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Dominique Clermont, Christine Harmant, Chantal Bizet

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Identification of Strains of *Alcaligenes* and *Agrobacterium* by a Polyphasic Approach

DOMINIQUE CLERMONT,* CHRISTINE HARMANT, AND CHANTAL BIZET

Collection de l'Institut Pasteur, 75724 Paris Cédex 15, France

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The number of stable discriminant biochemical characters is limited in the genera *Alcaligenes* and *Agrobacterium*, whose species are consequently difficult to distinguish from one another by conventional tests. Moreover, genomic studies have recently drastically modified the nomenclature of these genera; for example, *Alcaligenes xylosoxidans* was transferred to the genus *Achromobacter* in 1998. Twenty-five strains of *Achromobacter xylosoxidans*, three strains of an *Agrobacterium* sp., five strains of an *Alcaligenes* sp., and four unnamed strains belonging to the Centers for Disease Control and Prevention group IVc-2 were examined. These strains were characterized by conventional tests, including biochemical tests. The assimilation of 99 carbohydrates, organic acids, and amino acids was studied by using Biotype-100 strips, and rRNA gene restriction patterns were obtained with the automated Riboprinter microbial characterization system after cleavage of total DNA with *Eco*RI or *Pst*I restriction endonuclease. This polyphasic approach allowed the two subspecies of *A. xylosoxidans* to be clearly separated. Relationships between five strains and the *Ralstonia paucula* type strain were demonstrated. Likewise, three strains were found to be related to the *Ochrobactrum anthropi* type strain. We showed that substrate assimilation tests and automated ribotyping provide a simple, rapid, and reliable means of identifying *A. xylosoxidans* subspecies and that these two methods can be used as alternative methods to characterize unidentified strains rapidly when discriminant biochemical characters are missing.

The taxonomic position of the genus *Alcaligenes* has been changing for a few years as a result of genomic studies. *Alcaligenes* species have been transferred to the genera *Carbophilus* (14), *Halomonas* (9), *Ralstonia* (18, 20), and *Variovorax* (19). *Alcaligenes xylosoxidans*, *Alcaligenes ruhlandii*, and *Alcaligenes piechaudii* were recently reassigned to the genus *Achromobacter* (21). The subspecies *Achromobacter xylosoxidans* subsp. *denitrificans* has been proposed and the subspecies *Achromobacter xylosoxidans* subsp. *xylosoxidans* was automatically created. Likewise, some *Agrobacterium* species now belong to the genera *Ruegeria* and *Stappia* (17).

A. xylosoxidans was found in aqueous environmental sources and isolated from a wide range of clinical samples. This organism is recognized as an opportunistic pathogen responsible for serious infections (8, 10). Medical equipment and solutions have been found to be contaminated with this organism (8).

Due to the limited number of stable discriminating characteristics, many *Alcaligenes* and *Agrobacterium* species remain difficult to distinguish from one another by conventional tests. Ribotyping was proposed as a taxonomic tool a few years ago and was shown to differentiate genera and species (4, 11, 13, 15, 16). More recently, a phenotypic approach based on an auxanogram using the Biotype-100 identification system revealed the taxonomic diversity of the pseudomonads (12) and also successfully distinguished *Rhodococcus* and *Gordonia* strains (2).

Twenty-five strains of *A. xylosoxidans* subsp. *xylosoxidans* or subsp. *denitrificans*, five *Alcaligenes* strains, three *Agrobacterium* strains, and four unnamed bacteria belonging to the Centers for Disease Control and Prevention (CDC) group IVc-2 from the Collection de l'Institut Pasteur (CIP) were identified to the species or subspecies level by a polyphasic approach based on conventional tests, auxanogram, and automated ribotyping.

* Corresponding author. Mailing address: Collection de l'Institut Pasteur, BP 52, 25 Rue du Docteur Roux, 75724 Paris Cédex 15, France. Phone: 01 40 61 31 04. Fax: 01 40 61 30 07. E-mail: dclermon@pasteur.fr.

MATERIALS AND METHODS

Bacterial strains. Twenty-five strains of *A. xylosoxidans*, three strains of *Agrobacterium*, five strains of *Alcaligenes*, and four unnamed strains belonging to CDC group IVc-2 were studied. All the strains belonged to the CIP (Table 1).

