) was performed by following the manufacturer's instructions. DNA Strider 1.4f13 (CEA, France), 4Peaks 1.6 (Mek&Tosj, The Netherlands), and NIH online analysis tools (http://www.ncbi.nlm.nih.gov) were used for sequence analysis.

Conjugation experiments were carried out in duplicate in BHI broth, with *E. coli* K-12 transformed with pB1000 as the donor. The strains were grown until the donor reached an optical density at 600 nm of 0.9. Then, 1 ml of the donor culture, 1 ml of the recipient culture, and 1 ml of fresh BHI broth were mixed in and incubated for 2 to 3 h at 37°C without shaking. Transconjugants were selected on BHI agar plates containing nalidixic acid (50 μg/ml) and ampicillin (25 μg/ml).

Southern blotting. Southern blotting was performed as described previously (25). For digested plasmids, the DIG DNA labeling and detection kit (Roche, Germany) was used. The DNA probe was synthesized using the MAP-4/rob-1F PCR (Table 1; see Fig. 2).

PFGE. PFGE was performed essentially as previously described (25), with the following modifications: DNA plugs were digested for 16 h with 10 U of ApaI (Takara Bio Inc., Japan) at 37°C. The electrophoresis parameters used were as follows: running time, 22 h; temperature, 14°C; field strength, 6 V/cm; included angle, 120°; initial pulse time, 1 s; final pulse time, 30 s.

Stability of pB1000. The curing rate of plasmid pB1000 in *P. multocida* and *E. coli* transformants was determined as previously described (26). The proportion of bacteria harboring pB1000 was determined by plating subculture steps on Columbia agar plus 5% sheep blood plates (bioMérieux, France) and further replication on BHI agar plates and BHI agar plates containing ampicillin (25 μg/ml). Colonies that had lost pB1000 were able to grow on BHI agar plates but not in BHI broth with ampicillin, while colonies harboring pB1000 were able to grow on both plates. The rate of plasmid curing was calculated.

Nucleotide sequence accession numbers. The nucleotide sequences obtained in this study have been deposited in GenBank under the following accession numbers: pB1001, EU252517; pB1002, EU283341; pB1003, EU360945; pB1005, FJ197818; pB1006, FJ234438.

RESULTS AND DISCUSSION

Characterization of β-lactam-resistant *P. multocida* clinical isolates. During the period between 2002 and 2005, 604 *P. multocida* clinical isolates from pigs with clinical symptoms were recovered from different farms in Spain. Among them, 13 (BB1034 to BB1046) showed high-level resistance to ampicillin. PFGE showed that nine strains had indistinguishable patterns (BB1035, BB1036, BB1038 to BB1043, and BB1045), two other isolates seem to be genetically related to these (BB1037 and BB1044), and strains BB1034 and BB1046 presented unique patterns (Fig. 1).

All 13 ampicillin-resistant isolates (MIC, >256 μg/ml) were also resistant to penicillin (MIC, >16 μg/ml) and cefaclor (MIC > 16 μg/ml) and susceptible to cefotaxime (Table 2). A total of 8 out of 13 isolates were concomitantly resistant to tetracycline (MIC, 16 to 32 μg/ml), 6 were high-level resistant to streptomycin (MIC, >256 μg/ml), and 1 was simultaneously resistant to β-lactams, tetracycline, and streptomycin. In addition, all strains were also high-level resistant to sulfonamides (Table 2). Overall, the 13 *P. multocida* isolates resistant to ampicillin represent 2.15% of the total *P. multocida* isolates analyzed between 2002 and 2005. These data are similar to those of recent studies with diseased swine in Spain and Ger-

