Molecular Distribution of ROB-1 β-Lactamase in Actinobacillus pleuropneumoniae

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The plasmid content and molecular distribution of the ROB-1 β-lactamase was investigated in 31 swine isolates of Actinobacillus pleuropneumoniae. Three types of plasmid patterns were observed in ampicillin-resistant isolates: a predominant one consisting of 2.6-, 2.9-, and 5.0-kb plasmids and two other patterns consisting of 4.2- and 5.5-kb plasmids and of a 6.8-kb plasmid. Plasmid DNA preparations were hybridized with a blaROB-1 intragenic fragment from the Haemophilus influenzae R100 plasmid. Positive hybridizations were observed with all ampicillin-resistant isolates. The blaROB-1 gene was found on 3 plasmids of 2.6, 5.5, and 6.8 kb. One swine isolate of Pasteurella multocida also had a 2.6-kb plasmid bearing blaROB-1. ROB-1 was confirmed in typical isolates by isoelectric focusing and blaROB-1 sequences were confirmed by polymerase chain reaction amplification with an intragenic set of primers. Plasmids bearing blaROB-1 were successfully electrotransfected in a susceptible A. pleuropneumoniae isolate. These results emphasize the importance of ROB-1 in A. pleuropneumoniae and identify a possible reservoir of β-lactam resistance.

The antimicrobial susceptibility of Actinobacillus pleuropneumoniae has been studied largely because of the economical importance of porcine pleuropneumonia. The ampicillin resistance of A. pleuropneumoniae is presumably increasing in Québec (Canada), as demonstrated by susceptibility studies which showed that 53% of isolates were resistant in 1981 and that 74% were resistant in 1986 (24). In Canada (Ontario), the United States (South Dakota and Iowa), Italy, and Japan, more than 80% of isolates were found susceptible to ampicillin (2, 7, 17-19). Plasmid-mediated ampicillin resistance due to a β-lactamase was first described in A. pleuropneumoniae by Hirsh et al. in 1982, and the bla gene was found associated with pVM105 (5.3 kb) (3). Later studies associated ampicillin resistance with 4.3-, 5.3-, and 8.3-kb plasmids (4).

The ROB-1 β-lactamase was first described in ampicillin-resistant Haemophilus influenzae type b (16). One year later, a plasmid-mediated β-lactamase was found in ampicillin-resistant A. pleuropneumoniae but was identified as a TEM-type enzyme (3). Isoelectric focusing and DNA hybridization confirmed that the plasmid-mediated β-lactamases from both H. influenzae and A. pleuropneumoniae were ROB-1 (10). Further studies indicated that the blaROB-1 gene did not cross-hybridize with other known bla genes (6). Recent analysis at the molecular level clearly showed that ROB-1 is an unusual class A β-lactamase that could have originated in gram-positive bacteria (5). The β-lactamase was also found in a porcine isolate of Pasteurella multocida and in diverse isolates of Pasteurella species (8). An epidemiological survey of the prevalence of the ROB-1 β-lactamase among ampicillin-resistant H. influenzae isolates in the United States showed that 92% produced the TEM-1 enzyme, while the remaining isolates produced the ROB-1 enzyme (1).

In this study, we report the plasmid content of a collection of A. pleuropneumoniae strains, the molecular distribution of the ROB-1 β-lactamase, and the transfer of ampicillin resistance.

MATERIALS AND METHODS

Bacterial strains and plasmids. A. pleuropneumoniae isolates were isolated at the Faculté de Médecine Vétérinaire de l’Université de Montréal, Ste-Hyacinthe, Québec, Canada, from swine that had died of pleuropneumonia in commercial farms. Isolates were collected between 1980 and 1986. Serotyping was performed by the ring precipitation method or the coagglutination test (12, 13). A total of 139 isolates were tested for ampicillin susceptibility and plasmid profiles. Ampicillin susceptibility was tested on brain heart infusion agar (Difco Laboratories, Detroit, Mich.) containing 1% IsoVitalex (BBL) and ampicillin (20 μg/ml). Positive controls producing ROB-1 were A. pleuropneumoniae SD1 (3), H. influenzae ROB F990 (16), and Escherichia coli HB101 (pMON401) (origin of the blaROB-1 probe) (6). A porcine isolate of P. multocida (10) confirmed to produce ROB-1 by isoelectric focusing was included. The negative control was E. coli HB101(pBR322) (23) or HB101(pACYC184) (15). The recipient for electroproporation was the ampicillin-susceptible strain A. pleuropneumoniae 4074 (14). A. pleuropneumoniae and H. influenzae were grown on chocolate tryptic soy agar (International Biotechnology Inc.) plates with 1% IsoVitalex. E. coli was grown on tryptic soy agar and the appropriate antibiotic.