Conventional identification. All strains were examined by the conventional tests described by Chester and Cooper (7).

Identification with Biotype-100 strips. The assimilation of 99 carbohydrates, organic acids, and amino acids was studied by using Biotype-100 strips (BioMérieux, La Balme-les Grottes, France). Growth after 1 and 4 days of incubation was compared with that of the control without carbon source. The Recognizer, Adanson, and Dendrograf programs of the Taxotron package were used according to the user's manual for numerical analysis of Biotype-100 data. The distance coefficient selected was the complement of the Jaccard coefficient, and clustering was done by the unweighted pair group method of averages.

Identification with ribotyping method. Ribotyping was carried out using the Riboprinter microbial characterization system (Qualicon, Inc., Wilmington, Del.). Colonies were picked from solid medium, suspended in sample buffer, and heat treated. The lysing agent was added, and the samples were transferred to the Riboprinter system. Restriction endonuclease digestion, gel separation, transfer, and hybridization with a chemiluminescence-labeled DNA probe containing the rRNA operon from *Escherichia coli* were carried out by the automated instrument in 8 h.

Gel images were exported in TIFF and analyzed with the RestrictoScan, Restrictotyper, Adanson, and Dendrograf programs of the Taxotron package. The cubic Spline algorithm was used to calculate fragment sizes. A fixed fragment size tolerance value of 4% was chosen.

Antibiotic susceptibility. Antibiotic susceptibility of the strains was tested by the agar diffusion method (5). The susceptibilities were determined according to the guidelines of the Comité de l'Antibiogramme de la Société Française de Microbiologie (1).

TABLE 1. Strains studied

Strain[a]	Origin
Agrobacterium sp.	
CIP 102250	Human (blood)
CIP 102460	Contamination (HPLC[b])
CIP 102731	Human (conjunctivitis)
Alcaligenes sp.	
CIP 100007	Unknown
CIP 100008	Unknown
CIP 100998	Human (blood)
CIP 101080	Human (blood)
CIP 102485	Human
A. xylosoxidans subsp. *denitrificans*	
CIP 60.83	Unknown
CIP 77.15^T	Soil
CIP 100012	Unknown
CIP 100013	Unknown
CIP 100015	Unknown
CIP 100020	Unknown
A. xylosoxidans subsp. *xylosoxidans*	
CIP 58.72	Sputum
CIP 61.20	Antiseptic solution (quaternary ammonium)
CIP 62.30	Unknown
CIP 68.19	Unknown
CIP 71.32^T	Ear discharge
CIP 100005	Human (blood)
CIP 100029	Human (blood)
CIP 101719	Human (blood)
CIP 101902	Human (blood)
CIP 102041	Human (blood)
CIP 102236	Human (sputum)
CIP 102274	Human (sputum)
CIP 102288	Human (skin)
CIP 102498	Human (bile)
CIP 102630	Human (sputum)
CIP 102744	Human (blood)
CIP 102768	Human (trachea)
CIP 103938	Blood
CIP 103961	Sepsis
CIP 104044	Skin
CIP 104045	Human (lung)
Unnamed bacteria	
CIP 104521	Blood
CIP 104522	Blood
CIP 104523	Blood
CIP 104524	Bronchial source
R. paucula CIP 105943^T	Human (respiratory tract)
O. anthropi CIP 82.115^T	Unknown

[a] All the strains were from the CIP.
[b] High-performance liquid chromatograph.

TABLE 2. Characteristics differentiating the *A. xylosoxidans* strains

Characteristics	No. of positive strains	
	A. xylosoxidans subsp. *xylosoxidans* (20 strains)	*A. xylosoxidans* subsp. *denitrificans* (5 strains)
Growth:		
At 41°C	20	2
At 45°C	1	0
In 8% NaCl	3	0
On Simmon's citrate	17	5
On malonate	1	2
Activity of:		
Gamma-glutamyltransferase	2	2
Urease	7	0
Xylose oxidative degradation	20	0
Hydrolysis of hippurate	14	5

RESULTS

Conventional identification. All the strains studied were gram-negative rods, motile, oxidase and catalase positive, and strictly aerobic. All the strains were negative for production of gelatinase, caseinase, β-galactosidase, DNase, and arginine-dihydrolase. Lysine and ornithine were also not decarboxylated.

