Genomic Heterogeneity of the Genus *Nitrobacter*

E. NAVARRO,^{1*} M. P. FERNANDEZ,¹ F. GRIMONT,² A. CLAYS-JOSSERAND,¹ AND R. BARDIN¹

Laboratoire d'Ecologie Microbienne, Université Claude Bernard Lyon 1, F-69622 Villeurbanne Cedex,¹
and Unité des Entérobactéries, Institut National de la Santé et de la Recherche
Médicale Unité 199, Institut Pasteur, F-75724 Paris Cedex 15,² France

The genomic diversity among 22 *Nitrobacter* strains was investigated by determining rRNA gene restriction patterns, DNA hybridization characteristics, and DNA base compositions. The guanine-plus-cytosine contents of the DNAs ranged from 58 to 61 mol%. As determined by DNA hybridization (S1 nuclease method), five DNA genomic groups were differentiated, and these groups formed three genomic species. Genomic species 1, which corresponded to *Nitrobacter winogradskyi*, was split into three subspecies. Subspecies 1a contained strain W (= ATCC 25391), the type strain of *N. winogradskyi*; subspecies 1b contained proposed reference strain 6R; and subspecies 1c contained a strain of "*Nitrobacter agilis*" (strain ATCC 14123). Genomic species 2, which has not been described previously and which contained proposed reference strain LL and four strains that were isolated from lake sediments, was distinct from *N. winogradskyi* and *Nitrobacter hamburgensis*. This species is not named in this paper since it could not be differentiated from *N. winogradskyi* and *N. hamburgensis* on the basis of phenotypic characteristics. Genomic species 3 corresponded to *N. hamburgensis* and was distantly related to the other genomic species.

Nitrification, the conversion of ammonium to nitrite and nitrate, is a key step in the nitrogen cycle. This process has been implicated in the supply of nitrate as a nitrogen source for plants and as a substrate for denitrification. Furthermore, it is of great importance in eutrophication and lixiviation. Therefore, nitrification is of fundamental importance in all ecosystems. Chemolithoautotrophic nitrifiers appear to be present in every environment in which nitrogen mineralization takes place (3).

Although nitrite-oxidizing bacteria were first isolated a long time ago (49), nitrification has been studied essentially as a chemical process, and the microbial populations involved have been studied only slightly (29). The reasons for this neglect are the common physiological characteristics of these autotrophic organisms, including their very slow growth rates (8 h to several days) (45) and their low growth yields (13, 52). These characteristics result in experimental difficulties in isolation, enumeration, and determination of specific activities (33) and hamper ecological and population dynamics studies of nitrite oxidizers (3, 23, 51).

So far, all of the bacteria from soil and freshwater that are able to complete the second step in autotrophic nitrification (i.e., the oxidation of nitrite to nitrate) have been placed in the genus *Nitrobacter* (45). Currently, the taxonomy of this genus is based primarily on morphological characteristics, and for a long time the existence of one or two species (*Nitrobacter winogradskyi* and "*Nitrobacter agilis*") has been discussed (7, 44). In *Bergey's Manual of Determinative Bacteriology*, 8th ed. (44), only one species, *N. winogradskyi*, was mentioned.

A century after the first isolation of nitrite-oxidizing bacteria (49), the taxonomy of the genus *Nitrobacter* has begun to be clarified. Recent investigations in which DNA-DNA hybridization was used have indicated the existence of new species, *Nitrobacter hamburgensis* (6, 45) and "*Nitrobacter vulgaris*" (5). These two species are phenotypically different from *N. winogradskyi* in some ways; for instance, they grow faster under chemoorganotrophic conditions than under chemolithotrophic conditions. In addition, "*N. vulgaris*" is

able to grow anaerobically by denitrification (12). Studies on the phylogeny of the genus *Nitrobacter* have indicated that this taxon is closely related to *Rhodopseudomonas palustris* (35), which is member of the alpha subdivision of the *Proteobacteria*. (41, 50).

Currently, serotyping is the reference method that is used for characterizing *Nitrobacter* isolates (5, 11, 21, 42). However, the subjectivity of the immunofluorescence technique hampers comparisons of results from different laboratories. Thus, other characterization methods are necessary.

In this work, we examined the genetic diversity of 22 *Nitrobacter* strains by using DNA base composition, DNA-DNA hybridization, and rRNA gene polymorphism and compared the results with results obtained by using serotyping. On the basis of our results we delineated three genomic species and split genomic species 1 into three genomic subspecies.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains which we studied are listed in Table 1. New isolates were obtained by using the petri dish isolation method (39) and were identified on the basis of their physiological properties and by using electron microscopy (21).

