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Comparison of 16S Ribosomal DNA Sequences of All *Xanthomonas* Species

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The phylogenetic relationships of all validly described species of the genus *Xanthomonas* and the type strain of *Stenotrophomonas maltophilia* were analyzed by sequencing and comparing 16S ribosomal DNAs (rDNAs). The two genera exhibited a mean sequence similarity value of 96.6%, corresponding to differences at 50 nucleotide positions on average. The species of the genus *Xanthomonas* exhibited relatively high levels of overall sequence similarity; the mean similarity value was 98.2%, which corresponds to an average of 14 mutual nucleotide differences. Within the genus *Xanthomonas*, a group containing *Xanthomonas albilineans*, *Xanthomonas hyacinthi*, *Xanthomonas theicola*, and *Xanthomonas translucens* clustered apart from the main *Xanthomonas* core, whereas *Xanthomonas sacchari* formed a third phylogenetic lineage. Due to the very restricted variability in 16S rDNA sequences within the genus *Xanthomonas*, rDNA signatures that have possible diagnostic value for differentiating the *Xanthomonas* species could not be determined with certainty. When sequence similarities were compared with DNA-DNA pairing data determined previously, there was only a limited correlation. This illustrates the different resolving powers of the techniques for determining phylogenetic hierarchies and for species delineation.

The phytopathogenic specialization and the broad host range of members of the genus *Xanthomonas* (30) have made these microorganisms the subject of numerous taxonomic studies (for a review, see reference 59). Traditional methods used for the detection and identification of xanthomonads, such as biochemical (55), serological, and pathogenicity tests (3, 4, 5, 31, 45, 48), have been extended by molecular methods based on protein profiling (57, 60) and fatty acid analysis (7, 63).

Currently, molecular approaches are being used increasingly in studies of the taxonomy and epidemiology of *Xanthomonas* species (15, 19). This has led to the development of different probes for detection and identification by hybridization or by PCR amplification (17, 18, 20, 21, 24, 25, 27, 32, 40) and to analyses of the genetic structures of field populations (1, 4, 26, 28, 29).

A polyphasic approach, such as the approach described by Vandamme et al. (54), could utilize all of these methods to contribute to the classification of the genus *Xanthomonas*. In practice, however, DNA reassociation, which provides a measure of the overall similarity of chromosomal genomes, is generally used for species delineation. This strategy has been used by Vauterin et al. (58), who recently reclassified the genus *Xanthomonas* and recognized and described 20 genomic species.

Ideally, a comparison of the complete nucleotide sequences of genomes would probably be the most informative technique and thus the best method for determining the overall taxonomic relatedness of bacterial taxa. As sequencing entire genomes on a routine basis for taxonomy purposes is still impractical, techniques such as the sequencing of "molecular chronometers" like rRNA genes have been developed during the last decade (62). A considerable part of the 16S rRNA gene is conserved in all bacterial genera, whereas a smaller part is variable and this enables workers to estimate genealogical distances, from which phylogenies are derived.

We report here the results of determinations and comparisons of 20 complete 16S ribosomal DNA (rDNA) sequences, as well as 26 partial sequences, of *Xanthomonas* strains representing all 20 species delineated by Vauterin et al. (58) and the complete 16S rDNA sequence of the type strain of *Stenotrophomonas maltophilia*. In analyzing the sequence data, our goals were to assess the interspecific variability within the genus *Xanthomonas* and to establish the phylogenetic relationships of *Xanthomonas* species based on all available 16S rDNA sequence data. Another goal of this work was to assess the compatibility of 16S rDNA sequence similarity values with the results of DNA-DNA pairing experiments. In addition, the 16S rDNA sequences were screened for discriminative regions for distinguishing between the species.

MATERIALS AND METHODS

Bacterial strains. Thirty-eight bacterial strains were obtained from the culture collection of the Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium (Table 1). The strains were grown overnight in 5 ml of Trypticase soy broth (1.7% Bacto Tryptone [Difco], 0.3% Bacto Soytone [Difco], 0.25% Bacto Dextrose [Difco], 0.5% sodium chloride, 0.25% dipotassium phosphate).

