Characterization of *Bordetella* Strains and Related Bacteria by Amplified Ribosomal DNA Restriction Analysis and Randomly and Repetitive Element-Primed PCR

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Amplified ribosomal DNA restriction analysis (ARDRA) in which the almost complete 16S rRNA gene was used as the target of the PCR assay and randomly and repetitive element-primed PCR were used to characterize 67 strains belonging to the family *Alcaligenaceae*. Particular emphasis was placed on strains of *Bordetella pertussis* (13 strains), *Bordetella parapertussis* (10 strains), and *Bordetella bronchiseptica* (10 strains) (these organisms were referred to as the *B. bronchiseptica* group), as well as *Bordetella avium* (19 strains). Our data indicate that strains belonging to the *B. bronchiseptica* group behave as a single species comparable to strains of *B. avium*. ARDRA allowed us to differentiate among all other species as well but was not useful for infraspecific typing. Randomly and repetitive element-primed PCR could be used to distinguish several infraspecific types within *B. avium* and the *B. bronchiseptica* group and allowed us to differentiate these two taxa.

A multitude of DNA-based typing methods are gradually replacing or augmenting classical typing methods (13, 17). Most of these methods are based on specific or random amplification of DNA fragments, digestion of genomic or amplified DNA with restriction enzymes, or both. The majority of these methods were developed primarily to generate highly specific (if possible, strain-specific) fingerprints. Some, however, have proved to be useful for both species identification and strain characterization.

In the present study, we examined the discriminatory abilities of two of these genome-orientated methods for strains belonging to the family *Alcaligenaceae*, particularly *Bordetella pertussis*, *Bordetella parapertussis*, *Bordetella bronchiseptica*, and *Bordetella avium* strains. The population structure of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* is considered clonal (14, 15), and the taxonomic status of these taxa has been the subject of debate, as they exhibit greater than 80% DNA-DNA binding despite considerable phenotypic and chemotaxonomic differences (11, 15, 18, 21, 24). We selected about 10 strains of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* and compared the results of genomic typing analyses with the results obtained for 19 *B. avium* strains. Reference strains of all other *Bordetella* and *Alcaligenes* species were included for comparison.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All of the strains studied and their sources are listed in Table 1. The majority of these strains are reference strains which were included in several polyphasic taxonomic studies (9, 18, 20, 21). Five *B. pertussis* isolates from a recent outbreak in Cincinnati, Ohio (3) (isolates kindly provided by S. Reising, Children's Hospital Medical Center, Cincinnati, Ohio), and eight sporadic *B. pertussis* isolates were included. Sporadic isolates of *B. parapertussis*, *B. bronchiseptica*, and *B. avium* were also included. The *B. bronchiseptica* and *B. avium* strains were from a wide diversity of hosts; the *B. bronchiseptica* hosts included humans (4 strains), dogs (2 strains), a ferret, a guinea pig, a turkey, and a rabbit, and the *B. avium* hosts included turkeys (12 strains isolated in five countries), chickens (6 strains), and a duck.

Bacteriological purity was checked by plating the organisms and examining

living cells by phase-contrast microscopy and Gram-stained cells. *B. pertussis* strains were grown on charcoal agar (Oxoid, Basingstoke, United Kingdom) supplemented with 10% horse blood. All other strains were grown on Trypticase soy agar (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.) and were incubated aerobically at 36 to 37°C.

Preparation of DNA. Extraction of DNA for PCR analysis was performed as described by Pitcher et al. (16) or by a slightly modified procedure, as described previously (8).

Thermocycler. A Perkin-Elmer model 9600 thermocycler was used for all PCR.

Amplified rDNA restriction analysis (ARDRA). Amplification of the 16S rRNA genes, restriction digestion of the amplified 16S ribosomal DNA (rDNA) with the enzymes *Hae*III (Boehringer, Mannheim, Germany), *Hha*I (Pharmacia, Biotech Benelux, Roosendaal, The Netherlands), *Alu*I (Pharmacia), *Taq*I, and *Bst*UI (New England Biolabs; purchased from Westburg, Leusden, The Netherlands), and agarose gel electrophoresis were performed as described previously (8).

