ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1986, p. 212–215
0066-4804/86/020212-04$02.00/0

Vol. 29, No. 2

An Animal Source for the ROB-1 β-Lactamase of *Haemophilus influenzae* Type b

ANTONE A. MEDEIROS,[1]* ROGER LEVESQUE,[2]† AND GEORGE A. JACOBY[2]

Miriam Hospital, Brown University, Providence, Rhode Island 02906,[1] and Massachusetts General Hospital, Boston, Massachusetts 02114[2]

Received 2 August 1985/Accepted 14 November 1985

The most common cause of ampicillin resistance in *Haemophilus influenzae* type b is production of TEM-1 β-lactamase; however, a novel enzyme with a similar substrate profile but a quite different isoelectric point has also been described. This β-lactamase, designated ROB-1, has not been found previously in any other organism. In a survey of 46 ampicillin-resistant *H. influenzae* type b isolates, we found a second human isolate that produces ROB-1 and discovered that ampicillin-resistant isolates of the porcine pathogen *Haemophilus pleuropneumoniae* also produced ROB-1. In both *Haemophilus* species ROB-1 production was determined by plasmids that had considerable DNA sequence homology. However, the ROB-1 and TEM-1 β-lactamase genes were not related. Our findings suggest that this form of ampicillin resistance has an animal reservoir and that conditions fostering its prevalence in animal strains may play a role in the spread of resistance to human pathogens.

Since their appearance in 1974, β-lactamase-producing type b *Haemophilus influenzae* isolates have increased in prevalence; currently more than 20% of the *H. influenzae* type b strains isolated in the United States have this property (2). In most strains the β-lactamase produced is TEM-1 (15), but one strain which produces a plasmid-determined enzyme with broad-spectrum activity like that of TEM-1 but with an isoelectric point of 8.1 (quite different from the TEM-1 pI of 5.4) has been described (17). While TEM-1 is the most common type of plasmid-determined β-lactamase in a variety of gram-negative pathogens (12), the origin of the other enzyme, which has been designated ROB-1 (13), has not been elucidated.

Haemophilus pleuropneumoniae causes porcine pneumonia and pleuritis (18). In 1982 6% of the *H. pleuropneumoniae* isolates from South Dakota were resistant to ampicillin (9). Hirsh et al. (6) found that ampicillin resistance in one South Dakota isolate was mediated by 5.4-kilobase plasmid pVM105, which determined a β-lactamase with a substrate profile similar to that of TEM-1 and also resistance to sulfonamide. Similar plasmids have been detected in two β-lactamase-producing *H. pleuropneumoniae* strains from Canada by Gilbride et al. (K. Gilbride, J. Brunton, and S. Rosendal, Program Abstr. 23rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 429, 1983), who found that the β-lactamase of these strains had an alkaline pI which was distinct from that of TEM-1.

We found a second human isolate of *H. influenzae* that produces ROB-1 β-lactamase and discovered that ampicillin-resistant *H. pleuropneumoniae* strains which cause disease in pigs produce ROB-1 and carry plasmids very similar to those found in ROB-1-producing *H. influenzae* strains from humans. These findings bear on the continuing concern about the spread of antibiotic resistance from animals to humans (8).

* Corresponding author.
† Present address: Department of Microbiology, Faculty of Medicine, Laval University, Quebec, Canada G1K 7P4.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Haemophilus* strains were identified by using standard techniques (7) and were typed with specific antisera (Centers for Disease Control, Atlanta, Ga.). *H. influenzae* T2494 was isolated from the spinal fluid of a 1-year-old girl from Tennessee and was provided by L. G. Rubin, Long Island Jewish-Hillside Medical Center, New Hyde Park, N.Y. *H. influenzae* F990, the prototype strain for ROB-1 β-lactamase, has been described previously (17). *H. pleuropneumoniae* strain SD-1 and strain M62(pVM105), which contained a plasmid from strain SD-1, were supplied by D. C. Hirsch (6). Ampicillin-resistant *H. pleuropneumoniae* strains 83-14204 and 83-14781 were provided by M. C. Libal (South Dakota State University, Brookings) and came from pigs that were dying of hemorrhagic pneumonia in South Dakota and Minnesota. *Escherichia coli* HB101 (1) was used for transformations, and *E. coli* J53(R6K) (12) was used as a source of TEM-1 β-lactamase. Plasmid pACYC184 (1) was used as a cloning vector, and plasmid pBR322 (1) was used for DNA hybridization.