DNA preparation. Bacterial cells were pelleted by centrifugation, resuspended in 500 μl of Tris-EDTA buffer, and treated with 6 μl of RNase (1%, wt/vol), 10 μl of proteinase K (1%, wt/vol), and lysozyme before overnight incubation at 37°C. Then 30 μl of sodium dodecyl sulfate (10%, wt/vol) was added, and this was followed by 1 h of incubation at 37°C. After 80 μl of cetyltrimethylammonium bromide (10%, wt/vol) and 100 μl of NaCl (5 M) were added, the solutions were incubated for 10 min at 65°C. DNA was purified by three 1:1 extractions in which we used (i) chloroform, (ii) chloroform-phenol (1:1), (iii) chloroform and then was precipitated with isopropanol (60%, vol/vol), washed with ethanol (70%), and dissolved in Tris-EDTA buffer (31). The quality and concentration of the DNA were checked by measuring optical density.

PCR amplification. The two primers used for PCR amplification (46) are listed in Table 2. Bacterial DNA (1 μg) was added to a solution (total volume, 100 μl) containing 10 μl of 10× *Taq* buffer (10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, 50 mM KCl [0.01%, wt/vol], 0.1 mg of gelatin, 2.0 μl of formamide), 200 μM dATP, 200 μM dGTP, 200 μM dCTP, 200 μM dTTP, each of the two primers at a concentration of 0.6 μM, and 0.5 U of *Taq* DNA polymerase (Perkin-Elmer, Norwalk, Conn.). The samples were processed through 25 amplification cycles consisting of 1 min of denaturation at 94°C, 1 min of annealing at the primer-specific temperature (55°C), and 2 min of primer extension at 72°C (22). The amplification product was purified by filtration by using Microcon 100 spin

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TABLE 1. *Xanthomonas* strains investigated and accession numbers of their 16S rDNA gene sequences in the EMBL Data Library[a]

Taxon	Strain	No. of nucleotides different from the nucleotides of the type strain	EMBL accession no.
X. fragariae	LMG 708^{Tb}		X95920
X. fragariae	LMG 706	0	
X. hortorum pv. hederae	LMG 733^{T}		Y10759
X. hortorum pv. pelargonii	LMG 7314	1	
X. populi	LMG 5743^{Tb}		X95922
X. populi	LMG 974	0	
X. arboricola pv. juglandis	LMG 747^{T}		Y10757
X. arboricola pv. corylina	LMG 689	0	
X. arboricola pv. populi	LMG 12141	0	
X. arboricola pv. pruni	LMG 852	0	
X. cassavae	LMG 673^{T}		Y10762
X. cassavae	LMG 5264	1	
X. codiaei	LMG 8678^{T}		Y10765
X. codiaei	LMG 8677	3	
X. bromi	LMG 947^{T}		Y10764
X. bromi	LMG 8269	3	
X. cucurbitae	LMG 690^{T}		Y10760
X. cucurbitae	LMG 8662	0	
X. axonopodis pv. axonopodis	LMG 538^{Tb}		X95919
X. axonopodis pv. begoniae	LMG 7303	7	
X. axonopodis pv. citri	LMG 682	7	
X. axonopodis pv. coracanae	LMG 686	7	
X. axonopodis pv. dieffenbachiae	LMG 695	8	
X. oryzae pv. oryzae	LMG 5047^{Tb}		X95921
X. oryzae pv. oryzicola	LMG 665	2	
X. vasicola pv. holcicola	LMG 736^{T}		Y10755
X. vasicola pv. holcicola	LMG 7416	0	
X. vasicola pv. vasculorum	LMG 902	1	
X. pisi	LMG 847^{T}		Y10758
X. melonis	LMG 8670^{T}		Y10756
X. melonis	LMG 8672	0	
X. vesicatoria	LMG 911^{T}		Y10761
X. vesicatoria	LMG 920	0	
X. campestris pv. campestris	LMG 568^{Tb}		X95917
X. campestris pv. barbareae	LMG 547	0	
X. translucens pv. translucens	LMG 876^{T}		X99299
X. translucens pv. graminis	LMG 726[b]	2	
X. translucens pv. poae	LMG 728	0	
X. hyacinthi	LMG 739^{T}		Y10754
X. hyacinthi	LMG 742	1	
X. hyacinthi	LMG 8041	0	
X. theicola	LMG 8684^{T}		Y10763
X. sacchari	LMG 471^{T}		Y10766
X. sacchari	LMG 476	6	
X. albilineans	LMG 494^{Tb}		X95918
X. albilineans	LMG 482	1	

