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Molecular Epidemiology of Ampicillin-Resistant Non-β-Lactamase-Producing *Haemophilus influenzae*

LOUISE GAZAGNE,[1] CATHERINE DELMAS,[1] EDOUARD BINGEN,[2] AND HENRI DABERNAT[1]*

Laboratoire de Microbiologie, Centre Hospitalier Universitaire Purpan, Toulouse,[1] and Laboratoire de Microbiologie, Centre Hospitalier Universitaire R. Debré, Paris,[2] France

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Resistance to ampicillin without β-lactamase production is not a frequent occurrence among *Haemophilus influenzae* strains. This kind of resistance is encountered in unencapsulated strains isolated from bronchial secretions and ear, nose, and throat specimens and is exceptional in *H. influenzae* type b. We studied 29 of these strains from various areas in France and 2 reference strains. Strains were compared by using ribotyping, arbitarily primed PCR with two primers, and pulsed-field gel electrophoresis. Each technique enabled the identification of 20 to 23 different patterns among the 31 strains. The combination of the different patterns for the strains obtained by the different techniques provided 27 distinct profiles. According to these results, it seems that the clonal propagation of these resistant strains does not occur.

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The resistance to antimicrobial agents in *Haemophilus influenzae* has evolved significantly during the last 20 years. This resistance is essentially to β-lactam antibiotics as a result of β-lactamase production. The β-lactamase involved is frequently of the TEM type and is more rarely of the ROB type (9, 31, 33). About 50% of the strains isolated from patients with meningitis in France produce β-lactamase (8, 9).

In *H. influenzae* strains, resistance to β-lactam antibiotic can also result from the alteration of the antibiotic target: penicillin-binding protein (5, 17, 18, 21, 24, 27, 28, 32). This resistance mode is less frequent. It generally occurs among nonencapsulated strains from nonsystemic specimens (3, 9, 19, 20, 29, 35, 36).

Ampicillin-resistant non-β-lactamase-producing strains (AMP^r β−) are more difficult to detect because of their low incidence rate. The usual disk diffusion method with a 10- or 25-μg amoxicillin disk does not permit the detection of this mode of resistance. Detection of this kind of resistance (diameter of inhibition, <20 mm, corresponding to an MIC of ≥2 mg/liter) is obtained with a 2-μg ampicillin disk. In France these disks are rarely used because this infrequent mode of resistance is often disregarded. Epidemiological studies with these types of strains are rare (23). We have studied clinical AMP^r β− *H. influenzae* isolates using molecular biology tools. This allowed us to compare these strains and look for a possible clonal diffusion.

MATERIALS AND METHODS

Bacterial strains. In France, *H. influenzae* strains are sent to the National Center on *H. influenzae* for different reasons: generally for active surveillance for microbiology laboratories, sometimes for a problem with identification, or when this bacterium is isolated from cerebrospinal fluid. Between 1987 and 1994 about 3,000 strains were sent to the National Center, and 29 of these strains were AMP^r β−. They came from different French metropolitan areas. They have been isolated from various clinical specimens: purulent bronchial secretions; specimens from patients with otitis media, sinusitis, and conjunctivitis; and rhinopharyngeal specimens. The strains have been identified by typical Gram staining appearance, the shapes of the colonies, and their requirements for X factor and V factor. The characteristics of these strains are presented in Table 1. Biotyping was determined by the method of Kilian (15a). Capsular serotyping was performed by slide agglutination with specific antisera (antisera a to f; Difco); all strains were nontypeable. By an agar dilution method with *Haemophilus* Test Medium (Unipath), the MICs of ampicillin, cefaclor, and cefotaxime were determined. If isolates did not produce β-lactamase according to the results of a chromogenic cephalosporin test (Nitrocefin; Cefinase; Biomerieux, Marcy l'Etoile, France), if the 2-μg ampicillin disk diffusion test (Becton Dickinson) gave a zone of inhibition of <20 mm, and if the MIC of ampicillin was ≥2 mg/liter, strains were considered AMP^r β−. We simultaneously studied two quality control strains: the ampicillin-susceptible strain *H. influenzae* ATCC 49766 and the tetracycline-resistant and AMP^r β− strain *H. influenzae* ATCC 49247.

