Taxonomy of the *Azotobacteraceae* Determined by Using Immunoelectrophoresis

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The similarities of various strains of *Azotobacter* spp. and *Azomonas* spp. to reference strains of *Azotobacter paspali*, *Azotobacter vinelandii*, *Azotobacter chroococcum*, *Azomonas agilis*, *Azomonas insignis*, and *Azomonas macrocytogenes* were determined by rocket line immunoelectrophoresis. The strains of *Azotobacter paspali* and *Azotobacter vinelandii* used were immunologically more homogeneous than the strains of *Azotobacter chroococcum* studied, possibly due to the more diverse geographical origins of the *Azotobacter chroococcum* strains. Low values were obtained for the mean immunological distances (1 – proportion of immunoprecipitation bands shared between strains) between *Azotobacter paspali* and *Azotobacter vinelandii* strains, suggesting that these two species are immunologically closely related. Immunological distances from the *Azotobacter chroococcum* reference strain were similar for *Azotobacter paspali* and for other undisputed members of the genus *Azotobacter*, which makes it reasonable to retain *Azotobacter paspali* in this genus. When the three *Azotobacter* antisera were used, all *Azotobacter* species had mean immunological distances of less than 0.5, whereas the *Azomonas* species were immunologically more distant, showing that the six species of *Azotobacter* form an immunologically related group which is distinct from the *Azomonas* species. Our results with the three *Azomonas* antisera show that each species of *Azomonas* is immunologically distant from the other species, as well as from the *Azotobacter* species. We compare our immunoelectrophoretic results with the molecular biological results of De Smedt et al. (Int. J. Syst. Bacteriol. 30:106–122, 1980) and the numerical taxonomic analysis of Thompson and Skerman (*Azotobacteraceae: the Taxonomy and Ecology of the Aerobic Nitrogen-Fixing Bacteria*, Academic Press, Inc., London, 1979).

Controversy concerning the taxonomy of the *Azotobacteraceae* has recently arisen after the publication of results of two different types of investigation. On the basis of a numerical taxonomy study, Thompson and Skerman (11, 12) proposed two new genera, *Azorhizophilus* Thompson and Skerman 1981 to accommodate *Azotobacter paspali* Döbereiner 1966 (5) and *Azomonotrichon* Thompson and Skerman 1981 to accommodate the organism previously known as *Azomonas macrocytogenes* (H. Jensen 1953) Baillie, Hodgkiss, and Norris 1962 (1).

This proposal is not supported by the results of De Smedt et al. (3), who found that *Azotobacter paspali* is indistinguishable from other *Azotobacter* species on the basis of ribosomal ribonucleic acid (rRNA) hybridization and the guanine-plus-cytosine contents of their deoxyribonucleic acids (DNAs). Using the same criteria, De Smedt et al. (3) found that *Azomonas agilis*, *Azomonas insignis*, and *Azomonas macrocytogenes* are as distant from each other as they are from members of the genus *Azotobacter*. These

authors stated that new genera should be established to accommodate each of the three species of *Azomonas* or, alternatively, all three species should be retained in the genus *Azomonas* until further research clarifies the situation.

Rocket line immunoelectrophoresis has previously revealed the presence of certain family-specific and species-specific antigens in members of the *Azotobacteraceae* (10). In this paper we present data on the immunological affinities among various species in the family *Azotobacteraceae* and compare our results with those of Thompson and Skerman (11) and De Smedt et al. (3).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. All cultures were grown at 30°C on Winogradsky nitrogen-free agar medium (10) and washed off the agar surface for preparation of antisera or antigens for immunoelectrophoresis.

Preparation of antisera. The method of Tchan et al.