[a] For each species the complete 16S rDNA sequence of the type strain was determined and partial 16S rDNA sequences were determined for the additional strains. The number of nucleotide differences between the type strain of each species and each additional strain was calculated after all ambiguous and unknown base positions and all gaps were removed.

[b] Data from reference 38.

columns (Amicon) and was analyzed by 1% agarose gel electrophoresis and subsequent staining with ethidium bromide.

Sequencing of 16S rRNA genes. The PCR products were sequenced by using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, Calif.) and a model ABI 373A automatic sequencer. Nearly complete 16S rDNA sequences were determined by using all of the sequencing primers listed in Table 2. Partial sequences were determined by using the following five sequencing primers: 16R343, 16R519, 16F530, 16F946, and 16R1389 (Table 2).

16S rRNA gene sequence comparison and phylogenetic analysis. Sequences were aligned by using the sequence editing and analysis program of G. J. Olsen with reference data for members of the *Proteobacteria* from the Ribosomal RNA Database Project (34). Conserved primary sequence regions were used for initial alignments. Alignment of variable regions was aided by the results of an analysis of secondary structure. The 16S rDNA sequences of *Xanthomonas campestris* LMG 568^{T} (T = type strain), *Xanthomonas albilineans* LMG 494^{T}, *Xanthomonas axonopodis* LMG 538^{T}, *Xanthomonas fragariae* LMG 708^{T}, *Xanthomonas oryzae* LMG 5047^{T}, *Xanthomonas populi* LMG 5743^{T}, and *Xanthomonas translucens* LMG 726 were obtained from EMBL; the accession numbers of these sequences are given in Table 1. The 16S rDNA sequence of *S. maltophilia* LMG 958^{T} was also obtained from EMBL (accession number, X95923). Similarity values were calculated and a cluster analysis was performed by using the GeneCompar software (Applied Maths, Kortrijk, Belgium); only unambiguous and homologous nucleotide positions were used in this analysis. Dendrograms were constructed by using the same software package and the neighbor-joining method (47).

Nucleotide sequence accession numbers. The nucleotide sequences of 12 complete 16S rDNAs have been deposited in the EMBL Data Library under the accession numbers given in Table 1.

RESULTS AND DISCUSSION

Direct sequencing of the PCR-amplified 16S rDNAs allowed us to determine a continuous stretch of 1,498 to 1,502 bases, ranging from position 28 to position 1524 of the 16S rRNA gene sequence of *Escherichia coli*. These sequences corresponded to an estimated 98.3 to 98.6% of the total 16S rDNA primary sequence. The partial sequences determined, comprising 1,139 to 1,357 nucleotides, corresponded to 74.7 to 89.0% of the total 16S rDNA primary sequence. The sequence data were confirmed by sequencing the 16S rDNA PCR products, for the most part, in both the forward and reverse directions.

The presence of signature nucleotides at *E. coli* positions 170 (CTAATACCG), 315 (YCACAYYG), 510 (CTAACT YYG), and 1410 (TCACACCATG) (52) in all of the strains examined supported the results of previous analyses which demonstrated that the *Xanthomonas* species cluster in the gamma subdivision of the *Proteobacteria* (14, 38, 42, 43).

The levels of sequence similarity among the nearly complete 16S rDNA sequences of the type strains of the 20 *Xanthomonas* species and *S. maltophilia* are presented in a similarity matrix in Table 3; the data revealed that the levels of sequence similarity among all of the *Xanthomonas* species were relatively high and that the mean level of sequence similarity ± standard deviation was 99.0% ± 1.0%.