The gels were scanned under UV illumination with a Foto/Analyst Visionary digital documentation system (Fotodyne, Hartland, Wis.) and were digitized with an IRIS video digitizer card and Colorvision software (Inside Technology, Amersfoort, The Netherlands). An *Alu*I (Pharmacia) digest of plasmid pBR322 and a 128-bp fragment amplified from plasmid pGEM11Zf with phage promoter T7 and SP6 were used as normalization references (8). For each strain, the normalized restriction patterns obtained with the five restriction enzymes were assembled one after the other in the order *Hae*III-*Hha*I-*Alu*I-*Taq*I-*Bst*UI into a combined profile and were analyzed by using the Dice similarity coefficient and the unweighted pair group with mathematical average clustering algorithm.

Randomly and repetitive element-primed PCR. DNA was amplified by using 10 random primers (five 17-mers [primers D9355, D14216, D11344, D8635, and D14307] and five 10-mers [primers 1254, 1247, 1281, 1283, and 1290]) (1), repetitive primers ERIC1R and ERIC2, and the primer pair ERIC1R-ERIC2 (22). Each PCR was performed in a 100- μ l mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 2.5 mM MgCl₂, 0.1% (vol/vol) Triton X-100, 0.01% (wt/vol) gelatin, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 50 or 100 pmol of primer, 0.6 U of Goldstar polymerase (Eurogentec, Seraing, Belgium), and 100 ng of template DNA. The thermal cycling conditions used for the random primers were the conditions described by Akopyanz et al. (1); the thermal cycling conditions used for primers ERIC1R and ERIC2 have been described previously (19).

The gels were scanned under UV illumination, visualized, and digitized with the Gel Doc 1000 documentation system (Bio-Rad Laboratories, Nazareth, Belgium). The DNA band pattern of *Enterococcus faecium* LAB 1265 generated with random primer D14307 was included on each gel as a standard for normalization. The Pearson product moment correlation coefficient was used to calculate similarity values, and a cluster analysis was performed by using the unweighted pair group with mathematical average clustering algorithm.

Pattern analysis. Conversion, normalization, and numerical analysis of the DNA band patterns were performed with the GelCompar software package (Applied Maths, Kortrijk, Belgium).