TABLE 1. Immuno-electrophoretic properties of members of the *Azotobacteraceae*

Cultures used for antigen preparation			Immuno-electrophoretic similarity to reference strain when the following antisera were used: ^a					
Species	Strain ^b	Source ^c	<i>Azotobacter paspali</i> Ax12(3)	<i>Azotobacter vinelandii</i> AVO ₂	<i>Azotobacter chroococcum</i> Q ₄	<i>Azomonas macrocytogenes</i> LH ₂	<i>Azomonas insignis</i> D	<i>Azomonas agilis</i>
<i>Azotobacter paspali</i>	Ax12(3)	J.D.	1.00	0.69	0.7	0.45	0.25	0.14
	23A	J.D.	0.86	0.69	0.7			
	DSM 383	J.D.L.	0.86	0.61	0.7	0.54	0.25	0.14
	DSM 391	J.D.L.	1.00	0.69	0.7	0.54		
	DSM 400	J.D.L.	1.00	0.69	0.77	0.45	0.08	0.14
	Ax22	J.D.						
	DSM 376	J.D.L.	1.00	0.69	0.7	0.45		
	8A ^T	J.D.	1.00	0.69	0.7			
	DSM 88	J.D.L.	1.00	0.69	0.6	0.36		
	22B	J.D.	1.00	0.69	0.7		0.17	0.14
	15B	J.D.	1.00	0.69	0.7			
	WR 129 ^{Td}	J.P.T.	1.00	0.69	0.7			
	WR 130	J.P.T.	1.00	0.69	0.7			
	WR 131	J.P.T.	1.00	0.69	0.6			
	WR 132	J.P.T.	0.86	0.69	0.6			
	WR 133	J.P.T.	0.86	0.69	0.7			
	WR 134	J.P.T.	0.80	0.61	0.7			
<i>Azotobacter paspali</i> mean	X	?	1.00	0.69	0.7			
	Y	?	1.00	0.69	0.7			
	Ax52	J.D.	0.86	0.69	0.7		0.25	0.14
			0.94	0.68	0.69	0.47	0.20	0.14
			(0.06) ^e	(0.32)	(0.31)	(0.53)	(0.80)	(0.86)
<i>Azotobacter vinelandii</i>	AVO ₂	O.W.	0.80	1.00	0.70	0.27	0.33	0.14
	A ₁₁	O.W.	0.66	0.92	0.80		0.5	0.29
	A ₁₂	O.W.	0.73	0.92	0.70	0.18		
	A ₂₂	O.W.	0.60	1.00	0.70			
	A ₃₁	O.W.	0.60	1.00	0.70		0.50	
	Holland	A.J.K.	0.80	0.92	0.70	0.36		
	AVO	P.W.W.	0.66	1.00	0.70			
	AVO ₁	O.W.	0.85	0.92	0.70			
	12837	A.T.C.C.	0.71	1.00	0.60	0.36		
			0.71	0.96	0.70	0.29	0.44	0.22
<i>Azotobacter vinelandii</i> mean			(0.29)	(0.04)	(0.30)	(0.71)	(0.56)	(0.78)
<i>Azotobacter chroococcum</i>	Q ₄	Y.T.T.	0.60	0.61	1.00	0.36	0.08	0.14
	IP ₁	I.P.	0.80	0.69	0.5	0.45		
	IP ₂	I.P.	0.66	0.69	0.6			