A dendrogram depicting the estimated phylogenetic relationships (Fig. 1) was constructed by the neighbor-joining clustering method (47); this dendrogram was based on pairwise comparisons of all of the available 16S rDNA sequence data for the genus *Xanthomonas* and the type strain of *S. maltophilia*. All of the xanthomonads clustered together with sequence similarities greater than 97.2%, and the maximum number of base differences between any species pair was 42. Within the genus *Xanthomonas*, the following two main clusters could be distinguished: cluster 1 around *X. campestris*, the type species of the genus *Xanthomonas*; and cluster 3 around *X. albilineans* (Fig. 1). These clusters are referred to below as the *X. campestris* core and the *X. albilineans* core, respectively.

The levels of 16S rDNA sequence similarity between the species in the *X. campestris* core ranged from 98.9 to 100%, and the number of nucleotide differences ranged from 0 to 16. Cluster 1, consisting of *Xanthomonas arboricola*, *X. axonopodis*,

TABLE 2. Oligonucleotide primers used in this study for 16S rDNA amplification and sequencing of *Xanthomonas* spp.

Primer[a]	Sequence	Target positions[b]	Application
16F27	5′ AGAGTTTGATCMTGGCTCAG 3′	8-27	Amplification
16R343	5′ ACTGCTGCCTCCCGTA 3′	358-343	Sequencing
16F355	5′ ACTCCTACGGGAGGCAGC 3′	337-355	Sequencing
16R519	5′ GTATTACCGCGGCTGCTG 3′	536-519	Sequencing
16F530	5′ TTCGTGCCAGCAGCCGCGG 3′	512-530	Sequencing
16R685	5′ TCTACGCATTTCACCGCTAC 3′	704-685	Sequencing
16F704	5′ GTGTAGCGGTGAAATGCGTAGA 3′	685-704	Sequencing
16F946	5′ CCCGCACAAGCGGTGGA 3′	930-946	Sequencing
16R1087	5′ CTCGTTGCGGGACTTAACCC 3′	1206-1087	Sequencing
16F1195	5′ AGGAAGGTGGGGATGACGTC 3′	1195-1214	Sequencing
16R1389	5′ ACGGGCGGTGTGTACAAG 3′	1389-1372	Sequencing
16R1525	5′ TTCTGCAGTCTAGAAGGAGGTGWTCCAGCC 3′	1525-1496	Amplification

[a] F, forward primer; R, reverse primer.
[b] The numbering of target positions is based on the numbering of the *E. coli* 16S rRNA sequence (6).

Xanthomonas bromi, *X. campestris*, *Xanthomonas cassavae*, *Xanthomonas codiaei*, *Xanthomonas cucurbitae*, *X. fragariae*, *Xanthomonas hortorum*, *Xanthomonas melonis*, *X. oryzae*, *Xanthomonas pisi*, *X. populi*, *Xanthomonas vasicola*, and *Xanthomonas vesicatoria*, was a very homogeneous group within the genus *Xanthomonas*. Although all 15 species belonging to this cluster have been described as genospecies on the basis of DNA-DNA hybridization data (58), they did not show any significant 16S rRNA gene sequence differences which would allow definitive conclusions to be derived concerning their internal phylogenetic relationships.

Xanthomonas sacchari LMG 471^T (cluster 2 in Fig. 1) branched outside the *X. campestris* core; this organism exhibited 98.9% 16S rDNA sequence similarity and had 19 ± 2 nucleotide differences with cluster 1 organisms. Its mean level of sequence similarity with the *X. albilineans* core is 98.2%, and the numbers of nucleotide differences range from 26 to 28.