The *A. xylosoxidans* strains were characterized by their capacity to grow anaerobically with nitrate as the electron acceptor and by the absence of enzymatic activities as well as by the lack of production of H_2S and hydrolysis of esculin and tributyrin. Characteristics differentiating the 25 strains of *A. xylosoxidans* are shown in Table 2. Xylose oxidative degradation was the only positive test for the 20 *A. xylosoxidans* subsp. *xylosoxidans* strains. This test was negative for the five *A. xylosoxidans* subsp. *denitrificans* strains.

For the 12 remaining strains, the results obtained for conventional tests are shown in Table 3. *Alcaligenes* strains showed great variability in their conventional tests, whereas *Agrobacterium* strains had a lot of common traits. The unnamed bacteria all gave identical responses to the tests carried out.

Biotype data. The 37 strains studied could all use a wide range of organic compounds as their sole energy and growth sources. The use of L-tartrate, *trans*-aconitate, D-gluconate, and caprate as carbon sources allowed us to differentiate the two subspecies of *A. xylosoxidans*. Whereas *A. xylosoxidans* subsp. *xylosoxidans* was able to grow on *trans*-aconitate, D-gluconate, and caprate, *A. xylosoxidans* subsp. *denitrificans* was not. In contrast, the *A. xylosoxidans* subsp. *denitrificans* used L-tartrate while *A. xylosoxidans* subsp. *xylosoxidans* did not. The phenogram in Fig. 1 shows the relationships, in terms of carbon source utilization, between these strains and the *A. xylosoxidans* subsp. *xylosoxidans*, *A. xylosoxidans* subsp. *denitrificans*, *Ralstonia paucula*, and *Ochrobactrum anthropi* type strains. Examination of the phenogram gave clear groupings and allowed us to define four phenogroups (A, B, C, and D) for the 41 strains tested. Phenogroup A was composed of *A. xylosoxidans* subsp. *xylosoxidans* strains. Two strains, CIP 104044 and CIP 104045, were more closely related to the type strain of *A. xylosoxidans* subsp. *xylosoxidans* than the others. Phenogroup B included the *A. xylosoxidans* subsp. *denitrificans* strains. The five strains of *Alcaligenes* and the four unnamed bacteria fell into phenogroup C, as did the *R. paucula* type strain. Phenogroup D contained the three *Agrobacterium* strains studied and the *O. anthropi* type strain.

Ribotyping data. Ribotyping of the 41 strains listed in Table 1 was carried out by an automated system. The *Eco*RI and *Pst*I enzymes always generated an appropriate number of restriction fragments to allow a comparative analysis to be made. A schematic representation of the banding patterns of all the

TABLE 3. Characteristics differentiating unidentified strains

Characteristic	Result for strain[a]								
	CIP 100007	CIP 100008	CIP 100998	CIP 101080	CIP 102485	Unnamed bacteria	CIP 102250	CIP 102460	CIP 102731
Growth:									
At 5, 41, 45°C	−, −, −	−, +, −	−, −, −	−, +, −	−, +, −	−, +, −	−, −, −	−, −, −	−, −, −
On Simmon's citrate	+	+	+	+	+	+	−	−	+
On malonate	−	+	+	+	+	+	−	−	−
Denitrification	+	−	+	−	−	−	+	+	+
Hydrolysis of:									
Hippurate	−	+	+	+	+	+	+	+	+
Tributyrin	+	+	−	+	+	+	−	−	−
Esculin	−	−	−	−	−	−	−	+	+
Production of H_2S	−	−	−	−	−	−	+	+	+
Activity of:									
Gamma-glutamyltransferase	+	+	+	−	+	+	+	+	+
Urease	+	−	−	−	+	+	+	+	+
Phenylalanine desaminase	−	−	−	−	+	−	+	+	+
Tween 80-esterase	+	+	−	+	+	+	ND	ND	ND
Lipase	−	+	−	−	−	+	−	−	−
Lecithinase	−	−	−	−	+	+	−	−	−
Protease	−	−	−	−	−	+	−	−	−

[a] CIP 100007, CIP 100008, CIP 100998, CIP 101080, and CIP 102485 were identified as *Alcaligenes*; CIP 102250, CIP 102460, and CIP 102731 were identified as *Agrobacterium*; and the unnamed bacteria (CIP 104521, CIP 104522, CIP 104523, and CIP 104524) were not identified at all and had the same responses in the conventional tests. All the strains were from the CIP. ND, not determined.

strains and the deduced dendrograms are shown in Fig. 2 and 3.