During a period of 2 years, four strains, strains 4, 22, 23, and 31, were isolated from the same child with cystic fibrosis. Two pairs of strains were isolated from two children either at the same time (strains 28 and 29) or at the beginning and the end of an antibiotic treatment (strains 26 and 27).

Methods. (i) Ribotyping (RT). Genomic DNA was extracted by the method of Picard-Pasquier et al. (28a). We used the restriction endonuclease *Eco*RI (Boehringer Mannheim). After electrophoresis, we transferred the DNA to a nylon membrane (HYBOND-N; Amersham) by the Southern blotting method. Hybridization was performed with an *Escherichia coli* DNA probe complementary to 16S-23S RNA, and the probe was labelled with digoxigenin. Hybridization signals were detected with the DIG DNA Labelling and Detection Kit (Boehringer Mannheim).

Ribotypes were considered identical only if all bands in their patterns were of the same number and size.

(ii) AP-PCR. Genomic DNA was extracted by the method of Picard-Pasquier et al. (28a). We amplified the DNA with two oligonucleotide primers, 21762 (5′→3′ gCC CCC Agg ggC ACA gT; Genset) and RapIV (5′→3′ TCA CgA TgC A; Genset). Amplification was carried out with a Perkin-Elmer DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, Conn.). Randomly amplified products were separated by electrophoresis and were visualized with ethidium bromide.

The patterns generated by arbitrarily primed PCR (AP-PCR) were considered identical on the basis of similar numbers and the matching positions of all bands. With this in mind, specific patterns were established on the basis of a single band difference.

(iii) Pulsed-field gel electrophoresis (PFGE). After the colonies were grown, washed, and centrifuged, agarose plugs were made from a 1-1 mixture of 2% low-melting-point agarose and the cell suspension. The plugs were lysed with proteinase K (Boehringer Mannheim) overnight and were then washed with Tris-EDTA buffer. After three washes the agarose plugs were incubated with the restriction enzyme *Sma*I (Gibco BRL). The resulting DNA fragments were subjected to field inversion gel electrophoresis, and then the bands were visualized with ethidium bromide.

Strains with one band shift were considered to represent unique strains.

Software. The results were analyzed with Taxotron software (P. A. D. Grimont, Institut Pasteur, 1994). The patterns were obtained after transfer of the data into Mac Draw Pro.

* Corresponding author. Mailing address: Laboratoire de Microbiologie, Hôpital PURPAN, Place du Dr. Baylac, 31059 Toulouse Cedex, France. Phone: 33-5 61 77 23 57. Fax: 33-5 61 77.23 33. E-mail: dabernat.h@chu-toulouse.fr.

TABLE 1. Characteristics of AMP[r] β− *H. influenzae*: patterns obtained by different molecular biology techniques[a]

Strain	Area[b]	Age	Specimen, specimen source, or illness	Biotype	Resistance phenotype	AMP MIC (mg/liter)	Pattern by the following technique:				Roman numeral code
							RT	AP PCR (217δ2)	AP PCR (RapIV)	PFGE	
1	ATCC 49247			III	AMP T	2–4	A	a	α	*a*	I
2	ATCC 49766			II		0.25	B	b	β	*b*	II
3	31	55 yr	Br sec	I	AMP	4	C	c	χ	*c*	III
4	33	18 yr	Br sec CF	I	AMP RA	8	D	d	δ	*d*	IV
5	33	5 yr	Br sec CF	I	AMP RA	8	E	e	ε	*d*	V
6	44	70 yr	Sinus	II	AMP	8	F	f	ϕ	*e*	VI
7	13	11 yr	Conj	III	AMP RA TMP	2	A	a	α	*a*	I
8	31	29 yr	Br sec	II	AMP TMP	4	G	g	γ	*f*	VII
9	44	13 yr	Sinus CF	VII	AMP TMP	2	H	h	η	*g*	VIII
10	NK	NK	Br sec	III	AMP TMP	2	I	i	ι	*h*	IX
11	31	12 yr	Br sec	II	AMP TMP	2	B	b	φ	*b*	X
12	44	53 yr	Br sec	I	AMP	2	J	j	κ	*i*	XI
13	44	NK	Br sec	II	AMP	2	K	k	λ	*j*	XII
14	33	6 yr	Br sec	III	AMP	2	L	l	φ	*k*	XIII
15	91	<6 yr	Rhino	III	AMP	2	A	a	α	*l*	XIV
16	75	<6 yr	Rhino	II	AMP TMP	2	M	m	μ	*m*	XV
17	13	<6 yr	Rhino	III	AMP	2	N	n	ν	*n*	XVI
18	95	<6 yr	Rhino	III	AMP	2	O	a	ο	*o*	XVII
19	45	<6 yr	Rhino	III	AMP	2	A	o	α	*p*	XVIII
20	60	<6 yr	Rhino	II	AMP TMP	2	P	p	π	*q*	XIX
21	16	<6 yr	Rhino	V	AMP K	2	Q	q	θ	*r*	XX
22	33	18 yr	Br sec CF	I	AMP RA	8	R	r	ρ	*d*	XXI
23	33	18 yr	Br sec CF	I	AMP RA	4	S	q	ε	*d*	XXII
24	72	4 yr	Otitis	III	AMP	2	A	a	α	*a*	I
25	31	32 yr	Conj	IV	AMP	2	A	a	α	*a*	I
26	57	9 mo	Rhino	III	AMP TMP	2	T	s	σ	*k*	XXIII
27	57	9 mo	Rhino	III	AMP TMP	2	T	s	σ	*k*	XXIII
28	44	2 yr	Conj	III	AMP	2	U	t	τ	*s*	XXIV
29	44	2 yr	Rhino	III	AMP	2	U	t	υ	*s*	XXV
30	44	NK	Sinus	VII	AMP TMP	4	V	u	ϖ	*t*	XXVI
31	33	17 yr	Br sec CF	I	AMP RA	8	W	r	ω	*d*	XXVII
Total no. of patterns							23	21	23	20	27

