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Genetic Determinant of the ROB-1 β-Lactamase in Bovine and Porcine *Pasteurella* Strains

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The ROB-1 β-lactamase, previously described in *Haemophilus influenzae*, has been found in the genus *Pasteurella*. In three bovine strains of *Pasteurella multocida* and *Pasteurella haemolytica*, ROB-1 production was determined by plasmids of 4.4 kilobases. In one porcine strain of *Pasteurella aerogenes*, the enzyme seems to be chromosomally encoded.

Pasteurella spp. are important pathogens of animals. They can contribute to a variety of animal diseases, ranging from avian septicemic disease to pneumonia or shipping fever in cattle (16). Moreover, *Pasteurella multocida* has been associated with infections in humans with increasing frequency (7, 20).

In the last few years, many animal isolates of *Pasteurella* spp. were found to be resistant to tetracycline, streptomycin, or sulfonamide. The resistance genes were shown to be associated with small nonconjugal R plasmids (1, 9, 10, 19). Resistance to β-lactam antibiotics has occasionally been reported (4, 8).

Recently, we found bovine *P. multocida* and *P. haemolytica* strains resistant to amoxicillin and ticarcillin owing to production of a β-lactamase (17). The enzyme was periplasmic and was produced constitutively. It had a TEM-like substrate profile, characterized by the preferential hydrolysis of penicillins, such as ampicillin or amoxicillin, and was sensitive to inhibition by clavulanic acid. No inhibition was detected with the following antisera: anti-TEM-1, anti-TEM-2, anti-CARB-3, anti-OXA-1-like, and anti-OXA-2. The isoelectric point (pI) was compared with the pI of the ROB-1 reference β-lactamase produced by *Haemophilus influenzae* ROB-F990 (18). As determined by analytical isoelectric focusing (12), the pIs were identical (pI = 8.15). Moreover, comparison of the substrate and inhibition profiles of the enzymes confirmed their identity (data not shown). Production of ROB-1 β-lactamase by a porcine strain of *P. multocida* was previously reported in the United States by Medeiros et al. (13).

The ROB-1 β-lactamase, which has been described in human *H. influenzae* strains and porcine *Haemophilus pleuropneumoniae* strains, was shown to be plasmid mediated (13, 18). The purpose of the present study was to establish the genetic basis of ROB-1 β-lactamase production in bovine and porcine *Pasteurella* strains.

Four *Pasteurella* strains were studied. One *P. haemolytica* and two *P. multocida* strains were isolated between 1978 and 1985 from calf lungs and were collected at the Laboratoire National de Pathologie Bovine (Lyon, France). The porcine *Pasteurella aerogenes* ATCC 27883, isolated before 1973, was provided by F. Escande (Institut Pasteur, Paris, France). These strains were grown in tryptic soy broth (Bio-Mérieux, Lyon, France), supplemented with 5% horse blood at 37°C with shaking. By the standard disk diffusion assay on Mueller-Hinton agar (Diagnostics Pasteur), the four *Pasteurella* strains were found to be resistant at least to amoxicillin, ticarcillin, streptomycin, and sulfonamide. They were susceptible to amoxicillin-clavulanic acid (2 μg/ml) and to ticarcillin-clavulanic acid (2 μg/ml). Resistance patterns and MICs of β-lactam antibiotics are shown in Table 1.

Plasmid DNA was prepared from overnight cultures by the procedure of Birnboim and Doly (2) and was purified by isopycnic centrifugation in a cesium chloride-ethidium bromide gradient (11). Plasmid patterns of the four *Pasteurella* strains were analyzed by horizontal 1% agarose gel electrophoresis (14) (Fig. 1). *P. multocida* LNPB 9 showed three plasmid bands. The molecular sizes, determined by comparison with reference plasmids, were estimated at 10.3, 5.2, and 4.4 kilobases (kb). In *P. multocida* LNPB 86, two plasmids were observed, one of 5.0 kb and the other of 4.4 kb. Only one 4.4-kb plasmid was found in *P. haemolytica* LNPB 51. Thus, all these strains harbor a 4.4-kb plasmid. On the other hand, *P. aerogenes* ATCC 27883 failed to show a visible plasmid band.