N. winogradskyi and *Nitrobacter* sp. strains were cultivated in 2-liter Erlenmeyer flasks containing 800 ml of mineral medium supplemented with 2 g of NaNO₂ per liter (34). *N. hamburgensis* X14^T (T = type strain) was grown under mixotrophic conditions (6). The cultures were incubated at 28°C, and the purity of each culture was checked by spreading samples onto nutrient broth agar (Biomérieux, Marcy l'Etoile, France).

Serotyping. The procedure described by Josserand and Bardin (20) was used for immunological studies of *Nitrobacter* strains. Immunofluorescence tests were interpreted by using previously published recommendations (4, 31).

DNA extraction. DNA was extracted and purified by using the procedure of Brenner et al. (8), with the following modification: 1 mg of achromopeptidase (Walko Pure Chemical, Dallas, Tex.) per ml was added to the lysis solution to improve DNA extraction (36).

* Corresponding author.

TABLE 1. Origins of the *Nitrobacter* strains tested

Strain ^a	Other designation	Origin	Reference
<i>N. winogradskyi</i> W ^T (= ATCC 25391 ^T) ^b		Unknown	37
B1		Unknown	38
" <i>N. agilis</i> " ATCC 14123	AG	Unknown	43
<i>N. hamburgensis</i> X14 ^T		Soil, Hamburg, Germany	5
<i>Nitrobacter</i> spp.			
LL	L	Soil, Lyons, France	20
WBD1		Soil, Isère, France	25
WM1		Soil, Montpellier, France	25
LM1		Soil, Montpellier, France	25
LM2		Soil, Montpellier, France	25
6R		Soil, Rothamsted, United Kingdom	36
DE2		Lake water, Dombes, France	25
DE3		Lake water, Dombes, France	25
DE10		Lake water, Dombes, France	25
DE11		Lake water, Dombes, France	25
DE23		Lake water, Dombes, France	25
DE30		Lake water, Dombes, France	25
DS13		Lake sediments, Dombes, France	25
DS17		Lake sediments, Dombes, France	25
DS27		Lake sediments, Dombes, France	25
DS28		Lake sediments, Dombes, France	25
DS30		Lake sediments, Dombes, France	25
DS'16		Lake sediments, Dombes, France	25

^a The strains are in the culture collection of the Laboratoire d'Ecologie Microbienne, Lyons, France, or were supplied by E. L. Schmidt (St. Paul, Minn.) or E. Bock (Hamburg, Germany).

^b ATCC, American Type Culture Collection.

Gel electrophoresis of endonuclease-cleaved DNAs. Bacterial DNAs (2 to 5 µg) were cleaved by using restriction endonucleases *EcoRV* and *MluI* (5 to 15 U/µg of DNA) according to the instructions of the manufacturer (Appligène, Illkirch, France). The preparations were digested at 37°C for 5 h. Restriction fragments were separated by horizontal electrophoresis for 16 h at 2 V/cm, using a 0.8% (wt/vol) gel in Tris-borate buffer (89 mM Tris, 2.5 mM EDTA, 89 mM H₃BO₃).

Determination of base composition. The average guanine-plus-cytosine (G+C) content of a DNA was determined by using the chromatographic method of Peyret et al. (28). The Merck-Hitachi chromatography system used for quantification of nucleotides included a pump (model L-6200), a UV detector (model L-4000) linked to an integrator (model D-2500), and a 7-µm type RP18 chromatography column (catalog no. 15539); the mobile phase was 0.6 M NH₄H₂PO₄ (pH 4.25), the flow rate was 1.0 ml/min, and absorption was recorded at 260 nm.

DNA-DNA hybridization. Native DNA was labeled by nick translation (30) with tritium-labeled nucleotides (Amersham International, Amersham, England). Levels of DNA relatedness were determined by using the S1 nuclease-trichloroacetic method (18). DNA-DNA hybridization tests were carried out at 70°C by using labeled DNAs from strains W^T, LL, AG, DE30, DS27, and X14^T as probes.

Thermal stability of duplexes. The thermal denaturation midpoint (*T_m*) (the temperature at which 50% of the reassociated DNA become hydrolyzable by S1 nuclease) was determined by using the method of Crosa et al. (9), with

slight modifications (10). The ΔT_m was the difference between the *T_m* of the homoduplex and the *T_m* of the heteroduplex.

Radioactive labeling of RNA. 16+23S rRNA from *Escherichia coli* (Boehringer Mannheim France S. A., Neylan, France) was end-labeled by using [γ -³²P]ATP (Amersham) and a 5' DNA terminus labeling kit along with T4 polynucleotide kinase (Bethesda Research Laboratories, Gaithersburg, Md.); to do this, we used the exchange reaction procedure and instructions provided by the manufacturer.