<i>Azotobacter chroococcum</i> mean	N-1 NGNM ₂ NGNM7-2 Wheat plot Wheat root	Y.T.T. Y.T.T. Y.T.T. Y.T.T. Y.T.T.	0.66 0.60 0.60 0.73 0.73 0.67 (0.33)	0.69 0.79 0.61 0.68 (0.32)	0.7 0.6 0.6 0.66 0.70 0.67 (0.33)	0.45	0.25	0.14
<i>Azotobacter beijerinckii</i> mean	Sydney Peggy Veg2 Achromogenes A Achromogenes B	Y.T.T. Y.T.T. H.L.J. H.L.J.	0.64 0.57 0.50 0.64 0.59 (0.41)	0.54 0.69 0.42 0.69 0.59 (0.41)	0.55 0.55 0.55 0.55 0.59 (0.45)	0.36 0.18 0.27 0.36 0.29 (0.71)	0.33 0.08 0.21 (0.79)	0.29 0.29 0.29 (0.71)
<i>Azotobacter nigrans</i>	WR 128 ^T	J.P.T.	0.71 (0.29)	0.65 (0.35)	0.71 (0.29)	0.18 (0.82)	0.25 (0.75)	0.14 (0.86)
<i>Azotobacter armeniacus</i>	WR 136 ^T WR 139 WR 138 WR 137	J.P.T. J.P.T. J.P.T. J.P.T.	0.45 0.45 0.64 0.55 (0.45)	0.85 0.43 0.85 0.85 (0.25)	0.80 0.56 0.67 0.68 (0.32)	0.36 0.09 0.27 0.27 (0.75)	0.25 0.34 0.25 0.28 (0.72)	0.28 0.28 0.28 0.28 (0.72)
<i>Azomonas macrocytogenes</i>	LH ₂ NCIB 8701 NCIB 8700 ^T NCIB 8702 NCIB 9128 (61) NCIB 9129 WR 46 WR 125	H.L.J. J.D.L. J.D.L. J.D.L. J.D.L. J.D.L. J.P.T. J.P.T.	0.33 0.40 0.53 0.36 0.33 0.36 0.3 0.37 (0.63)	0.20 0.69 0.53 0.61 0.30 0.61 0.38 0.46 (0.54)	0.4 0.4 0.6 0.4 0.4 0.5	1.00 0.72 1.00 1.00 1.00 0.72 0.90 0.91 (0.09)	0.27 0.27 0.27 0.27 0.36 0.27 0.29 (0.71)	0.1 0.12 0.14 0.20 0.1 0.1 0.14 0.13 (0.87)
<i>Azomonas macrocytogenes</i> mean	10EM	H.L.J.	0.6	1.00	0.53	0.18	0.45	0.2
<i>Azomonas agilis</i> <i>Azomonas agilis</i> mean	A.J.K.	A.J.K.	0.33 0.33 (0.67)	0.38 0.38 (0.62)	0.33 0.33 (0.67)	0.36 0.36 (0.64)	0.33 0.33 (0.67)	1.00 1.00 (0)
<i>Azomonas insignis</i> C D	C D	H.L.J. H.L.J.	0.266 0.20	0.30 0.38	0.18 0.18	0.27 0.27	1.00 0.75	0.14 0.14

TABLE 1—Continued

Cultures used for antigen preparation		Immuno-electrophoretic similarity to reference strain when the following antisera were used: ^a						
Species	Strain ^b	Source ^c	<i>Azotobacter paspali</i> Ax12(3)	<i>Azotobacter vinelandii</i> AVO ₂	<i>Azotobacter chroococcum</i> Q ₄	<i>Azomonas macrocytogenes</i> LH ₂	<i>Azomonas insignis</i> D	<i>Azomonas agilis</i>
<i>Azomonas insignis</i> mean			0.23 (0.77)	0.34 (0.66)	0.18 (0.82)	0.27 (0.73)	0.88 (0.12)	0.14 (0.86)

^a Calculated as follows: number of bands with test antigen/number of bands with reference antigen.

^b The following pairs of strains were identical but were obtained from different sources: *Azotobacter paspali* Ax12(3) and WR 130; *Azotobacter paspali* 8A and WR 129; and *Azotobacter paspali* DSM 88 and WR 131. Immunological data from only one strain in each pair were used in calculating means.

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^d Type strain.

^e The numbers in parentheses are mean immunological distances, which were calculated as follows: 1 - mean immuno-electrophoretic similarity.

(10) was used to produce antisera in rabbits against whole cells of the following reference strains, which were suspended in 0.85% NaCl: *Azotobacter vinelandii* AVO₂, *Azotobacter paspali* Ax12(3), *Azotobacter chroococcum* Q₄, *Azomonas macrocytogenes* LH₂, *Azomonas agilis*, and *Azomonas insignis* D.

Preparation of antigens for immuno-electrophoresis. Bacterial cells suspended in distilled water were broken by ultrasonic disintegration for 3 to 10 min with a Biosonik II sonicator (Bronwill Scientific Inc., Rochester, N.Y.) with the power output control set at 90. An ice bath was used to maintain a low temperature during sonication, and cell breakage was checked regularly by phase-contrast microscopy until 80 to 90% cell breakage was achieved.