[a] Abbreviations: AMP, ampicillin; T, tetracycline; K, kanamycin; RA, rifampin; TMP, trimethoprim; Br sec, bronchial secretions; CF, cystic fibrosis; Rhino, rhinopharynx; Conj, conjunctivitis; NT, nontypeable; NK, not known.
[b] Geographic origin by French department number.

RESULTS

Ampicillin MICs were ≥2 mg/liter for all strains. The cefotaxime MICs were between 0.015 and 0.25 mg/ml, and the cefaclor MICs were between 1 and 32 mg/ml.

All *H. influenzae* strains were typeable by RT, PFGE, and AP-PCR. The DNA profiles of the same strain generated by each of the three molecular techniques were found to be stable and reproducible on two or more separate occasions.

RT. The RT patterns obtained with *Eco*RI had 10 to 13 bands ranging between 1 and 15 kb (Fig. 1). The output repeatedly contained two bands of between 1 and 2 kb.

Among the 29 strains of *H. influenzae*, digestion with *Eco*RI, gave 23 RT patterns.

AP-PCR. AP-PCR with two different primers, 217δ2 and RapIV (Fig. 2 and 3, respectively), gave 21 and 23 independent patterns, respectively. Both primers yielded 2 to 11 amplified products ranging in size from 0.2 to 2 kb.

The 21 different patterns obtained with primer 217δ2 (Fig. 2) are very heterogeneous; nevertheless, several groups could be identified.

The 23 patterns obtained with RapIV (Fig. 3) were extremely varied. Among the 31 strains, 12 were weakly linked.

PFGE. PFGE with *Sma*I restriction endonuclease digestion generated 20 unique DNA fragment patterns (Fig. 4).

The patterns were easy to interpret, with 5 to 10 bands in each one.

Using these different techniques, we spotted several groups of strains, as described Table 2.

Overall analysis. We assigned a letter to each different profile obtained by each technique. We obtained a code of four letters. We assigned a Roman numeral to each different code. When strains had the same Roman numeral, they had the same profile as determined by the four techniques. Twenty-seven different profiles were identified. One group consists of four strains (strains 1, 7, 24, and 25), and another group consists of two strains (strains 26 and 27). All the other strains had a unique code.