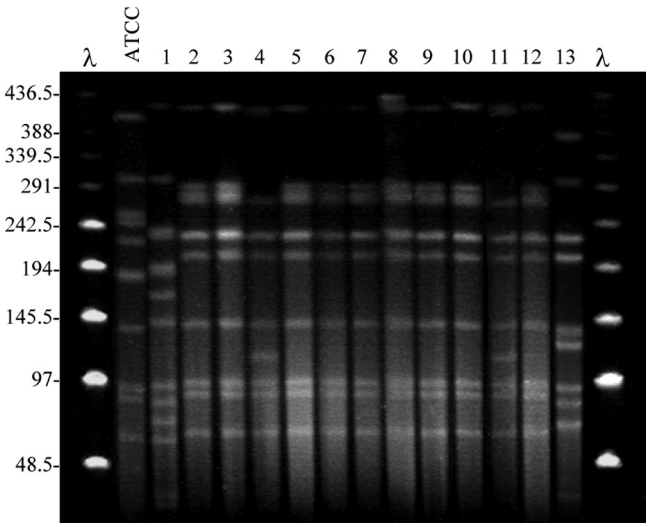

FIG. 1. PFGE fingerprint patterns of *P. multocida.* "λ" stands for lambda ladder molecular marker, and the numbers on the left show molecular mass in kb. ATCC stands for β-lactam-susceptible *P. multocida* strain ATCC 43137. Lane 1, BB1034; lane 2, BB1035; lane 3, BB1036; lane 4, BB1037; lane 5, BB1038; lane 6, BB1039; lane 7, BB1040; lane 8, BB1041; lane 9, BB1042; lane 10, BB1043; lane 11, BB1044; lane 12, BB1045; lane 13, BB1046.

many, where high-level resistance to ampicillin was 3% and 3.11%, respectively (17, 22).

Resistance genes and plasmid profiles in *P. multocida*-resistant isolates. PCR assays showed that all 13 isolates carried bla_{ROB-1} (Table 1). Among the eight *P. multocida* strains concomitantly resistant to tetracycline (Table 2), six strains carried *tet*(H) (BB1039 to BB1043 and BB1045) and one bore *tet*(B) (BB1034), genes encoding for major facilitator superfamily efflux pumps. Strain BB1044 carried the *tet*(O) gene, encoding a ribosomal protection protein. This is, to our knowledge, the first report of this resistance gene in *P. multocida*. All six streptomycin-resistant strains (BB1035 to BB1038, BB1044, and BB1046) bore the *strA* gene encoding a streptomycin phosphotransferase (Table 2).

Agarose gel electrophoresis of plasmid preparations and plasmid digestion with S1 nuclease were performed in all strains (Fig. 2). Plasmid digestion with S1 gives rise to a single band per plasmid, showing the size in base pairs of the replicon after electrophoresis (2). Each strain carried more than one small-size replicon, ranging from 4 to 6 kb (Fig. 2). A direct association between plasmid profile and antimicrobial resistance phenotype and genotype was observed in all strains (Fig. 2; Table 2). In order to assess the location of the antimicrobial resistance determinants, inverted PCR was used. Sets of primers hybridizing within the resistance genes and facing outward were designed and used to amplify the replicons by PCR (Table 1). PCR products for every resistance gene were obtained from all *P. multocida* isolates, indicating that the genes were located in small plasmids. Inverted PCR amplicons agreed with S1 nuclease plasmid digestions in size when electrophoresed in agarose gel (data not shown). Moreover, Southern blot hybridizations confirmed the plasmid location of the bla_{ROB-1} gene in all strains (Fig. 2). BB1034 to BB1045 revealed the same replicon of ca. 4.6 kb as the carrier of this gene. In contrast, in BB1046, the probe hybridized with a plasmid of approximately 5.7 kb.

Complete nucleotide sequencing and genetic organization of plasmids. (i) Resistance to β-lactams. A total of 12 *P. multocida* strains out of 13 carried plasmid pB1000, a 4,613-bp replicon bearing bla_{ROB-1} that was first described in *Haemophilus parasuis* (25). pB1000 belongs to the ColE1 superfamily (MOB_{HEN} family) (10) (Fig. 3). In *H. parasuis*, clonal dissemination of a strain carrying pB1000 was responsible for β-lactam resistance, as exactly the same pulsotype was found in all isolates tested, even if they came from different geographical regions in Spain (25). In the case of *P. multocida*, at least three divergent PFGE patterns were detected. One isolate, BB1046, bore a bla_{ROB-1}-carrying plasmid that was ca. 1 kb larger than pB1000. This replicon, pB1002, was completely sequenced and found to be composed by a segment of

TABLE 2. Antimicrobial susceptibility, genes, and plasmid profiles of the *P. multocida* isolates