Each antigen was freeze-dried and suspended in saline to a concentration of 40 mg (dry weight) per ml.

Immuno-electrophoresis. Immunological similarities to the reference strains were determined after rocket line immuno-electrophoresis by the method of Krøll (7), in which antigens of the various strains were placed in wells. The antigens prepared from the reference strain were incorporated in the reference strip, as well as in one of the wells. All wells contained 10 µl of antigen preparation. The following concentrations were used for the reference antigen strips (milligrams [dry weight] of antigen per cubic centimeter of gel): *Azotobacter paspali*, 5.2; *Azotobacter vinelandii*, 1.2; *Azotobacter chroococcum*, 4; *Azomonas macrocytogenes*, 3.5; *Azomonas agilis*, 8; *Azomonas insignis*, 8. The antigens were electrophoresed into a gel containing antiserum prepared against the reference strain. The following concentrations of antisera were used (microliters of antiserum per square centimeter of gel): *Azotobacter paspali*, 6; *Azotobacter vinelandii*, 8; *Azotobacter chroococcum*, 12; *Azomonas macrocytogenes*, 7; *Azomonas agilis*, 18; *Azomonas insignis*, 12.

Agarose A (Pharmacia Fine Chemicals A.B., Uppsala, Sweden) was used to prepare all gels except the reference antigen strip, which was prepared from low-temperature-gelling agarose B (Pharmacia) to avoid heating the antigens above 40°C.

Electrophoresis was carried out with a total output of 80 V (corresponding to 1 V/cm) for 16 h. Coomassie brilliant blue R was used for plate staining.

Immuno-electrophoretic similarity was calculated from the proportion of antigen-antibody precipitation bands shared by a reference strain and another strain, when they were electrophoresed against antiserum prepared against the reference strain (Fig. 1 and 2). This result was used to calculate the immunological distance from the reference strain (immunological distance is defined as follows: 1 - proportion of shared bands). For example, in calculating the immunological relatedness between reference strain *Azotobacter paspali* Ax12(3) and *Azomonas macrocytogenes* LH₂, the following data were obtained: the homologous antigen produced 15 bands, and strain LH₂ produced 5 bands. Thus, the immuno-electrophoretic similarity is 5/15 = 0.33, and the immunological distance is 1 - 0.33 = 0.67.

Since formation of precipitation bands or peaks is a function of the ratio of antigen molecules to antibody molecules, the absence of a particular band does not necessarily imply complete absence of the antigen, but rather shows that the concentration of this antigen in the strain being tested is significantly lower than the

FIG. 1. Rocket line immunoelectrophoresis of members of the *Azotobacteraceae*. The gel contained anti-*Azotobacter paspali* Ax12(3) serum. r g s, Reference gel strip containing *Azotobacter paspali* Ax12(3) antigen. The following antigens were in the wells: ♦ P, *Azotobacter paspali* Ax12(3); P, *Azotobacter paspali* DSM 383; V, *Azotobacter vinelandii* AVO₂; C, *Azotobacter chroococcum* Q₄; M, *Azomonas macrocytogenes* LH₂; Ag, *Azomonas agilis*; I, *Azomonas insignis* D. Even though all precipitation lines are not visible in the photograph, it is evident that more horizontal precipitation lines are elevated to form peaks above the wells containing *Azotobacter* antigens than above the wells containing *Azomonas* antigens. The close immunological relationship between *Azotobacter paspali* and *Azotobacter vinelandii* is apparent.

concentration in the reference strain against which the antiserum was produced. We believe that a concentration difference large enough to cause the nonappearance of a band should be counted as an absolute difference for the purpose of taxonomic comparison under our standardized conditions. It should be appreciated that two strains having similar immunological distances from a common reference strain may not

necessarily be closely related, since they may have different antigens in common with the reference strain. For example, two strains with immunological distances of more than 0.5 from the same reference strain theoretically may have no common bands when they are tested against the reference antiserum. For two strains with immunological distances of less than 0.5, the maximum possible distance apart (based on anti-