The dendrogram obtained from *Eco*RI patterns revealed four ribogroups (Fig. 2). Ribogroup I included all the strains of *A. xylosoxidans* subsp. *xylosoxidan*s. It was homogeneous and composed mostly of strains with a pattern identical to that of the species type strain. Most of the differences observed were within the smallest restriction fragments. Ribogroup III was composed of strains with a pattern similar or identical to that of the *A. xylosoxidans* subsp. *denitrificans* type strain. Ribogroup II, including the *Alcaligenes* strains and the four unnamed bacteria, could be subdivided into three clusters. In the first cluster the profiles observed for each strain were very similar to that of the *R. paucula* type strain and differed significantly from those of the two other clusters. Ribogroup IV was subdivided into two clusters, one containing two *Agrobacterium* strains and the other including the *O. anthropi* type strain and *Agrobacterium* sp. strain CIP 102731. These last two strains had identical ribotyping patterns. It is interesting that resistance to ticarcillin, piperacillin, cephalothin, cefotaxime, ceftazidime, kanamycin, and erythromycin and susceptibility to gentamicin and nalidixic acid were observed for *Agrobacterium* strains and for the *O. anthropi* type strain.

To verify whether *Eco*RI groupings were of taxonomic significance for the unidentified strains, they were ribotyped after digestion with *Pst*I. The resulting dendrogram (Fig. 3) shows that *Alcaligenes* and *Agrobacterium* strains were separated into two groups. The *R. paucula* type strain was found in one of these groups, and the *O. anthropi* type strain was in the other. A significant distance between three of the *Alcaligenes* strains and *R. paucula* type strain was observed. This result was in agreement with that obtained with *Eco*RI for two of the strains. The third strain, CIP 101080, appeared to be more distant from *R. paucula* when cut with *Pst*I than with *Eco*RI. Conversely, CIP 100008 appeared to be closer to *R. paucula* when cut with *Pst*I than with *Eco*RI. The *Eco*RI and *Pst*I results both gave the same groupings for the *Agrobacterium* strains.

DISCUSSION

We carried out a polyphasic taxonomic study to identify 37 strains. The strains were characterized by use of an auxanogram and ribotyping. The results were analyzed by computer and compared to the results given by conventional tests.

The oxidative degradation of xylose was the only biochemical characteristic found to differentiate the two *A. xylosoxidans* subspecies. Due to the lack of discriminating biochemical features, reliable tests were needed to distinguish the two subspecies. Our investigation demonstrated that although ribotyping cannot delineate the *Staphylococcus* subspecies (6), it can clearly discriminate the two *A. xylosoxidans* subspecies. The ribogroups obtained were homogeneous with respect to current nomenclature, and it is noteworthy that most *A. xylosoxidans* subspecies strains displayed identical ribotyping patterns. Moreover, ribogroups were consistent with phenogroups. However, the biotype data suggested that the two subspecies were more closely related than the ribotyping data.

Without consideration of the biochemical behavior, ribotyping and substrate assimilation tests grouped one strain of *Alcaligenes*, CIP 102485, and the four unnamed strains (CIP 104521, CIP 104522, CIP 104523, and CIP 104524) to *R. paucula*. Likewise, three strains of *Agrobacterium*, CIP 102460, CIP