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TABLE 1. Strains examined

Strain ^a	Other designation ^a	Source ^b
<i>Alcaligenes denitrificans</i> LMG 1231 ^T	ATCC 15173 ^T	Soil
<i>Alcaligenes denitrificans</i> LMG 1860	CIP 60.83	Urine (France)
<i>Alcaligenes faecalis</i> LMG 1229 ^T	NCIB 8156 ^T	
<i>Alcaligenes faecalis</i> LMG 1230	CCEB 554	Feces (Czechoslovakia)
<i>Alcaligenes piechaudii</i> LMG 1861	NCMB 1051	Soil
<i>Alcaligenes piechaudii</i> LMG 1873 ^T	CIP 60.75 ^T	Pharynx
<i>Alcaligenes xylosoxidans</i> LMG 1863 ^T	ATCC 27061 ^T	Human, ear discharge (Japan)
<i>Alcaligenes xylosoxidans</i> LMG 1866	ATCC 15749	Soil
<i>Bordetella avium</i> LMG 1851	363-78	Turkey coryza, respiratory tract (Germany)
<i>Bordetella avium</i> LMG 1852 ^T	591-77 ^T	Turkey, air sac (Germany)
<i>Bordetella avium</i> LMG 1854	P-4085	Turkey (United States)
<i>Bordetella avium</i> LMG 1855	XX14	Turkey (Spain)
<i>Bordetella avium</i> LMG 1858	NC	Turkey (United States)
<i>Bordetella avium</i> LMG 3524	946-77	Turkey, lung (Germany)
<i>Bordetella avium</i> LMG 3549	2-81	Turkey (United Kingdom)
<i>Bordetella avium</i> LMG 3550	3-81	Turkey (United Kingdom)
<i>Bordetella avium</i> LMG 3557	298-81	Duck, trachea (Germany)
<i>Bordetella avium</i> LMG 3559	897	Turkey (Israel)
<i>Bordetella avium</i> LMG 3560	1106	Turkey (Israel)
<i>Bordetella avium</i> LMG 3561	XX15	Turkey (Spain)
<i>Bordetella avium</i> LMG 10973	15	Broiler, trachea (Belgium, 1989)
<i>Bordetella avium</i> LMG 10974	16	Chicken (Belgium)
<i>Bordetella avium</i> LMG 10975	17	Broiler, trachea (Belgium, 1988)
<i>Bordetella avium</i> LMG 10976	18	Chicken, spleen (Belgium, 1989)
<i>Bordetella avium</i> LMG 10977	19	Broiler, trachea (Belgium, 1988)
<i>Bordetella avium</i> LMG 10978	20	Broiler, trachea (Belgium, 1988)
<i>Bordetella bronchiseptica</i> LMG 1232 ^T	NCTC 452 ^T	Dog, lung (before 1932)
<i>Bordetella bronchiseptica</i> LMG 1807	NCTC 455	Ferret, trachea (1913)
<i>Bordetella bronchiseptica</i> LMG 1809	NCTC 8751	Guinea pig, lung (before 1951)
<i>Bordetella bronchiseptica</i> LMG 1811	NCTC 10540	Whooping cough (United States, 1949)
<i>Bordetella bronchiseptica</i> LMG 3521	40-81	Turkey, trachea (Germany, before 1981)
<i>Bordetella bronchiseptica</i> LMG 3531	NCTC 458	Sputum (1913)
<i>Bordetella bronchiseptica</i> LMG 3535	CCM 6048	Rabbit (before 1982)
<i>Bordetella bronchiseptica</i> LMG 3538	CCM 6155	Dog (before 1982)
<i>Bordetella bronchiseptica</i> LMG 3542	CCUG 7865	Whooping cough (Sweden, 1979)
<i>Bordetella bronchiseptica</i> LMG 14526	CCUG 31300	Human, pharynx (Sweden)
<i>Bordetella hinzii</i> LMG 13501 ^T	TC58 ^T	Chicken, trachea (Australia)
<i>Bordetella hinzii</i> LMG 14052	TC104	Blood culture, HIV patient ^c
<i>Bordetella holmesii</i> LMG 15945 ^T	CCUG 34073 ^T	Human, blood (United States)
<i>Bordetella holmesii</i> LMG 15946	CCUG 34074	Human, blood (United States)
<i>Bordetella parapertussis</i> LMG 1816	NCTC 7385	Before 1949
<i>Bordetella parapertussis</i> LMG 1818	NCTC 10853	Whooping cough (before 1973)
<i>Bordetella parapertussis</i> LMG 1819	CNCTC Hpp 5/59	Whooping cough (before 1979)
<i>Bordetella parapertussis</i> LMG 1820	NCTC 10520	Whooping cough (before 1968)
<i>Bordetella parapertussis</i> LMG 1825	NCTC 10525	Whooping cough (before 1961)
<i>Bordetella parapertussis</i> LMG 1827	CNCTC Hpp 1/58	Whooping cough (before 1982)
<i>Bordetella parapertussis</i> LMG 1833	NCTC 8250	Whooping cough (before 1953)
<i>Bordetella parapertussis</i> LMG 14449 ^T	CCUG 413 ^T	Whooping cough (before 1940)
<i>Bordetella parapertussis</i> LMG 14450	CCUG 882A	Whooping cough (before 1949)
<i>Bordetella parapertussis</i> LMG 14451	CCUG 4099	Whooping cough (Sweden, 1974)
<i>Bordetella pertussis</i> LMG 14452	CCUG 699	Sweden, 1970
<i>Bordetella pertussis</i> LMG 14453	CCUG 9818	Sweden, 1980
<i>Bordetella pertussis</i> LMG 14454	CCUG 17283	Whooping cough (before 1982)
<i>Bordetella pertussis</i> LMG 14455 ^T	CCUG 30873 ^T	Whooping cough (before 1947)
<i>Bordetella pertussis</i> LMG 14522	CCUG 32523	Human (Sweden, 1994)
<i>Bordetella pertussis</i> LMG 15140	CCUG 13475	Whooping cough (before 1984)
<i>Bordetella pertussis</i> LMG 15141	CCUG 15610	Whooping cough (before 1984)
<i>Bordetella pertussis</i> LMG 15142	CCUG 23702	Whooping cough (Sweden, 1988)
<i>Bordetella pertussis</i> LMG 15585	CHMC 237-731	Whooping cough (United States)
<i>Bordetella pertussis</i> LMG 15586	CHMC 212-422	Whooping cough (United States)
<i>Bordetella pertussis</i> LMG 15587	CHMC 266-1003	Whooping cough (United States)
<i>Bordetella pertussis</i> LMG 15588	CHMC 221-1047	Whooping cough (United States)
<i>Bordetella pertussis</i> LMG 15589	CHMC 244-889	Whooping cough (United States)
<i>Bordetella trematum</i> LMG 13506 ^T	1779 ^T	Human, chronic otitis media (Germany)
<i>Bordetella trematum</i> LMG 14446	CCUG 13902	Leg wound (1980)