Southern transfer and hybridization. The exact procedures which we used to transfer DNA fragments to Hybond-N nylon membranes (Amersham) and for prehybridization, hybridization with labeled rRNA at 65°C for 17 h, washing, and autoradiography have been described previously (15).

DNA fragment size determination. The DNA fragment size marker which we used was DNA from *Xenorhabdus* sp. strain 278 cleaved with *EcoRI*. The rRNA gene restriction pattern of this DNA contained 15 fragments (13.1, 11.6, 10.1, 8.5, 5.8, 4.6, 3.8, 2.9, 2.6, 2.4, 2.2, 2.1, 1.5, 1.4, and 0.8 kb) (14a). Fragment sizes were interpolated by using the algorithm of Schaffer and Sederoff (19, 32).

RESULTS

G+C contents of DNAs. The G+C contents of the *Nitrobacter* strains ranged from 58 to 61 mol% (Table 2).

DNA-DNA hybridizations. The unstandardized reassociation values for homoduplexes ranged from 75 to 100%. The levels of reassociation in control tubes containing only labeled DNAs ranged from 3 to 7%. The *T_m* values of homoduplexes (in 0.2 M NaCl) ranged from 88 to 93.4°C; this corresponded to 93 to 98.4°C in 0.042 M NaCl.

The percentages of relative DNA homology at 70°C with reference DNAs from strains W^T, AG, DE30, DS27, LL, and X14^T are shown in Table 2 for 22 strains. These strains fell into five discrete DNA relatedness groups. Group 1a contained eight strains that were 71 to 88% related to strain W^T and exhibited low levels of divergence (ΔT_m , about 1°C). Group 1b contained seven strains that were more than 90 and 86% related to strains DE30 and DS27, respectively; these strains were 47 to 67% related to genomic group 1a strains, with ΔT_m values between 4 and 6°C. Strain AG was 53 to 59% related to group 1a strains, with ΔT_m values between 3 and 5°C, and 49 to 62% related to group 1b strains, with ΔT_m values between 4.5 and 5°C. Strain AG was the only representative of group 1c. The ΔT_m values among group 1a, 1b, and 1c strains ranged from 3 to 6°C (most often 4 to 5°C). Thus, groups 1a, 1b, and 1c belonged to a single genomic species (genomic species 1) (17). Group 2 contained five strains that were 62 to 73% related to strain LL, with a ΔT_m value of 3°C; these strains were 31 to 47% related to the other genomic groups, with ΔT_m values of more than 8°C. Thus, strains in this group constituted genomic species 2. Strain X14^T, which belonged to group 3, yielded low relative binding ratios (less than 30%) with members of the other genomic groups; this strain was the only representative of genomic species 3.

rRNA gene restriction patterns. The diverse patterns obtained after digestion with *MluI* or *EcoRV* are shown in Fig. 1. Actual results obtained for some strains are shown in Fig. 2. Six rRNA gene restriction patterns (patterns M1 to M6) and four rRNA gene restriction patterns (patterns E1 to E4) were obtained with *MluI* and *EcoRV*, respectively (Fig. 1). Each pattern contained one or two fragments, whose sizes ranged from 2 to 13 kb (*MluI*) or from 6.1 to 19 kb (*EcoRV*).

TABLE 2. Levels of DNA relatedness among strains of *Nitrobacter* species

Source of unlabeled DNA	G+C content (mol%)	% Reassociation at 70°C with labeled DNA from:					
		Strain W ^T	Strain DE30	Strain DS27	Strain AG	Strain LL	Strain X14 ^T
Genomic species 1 (<i>N. winogradskyi</i>)							
Subspecies 1a							
W ^T (= ATCC 25391 ^T)	60	100 ^a	65 (4) ^b	53	59 (3)	35 (11)	26
B1	— ^c	83	47	—	—	45	—
WBD1	—	84	67	—	55	—	—
WM1	58	75	—	—	—	36	—
LM1	60	83	—	—	—	—	—
LM2	—	85	—	—	—	44	—
DE2	—	71	54	—	—	—	—
DE11	61	88 (1)	57 (5.5)	47	53 (5)	40 (8.5)	24
Subspecies 1b							
6R	61	55 (5)	90 (1)	86	51 (4.5)	36 (8)	27
DE3	—	58 (5.5)	100	—	49	46	30
DE10	—	50	96	—	—	38 (11)	—
DE23	—	58	100	—	52	47	27
DE30	61	53 (5.5)	100	101	51 (5)	38 (11.5)	26
DS27	—	57	—	100	—	34	—
DS30	60	53 (6)	100	—	—	35	31
Subspecies 1c strain AG (= ATCC 14123)	60	59 (5)	62 (5)	62	100	40 (9)	26
Genomic species 2							
LL	59	33	40	32	28	100	28
DS13	—	—	—	—	32	70	—
DS17	59	33	38	—	31	73	26
DS28	—	31	—	—	—	62 (3)	—
DS'16	58	34	—	—	—	62	—
Genomic species 3 (<i>N. hamburgensis</i>) strain X14 ^T	61	30	30	23	22	31	100

^a Level of relatedness at 70°C.^b The numbers in parentheses are ΔT_m values (in degrees Celsius).^c —, no data.