The members of the *X. albilineans* core (cluster 3 in Fig. 1), comprising *X. albilineans* LMG 494^T, *X. translucens* LMG 876^T, *Xanthomonas hyacinthi* LMG 739^T, and *Xanthomonas theicola* LMG 8684^T, exhibited only 0.3% 16S rDNA sequence differences (three to seven nucleotide differences). As observed for the members of the *X. campestris* core, the differences in 16S rDNA sequence similarity among the type strains of *X. translucens*, *X. albilineans*, *X. theicola*, and *X. hyacinthi* are too small to discriminate these species. As a group though, these species are clearly separated from the other xanthomonads, since they have a mean level of 16S rDNA sequence similarity of only 97.8% with *X. campestris*, corresponding to 32 to 42 nucleotide differences.

Particularly within the *X. campestris* core but also within the *X. albilineans* core, the levels of interspecific sequence similarity are remarkably high. No nucleotide differences were observed among the sequences of the type strains of *X. cucurbitae*, *X. vesicatoria*, and *X. pisi*, between the sequences of the type strains of *X. campestris* and *X. arboricola*, and between the sequences of the type strains of *X. vasicola* and *X. bromi*, although DNA-DNA hybridization values indicate that these taxa are distinct species. Effective identity of the 16S rDNA sequences of different species has been described for the genera *Aeromonas* (37), *Bacillus* (2, 16), and *Saccharomonospora* (23). There are several other genera in which the species have been determined to have similarly small numbers of nucleotide differences; these genera include the genus *Enterococcus* (36), the genera *Aerococcus* and *Leuconostoc* (35), and the genus *Serratia* (10). It is now generally accepted that 16S rDNA sequence identity is not sufficient to ensure species identity (53).

The 16S rDNA sequence of *S. maltophilia* LMG 958^T differs from the sequences of the *Xanthomonas* type strains; the average level of sequence similarity is 96.6% ± 0.7%, which corresponds to 50 ± 11 nucleotide differences. *S. maltophilia* LMG 958^T has average levels of sequence similarity of 95.2 to 95.4% with members of the *X. albilineans* core and 96.5 to 97.1% with members of the *X. campestris* core. These values are lower than the levels of similarity between the *X. campestris* core and the *X. albilineans* core (97.2 to 97.9%), which may support the opinion that *S. maltophilia* should be classified outside the genus *Xanthomonas* (42, 56).

However, the present data raise the question whether the levels of 16S rDNA sequence difference between *S. maltophilia* LMG 958^T and *Xanthomonas* spp. (2.9 to 4.8%) are significant enough to classify these taxa in two separate genera. Examples of genera with smaller internal 16S rDNA sequence differences are the genus *Acinetobacter* (with 0.0 to 4.6% differences) (44), the genus *Saccharomonospora* (with 0.1 to 3.7% sequence differences) (23), and the genus *Aeromonas* (with only 0.1 to 2.2% differences) (37). Other genera, however, are much more heterogeneous in terms of intrageneric 16S rDNA relatedness; within the genus *Nocardia* 1.6 to 9.6% difference was found (8), 0.4 to 5.5% difference was found within the genus *Acetobacter* (49), and 0.1 to 8.7% difference was found within the genus *Vibrio* (61).

A second question which arose because of the observed sequence data concerns the cluster around *X. albilineans*. The 16S rDNA sequences of the species in this cluster differ by 2.1 to 2.8% from the 16S rDNA sequence of the *X. campestris* core, and these organisms were also identified as a separate group in a previous study based on the restriction fragment length polymorphism of PCR-amplified 16S rDNAs of 33 *Xanthomonas* strains (41). When the data are expressed as number of nucleotide differences, the *X. albilineans* group and the *X. campestris* group differ at 36 nucleotide positions on average (Table 4). This value is rather high compared to the five nucleotide differences within each group separately. One may wonder whether these data are not significant enough to justify classification of the clusters in two distinct genera. Considering the clear delineation of the two groups, it would be very difficult for a taxonomic splitter to resist creating another genus for the *X. albilineans* core. However, we could not find any common feature that separated the *X. albilineans* core from the *X. campestris* core. *X. translucens*, *X. hyacinthi*, and *X. theicola* had

TABLE 3. Levels of sequence similarity and numbers of nucleotide differences for a 1,498- to 1,503-nucleotide region of 16S rDNAs of the type strains of *Xanthomonas* species after ambiguous and unknown base positions (0.07% of the positions) and all gaps were removed