To determine if the plasmids found in three strains were involved in ROB-1 β-lactamase production, DNA extracts from strains LNPB 9, LNPB 86, and LNPB 51 were used to transform *Escherichia coli* K-12 HB101 (3) by the method of Cohen et al. (6). Transformants were selected on tryptic soy agar plates containing ticarcillin (128 μg/ml). By the standard disk diffusion assay, these transformants were found to be resistant to amoxicillin and ticarcillin and susceptible to other antimicrobial agents, including streptomycin and sulfonamides. The MICs of amoxicillin and ticarcillin were strikingly elevated (>1,024 μg/ml), i.e., five times higher

TABLE 1. Susceptibility to antimicrobial agents

Strain	Resistance pattern[a]	MIC[b] (μg/ml)	
		Amoxicillin	Ticarcillin
P. multocida LNPB 9	Amo Tic Sm Su	128	256
P. multocida LNPB 86	Amo Tic Sm Su Cm Nal	128	256
P. haemolytica LNPB 51	Amo Tic Sm Su Tc	128	128
P. aerogenes ATCC 27883	Amo Tic Sm Su Cm Nal	512	1,024

[a] Abbreviations: Amo, amoxicillin; Tic, ticarcillin; Sm, streptomycin; Su, sulfonamide; Tc, tetracycline; Cm, chloramphenicol; Nal, nalidixic acid.
[b] The MICs of amoxicillin plus clavulanic acid (2 μg/ml) and ticarcillin plus clavulanic acid (2 μg/ml) were <1 for all strains shown.

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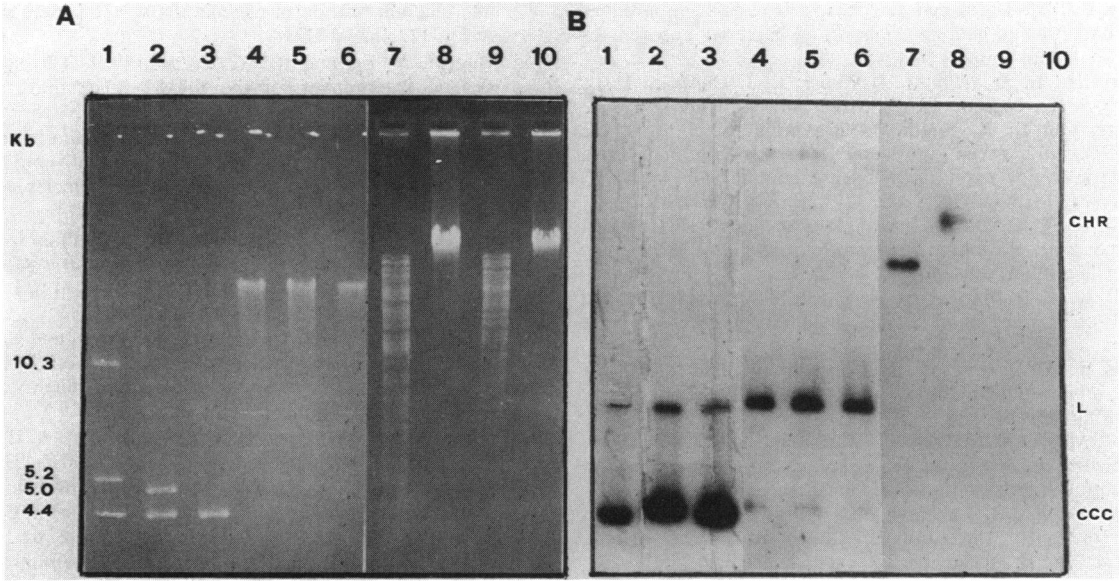