FIG. 2. Mean immunological distance of species of *Azotobacter* (●) and *Azomonas* (○) from reference strains. Ag, *Azomonas agilis*; I, *Azomonas insignis*; M, *Azomonas macrocytogenes*; AR, *Azotobacter armeniacus*; B, *Azotobacter beijerinckii*; C, *Azotobacter chroococcum*; N, *Azotobacter nigricans*; P, *Azotobacter paspali*; V, *Azotobacter vinelandii*.

gens in common with the reference strain) is the sum of their respective immunological distances from the reference strain.

Comparison between immunological data and molecular biological results. To investigate groupings by the different methods, plots of mean denaturation temperature of DNA-rRNA hybrids [$T_{m(e)}$] versus mean percent binding of rRNA to DNA (rRNA binding percentage) were superimposed on plots of $T_{m(e)}$ versus mean immunological distance. Values of $T_{m(e)}$ and rRNA binding percentage were taken from the data of De Smedt et al. (3) by averaging the data for the strains tested in our study; if no common strains were tested, the mean for the species was based upon all of the strains tested by De Smedt et al. (3). The scales for rRNA binding percentage and mean immunological distance were chosen so that the data points would span approximately the same horizontal range.

RESULTS

Because of the large number of antigens tested, many immunoelectrophoretic plates containing reference antigen were used for each reference antiserum. The number of precipitation peaks formed by the reference antigen against its homologous antiserum was highly reproducible, with a maximum difference of one precipitation peak between plates (Table 2).

Our data indicate that all members of the *Azotobacteraceae* have some taxonomic relationship, since they all share some antigens with the reference strains, whereas other nitrogen-fixing bacteria (*Beijerinckia* and *Rhizobium*) show no cross-reaction (10). The mean immunological distances of the various species from the reference strains are shown in Table 1 and Fig. 2.

It is apparent that members of *Azotobacter paspali* and *Azotobacter vinelandii* are immunologically homogeneous within species, as the immunological distances from the corresponding reference strains ranged from 0 to 0.2 for *Azotobacter paspali* and from 0 to 0.08 for *Azotobac-*

ter vinelandii. When the strains of *Azotobacter paspali* and *Azotobacter vinelandii* were tested against the reference antiserum of the other species (*Azotobacter paspali* strains versus *Azotobacter vinelandii* reference antiserum and vice versa), *Azotobacter paspali* and *Azotobacter vinelandii* had mean immunological distances of 0.31 and 0.32, respectively.

Azotobacter chroococcum strains may be considerably less homogeneous, as their immunological distances from the reference strain, strain Q₄, ranged from 0.50 to 0.30. Although this could have been due to the choice of the reference strain, the greater diversity of the *Azotobacter chroococcum* strains probably also reflects the geographical diversity of their origins, whereas all strains of *Azotobacter paspali* were isolated from Brazil and the majority of *Azotobacter vinelandii* strains originated from the eastern United States (10).

When the anti-*Azotobacter chroococcum* serum was used, five of the six *Azotobacter* species tested had the same degree of immunological similarity to the reference strain, based on mean immunological distance. Although the mean immunological distance of *Azotobacter beijerinckii* was greater, the individual distances of all of its strains from the reference strain lay within the range of variation of *Azotobacter chroococcum* strains (Table 1). Among the *Azotobacter* species, *Azotobacter beijerinckii* had the lowest similarity to the *Azotobacter* reference strains, except in the tests against *Azotobacter paspali* antiserum, where the value for *Azotobacter armeniacus* was lower (Table 1).