Media and susceptibility testing. *Haemophilus* strains were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) containing 10 μg of hemin per ml and 2 μg of diphosphopyridine nucleotide per ml or on chocolate agar plates. *E. coli* strains were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). Transformants were selected on Trypticase soy agar (BBL) plates containing 10 μg of ampicillin per ml and were tested for antibiotic susceptibility by using disks.

β-Lactamase characterization. Sonic extracts were prepared as described previously (15). Those extracts with low β-lactamase activity were concentrated by using a Micro-ProDiCon apparatus (Bio-Molecular Dynamics, Beaverton, Oreg.) and membranes with a 10,000-molecular-weight cutoff (14). Analytical isoelectric focusing (11) was performed on polyacrylamide gels as described previously (14). β-Lactamase activity was assayed alkalimetrically (4). Inhibitors were preincubated with enzyme at 37°C for 5 min, and residual activity was determined with nitrocefin as the sub-

TABLE 1. Substrate and inhibition profiles of TEM-1 and ROB-1 β-lactamases produced by *H. influenzae* and *H. pleuropneumoniae*

Strain	β-Lactamase type	Relative rates of hydrolysis[a]					% Inhibition by:[b]		
		Ampicillin	Carbenicillin	Cloxacillin	Cephalothin	Cephaloridine	NaCl (100 mM)	Cloxacillin (0.1 mM)	Clavulanate (0.006 mM)
E. coli J53 (R6K)	TEM-1	81	11	≤0.2	15	81	2	0	99
H. influenzae Rob F990	ROB-1	107	19	≤0.2	4.5	37	0	69	95
H. influenzae T2494	ROB-1	103	18	≤0.2	4.6	31			
H. pleuropneumoniae SD-1	ROB-1	105	19	≤0.2	4.6	36	0	76	98
H. pleuropneumoniae M62(pVM105)	ROB-1	95	16	≤0.2	3.8	38			

[a] Activity relative to penicillin G activity (defined as 100).
[b] Expressed as follows: 100% − the percentage of activity against nitrocefin at the concentration of inhibitor indicated.

strate (16) by monitoring absorbance at 482 nm, using a Hitachi model 100-60 double-beam spectrophotometer.

DNA techniques. Plasmids were purified by cesium chloride-ethidium bromide gradient ultracentrifugation (3) and were cleaved with restriction endonucleases under the conditions specified by the manufacturers (Bethesda Research Laboratories, Inc., Gaithersburg, Md., and New England BioLabs, Inc., Beverly, Mass.). Fragments were separated by electrophoresis on 1.2% agarose. Plasmid DNA was labeled with ^{32}P by nick translation (10). Hybridization was performed as described by Southern (20), using conditions of high stringency (50% formamide at 42°C, followed by washing at 60°C) (19). Cloning of the ROB-1 gene and transformation of DNA were carried out as described previously (19).

RESULTS

A second *H. influenzae* type b strain (strain T2494) producing the ROB-1 β-lactamase was isolated from a 1-year-old girl with meningitis in Tennessee. As determined by analytical isoelectric focusing, this strain (Fig. 1, lane 1) produced a β-lactamase with an isoelectric point of 8.1 which was identical to the enzyme produced by ROB-1 prototype *H. influenzae* strain F990, which was isolated from a child with meningitis in Maryland (17) (lane 2). Figure 1 also shows the residual β-lactamase activity remaining at the loading site, which is characteristic of this enzyme (17). Furthermore, the β-lactamases from these two strains had identical substrate and inhibition profiles (Table 1). A total of 45 other β-lactamase-producing *H. influenzae* type b strains from medical centers in Chicago, Ill., Dallas, Tex., Rhode Island, St. Louis, Mo., and Tennessee all produced TEM-1 enzyme similar to that shown in Fig. 1, lane 6.