Strain	MIC (mg/liter)[a]							Resistance genes	Plasmids (sizes in kb)	Plasmid profile[b]
	AMX	PEN	CEC	CTX	TET	STR	SMX			
ATCC 43137	≤1	≤0.03	≤1	≤0.06	2	8	>256	ND[c]	ND	ND
BB1034	>256	>16	>16	1	16	32	>256	bla_{ROB-1}, *tet*(B)	pB1000/pB1001 (4.6/5.1)	I
BB1035	>256	>16	>16	0.5	2	>256	>256	bla_{ROB-1}, *strA*, *sul2*	pB1000/pB1005 (4.6/4.2)	II
BB1036	>256	>16	>16	1	2	>256	>256	bla_{ROB-1}, *strA*, *sul2*	pB1000/pB1005 (4.6/4.2)	II
BB1037	>256	>16	>16	1	2	>256	>256	bla_{ROB-1}, *strA*, *sul2*	pB1000/pB1005 (4.6/4.2)	II
BB1038	>256	>16	>16	1	2	>256	>256	bla_{ROB-1}, *strA*, *sul2*	pB1000/pB1005 (4.6/4.2)	II
BB1039	>256	>16	>16	1	32	8	>256	bla_{ROB-1}, *tet*(H)	pB1000/p9956 (4.6/5.6)	III
BB1040	>256	>16	>16	1	32	16	>256	bla_{ROB-1}, *tet*(H)	pB1000/p9956 (4.6/5.6)	III
BB1041	>256	>16	>16	1	32	16	>256	bla_{ROB-1}, *tet*(H)	pB1000/p9956 (4.6/5.6)	III
BB1042	>256	>16	>16	1	32	8	>256	bla_{ROB-1}, *tet*(H)	pB1000/p9956 (4.6/5.6)	III
BB1043	>256	>16	>16	1	32	8	>256	bla_{ROB-1}, *tet*(H)	pB1000/p9956 (4.6/5.6)	III
BB1044	>256	>16	>16	1	32	>256	>256	bla_{ROB-1}, *tet*(O), *strA*, *sul2*	pB1000/pB1006/pB1005 (4.6/6.0/4.2)	IV
BB1045	>256	>16	>16	1	32	16	>256	bla_{ROB-1}, *tet*(H)	pB1000/p9956 (4.6/5.6)	III
BB1046	>256	>16	>16	0.5	1	>256	>256	bla_{ROB-1}, *strA*, *sul2*	pB1003/pB1002 (5.0/5.7)	V

[a] AMX, amoxicillin; CEC, cefaclor; CTX, cefotaxime; STR, streptomycin; SMX, sulfamethoxazole; TET, tetracycline; PEN, penicillin.
[b] Plasmid profile roman numbers are also used in Fig. 2.
[c] ND, not determined.