The patterns associated with each strain are shown in Table 3. Strains which produced *EcoRV* pattern E1 produced one of four *MluI* patterns (pattern M1, M2, M3, or M4). Strains which produced *MluI* pattern M5 produced one of two *EcoRV* patterns (pattern E2 or E3). Strain X14^T produced specific patterns with the two enzymes (patterns E4 and M6).

Our results suggest that only one copy of the rRNA operon is present in the *Nitrobacter* chromosome. This

agrees with the concept that there is a correlation between the number of operons and the growth rate (16).

Serotyping. Nine *Nitrobacter* strains were examined serologically by using the indirect immunofluorescence technique. These strains fell into five serogroups (Table 4). Strain W^T, WBD1, and WM1 belonged to serogroup *N. winogradskyi* of Stanley and Schmidt (42). Serogroup LL contained three strains (strains LL, LM1, and LM2). Strains DE2 and DE11 belonged to the same serogroup. Another serogroup contained a single strain, strain AG.

DISCUSSION

The G+C contents of the *Nitrobacter* DNAs (58 to 61 mol%) agreed with the range of values reported previously (5, 46).

It is currently accepted that a species includes strains that exhibit approximately 70% or greater DNA-DNA relatedness and have ΔT_m values of less than 5°C (48). ΔT_m values are important since they are less subject to variation because of genome size or methodological choice (17). Strains that have ΔT_m values of 3 to 5°C often belong to different subspecies within a species (17). Thus, our DNA hybridization results led us to delineate three species and to split genomic species 1 into three genomic subspecies (Table 2).

Genomic species 1 corresponds to *N. winogradskyi* since it contains the type strain of this species, strain W (= ATCC 25391) (37). A total of 15 other strains were grouped in this species. All of the strains in this species produced the same *EcoRV* rRNA gene restriction pattern. This species con-

FIG. 1. Normalized graph showing migration patterns of rRNA gene restriction fragments after cleavage by *MluI* (patterns M1 to M6) or *EcoRV* (patterns E1 to E4) and hybridization with labeled *E. coli* 16+23S rRNA.

FIG. 2. rRNA restriction patterns of *Nitrobacter* DNAs cleaved by *Mlu*I (A) or *Eco*RV (B). Lane 1, strain WBD1; lane 2, strain B1; lane 3, strain WM2; lane 4, strain LM2; lane 5, strain DE11; lane 6, strain X14^T; lane 7, strain 6R; lane 8, strain LL; lane 9, strain DS30; lane 10, strain LL; lane 11, strain DS28; lane 12, strain X14^T.

tained three subspecies, which were 47 to 67% related (ΔT_m values, 3 to 6°C).

Subspecies 1a contained eight closely related strains (strains W^T, B1, WBD1, WM1, LM1, LM2, DE2, and DE11), as shown by DNA relatedness values (Table 2). However, some heterogeneity in *Mlu*I rRNA gene polymorphism was observed (Table 3). Subspecies 1a contained type strain ATCC 25391 of *N. winogradskyi* (37).

Subspecies 1b contained seven closely related strains (strains 6R, DE3, DE10, DE23, DE30, DS27, and DS30), which were 47 to 67% related to subspecies 1a strains. The homogeneity of this DNA genomic group was shown by the single *Mlu*I rRNA pattern (Table 3). Subspecies 1b differed from subspecies 1a in *Mlu*I rRNA gene restriction pattern (Table 3). Strain 6R is proposed as the reference strain for subspecies 1b.

Strain AG was the only representative of subspecies 1c. This strain could not be differentiated from subspecies 1a strains on the basis of polymorphism of rRNA genes by using the two restriction enzymes. Strain AG (= ATCC 14123) is a reference strain of "*N. agilis*" (1). "*N. agilis*" did not appear on the Approved Lists of Bacterial Names (37). Our data suggested that "*N. agilis*" may be a subspecies of *N. winogradskyi*, although there are few phenotypic traits to support this hypothesis.