The immunological distances of some *Azomonas macrocytogenes* strains from the reference *Azotobacter* strains were less than the immunological distances of some strains of the least similar *Azotobacter* species, especially when *Azotobacter vinelandii* was used. *Azomonas macrocytogenes* strain 10EM was immu-

TABLE 2. Number of precipitation peaks produced by reference antigen against homologous antiserum or immunoelectrophoretic plates

Reference antiserum	No. of peaks with reference antigen	Species tested
<i>Azotobacter paspali</i> Ax12(3)	15	<i>Azotobacter paspali</i> , <i>Azotobacter vinelandii</i> , <i>Azotobacter chroococcum</i> , <i>Azomonas macrocytogenes</i> , <i>Azomonas agilis</i> , <i>Azomonas insignis</i>
<i>Azotobacter paspali</i> Ax12(3)	14	<i>Azotobacter armeniacus</i> , <i>Azotobacter beijerinckii</i>
<i>Azotobacter vinelandii</i> AVO ₂	26	All species
<i>Azotobacter chroococcum</i> Q ₄	10	<i>Azotobacter chroococcum</i> , <i>Azotobacter vinelandii</i> , <i>Azomonas macrocytogenes</i>
<i>Azotobacter chroococcum</i> Q ₄	9	<i>Azotobacter armeniacus</i> , <i>Azotobacter beijerinckii</i> , <i>Azomonas agilis</i> , <i>Azomonas insignis</i>
<i>Azomonas macrocytogenes</i> LH ₂	11	All species
<i>Azomonas agilis</i>	7	All species
<i>Azomonas insignis</i> D	12	All species

TABLE 3. Comparison between data obtained by immunoelectrophoresis and by molecular biological methods

Species	Guanine-plus-cytosine content (mol %) ^a	Similarity to reference strain											
		<i>Azotobacter chroococcum</i>		<i>Azotobacter paspali</i>		<i>Azomonas agilis</i>		<i>Azomonas insignis</i>					
		Hybridization with rRNA ^b	Immunological dis-tance	Hybridization with rRNA ^b	Immunological dis-tance	Hybridization with rRNA ^b	Immunological dis-tance	Hybridization with rRNA ^b	Immunological dis-tance				
		<i>T_{m(c)}</i> (°C)	% rRNA binding	<i>T_{m(c)}</i> (°C)	% rRNA binding	<i>T_{m(c)}</i> (°C)	% rRNA binding	<i>T_{m(c)}</i> (°C)	% rRNA binding				
<i>Azotobacter paspali</i>	63.7	78.0	0.156	0.31	81.5	0.144	0.06	76.0	0.149	0.86	75.0	0.167	0.80
<i>Azotobacter vine-landii</i>	(65.8)	(78.4)	(0.151)	0.30	(81.2)	(0.115)	0.29	(75.0)	(0.142)	0.78	(75.0)	(0.144)	0.56
<i>Azotobacter chroococcum</i>	(66.3)	(80.9)	(0.154)	0.33	(78.0)	(0.104)	0.33	(76.3)	(0.137)	0.86	(76.5)	(0.142)	0.83
<i>Azotobacter beijerinckii</i>	(66.0)	(80.0)	(0.153)	0.45	(77.8)	(0.104)	0.41	(76.0)	(0.138)	0.71	(76.0)	(0.152)	0.79
<i>Azotobacter nigricans</i>	64.5	79.0	0.161		79.0	0.124	0.29			0.86			0.75
<i>Azotobacter armeniacus</i>	64.4	79.7	0.164	0.32	78.5	0.103	0.45	75.0	0.148	0.72	75.5	0.154	0.72
<i>Azomonas macrocytogenes</i>	58.8	76.3	0.126	0.55	76.8	0.099	0.63	76.2	0.127	0.87	76.7	0.116	0.71
<i>Azomonas agilis</i>	(52.7)	(76.6)	(0.091)	0.67	(76.0)	(0.078)	0.67	(78.5)	(0.151)	0	(76.4)	(0.108)	0.67
<i>Azomonas insignis</i>	55.3	76.3	0.112	0.82	(76.5)	(0.099)	0.77	76.0	0.121	0.86	80.5	0.130	0.12

^a The guanine-plus-cytosine contents and rRNA hybridization data were obtained from De Smedt et al (3).