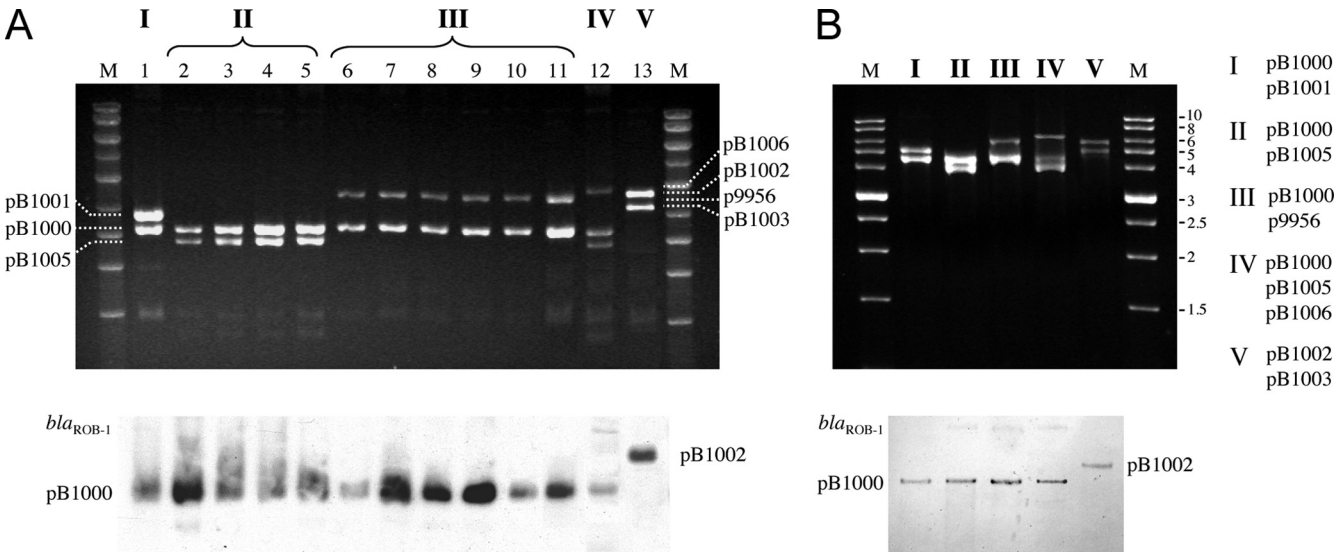

FIG. 2. (A, top) Plasmid profile of *P. multocida* strains. Lane 1, BB1034; lane 2, BB1035; lane 3, BB1036; lane 4, BB1037; lane 5, BB1038; lane 6, BB1039; lane 7, BB1040; lane 8, BB1041; lane 9, BB1042; lane 10, BB1043; lane 11, BB1045; lane 12, BB1044; lane 13, BB1046. Roman numbers above the lanes indicate different plasmid profiles. "M" stands for molecular weight marker. (Bottom) Hybridization of plasmid extractions from the top panel using a bla_{ROB-1}-specific probe. (B, top) Plasmid restriction profile using S1 nuclease for each different plasmid profile. Roman numbers indicate plasmid profiles. Replicons belonging to each profile are detailed on the right. "M" stands for molecular weight marker, and the numbers on the right designate size in kb. Note that BB1044, profile IV, bears up to three different plasmids. (Bottom) Hybridization of plasmids digested with S1 nuclease from the top panel using a bla_{ROB-1}-specific probe.

4,613 bp, 99.7% identical to pB1000, and by the 1,070-bp-long insertion sequence IS*Apl1* located downstream of bla_{ROB-1} (Fig. 3). IS*Apl1* was recently described in *Actinobacillus pleuropneumoniae* downstream of the bla_{ROB-1} gene (28). Sequence analysis of pB1002 showed that the putative recombination site we proposed for the bla_{ROB-1} gene in pB1000 (25) could also be the recombination site for the insertion of IS*Apl1* in pB1002. In the first report of pB1000, we proposed the GACTT/CTGAA sequence for being the potential recombination site of bla_{ROB-1}. Interestingly, in pB1002, IS*Apl1* is also bracketed by CTGAA direct repeats. Whether this IS*Apl1* is involved in the mobilization of bla_{ROB-1} is currently under investigation.

(ii) Resistance to tetracycline. Eight β-lactam-resistant *P. multocida* strains were resistant to tetracycline and carried three different plasmids bearing *tet*(H), *tet*(B), or *tet*(O) (Fig. 2). The complete nucleotide sequences of the three plasmids was determined. BB1034 bore the new *tet*(B)-bearing plasmid pB1001, a 5,128-bp replicon with a 1,206-bp *tet*(B) gene coding for the major facilitator superfamily efflux pump Tet(B). The Tet(B) sequence is novel in that it differs by one residue (L14F) from that of plasmid R100 from *Shigella flexneri* (GenBank accession no. NP 052931). pB1001 bears no mobilization genes but presents a replication gene encoding a 325-amino-acid Rep protein, presenting 78% identity with pFA3 and pAB6 from *Neisseriaceae* (1, 12). pB1001 from BB1034 is the only plasmid described in this work not possessing mobilization genes and also the sole plasmid encoding a replication protein. Plasmids carrying *tet*(H) and *tet*(O) were sequenced and shown to be the 5,674-bp plasmid p9956 (BB1039 to BB1043 and BB1045), reported recently in *A. pleuropneumoniae* isolates from Spain (3), and a 6,033-bp plasmid that was named pB1006 (BB1044), respectively. p9956 possesses functionally active relaxase proteins (3) and *tet*(H) and *tet*(R) genes similar to those from pPMT1 (18). The resistance fragment of pB1006 bore the *tet*(O) gene coding for a 639-amino-acid Tet(O) protein identical to that described in *A. pleuropneumoniae* on plasmid p13142 (GenBank accession no. AY987963.1).