From 1931 to 1974, all motile *Nitrobacter* strains were named "*N. agilis*" (26). This name appeared in *Bergey's Manual of Determinative Bacteriology*, 7th ed. (7). It has been demonstrated that the motility of *Nitrobacter* strains varies with the growth phase (40, 47). Moreover, *N. winogradskyi* and "*N. agilis*" could not be differentiated on

TABLE 3. Results obtained with different approaches

Strain	Sero-group	G+C content (mol%)	Genomic group	rRNA gene polymorphism ^a	
				<i>Mlu</i> I pattern	<i>Eco</i> RV pattern
W ^T	W	60	Subspecies 1a	M1	E1
B1	W ^b		Subspecies 1a	M1	E1
WBD1	W		Subspecies 1a	M2	E1
WM1	W	58	Subspecies 1a	M1	E1
LM1	L	60	Subspecies 1a	M1	E1
LM2	L		Subspecies 1a	M1	E1
DE2	DE11		Subspecies 1a	M3	E1
DE11	DE11	61	Subspecies 1a	M3	E1
AG	AG	60	Subspecies 1c	M1	E1
6R	6R ^b	61	Subspecies 1b	M4	E1
DE3			Subspecies 1b	M4	E1
DE10			Subspecies 1b	M4	E1
DE23			Subspecies 1b	M4	E1
DE30		61	Subspecies 1b	M4	E1
DS27			Subspecies 1b	M4	E1
DS30		60	Subspecies 1b	M4	E1
LL	L	59	Genomic species 2	M5	E2
DS13			Genomic species 2	M5	E3
DS17		59	Genomic species 2	M5	E3
DS28			Genomic species 2	M5	E3
DS'16		58	Genomic species 2	M5	E3
X14 ^T	X14 ^c	61	Genomic species 3	M6	E4

^a Pattern associated with each strain.

^b See reference 42.

^c See reference 5.

TABLE 4. Cross-reactivity of *Nitrobacter* strains

<i>Nitrobacter</i> strain	Genomic group	Cross-reactivity with antiserum to:			
		Strain W ^T	Strain LL	Strain AG	Strain DE11
W ^T	Subspecies 1a	4+ ^a	—	—	
WBD1	Subspecies 1a	3+	(+)		
WM1	Subspecies 1a	3+	—		
LL	Genomic species 2	—	4+	(+)	
LM1	Subspecies 1a	—	3+		
LM2	Subspecies 1a	—	3+		
AG	Subspecies 1c	—	—	4+	
DE2	Subspecies 1a	—	—	(+)	4+
DE11	Subspecies 1a	—	—	(+)	4+

^a —, no fluorescence; (+), bacteria visible but not distinct; 3+, moderate fluorescence; 4+, maximum fluorescence.

the basis of DNA G+C content or cellular fatty acid content (2, 46), and studies on the growth of these organisms in presence of organic matter gave conflicting results because of the diversity of the strains compared (22, 27, 38, 43, 47). Pan (27) could not separate "*N. agilis*" from *N. winogradskyi* by using phenotypic characteristics. Consequently, in *Bergey's Manual of Determinative Bacteriology*, 8th ed. (44), a single *Nitrobacter* species, *N. winogradskyi*, was included.

Strains belonging to genomic group 2 (Table 2) were clearly separated from strains belonging to the other genomic groups, as shown by DNA relatedness and ΔT_m values (Table 2). These strains constituted a new, separate genomic species (genomic species 2). Our results were confirmed by rRNA gene polymorphism data (Table 3). Previous data on the physiology, serology, and protein contents (13, 14, 19a, 21, 24) of strains W^T and LL showed that these two strains differ considerably. Strains, DS13, DS17, DS28, and DS'16, which belong to genomic species 2, were indistinguishable as determined by rRNA polymorphism (Table 3), polymorphism of the intergenic spacer region of the ribosomal operon (25), and restriction length polymorphism of the total DNA (data not shown). These data led us to believe that strains DS28 and DS'16 are members of genomic species 2, even though they exhibited only 62% DNA-DNA homology with strain LL. Among the genomic species 2 strains, strain LL produced a specific *EcoRV* rRNA restriction pattern.

In this study we did not include "*N. vulgaris*," a species described by Bock et al. (5), since our experimental work was finished when the strains of this species were received and it was not practical to cultivate them given the long time necessary. Nevertheless, clear physiological differences led us to separate "*N. vulgaris*" from genomic species 2. "*N. vulgaris*" strains often grow more slowly under lithoautotrophic conditions than under chemoorganotrophic conditions (5). In contrast, strain LL, a member of genomic species 2, grew better under lithoautotrophic conditions (14).