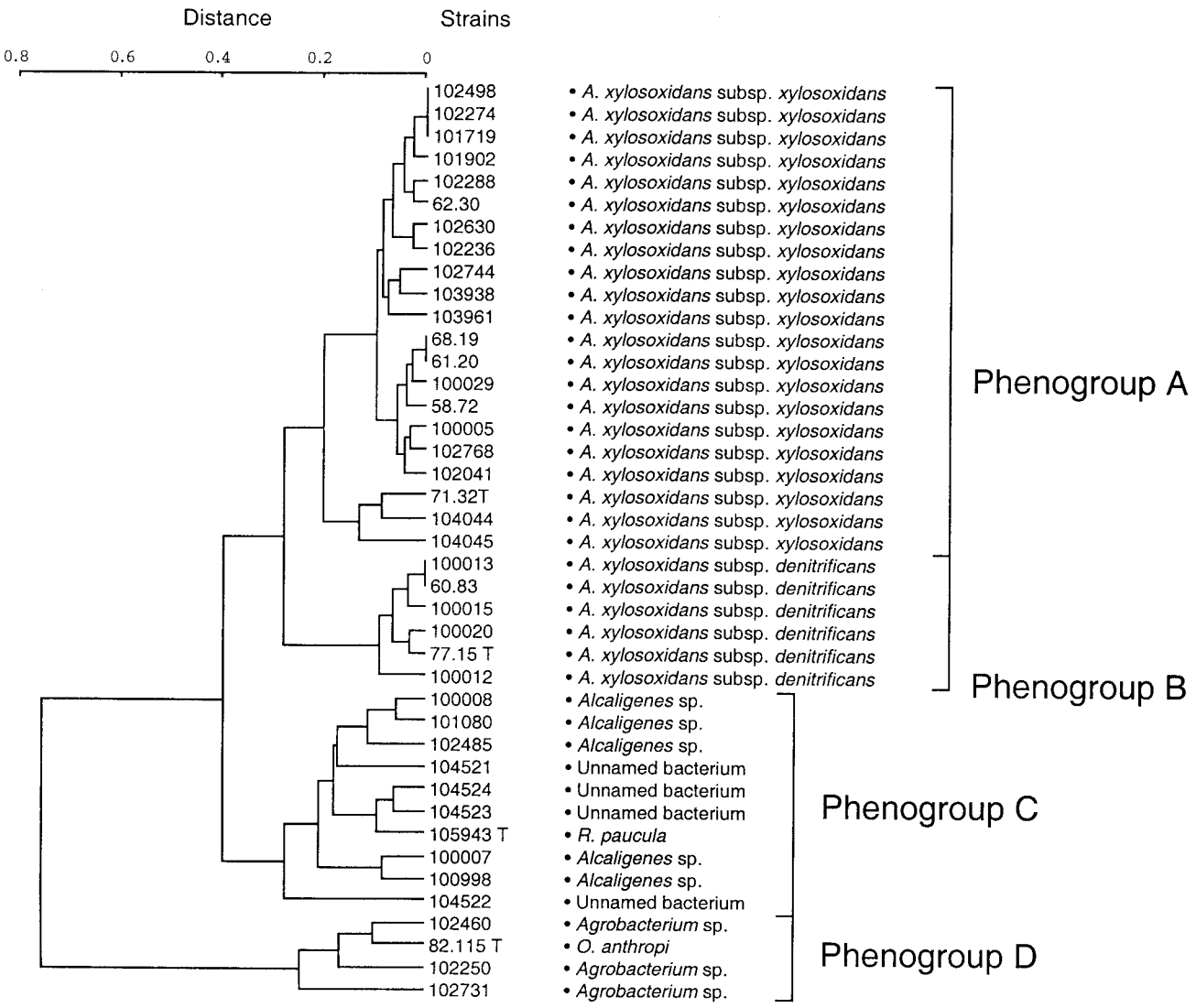

FIG. 1. Phenogram based on the carbon source utilization test.

102731, and CIP 102250, were related to the *O. anthropi* type strain.

It was not surprising that the unnamed bacteria were found to be related to *R. paucula*, because they belong to the same CDC group as CIP 105943^T (18). The observation that the ribotypes of unidentified strains fall into the same cluster as the *R. paucula* or *O. anthropi* type strains in both ribotyping assays strongly suggests that they belong to the corresponding species. Using two endonucleases instead of one reduces the probability of two unrelated strains having similar patterns. CIP 102460, CIP 102731, and CIP 102250 also had drug resistance profiles similar to that of *O. anthropi*, which also suggested that these strains belong to this species (3).