DISCUSSION

H. influenzae AMP[r] β− strains are not frequently encountered. According to the accepted limit of the MIC, their incidence varies. The frequencies of occurrence of resistant strains are <1 to 2.5% (MICs, ≥2 mg/liter) in Canada and the United States (1, 2, 5, 10, 11, 34), 5 to 7% (MICs, ≥1 mg/liter) in the United Kingdom (29–31), <1 to 7% (MICs, ≥1 or ≥2 mg/liter) in Australia (3, 6), 13% (MICs, ≥1 mg/liter) in Greece (14), 1% (MICs, ≥2 mg/liter) in France (7), and 0.3% (MICs, ≥4 mg/liter) in an international study (15). This variability in the incidence of AMP[r] β− strains illustrates the difficulties encountered in highlighting this kind of resistance. A 20-μg ampicillin disk cannot detect AMP[r] β− *H. influenzae* strains. An inhibition zone diameter of <20 mm obtained with a 2-μg

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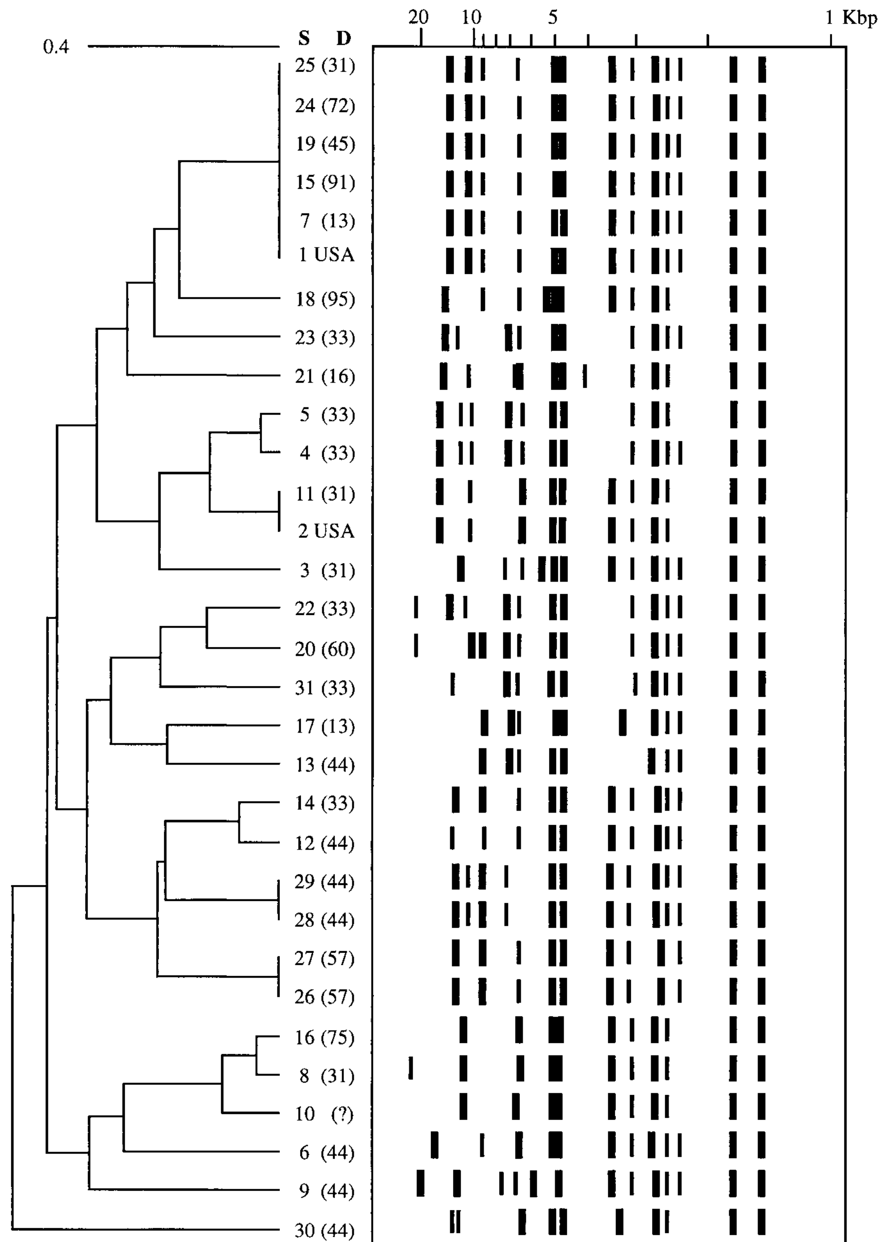

FIG. 1. Patterns for AMP^r β− *H. influenzae* strains obtained by RT. The dendrogram and RT patterns of *Eco*RI-restricted *H. influenzae* DNA were analyzed with Taxotron software. Strains 1 to 31 are described in Table 1. S, strains; D, areas.