^a ATCC, American Type Culture Collection, Rockville, Md.; CCEB, Culture Collection of Entomogenous Bacteria, Department of Insect Pathology, Institute of Entomology, Prague, Czech Republic; CCM, Czech Collection of Microorganisms, Brno, Czech Republic; CCUG, Culture Collection, Department of Clinical Bacteriology, University of Göteborg, Göteborg, Sweden; CIP, Collection bactérienne de l'Institut Pasteur, Paris, France; CNCTC, Czechoslovak National Collection of Type Cultures, Institute of Hygiene and Epidemiology, Prague, Czech Republic; LMG, Laboratorium voor Microbiologie Gent Culture Collection, Universiteit Gent, Ghent, Belgium; NCIB, National Collection of Industrial Bacteria, NCIMB, Ltd., Aberdeen, Scotland, United Kingdom; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdom; CHMC, Children's Hospital Medical Center, Cincinnati, Ohio.

^b If known.

^c HIV, human immunodeficiency virus.

RESULTS

Reproducibility. In general, the level of reproducibility for computer-assisted comparisons of combined ARDRA patterns obtained on different gels was at least 95%. Occasional low values were due to electrophoretic anomalies, and these data were not used in the numerical analysis.

For randomly and repetitive element-primed PCR, reproducibility values greater than 92% were obtained for patterns generated with a single batch of reagents. The use of another brand of DNA polymerase introduced considerable differences in at least some of the patterns. We therefore compared only data which were generated with the same batches of reagents.

ARDRA. In the numerical analysis of the combined ARDRA patterns (Fig. 1), *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* strains formed a single cluster above a similarity level of 97%, which is within the reproducibility range of the method. All *B. avium* strains clustered together above a similarity level of 98%. The *Alcaligenes xylosoxidans* and *Alcaligenes denitrificans* strains (20) formed a single cluster at a similarity level of 97%. The combined ARDRA patterns of *B. avium*, *Bordetella hinzii*, and *Bordetella trematum* were very similar and clustered together at a similarity level of 92%. *Alcaligenes piechaudii*, *Bordetella holmesii*, and *Alcaligenes faecalis* occupied clearly distinct positions on the dendrogram, and the latter organism was the most remote member of the group.

Although the combined pattern analysis in which five restriction enzymes were used revealed high overall levels of similarity between *A. xylosoxidans* and *A. denitrificans* and among *B. avium*, *B. hinzii*, and *B. trematum*, the patterns generated with individual enzymes allowed us to differentiate each of these species. For instance, the *Hae*III digests of *A. denitrificans* and *A. xylosoxidans* strains resulted in clear differentiation on the basis of an additional DNA band (approximate molecular weight, 785 bp) present in the patterns of strains of the former species (Fig. 2). Similarly, *Alu*I digests generated different band patterns for *B. avium*, *B. hinzii*, and *B. trematum* strains (Fig. 2). For rapid identification purposes, digestion of the amplicon with *Alu*I followed by digestion with *Hae*III (for differentiation of *A. denitrificans*, *A. xylosoxidans*, and *B. hinzii*) or *Hha*I (for differentiation of *A. piechaudii* and *B. avium*) could be used to differentiate all of the species (Fig. 2).