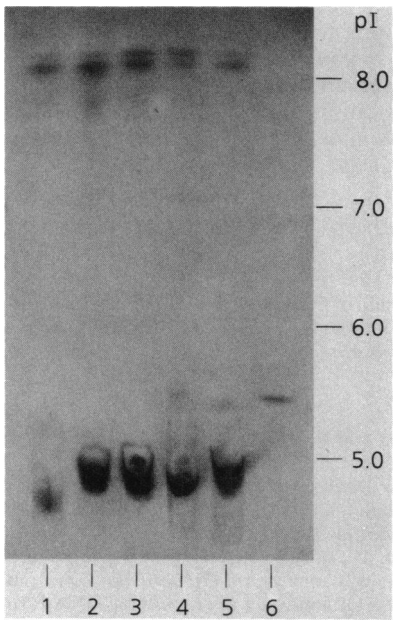

FIG. 1. Isoelectric focusing gel showing the band patterns of β-lactamases from *H. influenzae* T2494 (lane 1), *H. influenzae* Rob F990 (the ROB-1 β-lactamase prototype) (lane 2), *H. pleuropneumoniae* M62(pVM105) (lane 3), *H. pleuropneumoniae* 83-14204 (lane 4), *H. pleuropneumoniae* 83-14781 (lane 5), and *E. coli* J53(R6K) (the TEM-1 prototype) (lane 6). β-lactamase activity was detected by using the chromogenic substrate nitrocefin.

Ampicillin-resistant *H. pleuropneumoniae* strains also produced the ROB-1 β-lactamase. The enzymes from *H. pleuropneumoniae* M62(pVM105) (6) (Fig. 1, lane 3) and *H. pleuropneumoniae* isolates from South Dakota and Minnesota (lanes 4 and 5) focused with pIs of 8.1 (identical to the pI of ROB-1). The substrate and inhibition profiles of the *H. pleuropneumoniae* β-lactamases were also identical to those of ROB-1 (Table 1). Biochemical and serological tests confirmed that the *H. pleuropneumoniae* and *H. influenzae* strains were distinct.

The original ROB-1 β-lactamase-producing *H. influenzae* strain (strain F990) and strain T2494 contained 4.4-kilobase plasmids, which were designated R_{Rob} and pMG301. These plasmids and plasmid pVM105 found in *H. pleuropneumoniae* could be transformed into *E. coli* HB101, in which they were unstable in the absence of continued ampicillin selection. Plasmids R_{Rob} and pMG301 did not provide sulfonamide resistance, unlike pVM105. When the three plasmids were purified and cleaved with various restriction endonucleases, identical fragments were produced from R_{Rob} and pMG301 by *Alu*I (nine fragments), *Sau*3A (seven fragments), and *Taq*I (five fragments), while pVM105 yielded nine, seven, and eight fragments, respectively, with these enzymes (Fig. 2a). Many fragments produced from pVM105 were identical in size to the fragments produced from R_{Rob} and pMG301, suggesting a close relationship. When plasmid R_{Rob} was labeled with ^{32}P by nick translation and hybridized with the fragments produced from pMG301 and pVM105 by using *Alu*I, multiple homologous segments were evident (Fig. 2b). Hence, R_{Rob} and pMG301 appeared to be identical, while pVM105 had many DNA sequences in common but had additional restriction fragments that were presumably related to the extra sulfonamide resistance which it carried.

The ROB-1 β-lactamase gene was cloned on a 2-kilobase fragment produced by partial *Sau*3A digestion of R_{Rob} into the *Bam*HI site of pACYC184 to yield plasmid pMON401.