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ampicillin disk would indicate the need to measure the ampicillin MIC.

Although the MICs of other β-lactam antibiotics were increased (2, 13, 22), the clinical consequences of these types of strains did not seem to be very important. Three infections with AMP^r β− *H. influenzae* were described. Two cases, a case of endocarditis (18) and a case of septicemia (27), were due to a serotype b strain; one case (a case of meningitis) was caused by a nonserotypeable strain (22).

Studies of the epidemiology of nontypeable strains prove the important heterogenieity of these strains (4, 12, 16, 26). This heterogenieity is the same as that in AMP^r β− *H. influenzae* strains, and Mendelman et al. (23) have deduced that this resistance originated long ago. Our results agree with their conclusions. The four techniques that we used gave 27 different profiles for 31 strains.

In our study, using these different techniques we spotted several groups of strains. The most important was made up of strains 1 (ATCC 49247), 7, 15, 24, 25, and ±19. These strains came from various areas, and their resistance to antibiotics was heterogeneous. Five strains were biotype III, and one was biotype IV. Their only similarity was their biotype, and biotype III is very common among nontypeable *H. influenzae* strains.

The second group was consisted of two strains that exhibited the same biotype. Strain 11 was paired with strain 2 (ATCC 49766) by all methods except AP-PCR with primer RapIV; by this technique strain 11 was identical to strain 14, but their antibiotic susceptibilities, origins, and biotypes were different.