Preparation of DNA and related techniques. Plasmids pMON401, pBR322, and pACYC184 were prepared by the cleared-lysate method and purified by cesium chloride-ethidium bromide gradient ultracentrifugation (20). Rapid preparation of plasmids from A. pleuropneumoniae was done by the alkaline lysis method and by the boiling method (9). Restricted DraI fragments from pMON401 were separated by polyacrylamide gel (15%) electrophoresis. The 240-bp DraI intragenic restriction fragment was purified by elec-
elution (9). The probe was radiolabeled with the Prime Time C kit (IBI; Terochem Scientific, Montréal, Québec, Canada). Hybridization was done at high stringency (42°C, 50% formamide–5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as described elsewhere (11). Southern transfer of DNA to nitrocellulose membranes was done after electrophoresis of DNA on a 0.7% agarose gel (9). Electroporation of plasmid DNA minipreparations in A. pleuropneumoniae 4074 was done as described by Sirois et al. (21). Bacterial cell extracts were prepared by freezing and boiling (22), and an aliquot of 2 μl was used with the GeneAmp kit (Cetus Perkin Elmer, Montréal, Québec, Canada). Polymerase chain reaction (PCR) amplification conditions were those recommended by the manufacturer; two intragenic primers (5' TGTTTGCAATCGCTGCC 3' and 5' TTATCGTACACT TTCCA 3') yielded a specific $bla_{ROB-1}$ DNA amplification product of 400 bp.

**Biochemical characterization of the β-lactamase.** β-Lactamase preparation and isoelectric focusing were done as described before (10).
RESULTS

Strain susceptibility and plasmid content. Of the 139 A. pleuropneumoniae isolates tested for ampicillin susceptibility, 42% were resistant to 20 μg/ml. The plasmid profile analysis showed that 113 isolates (81%) contained plasmids irrespective of ampicillin susceptibility. We noted that 52 ampicillin-resistant isolates (88%) and 61 ampicillin-susceptible isolates (76%) contained plasmids. On the basis of these data, we chose 24 ampicillin-resistant isolates for further study. These isolates were selected as representatives of the typical plasmid patterns observed. As controls, we chose six ampicillin-susceptible isolates containing plasmids and one isolate with no detectable plasmid DNA.

The plasmid patterns obtained from selected A. pleuropneumoniae isolates are shown in Fig. 1 (parts I, II, and III, panel A) and listed in Table 1. For ampicillin-resistant A. pleuropneumoniae, three different plasmid patterns were observed. The most common pattern included plasmids of 2.6, 2.9, and 5.0 kb (Fig. 1, part I, panel A, lanes H to J and L to O; Fig. 1, part II, panel A, lanes C to I and L to N; and Fig. 1, part III, panel A, lanes C to E) and was found in 20 of the 24 resistant isolates. Some of these patterns had an "extra" DNA band which migrated at 9 kb (Fig. 1, part I, panel A, lanes J to L; Fig. 1, part II, panel A, lanes D to H; and Fig. 1, part III, panel A, lanes C, E, and F) and which may represent an additional plasmid but which may also represent a multimer. Plasmids of 4.2 and 5.5 kb (Fig. 1, part II, panel A, lane K, and Fig. 1, part III, panel A, lane H) for two isolates and 6.8 kb (Fig. 1, part II, panel A, lane J, and Fig. 1, part III, panel A, lane G) for two other isolates were also identified. For ampicillin-susceptible isolates, three plasmid patterns were observed. The most common was carried by four isolates and included plasmids of 2.9 and 5.0 kb (Fig. 1, part I, panel A, lane K, and Fig. 1, part III, panel A, lanes I and J). There was no clear correlation between ampicillin resistance and the presence of typical plasmids in any of the tested isolates. Small size differences between the plasmids reported here and those previously described (pVM105 and Rrob) were due to the use of different size markers (supercoiled DNA ladder).

Construction of a blaROB-1 probe. As a first step in developing a specific blaROB-1 probe, we validated the specificity and possible cross-hybridization of the 240-bp Dral restriction fragment probe with other bla genes (data not shown). At high stringency, the probe did not cross-hybridize with other bla genes (11). The results of screening by nucleic acid hybridization with the blaROB-1 probe are shown in Fig. 1 (parts I, II, and III, panel B) and listed in Table 1. The blaROB-1 probe hybridized with a specific plasmid DNA band for every resistant isolate tested. No hybridization was observed with plasmid DNA from ampicillin-susceptible isolates, while positive hybridization was detected with pMON401, H. influenzae ROB F990 (Rrob), A. pleuropneumoniae SD1(pVM105), and P. multocida (2.6-kb plasmid). The most common plasmid found hybridizing with the blaROB-1 probe was the 2.6-kb plasmid found in the A. pleuropneumoniae and P. multocida swine isolates. An extra band was also picked up by the probe at about 6 kb and presumably represents an open circular form of the 2.6-kb plasmid. Preparations of P. multocida plasmid DNA contained multiple bands; not only did a 2.6-kb plasmid hybridize with the probe, but also a series of multimer bands did so. We also observed in P. multocida a band migrating at 4.4 kb and hybridizing with the probe. This band may have been due to the presence of a second plasmid carrying blaROB-1 and similar to Rrob. Hybridization also confirmed that the 5.5- and 6.8-kb plasmids were blaROB-1 carriers.