(iii) Resistance to streptomycin. Six ampicillin-resistant *P. multocida* strains were concomitantly high-level resistant to streptomycin (BB1035 to BB1038, BB1046, and BB1044), with the latter also being resistant to tetracycline due to plasmid pB1006 (Table 2). Five strains shared the same *strA*-carrying plasmid, with an approximate size of 4.2 kb, whereas in BB1046, *strA* was borne in a ca. 5-kb replicon. In pB1003 (5,057 bp) (Fig. 3) from BB1046, the locus containing *sul2* and *strA* was identical to that described in pMS260 from *A. pleuropneumoniae* (15). Overlapping the 3′ end of *strA*, a fragment of 302 nucleotides from the 5′ region of the *strB* gene was located (Fig. 3). The plasmid encoding streptomycin resistance in the remaining five strains was named pB1005 (Fig. 3). This replicon shared 99.8% and 98.8% identity with plasmids ABB7_B (GenBank accession no. CP001093) and pTYM1 (5) from *A. pleuropneumoniae*, respectively, and 99.4% nucleotide identity with pYFC1 from *Mannheimia haemolytica* (6). The resistance region of pB1005 is composed of the overlapped *sul2-strA* genes and is 100% identical to the locus in plasmid ABB7_B from *A. pleuropneumoniae*.

Plasmids pB1000, pB1002, pB1003, pB1005, pB1006, and p9956 showed mobilization genes homologous to those from the MOB_{HEN} family, encoding proteins similar to others described previously in *Pasteurellaceae* (3, 21, 30). These plasmids showed a region of approximately 800 bp, located upstream of the mobilization genes, with an identity between 84.5% and 99.4% with the *oriV* region described previously for the *Haemophilus ducreyi* plasmid pLS88 (8) (Fig. 3). This region of 823