Genomic species 3 corresponds to *N. hamburgensis* since it contains the type strain of this species (strain X14) (6, 45). As expected, this species was not closely related to *N. winogradskyi* and to genomic species 2 (Table 2). Strain X14^T produced a specific rRNA pattern with each of the enzymes which we used (Table 3). Thus, we confirmed with these results the status of *N. hamburgensis* as a species.

Serotyping by using the immunofluorescence technique was the first method that was used to characterize *Nitrobac-*

ter strains, and this method has remained the reference method since phenotypic data for these slowly growing bacteria are often difficult to obtain. Nevertheless, the level of discrimination of serotyping has not been clearly established yet. Bock et al. (5) found concurrent results when they used serology and DNA-DNA homology to study *N. winogradskyi*, *N. hamburgensis*, and "*N. vulgaris*." In contrast, our results did not show a clear correlation between serogroups and genomic groups. Diverse serogroups were found in subspecies 1a, and cross-reactions were observed between strains belonging to two different genomic species (*N. winogradskyi* and genomic species 2) (Table 3).

Strains obtained from widely separated geographical areas were found to be very closely related in our study, whereas strains which coexist in the same lake belonged to different genomic species. It seems that geographical origin has little bearing on the genetic properties of *Nitrobacter* strains, and this may be related to the presence of locally coexisting ecological niches.

In conclusion, we propose a new taxonomic scheme for the genus *Nitrobacter* based on DNA relatedness and rRNA gene polymorphism. We delineated three genomic species. Genomic species 1, corresponding to the previously described species *N. winogradskyi* (45), was split into three subspecies. This is the first time that subspecies have been described for *N. winogradskyi*. As previously noted (45), *N. winogradskyi* strains differed genotypically, but no clear correlation was found with phenotypic characteristics. Genomic species 2, which has not been described previously, was distinct from *N. winogradskyi* and *N. hamburgensis*; strain LL is proposed as the reference strain of this taxon. We confirmed that genomic species 3, which corresponds to *N. hamburgensis*, is distantly related to the other genomic groups.

Because of the extremely slow growth of *Nitrobacter* isolates, phenotypic tests are not always discriminating enough for identification purposes. In agreement with the recommendations published previously by a group of experts (48), the new subspecies and genomic species cannot be named at this time.

ACKNOWLEDGMENTS

We thank D. Bernillon and M. Lefèvre for technical assistance, L. Gay for scintillation counting, H. Meugnier for the use of the Merck-Hitachi chromatography system, and P. A. D. Grimont for critically reading the manuscript.

REFERENCES

1. American Type Culture Collection. 1989. Catalogue of bacteria and bacteriophage. 17th ed. American Type Culture Collection, Rockville, Md.
2. Auran, T. B., and E. L. Schmidt. 1972. Similarities between *Hyphomicrobium* and *Nitrobacter* with respect to fatty acids. J. Bacteriol. 109:450-451.
3. Belser, L. W. 1979. Population ecology of nitrifying bacteria. Annu. Rev. Microbiol. 33:309-333.
4. Belser, L. W., and E. L. Schmidt. 1978. Serological diversity within a terrestrial ammonia-oxidizing population. Appl. Environ. Microbiol. 36:589-593.
5. Bock, E., H.-P. Koops, U. C. Möller, and M. Rudert. 1990. A new facultatively nitrite oxidizing bacterium, *Nitrobacter vulgaris* sp. nov. Arch. Microbiol. 153:105-110.
6. Bock, E., H. Sundermeyer-Klinger, and E. Stackebrandt. 1983. New facultative lithotrophic nitrite-oxidizing bacteria. Arch. Microbiol. 136:281-284.