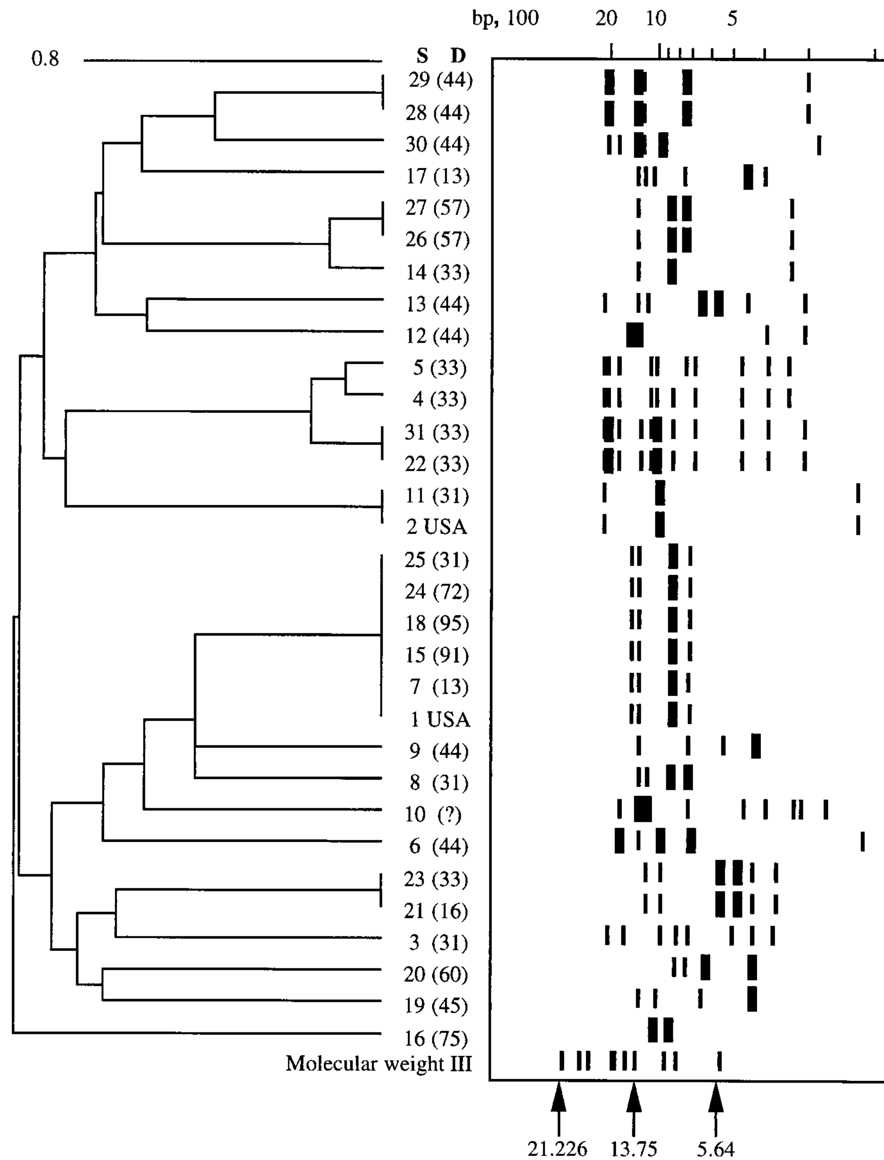

FIG. 2. Patterns for AMP[r] β− *H. influenzae* strains obtained by AP-PCR with primer 217δ2. The dendrogram and AP-PCR profiles obtained with primer 217δ2 of *H. influenzae* were analyzed with Taxotron software. Strains 1 to 31 are described in Table 1. The Molecular Weight III marker was from Boehringer Mannheim. S, strains; D, areas.

It seems that the different patterns obtained did not permit us to make a deduction about the resistance of strain 11 (ATCC 49766 is ampicillin susceptible).

The third group consisted of strains 26, 27, and ±14. Strains 26 and 27 were isolated from the rhinopharynx of a 9-month-old child at the beginning and at the end of antibiotic treatment and were absolutely identical. Strain 14 had the same biotype as strains 26 and 27 but it was isolated in a different geographic area and had an antibiotic susceptibility profile different from those of strains 26 and 27.

TABLE 2. Different strain groups obtained by four molecular biology techniques[a]

Strains clustered in the same group according to the results of the following technique[a]:			
RT	AP-PCR (217δ2)	AP-PCR (RapIV)	PFGE
1 (ATCC 49247), 7, 15, 19, 24, 25	1 (ATCC 49247), 7, 15, 18, 24, 25	1 (ATCC 49247), 7, 15, 19, 24, 25	1 (ATCC 49247), 7, 24, 25
2 (ATCC 49766), 11	2 (ATCC 49766), 11	11, 14	2 (ATCC 49766), 11
26, 27	26, 27, ±14 (one band difference)	26, 27	26, 27, 14
28, 29	28, 29	28, ±29 (one band difference)	28, 29
4, ±5 (one band difference)	4, ±5 (one band difference), 22, 31	±4, 5, 23 (one band difference)	4, 5, 22, 23, 31

[a] ±, the strain shared only some characteristics with the other strains in the group.