^b Mean values based only on those strains of each species which are common to the study of De Smedt et al. (3) and the present study. Values in parentheses indicate that the mean is based upon all strains of that species used by De Smedt et al. (3) since different strains were used in the present study.

FIG. 3. Maps of similarity of species of *Azotobacter* and *Azomonas* to *Azotobacter paspali* (A) and *Azotobacter chroococcum* (B). $T_{m(e)}$ of DNA-rRNA hybrids is plotted against mean percent binding of rRNA to DNA (rRNA binding percentage) (○) and against mean immunological distance from the reference strain (●). $T_{m(e)}$ and rRNA binding percentages were taken from the data of De Smedt et al. (3). (A) The reference strains used were *Azotobacter paspali* 8A (for $T_{m(e)}$ and rRNA binding percentage) and Ax12(3) (for immunological distance). (B) The reference strains used were *Azotobacter chroococcum* NCIB 8002 (for $T_{m(e)}$ and rRNA binding percentage) and Q₄ (for immunological distance). AG, *Azomonas agilis*; I, *Azomonas insignis*; M, *Azomonas macrocytogenes*; AR, *Azotobacter armeniacus*; B, *Azotobacter beijerinckii*; C, *Azotobacter chroococcum*; N, *Azotobacter nigricans*; P, *Azotobacter paspali*; V, *Azotobacter vinelandii*.

nologically distant from the reference *Azomonas macrocytogenes* strain, but very similar to *Azotobacter vinelandii*, as previously noted by Tchan et al. (10). When tested against anti-*Azotobacter paspali* serum, strain 10EM gave a similar result to the *Azotobacter vinelandii* AVO₂ antigens. For this reason, strain 10EM was excluded from our calculation of mean immunological distance for *Azomonas macrocytogenes* to avoid distorting the value toward

Azotobacter vinelandii. However, it was not considered desirable to include strain 10EM in the calculations for *Azotobacter vinelandii* in the absence of supporting phenotypic or molecular biological data.

When the three *Azotobacter* antisera were used, the mean immunological distance of the three *Azomonas* species was always greater than the value for any *Azotobacter* species, supporting the separation of this genus from *Azotobacter*. The results obtained with anti-*Azomonas agilis*, anti-*Azomonas insignis*, and anti-*Azomonas macrocytogenes* reference sera confirmed the separation of the three *Azomonas* species from all species of *Azotobacter*, but also clearly showed that the *Azomonas* species are immunologically distant from one another (Table 1 and Fig. 2).

DISCUSSION

Vincent (13, 14) has previously pointed out that groupings of the rhizobia obtained by serology agree well with groupings obtained by molecular biological methods. However, the immunodiffusion technique used by this author was able to resolve only a limited number of precipitation bands, which were inadequate for mathematical analysis. The immunoelectrophoretic technique, with its higher resolving power, is better suited to make comparisons between immunological results and molecular biological data. For this reason, we compared our immunological results for the *Azotobacteraceae* with the molecular biological results of De Smedt et al. (3) (Table 3), who studied the similarities between rRNA cistrons of several reference strains and a variety of strains of *Azotobacter* and *Azomonas*. rRNA is regarded as a conservative molecule (6, 8, 9), and the degree of rRNA similarity correlates well with overall phenotypic similarity between groups of bacteria which are too different from one another to be compared by DNA-DNA hybridization (2-4).

When *Azotobacter paspali* reference strains are used, there is good agreement between superimposed maps (Fig. 3A), indicating good correspondence between immunological distance and rRNA binding percentage; in fact, the correlation coefficients (r) between these parameters are highly significant ($P < 0.01$) for the *Azotobacter* species ($r = -0.94$) and for the *Azotobacter* plus *Azomonas* species ($r = -0.86$). The correlation between $T_{m(e)}$ and immunological distance is also significant for the *Azotobacter* plus *Azomonas* species ($r = -0.89$, $P < 0.01$), but not for the *Azotobacter* species alone. The superimposed maps do not agree as well for the *Azomonas* species, and the immunological data provide a greater separation between the *Azomonas* and *Azotobacter* groupings.