7. Breed, R. S., E. G. D. Murray, and N. R. Smith (ed.). 1957. *Bergey's manual of determinative bacteriology*, 7th ed., p. 1094. The Williams & Wilkins Co., Baltimore.
8. Brenner, D. J., A. C. McWorter, J. K. Leete Knutson, and A. G. Steigerwalt. 1982. *Escherichia vulneris*: a new species of *Enterobacteriaceae* associated with human wounds. *J. Clin. Microbiol.* **15**:1133-1140.
9. Crosa, J. M., D. J. Brenner, and S. Falkow. 1973. Use of a single-strand-specific nuclease for analysis of bacterial and plasmid deoxyribonucleic acid homo- and heteroduplexes. *J. Bacteriol.* **115**:904-911.
10. Fernandez, M. P., H. Meugnier, P. A. D. Grimont, and R. Bardin. 1989. Deoxyribonucleic acid relatedness among members of the genus *Frankia*. *Int. J. Syst. Bacteriol.* **40**:424-429.
11. Fliermans, C. B., B. B. Bohlool, and E. L. Schmidt. 1974. Autecological study of chemoautotroph *Nitrobacter* by immunofluorescence. *Appl. Microbiol.* **27**:124-129.
12. Freitag, A., M. Rudert, and E. Bock. 1987. Growth of *Nitrobacter* by dissimilatory nitrate reduction. *FEMS Microbiol. Lett.* **48**:105-109.
13. Gay, G., and A. Corman. 1984. Comparative study of the growth of two strains of *Nitrobacter* in batch and continuous culture. *Microb. Ecol.* **10**:99-105.
14. Gay, G., A. Josserand, and R. Bardin. 1983. Growth of two serotypes of *Nitrobacter* in mixotrophic and chemoorganotrophic conditions. *Can. J. Microbiol.* **29**:394-397.
- 14a. Grimont, F. Unpublished data.
15. Grimont, F., and P. A. D. Grimont. 1986. Ribosomal ribonucleic acid gene restriction patterns as potential taxonomic tools. *Ann. Inst. Pasteur/Microbiol. (Paris)* **137B**:165-175.
16. Grimont, F., and P. A. D. Grimont. 1990. La carte d'identité génétique des bactéries. *Biofutur* **95**:50-52.
17. Grimont, P. A. D. 1988. Use of DNA reassociation in bacterial classification. *Can. J. Microbiol.* **34**:541-546.
18. Grimont, P. A. D., M. Y. Popoff, F. Grimont, C. Coynault, and M. Lemelin. 1980. Reproducibility and correlation study of three deoxyribonucleic acid hybridization procedures. *Curr. Microbiol.* **4**:325-330.
19. Irino, K., F. Grimont, I. Casin, P. A. D. Grimont, and The Brazilian Purpuric Fever Study Group. 1988. rRNA gene restriction patterns of *Haemophilus influenzae* biogroup aegyptius strains associated with Brazilian purpuric fever. *J. Clin. Microbiol.* **26**:1535-1538.
- 19a. Josserand, A. 1983. Ph.D. thesis. Université Lyon I, Villeurbanne, France.
20. Josserand, A., and R. Bardin. 1981. Nitrification en sol acide. I. Mise en évidence de germes autotrophes nitrifiants (genre *Nitrobacter*) dans un sol forestier sous résineux. *Rev. Ecol. Biol. Sol* **18**:435-445.
21. Josserand, A., and J. C. Cleyet-Marel. 1979. Isolation from soils of *Nitrobacter* and evidence for novel serotypes using immunofluorescence. *Microb. Ecol.* **5**:207-213.
22. Kalthoff, H., S. Fehr, H. Sundermeyer, L. Renwranz, and E. Bock. 1979. A comparison by means of antisera and lectins of surface structures of *Nitrobacter winogradskyi* and *N. agilis*. *Curr. Microbiol.* **2**:375-380.
23. MacDonald, R. M. 1986. Nitrification in soil: introductory history, p. 17-38. In J. I. Prosser (ed.), *Nitrification*. SGM IRL Press, Oxford.
24. Navarro, E., A. Josserand, B. Bernillon, and R. Bardin. 1987. Aspects microbiens de la nitrification: effets des variations quantitatives et diversité des souches de *Nitrobacter*. *Rev. Ecol. Biol. Sol* **24**:591-602.
25. Navarro, E., P. Simonet, P. Normand, and R. Bardin. 1992. Characterization of natural population of *Nitrobacter* spp. using PCR/RFLP analysis of the ribosomal intergenic spacer. *Arch. Microbiol.* **157**:107-115.
26. Nelson, D. H. 1931. Isolation and characterization of *Nitrosomonas* and *Nitrobacter*. *Zentralbl. Bakteriell. Parasitenkd. Infektionskr. Abt. 2* **83**:280-311.
27. Pan, P. H. C. 1971. Lack of distinction between *Nitrobacter agilis* and *Nitrobacter winogradskyi*. *J. Bacteriol.* **108**:1416-1418.
28. Peyret, M., J. Freney, H. Meugnier, and J. Fleurette. 1989. Determination of G+C content of DNA using high-performance liquid chromatography for the identification of staphylococci and micrococci. *Res. Microbiol.* **140**:467-475.