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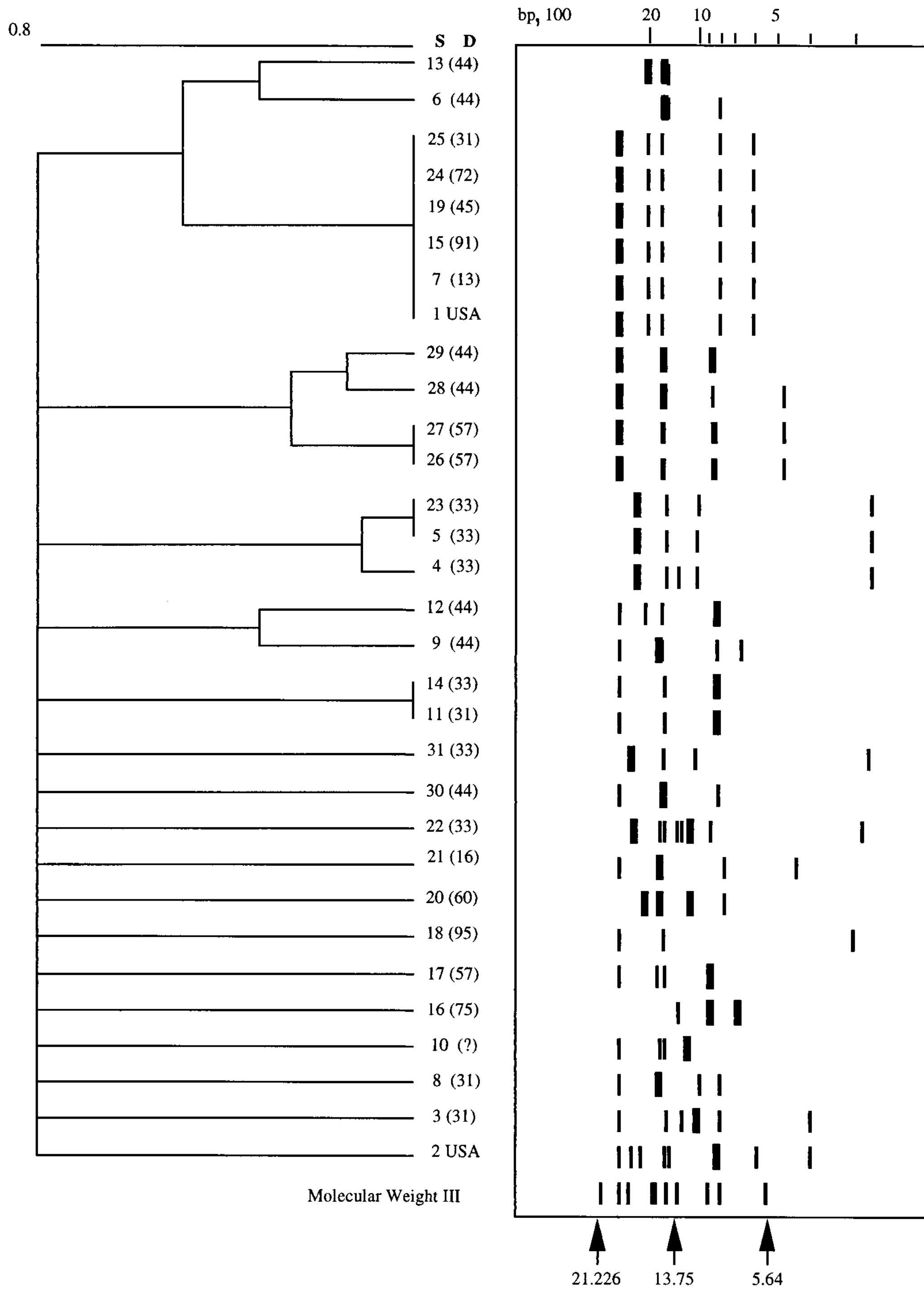

FIG. 3. Patterns for AMP^r β− *H. influenzae* strains obtained by AP-PCR with primer RapIV. The dendrogram and AP-PCR profiles obtained with primer RapIV of *H. influenzae* were analyzed with Taxotron software. Strains 1 to 31 are described in Table 1. The Molecular Weight III marker was from Boehringer Mannheim. S, strains; D, areas.

The fourth group consisted of a couple of identical strains (strains 28 and 29) simultaneously isolated from the eye and the rhinopharynx, respectively, of a 2-year-old child. The strains were identical by all techniques except AP-PCR with primer RapIV (one band difference). We cannot explain this result.

The fifth group consisted of strains that came from the same hospital (strains 4, 5, ±22, ±23, and ±31). All five strains had the same antibiotic susceptibility profiles and biotypes and were isolated from two patients with cystic fibrosis. Four of the strains (strains 4, 22, 23, and 31) came from an 18-year-old patient and were isolated over a period of 2 years. By our methods, these four strains had similar profiles, permitting us to presume a persistent infection with the same AMP^r β− *H. influenzae* strain. Nevertheless, this strain varied a little during this period. The other strain, strain 5, came from a 5-year-old child with cystic fibrosis and was linked to the four