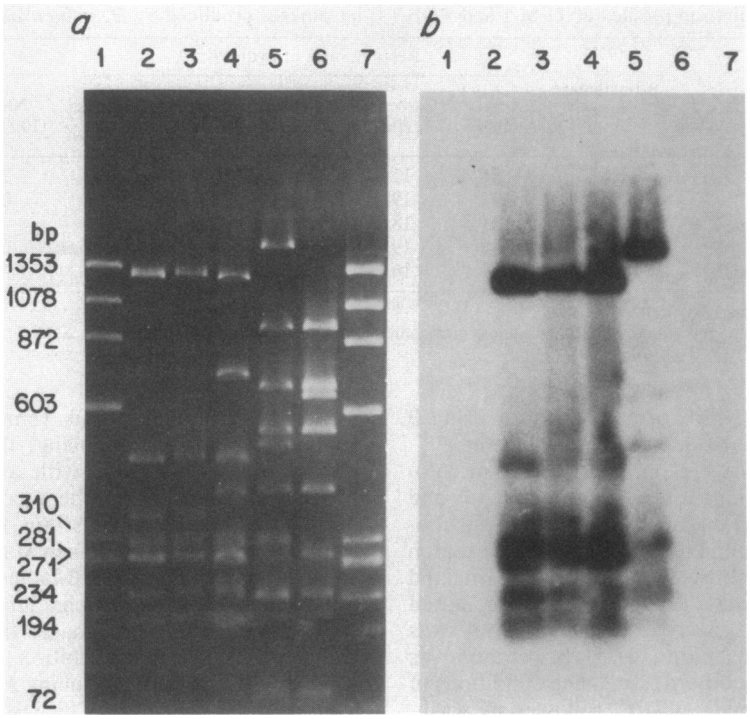

FIG. 2. Comparison of ROB-1-producing plasmids. (a) Agarose gel electrophoresis of fragments produced by *Alu*I digestion from R_{Rob} (lane 2), pMG301 (lane 3), pVM105 (lane 4), pMON401 (lane 5), and pACYC184 (lane 6). Lanes 1 and 7 contained marker *Hae*III-digested φX174 replicative-form DNA (sizes indicated on the left). bp, Base pairs. (b) Autoradiograph of a Southern blot of the same gel probed with ^{32}P-labeled R_{Rob}.

*Alu*I digests of the recombinant plasmid and the fragments homologous to R_{Rob} are also shown in Fig. 2. No sites for endonucleases *Bgl*I, *Hin*cII, and *Pst*I were detected in the cloned ROB-1 gene. These enzymes cleave the TEM-1 β-lactamase gene (5). Furthermore, a TEM-1 gene-specific probe produced by *Bgl*I-*Hin*cII digestion of pBR322 (5) did not hybridize with R_{Rob}. Consequently, although TEM-1 and ROB-1 had similar substrate profiles, they must differ considerably in DNA sequence.

DISCUSSION

The ROB-1 β-lactamase appears to be responsible for ampicillin resistance in *H. pleuropneumoniae* strains which cause disease in pigs and also is an unusual cause of ampicillin resistance in *H. influenzae* strains which produce meningitis in humans. Recently, Daum et al. (R. Daum, M. Willard, and M. Murphey-Corb, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 991, 1984) reported that plasmids in four ampicillin-resistant *H. influenzae* strains from New Orleans, La., were homologous to R_{Rob}, suggesting that in some regions ROB-1 β-lactamase production may be more common than is presently appreciated. Whatever the geographical origin of the ROB-1-producing *H. influenzae* strains, the plasmids which they contain seem to be strikingly similar.

Other animal pathogens may also produce the ROB-1 β-lactamase. An ampicillin-resistant strain of *Pseudomonas multocida* isolated from a pig was recently examined and found to produce a β-lactamase that appeared, as determined by isoelectric focusing, to be identical to ROB-1 (D. Weber, J. Wolfson, and A. A. Medeiros, unpublished data). More ampicillin-resistant pathogens will need to be examined in order to establish the prevalence of this β-lactamase type in the floras of various animal species.

The available information about the Maryland and Tennessee cases of meningitis due to *H. influenzae* indicated no direct exposure to pigs or other farm animals, but the potential for spread of this form of ampicillin resistance from an animal reservoir to human pathogens and the role of feed additives (8) in promoting ampicillin resistance in *H. pleuropneumoniae* deserve further investigation.