There is less similarity between the superimposed maps when *Azotobacter chroococcum* reference strains are used (Fig. 3B); however, the separation between the *Azotobacter* and *Azomonas* groups is similar. Correlations between rRNA binding percentage or $T_{m(e)}$ and immunological distance are not significant for *Azotobacter* species alone, although there are significant correlations with immunological distance for the *Azotobacter* plus *Azomonas* species [$r = -0.88$, $P < 0.01$ for rRNA; $r = -0.73$, $P < 0.05$ for $T_{m(e)}$].

Data were not plotted for *Azomonas agilis* or *Azomonas insignis* reference strains, since the means were based on fewer points and therefore were less reliable. For both species, $T_{m(e)}$ but not rRNA binding percentage is significantly correlated with immunological distance, based on *Azomonas* plus *Azotobacter* species (for *Azomonas agilis*, $r = -0.79$, $P < 0.05$; for *Azomonas insignis*, $r = -0.80$, $P < 0.05$). $T_{m(e)}$ gives the same general result as immunological distance, with the reference *Azomonas* species on its own (i.e., separated from all other *Azomonas* and *Azotobacter* species) (Fig. 2).

Thus, the results obtained by our immunoelectrophoretic investigation are in agreement with those obtained by molecular biological methods (3) despite the possible shortcoming of comparing our phenotypic results with the data of De Smedt et al. It is possible that differences in order of similarity between these sets of results may be due to the use of different reference strains in the case of *Azotobacter chroococcum*, *Azomonas agilis*, and *Azomonas insignis*, since very good agreement between the two methods was obtained for *Azotobacter paspali*, for which two immunologically identical reference strains were used; strain 8A, which was used by De Smedt et al. (3), is immunologically indistinguishable from strain Ax12(3).

Our results indicate that *Azotobacter paspali* is immunologically similar to *Azotobacter vinelandii* (immunological distances of 0.31 for *Azotobacter vinelandii* against *Azotobacter paspali* antiserum and 0.32 for *Azotobacter paspali* against *Azotobacter vinelandii* antiserum). When strains were tested against anti-*Azotobacter chroococcum* serum, the mean immunological distance and range of distances of *Azotobacter paspali* from the reference strain were similar to those of other undisputed members of the genus, including *Azotobacter chroococcum*, which is the type species of *Azotobacter*. Therefore, it seems reasonable to include *Azotobacter paspali* in the genus *Azotobacter*, a conclusion which is in agreement with the molecular biological evidence (3), but not in accord with the classification obtained by numerical taxonomy (11).

Azomonas macrocytogenes was not immunologically close to *Azotobacter paspali* (11) or to any other species of *Azotobacter*, although it is more closely related to *Azotobacter* than the other *Azomonas* species are. The DNA base composition of this species is also intermediate between the base compositions of the azotobacters and the other members of the genus *Azomonas* (guanine-plus-cytosine contents: *Azotobacter* species, 63.2 to 67.5 mol%; *Azomonas macrocytogenes*, 58.2 to 58.6 mol%; *Azomonas insignis*, 55.1 to 58.3 mol%; and *Azomonas agilis*, 52.0 to 53.2 mol%). Our results, together with the molecular biological data of De Smedt et al. (3), lend support to the establishment by Thompson and Skerman (11) of a separate genus for *Azomonas macrocytogenes*.

Although *Azomonas agilis* and *Azomonas insignis* are both immunologically distant from the reference strains of the *Azotobacter* species, it is not possible to conclude from this that these two *Azomonas* species are closely related. The results obtained with antisera against *Azomonas agilis* and *Azomonas insignis* show that these species are immunologically distinct and could even be placed in two distinct genera. Therefore, we have reservations concerning the retention of *Azomonas agilis* and *Azomonas insignis* in the same genus, as proposed by Thompson and Skerman (11). However, more strains of these species must be tested before a final conclusion can be reached. The establishment of a new genus to accommodate *Azomonas insignis* (3) should be given serious consideration if the present results are confirmed.

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