Randomly and repetitive element-primed PCR. The suitability of all primers for generating DNA band patterns was evaluated by using a minimal set of strains representing various species. Especially the primer pair ERIC1R-ERIC2 and primers ERIC2, D8635, D9355, and D11344 generated high levels of pattern diversity. The band patterns generated by the other primers were less useful or not useful for at least some of the strains examined. All of the isolates were subsequently characterized by using random primer D8635 and the repetitive primer pair ERIC1R-ERIC2.

With both of the primers examined, some variability was detected among the *B. avium* strains, but the overall DNA amplification patterns were similar and were distinct from the patterns of the other species examined. Comparable variability was found among the *B. bronchiseptica* strains (Fig. 3). *B. pertussis* and *B. parapertussis* strains, however, were each characterized by a single invariable DNA amplification pattern which was similar to the patterns of *B. bronchiseptica* strains (some patterns illustrating the diversity of the DNA amplification patterns are shown in Fig. 3).

Very different DNA amplification patterns were generated when we examined strains belonging to other species, but the numbers of strains included per taxon were too small to con-

clude whether the DNA amplification patterns were species specific (data not shown).

B. pertussis strains were further analyzed by using the remaining 11 primers. Except for strain LMG 14455^T, no differences were detected. Strain LMG 14455^T had a slightly aberrant DNA amplification pattern with some of the primers examined (D9355, D14216, D11344, D14307, and ERIC2).

DISCUSSION

The classification of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* as distinct species is controversial, and the relationship of these taxa is a taxonomic curiosity. As discussed previously, high levels of DNA-DNA binding suggest that all three organisms belong to a single species, but this suggestion is contradicted by the high degree of heterogeneity in their phenotypes (11, 18, 21, 24).

A variety of DNA-based typing methods have been developed during the last decade, and each has its own range of applications. A number of these methods have been shown to be useful for discriminating strains at various taxonomic levels. It was our aim in the present study to compare the genotypic diversity of strains of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* by using different genome-oriented typing methods and to compare the observed heterogeneity with the heterogeneity detected in *B. avium*. The latter species is genotypically and phenotypically homogeneous and differs both in genotype and in phenotype from related *Bordetella* and *Alcaligenes* species (9, 20, 21, 24).

ARDRA. Amplification and digestion of rDNA have been used for identifying and typing a wide variety of bacteria (7, 8). Methodological variability can be introduced by the amplified region selected (16S rDNA, 23S rDNA, and/or spacer region), which has a major impact on the resolution and hence the discriminatory power of the technique. In the present study, the target for the PCR was the almost complete 16S rRNA gene without spacer sequences. This approach has been shown to be useful primarily for species level identification, but not for intraspecific typing (7, 8). ARDRA patterns obtained with five different restriction enzymes were combined, and a numerical comparison was performed by using these combined patterns (Fig. 1). Although the patterns generated by individual enzymes were often useful for differentiating some species, they were never useful for differentiating all of the species. This is illustrated in Fig. 2, in which *Alu*I digests differentiated *B. holmesii* and *B. trematum*, but not *B. avium* and *A. piechaudii* or *A. denitrificans*, *A. xylosoxidans*, and *B. hinzii*. Conversely, numerical analysis of the combined patterns allowed us to distinguish each individual species except *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*, which formed a single homogeneous cluster (Fig. 1). These data indicate that numerical analysis of combined ARDRA patterns is an excellent tool for differentiating species within the family *Alcaligenaceae* and further support the genotypic evidence which indicates that *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* form a single species. The failure to differentiate the latter three species by this approach is not unexpected given the high levels of 16S rDNA sequence similarity (more than 99.7%) between the type strains of these species (24).

Randomly and repetitive element-primed PCR typing. Typing methods based on the amplification of DNA fragments in which random sequences are used as primers in the PCR have been referred to as DNA amplification fingerprinting, arbitrarily primed PCR, or randomly amplified polymorphic DNA analysis (2, 23, 25). Likewise, oligonucleotides complementary to fragments of repetitive elements (repetitive extragenic pal-

FIG. 1. Dendrogram derived from unweighed pair group average linkage of Dice similarity coefficients for the combined ARDRA patterns of all of the strains studied. *Pseudomonas fluorescens* LMG 1799 was used as an outgroup reference strain.