ACKNOWLEDGMENTS

We thank D. C. Hirsh, M. C. Libal, and L. G. Rubin for strains and information.

This work was supported by a fellowship from the Medical Research Council of Canada to R.L. and by grants from the National Science Foundation and National Institute of Allergy and Infectious Diseases to G.A.J.

LITERATURE CITED

1. **Bolivar, F., and K. Backman.** 1979. Plasmids of *Escherichia coli* as cloning vectors. Methods Enzymol. **68**:245–267.
2. **Centers for Disease Control.** 1984. Ampicillin and chloramphenicol resistance in systemic *Haemophilus influenzae* disease. Morbid. Mortal. Weekly Rep. **33**:35–37.
3. **Clewell, D. B., and D. R. Helinski.** 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. USA **62**:1159–1166.
4. **Hedges, R. W., A. A. Medeiros, M. Cohenford, and G. A. Jacoby.** 1985. Genetic and biochemical properties of AER-1, a novel carbenicillin-hydrolyzing β-lactamase from *Aeromonas hydrophila*. Antimicrob. Agents Chemother. **27**:479–484.
5. **Heffron, F., B. J. McCarthy, H. Ohtsubo, and E. Ohtsubo.** 1979. DNA sequence analysis of the transposon Tn3: three genes and

three sites involved in transposition of Tn3. Cell **18**:1153–1163.
6. **Hirsh, D. C., L. D. Martin, and M. C. Libal.** 1982. Plasmid-mediated antimicrobial resistance in *Haemophilus pleuropneumoniae*. Am. J. Vet. Res. **43**:269–272.
7. **Kilian, M.** 1985. *Haemophilus*, p. 387–392 *In* E. H. Lennette, A. Balows, W. J. Hauser, Jr., and H. J. Shadomy (ed.), Manual of clinical microbiology. American Society for Microbiology, Washington, D.C.
8. **Levy, S. B.** 1984. Playing antibiotic pool: time to tally the score. N. Engl. J. Med. **311**:663–665.
9. **Libal, M. C.** 1982. Antimicrobial sensitivity patterns of *Haemophilus pleuropneumoniae* isolates from pigs with pneumonia. J. Am. Vet. Med. Assoc. **180**:399.
10. **Maniatis, T., E. F. Firsch, and J. Sambrook.** 1982. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Matthew, M., A. M. Harris, M. J. Marshall, and G. W. Ross.** 1975. The use of analytical isoelectric focusing for detection and identification of β-lactamases. J. Gen. Microbiol. **88**:169–178.
12. **Matthew, M., and R. W. Hedges.** 1976. Analytical isoelectric focusing of R-factor-determined β-lactamases: correlation with plasmid compatibility. J. Bacteriol. **125**:713–718.
13. **Medeiros, A. A.** 1984. β-lactamase. Br. Med. Bull. **40**:18–27.
14. **Medeiros, A. A., R. W. Hedges, and G. A. Jacoby.** 1982. Spread of a "*Pseudomonas*-specific" β-lactamase to plasmids of enterobacteria. J. Bacteriol. **149**:700–707.
15. **Medeiros, A. A., and T. F. O'Brien.** 1975. Ampicillin resistant *Haemophilus influenzae* type b possessing a TEM-type β-lactamase but little permeability barrier to ampicillin. Lancet **i**:716–718.
16. **O'Callaghan, C. H., A. Morris, S. M. Kirby, and A. H. Shingler.** 1972. Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. **1**:283–288.
17. **Rubin, L. G., A. A. Medeiros, R. H. Yolken, and E. R. Moxon.** 1981. Ampicillin treatment failure of apparently β-lactamase-negative *Haemophilus influenzae* type b meningitis due to novel β-lactamase. Lancet **ii**:1008–1010.
18. **Shope, R. E.** 1964. Porcine contagious pleuropneumonia. I. Experimental transmission, etiology, and pathology. J. Exp. Med. **119**:357–368.
19. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.