Transfer of plasmid-mediated ampicillin resistance. To prove that plasmids hybridizing with the blaROB-1 probe encoded ampicillin resistance, we attempted to transform plasmid DNAs by electroporation with plasmid DNA preparations from typical isolates. As depicted in Fig. 2, the 2.6-, 6.8-, 4.4 (Rrob), and 5.5 (pVM105)-kb plasmids that could be transferred in A. pleuropneumoniae 4074 encoded ampicillin resistance. The 6.8-kb plasmid electrot transformed in A. pleuropneumoniae 4074 showed additional DNA bands that
were absent in the original isolates. These bands were probably due to the formation of multimers. The results obtained were further confirmed by β-lactamase identification by isoelectric focusing and blaROB-1 PCR amplification. The isolates had a β-lactamase with a pI of approximately 8.1, as expected (data not shown), and an amplification product of 400 bp (data not shown).

**DISCUSSION**

An overview of ampicillin susceptibility among 139 *A. pleuropneumoniae* isolates revealed less ampicillin resistance (42%) than did earlier studies done in Canada (Québec), the United States, and other countries (2, 7, 17–19, 24). In attempting to evaluate the molecular distribution of plasmids mediating the ROB-1 β-lactamase, we decided to select isolates representing all typical plasmid patterns. The most common pattern observed in ampicillin-resistant isolates included 3 plasmids of 5.0, 2.9, and 2.6 kb. One of the two other patterns included plasmids of 5.5 and 4.2 kb similar to those in strain SD1 from South Dakota (3). Interestingly, both isolates and strain SD1 were serotype 7. In other cases, we found no clear correlation between plasmid patterns and serotypes. For example, *A. pleuropneumoniae* isolates 131 and 132 possessed identical plasmid profiles but were different serotypes. Further studies are necessary to confirm whether a specific plasmid pattern correlates with a particular serotype.

To verify that ampicillin resistance was due to the ROB-1 β-lactamase, we hybridized plasmid DNAs with an intragenic blaROB-1 probe. Results were confirmed by isoelectric focusing and PCR amplification. PCR amplification opens new perspectives in large molecular epidemiological studies of β-lactamase distribution. Ampicillin resistance could also be transferred by transformation via electroporation. All resistant isolates bearing plasmids hybridized with the probe, indicating that the ampicillin resistance in our isolates was due to only the ROB-1 β-lactamase. The blaROB-1 gene was found to be distributed in *A. pleuropneumoniae* largely

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**TABLE 1. Bacterial strains used and relevant characteristics**

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<th>pl</th>
<th>Plasmid transferb</th>
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a Plasmid (kilobases) hybridizing with the 240-bp DraI fragment from blaROB-1.
b Plasmid (kilobases) for which transfer of ampicillin resistance was confirmed by electrophoresis in *A. pleuropneumoniae* 4074.
c +, positive; −, negative.
FIG. 2. Agarose gel (0.79%) electrophoresis of plasmid DNA mini-preparations of ampicillin-resistant isolates and electro-transformants obtained. Lanes: A, supercoiled DNA marker from Bethesda Research Laboratories; B, A. pleuropneumoniae 2; C, electro-transformant 2.6-kb (2); D, A. pleuropneumoniae 4; E, electro-transformant 6.8-kb (4); F, A. pleuropneumoniae SD1; G, electro-transformant 5.5-kb pVM105 (SD1); H, H. influenzae ROF F990; I, electro-transformant 4.4-kb Rrob (ROB F990); J, P. multocida; K, electro-transformant 2.6-kb (pm). Bacterial strains used for plasmid DNA preparations are indicated in parentheses.

REFERENCES


on three kinds of plasmid: the small and very common 2.6-kb plasmid; the 5.5-kb plasmid, like pVM105 from A. pleuropneumoniae SD1; and a 6.8-kb plasmid. In other members of the family Pasteurellaceae, the blaROB-1 gene was found in a 2.6-kb plasmid from P. multocida and on 4.4-kb Rrob from H. influenzae, the prototype for ROB-1 β-lactamase. The P. multocida swine isolate contained a 2.6-kb plasmid, like most isolates of A. pleuropneumoniae but unlike a bovine isolate described by Livrelli et al. (8) that contained a 4.4-kb plasmid similar to Rrob.

Since ROB-1 has been found only in the family Pasteurellaceae, it is interesting to compare its distribution in A. pleuropneumoniae and in H. influenzae. In contrast to what we observed in A. pleuropneumoniae, ROB-1 is not predominant in H. influenzae, supporting the previously suggested hypothesis of a ROB-1 reservoir for β-lactamase resistance in A. pleuropneumoniae (10). The fact that ROB-1 is not as common as TEM-1 in H. influenzae may be due to the coexistence of H. influenzae with other TEM-1 carriers, such as human Salmonella and Escherichia spp. For A. pleuropneumoniae, antibiotic usage and pressure in commercial farms could be the source for the maintenance of and increase in ROB-1. Moreover, the discovery of ROB-1 in distinct bacterial pathogens, such as A. pleuropneumoniae (animals) and H. influenzae (humans), shows that animal Pasteurellaceae isolates could be an important source of β-lactam resistance genes in human Pasteurellaceae isolates.

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