FIG. 2. Normalized computer profiles of the ARDRA patterns of a selection of the strains obtained with different restriction enzymes. Lane M contained the molecular weight marker.

indromic and enterobacterial repetitive intergenic consensus motifs) have also been used as primers in PCR assays (12). Although primarily used for strain differentiation, these PCR-based typing methods have proved to be useful for identification of species belonging to several genera, including the genera *Campylobacter*, *Capnocytophaga*, and *Enterococcus* (4, 6).

In the present study, several primers were useful for differentiating a selection of strains representing various species in the family *Alcaligenaceae*. Two of these primers were selected to examine all of the isolates, and this analysis revealed the presence of different types among the *B. avium* strains (Fig. 3). A comparable heterogeneity was found among the *B. bronchiseptica* strains, but was absent in *B. pertussis* and *B. parapertussis* strains. For each of the latter two species, only a single pattern was generated with the primer pair ERIC1R-ERIC2 and primer D8635. When *B. pertussis* strains were examined with additional primers, only the type strain occasionally differed from the other strains. This aberrant nature of the *B. pertussis* type strain was also demonstrated by whole-cell protein electrophoresis (18). These data indicate that random and repetitive element-based PCR typing can be useful for epidemiological investigations of *B. bronchiseptica* and *B. avium* strains. However, these methods failed to identify a reasonable number of infraspecific types within *B. pertussis* and *B. parapertussis* and therefore seem inadequate for epidemiological

FIG. 3. DNA amplification patterns of a selection of *Bordetella* strains generated by ERIC1R-ERIC2-primed PCR. The molecular weight marker used (lane MWM) comprised DNA bands at (from right to left) 910, 657, 521, 403, 281, 226, 128, and 57 bp.

studies of these species. DNA band patterns generated by these techniques were clearly useful for differentiating *B. avium* strains from strains belonging to the *B. bronchiseptica* group (including *B. pertussis* and *B. parapertussis*) or other *Bordetella* or *Alcaligenes* species. The observed diversity in the reference strains of the remaining species studied did not allow us to draw conclusions concerning the general suitability of the method for species differentiation within the entire family *Alcaligenaceae* (data not shown).

In conclusion, our data substantiate the conclusion that there is little genotypic evidence which justifies the present species status of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*. Strains of these three species, which together were referred to as the *B. bronchiseptica* group, behave as a single species comparable to strains of *B. avium*. The methods which we used did not allow us to identify strains of the *B. bronchiseptica* group at the species level, but clearly separated strains of the *B. bronchiseptica* group from *B. avium* strains, and the ARDRA results could be used to differentiate all or most other members of the family *Alcaligenaceae* (Fig. 1). In contrast to macrorestriction enzyme analysis (10), none of the techniques used in this study readily allowed us to differentiate sporadic isolates of *B. pertussis* and *B. parapertussis*. Our data therefore

suggest that there is considerably less genotypic variability in *B. pertussis* and *B. parapertussis* than in *B. bronchiseptica* and *B. avium*. The results of examinations of large numbers of strains by multilocus enzyme electrophoresis suggest that the *B. bronchiseptica* group can be subdivided on the basis of host specificity, with *B. pertussis*, *B. parapertussis*, and the porcine *B. bronchiseptica* isolates as major lineages (5) (*B. bronchiseptica* strains from other sources represent additional lineages [5, 14, 15]). The DNA fingerprints generated by ERIC1R-ERIC2- and D8635-primed PCR support this hypothesis.

ACKNOWLEDGMENTS

P.V., M.H., and P.D. are indebted to the Fund for Scientific Research - Flanders (Belgium) for positions as postdoctoral fellows (P.V. and M.H.) and a senior research associate (P.D.). K.K. is indebted to the Fund for Medical Scientific Research, Belgium, for research and personnel grants. Part of this research was performed in the framework of contract G. O. A. 91/96-2 with the Ministerie van de Vlaamse Gemeenschap, Bestuur Wetenschappelijk Onderzoek (Belgium).

We thank all depositors of strains listed in Table 1.

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