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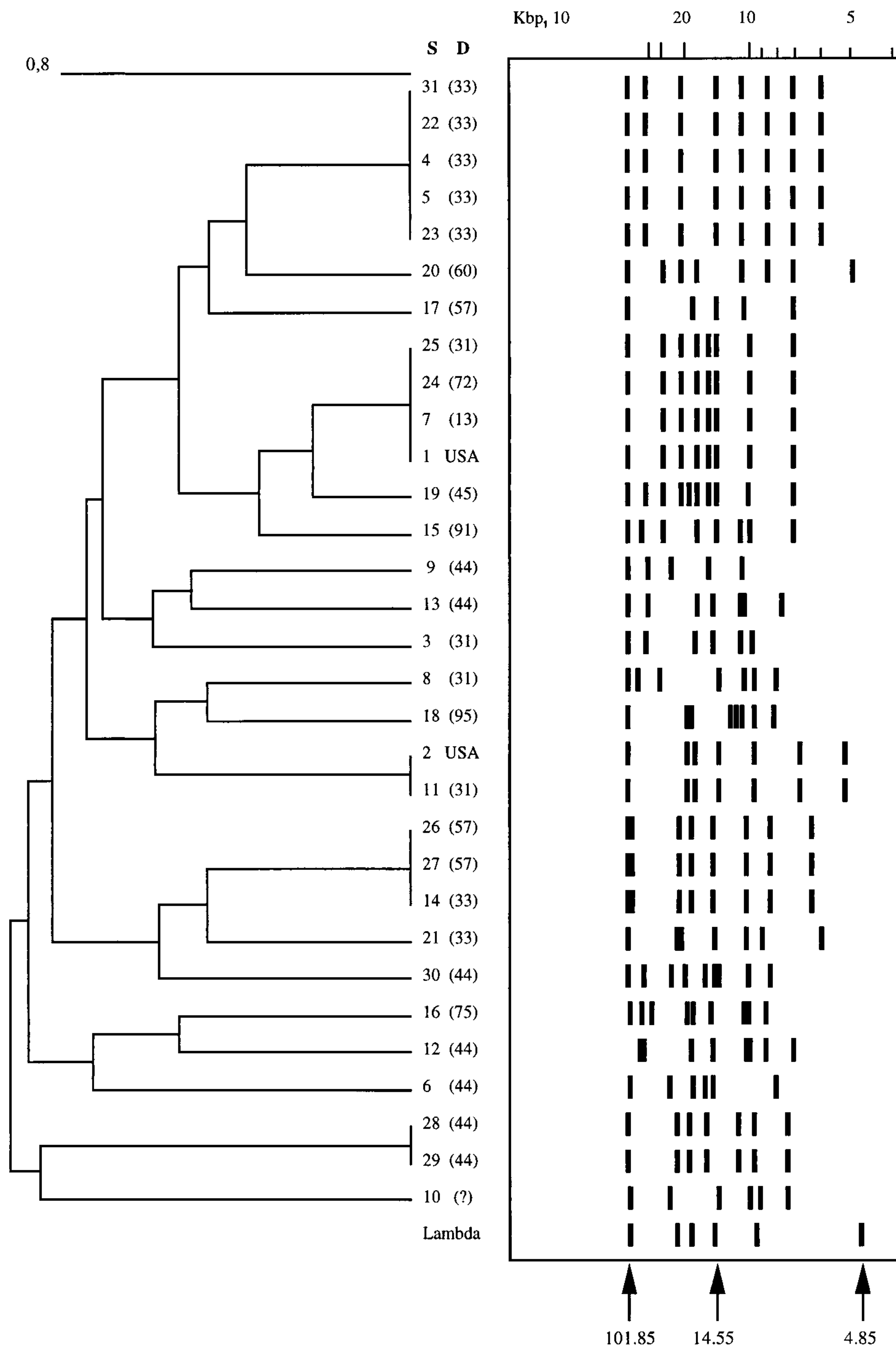

FIG. 4. Patterns for AMP^r β− *H. influenzae* strains obtained by PFGE. The dendrogram and PFGE profiles of *Sma*I-digested DNA of *H. influenzae* were analyzed with Taxotron software. Strains 1 to 31 are described in Table 1. The bacteriophage lambda Ladder PFG Marker was obtained from Biolabs. S, strains; D, areas.

previous strains according to its profile. A horizontal transmission of this strain seems possible.

The results obtained by the molecular biology techniques that we used were reproducible, and all techniques gave the same number of patterns. However, our impression is that AP-PCR and PFGE (this technique showed that the four strains from the same cystic fibrosis patient were identical) are more adaptable than RT to epidemiological studies of *H. influenzae*. Indeed, RT is more fastidious and time-consuming than AP-PCR and PFGE.

By four molecular techniques, we highlighted 27 different profiles among the 31 strains tested. The important number of patterns obtained by these methods, unlike the numbers of patterns obtained for multidrug-resistant *Streptococcus pneumoniae* strains (25), allowed us to eliminate the hypothesis of the clonal propagation of AMP^r β− *H. influenzae* strains in this study.

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Few studies on the virulence of AMP^r β− *H. influenzae* strains have been published. It seemed important to clarify the place of this type of *H. influenzae* in pathology and to suggest a consensus addressing the possible problem of failure of treatment. Moreover, a longer epidemiological study to specify their origins and their diffusion modes should be considered.

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