Although the ribotype and biotype groupings corresponded, the respective distribution of the strains within each group was slightly different. This explains why it was difficult to classify CIP 101080 and CIP 100008. Furthermore, the comparison with other type strains of neighboring species, like *Ralstonia pickettii*, *Ralstonia gilardii*, *Ralstonia eutropha*, and *Ralstonia solanacearum*, did not provide the best grouping for CIP 100998 and CIP 10007 (data not shown). Further analysis is required to resolve the taxonomic relationship of CIP 101080, CIP 100008, CIP 100998, and CIP 10007. Methods that may be used include 16S rRNA sequencing, followed by the comparison of the sequences obtained to those found in the databases.

This study emphasized the need for performant bacterial strain identification systems and the creation of databases which allow the strains identified by each system to be compared. The methods used in this study constitute a powerful tool for the rapid, simple, and reliable identification of strains which are difficult to separate using conventional tests. These methods cannot replace quantitative DNA-DNA hybridization studies for the estimation of relationships between the strains; however, they allow the rapid screening of unidentified strains for comparison with the type strains of species within the same genus.

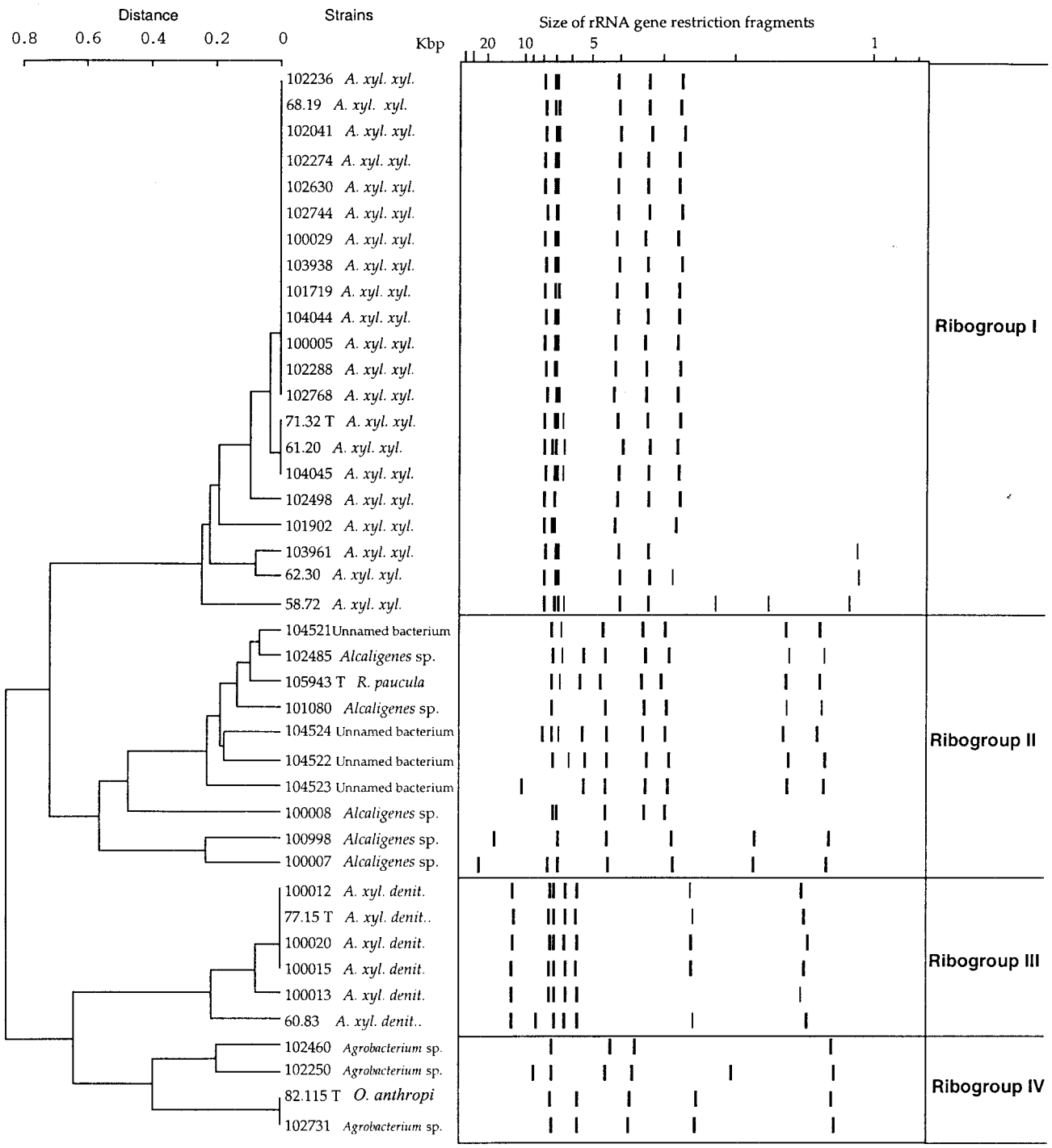

FIG. 2. Dendrogram based on the *Eco*RI ribotyping patterns of the 41 strains. *A. xyl. xyl.*, *A. xylosoxidans* subsp. *xylosidans*; *A. xyl. denit.*, *A. xylosoxidans* subsp. *denitrificans*.

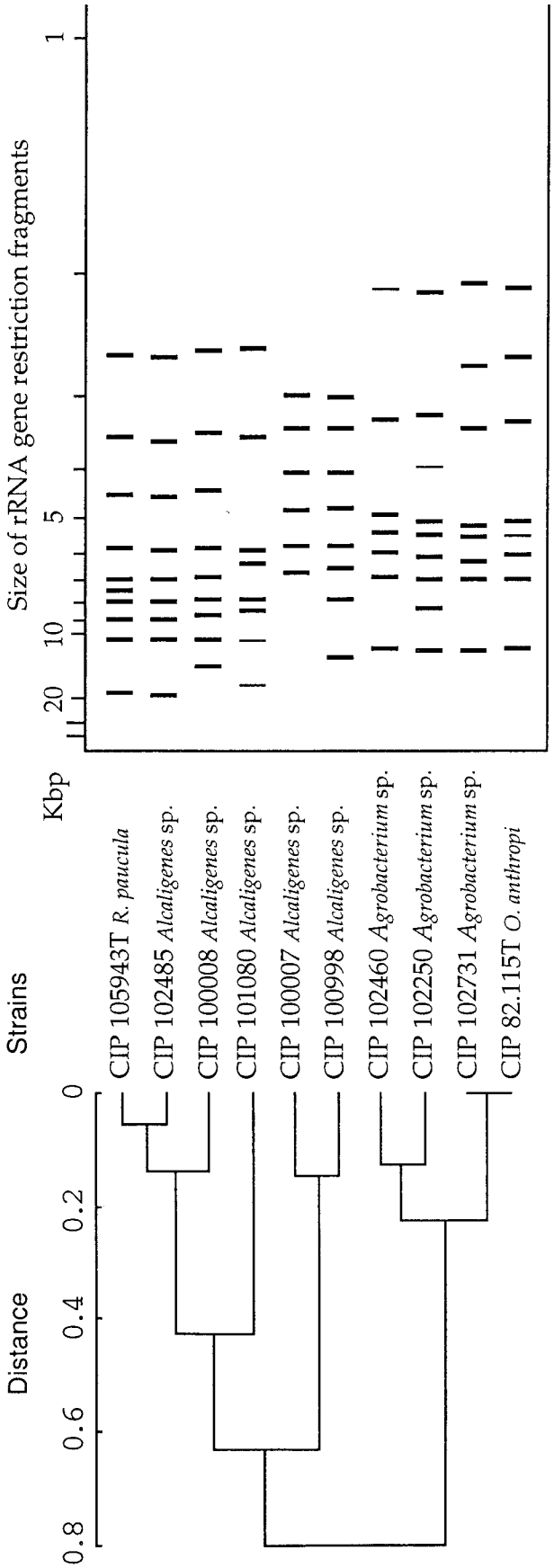

FIG. 3. Dendrogram based on the *Pst*I ribotyping patterns of the species unidentified strains.

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