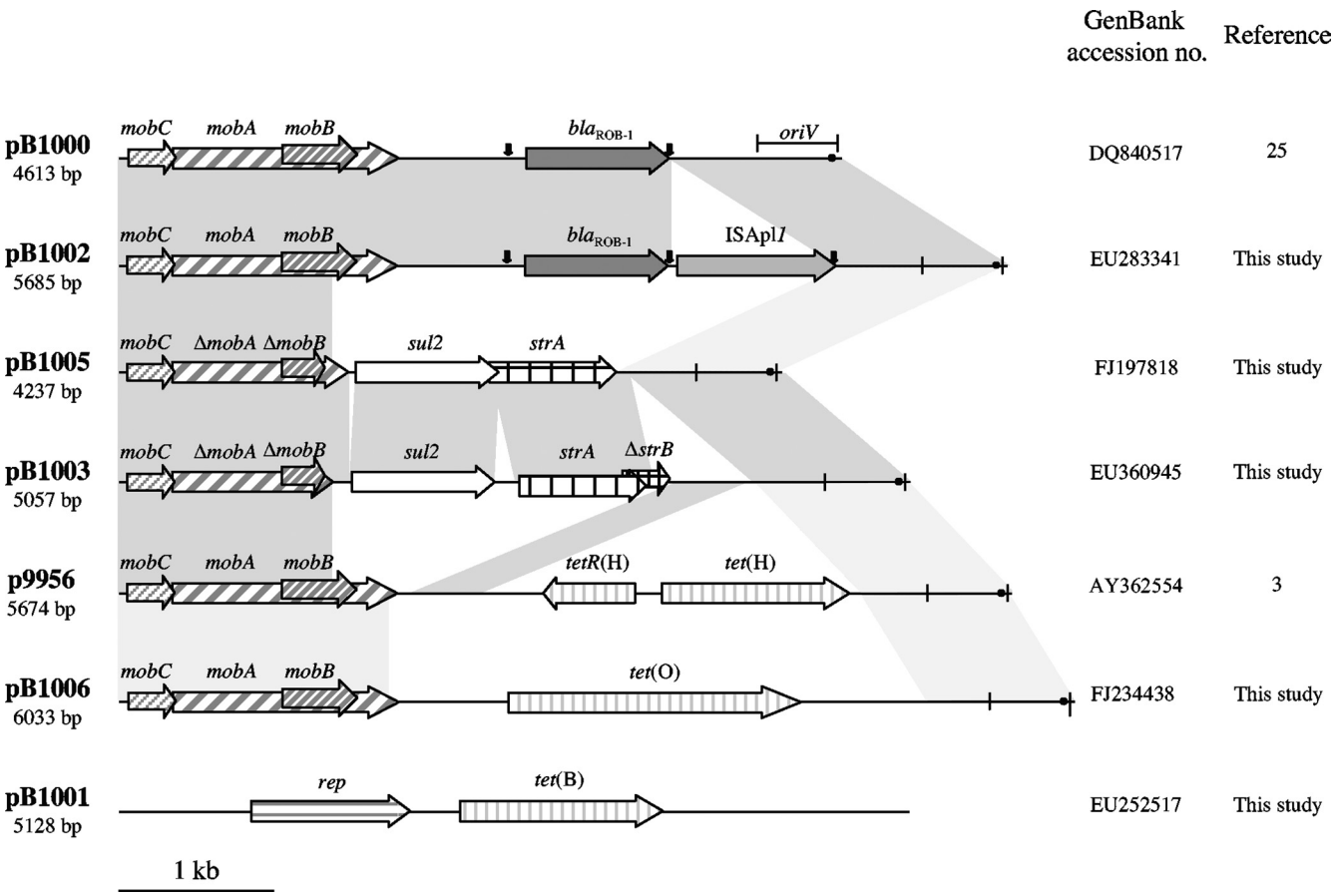

FIG. 3. Schematic representation of the plasmids described in this study. The reading frames for genes are shown as arrows, with the direction of transcription indicated by the arrowhead. Regions with more than 98% identity are dark shaded, and regions between 85% and 98% identity are light shaded. Vertical black arrows show the putative recombination site for bla_{ROB-1} and IS*Apl1*. Two vertical bars bracket the region containing the putative origin of replication (*oriV*). A black point indicates the putative transfer origin (*oriT*).

bp was isolated in pLS88 via deletion derivatives. The *oriV* region is usually responsible for the biologic properties of the plasmid, including replication, copy number control, incompatibility, and partitioning. None of the *oriV* regions in the plasmids of this study were identical, although they were highly similar. The exhibited differences seem to be enough to avoid incompatibility, as even three of them can stably coexist in the same cell (data not shown). pB1001 is similar to plasmids described previously in the genus *Neisseria* (1, 12). It has no mobilization genes and is the only replicon containing a *rep* gene. All plasmids, with the exception of pB1002 and pB1006, were successfully transformed into *E. coli*, conferring β-lactam, tetracycline, or streptomycin resistance.

(iv) pB1000 mobilization and stability. Plasmid pB1000 was successfully transformed into *E. coli* strain K-12 containing a chromosomal copy of the conjugation machinery of an IncP plasmid (4). From this strain, pB1000 was mobilized into *E. coli* K802N at a frequency of 3×10^{-3} colonies per donor CFU. PCR of bla_{ROB-1} confirmed transfer of this replicon. Hence, pB1000 is a mobilizable plasmid with active mobilization genes.

Furthermore, these experiments indicated that bla_{ROB-1} can be expressed in enterobacteria, as previously shown by others (11). Notwithstanding, the ROB-1 β-lactamase has been described only in clinical isolates of the *Pasteurellaceae* family. To determine whether the replicon is involved in this trait, the stability of plasmid pB1000 in *E. coli* and in *P. multocida* strains was assessed. Cultures were inoculated and then propagated every 24 h for 5 days in triplicate. For *P. multocida*, 100% of the colonies remained resistant to ampicillin after the five subcultures and conserved pB1000, as confirmed by PCR mapping. On the other hand, only 18% of *E. coli* colonies maintained ampicillin resistance after day 1, 2% after day 2, and 0% after day 3. The host stringency of the replicon seems thus to be a reason for which bla_{ROB-1} is present only in *Pasteurellaceae*. This, together with the fact that we were able to mobilize pB1000 through conjugation and transformation, indicates that vertical as well as horizontal transmission is responsible for dissemination of pB1000 within the *Pasteurellaceae* family.

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