29. Prosser, J. I., and D. J. Cox. 1982. Nitrification, p. 178-193. In R. G. Burns and J. H. Slater (ed.), *Experimental microbial ecology*. Blackwell Scientific Publications, Oxford.
30. Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *Int. J. Syst. Bacteriol.* **113**:237-251.
31. Robert, F. M., and E. L. Schmidt. 1985. Somatic serogroups among 55 strains of *Rhizobium phaseoli*. *Can. J. Microbiol.* **31**:519-523.
32. Schaffer, H. E., and R. R. Sederoff. 1981. Improved estimation of DNA fragment length from agarose gels. *Anal. Biochem.* **115**:113-122.
33. Schmidt, E. L., and L. W. Belser. 1982. Nitrifying bacteria, p. 1027-1042. In R. H. Miller and D. R. Keeney (ed.), *Methods of soil analysis*, 2nd ed. American Society of Agronomists, Madison, Wis.
34. Schmidt, E. L., J. A. E. Molina, and C. Chiang. 1973. Isolation of chemoautotrophic nitrifiers from Moroccan soils. *Bull. Ecol. Res. Comm. (Stockholm)* **17**:166-167.
35. Seewaldt, E., K. H. Schleifer, E. Bock, and E. Stackebrandt. 1982. The close phylogenetic relationship of *Nitrobacter* and *Rhodopseudomonas palustris*. *Arch. Microbiol.* **131**:287-290.
36. Simonet, P., A. Capellano, E. Navarro, R. Bardin, and A. Moiroud. 1984. An improved method for lysis of *Frankia* with achromopeptidase allows detection of new plasmid. *Can. J. Microbiol.* **30**:1292-1295.
37. Skerman, V. B. D., V. McGowan, and P. H. A. Sneath (ed.). 1980. Approved lists of bacterial names. *Int. J. Syst. Bacteriol.* **30**:225-420.
38. Smith, A. J., and D. S. Hoare. 1968. Acetate assimilation by *Nitrobacter agilis* in relation to its "obligate autotrophy." *J. Bacteriol.* **95**:844-855.
39. Soriano, S., and N. Walker. 1968. Isolation of ammonia oxidizing autotrophic bacteria. *J. Appl. Bacteriol.* **31**:493-498.
40. Soriano, S., and N. Walker. 1973. The nitrifying bacteria in soils from Rothamsted classical fields and elsewhere. *J. Appl. Bacteriol.* **36**:523-529.
41. Stackebrandt, E., R. G. E. Murray, and H. G. Trüper. 1988. *Proteobacteria* classis nov., a name for the phylogenetic taxon that includes the "purple bacteria and their relatives." *Int. J. Syst. Bacteriol.* **38**:321-325.
42. Stanley, P. M., and E. L. Schmidt. 1981. Serological diversity of *Nitrobacter* spp. from soil and aquatic habitats. *Appl. Environ. Microbiol.* **41**:1069-1071.
43. Steinmüller, W., and E. Bock. 1976. Growth of *Nitrobacter* in the presence of organic matter. I. Mixotrophic growth. *Arch. Microbiol.* **108**:299-304.
44. Watson, S. W. 1974. Family I. *Nitrobacteraceae*, p. 450-456. In R. E. Buchanan and N. E. Gibbons (ed.), *Bergey's manual of determinative bacteriology*, 8th ed. The Williams & Wilkins Co., Baltimore.
45. Watson, S. W., E. Bock, H. Harms, H.-P. Koops, and A. B. Hooper. 1989. Nitrifying bacteria, p. 1808-1834. In J. C. Holt, J. T. Staley, M. P. Bryant and N. Pfennig (ed.), *Bergey's manual of systematic bacteriology*, vol. 3. The Williams & Wilkins Co., Baltimore.
46. Watson, S. W., and M. Mandel. 1971. Comparison of the morphology and deoxyribonucleic acid composition of 27 strains of nitrifying bacteria. *J. Bacteriol.* **107**:563-569.
47. Watson, S. W., and J. B. Waterbury. 1971. Characteristics of two marine nitrite oxidizing bacteria, *Nitrospina gracilis* nov. gen. nov. sp. and *Nitrococcus mobilis* nov. gen. nov. sp. *Arch. Microbiol.* **77**:203-230.
48. Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Trüper. 1987. Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics. *Int. J. Syst.*

- Bacteriol. **34**:463–464.
49. **Winogradsky, S.** 1892. Contributions à la morphologie des organismes de la nitrification. Arch. Sci. Biol. St. Petersb. **1**:88–137.
50. **Woese, C. R., E. Stackebrandt, W. G. Weisburg, B. J. Paster, M. T. Madigan, V. J. Fowler, C. M. Hahn, P. Blanz, R. Gupta, K. H. Nealson, and G. E. Fox.** 1984. The phylogeny of purple bacteria: the alpha subdivision. Syst. Appl. Microbiol. **5**:315–326.
51. **Woldendorp, J. W., and H. J. Laanbroek.** 1989. Activity of nitrifiers in relation to nitrogen nutrition of plants in natural ecosystems. Plant Soil **115**:217–228.
52. **Wood, P. M.** 1986. Nitrification as a bacterial energy source, p. 39–62. In J. I. Prosser (ed.), Nitrification. SGM IRL Press, Oxford.