FIG. 1. (A) Ethidium bromide-stained agarose gel electrophoresis of plasmid DNA from *P. multocida* LNPB 9 (lane 1), *P. multocida* LNPB 86 (lane 2), *P. haemolytica* LNPB 51 (lane 3), LNPB 9-*E. coli* HB101 transformant (lane 4), LNPB 86 transformant (lane 5), LNPB 51 transformant (lane 6) and chromosomal DNA from *P. aerogenes* 27883 (lane 8) and *E. coli* HB101 (lane 10). Partially *Eco*RI-restricted chromosomal DNA from *P. aerogenes* 27883 (lane 7) and *E. coli* HB101 (lane 9) is also shown. (B) The corresponding autoradiograph after transfer onto nitrocellulose filter and hybridization with ^{32}P-labeled R_{Rob}. CHR, Chromosomal DNA; L, linear DNA; CCC, covalently closed circular DNA.

than in wild-type *Pasteurella* strains. Moreover, the specific activities of β-lactamase from amoxicillin-resistant transformants were at least four times greater than those from the original *Pasteurella* isolates. As determined by analytical isoelectric focusing, all the transformants produced ROB-1 β-lactamase (pI = 8.15). Analysis of plasmid DNA revealed a very weak band in all transformants which comigrated with the common plasmid band of 4.4 kb present in the wild strains. To verify where the ROB-1 resistance gene was located, either in transformants or in *P. aerogenes* 27883, R_{Rob} (plasmid DNA from F990, the original ROB-1-producing *H. influenzae* strain) was labeled with ^{32}P by nick translation, and hybridization experiments were performed under conditions of high stringency (11). The ROB-1 resistance gene was found to be associated with the 4.4-kb plasmid in the three bovine *Pasteurella* strains and in amoxicillin-resistant transformants (Fig. 1). Moreover, the ROB-1 resistance gene was identified in chromosomal DNA from *P. aerogenes* 27883.

We have demonstrated by agarose gel electrophoresis and transformation experiments that ROB-1 β-lactamase is plasmid mediated in three bovine *Pasteurella* strains. Until now, the presence of β-lactamase plasmids had not been described in the genus *Pasteurella*. Zimmerman found that ampicillin resistance of one *P. haemolytica* strain was β-lactamase mediated, but this resistance could not be conclusively shown to be plasmid encoded (21). The plasmids encoding for amoxicillin resistance in these strains are small (4.4 kb). This molecular size is identical to those of R_{Rob} and pMG301 from original ROB-1 β-lactamase-producing *H. influenzae* strains. Further studies using restriction endonucleases will be necessary to compare our plasmids and the R_{Rob} and pMG301 plasmids.

Two observations require comments. First, we found one porcine *P. aerogenes* strain in which ROB-1 β-lactamase production seems to be chromosomally encoded. This was an unexpected finding because, until now, ROB-1 β-lactamase had always been found to be plasmid mediated. However, such a property had already been reported for the SHV-1 β-lactamase. In *Klebsiella pneumoniae*, SHV-1 can be either chromosomal or plasmid mediated, and in *E. coli* and *Proteus mirabilis*, SHV-1 is plasmid mediated. The genes encoding this enzyme were thought to be part of a transposable element (15). Moreover, ROB-1 was first described in a human *H. influenzae*, isolated in 1980 (18). *P. aerogenes* 27883 was isolated before 1973, and *P. multocida* LNPB 9 was isolated in 1978. These findings support the hypothesis that ROB-1 may have an animal origin. The second observation is the high level of expression of β-lactamase for all transformants, in spite of the apparent low copy number of plasmid DNA. In a study by Chen and Clowes (5), transcriptional initiation may have occurred at different sites in the wild-type strain (*H. influenzae*) and in *E. coli* transformants, leading to a 6- to 10-fold increase in β-lactamase expression in *E. coli*.

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