Strain	% Sequence similarity or no. of nucleotide differences																				
	X. fragariae LMG 708^T	*X. hortorum* LMG 733^T	*X. populi* LMG 5743^T	*X. arboricola* LMG 747^T	*X. cassavae* LMG 673^T	*X. codiaei* LMG 8678^T	*X. bromi* LMG 947^T	*X. cucurbitae* LMG 690^T	*X. axonopodis* LMG 538^T	*X. oryzae* LMG 5047^T	*X. vasicola* LMG 736^T	*X. pisi* LMG 847^T	*X. melonis* LMG 8670^T	*X. vesicatoria* LMG 911^T	*X. campestris* LMG 568^T	*X. translucens* LMG 876^T	*X. hyacinthi* LMG 739^T	*X. theicola* LMG 8684^T	*X. sacchari* LMG 471^T	*X. albilineans* LMG 494^T	*S. maltophilia* LMG 958^T
X. fragariae LMG 708^{Tb}		99.5	99.5	99.6	99.5	99.2	99.5	99.6	98.9	99.5	99.5	99.6	99.6	99.6	99.5	97.3	97.4	97.2	98.4	97.4	96.7
X. hortorum LMG 733^T	8		99.9	99.7	99.8	99.5	99.8	99.9	99.2	99.7	99.8	99.9	99.9	99.9	99.9	97.7	97.6	97.6	98.8	97.7	97.1
X. populi LMG 5743^{Tb}	8	2		99.9	99.8	99.5	99.8	99.9	99.2	99.7	99.8	99.9	99.9	99.9	99.9	97.7	97.6	97.6	98.8	97.7	97.1
X. arboricola LMG 747^T	7	1	1		99.9	99.5	99.9	99.9	99.3	99.8	99.9	99.9	99.9	99.9	100	97.7	97.7	97.7	98.9	97.9	97.1
X. cassavae LMG 673^T	7	3	3	2		99.7	99.9	99.9	99.3	99.8	99.9	99.9	99.8	99.9	99.9	97.7	97.9	97.7	98.9	97.8	97.0
X. codiaei LMG 8678^T	12	8	8	7	5		99.5	99.6	98.9	99.5	99.5	99.6	99.5	99.6	99.5	97.4	97.3	97.4	98.6	97.5	96.9
X. bromi LMG 947^T	7	3	3	2	2	7		99.9	99.3	99.8	100	99.9	99.8	99.9	99.9	97.7	97.9	97.7	98.7	97.7	97.0
X. cucurbitae LMG 690^T	6	2	2	1	1	6	1		99.2	99.9	99.9	100	99.9	100	99.9	97.7	97.8	97.6	98.8	97.7	97.1
X. axonopodis LMG 538^{Tb}	16	12	12	11	11	16	11	12		99.1	99.3	99.2	99.3	99.2	99.3	97.8	97.9	97.9	98.4	97.9	96.5
X. oryzae LMG 5047^{Tb}	8	4	4	3	3	8	3	2	14		99.8	99.9	99.7	99.9	99.8	97.6	97.5	97.5	98.7	97.7	96.9
X. vasicola LMG 736^T	7	3	3	2	2	7	0	1	11	3		99.9	99.8	99.9	99.9	97.7	97.7	97.7	98.7	97.7	97.0
X. pisi LMG 847^T	6	2	2	1	1	6	1	0	12	2	1		99.9	100	99.9	97.7	97.6	97.6	98.8	97.7	97.1
X. melonis LMG 8670^T	6	2	2	1	3	8	3	2	10	4	3	2		99.9	99.9	97.7	97.6	97.6	98.8	97.7	97.1
X. vesicatoria LMG 911^T	6	2	2	1	1	6	1	0	12	2	1	0	2		99.9	97.7	97.6	97.6	98.8	97.7	97.1
X. campestris LMG 568^{Tb}	7	1	1	0	2	7	2	1	11	3	2	1	1	1		97.7	97.9	97.7	98.9	97.8	97.1
X. translucens LMG 876^{Tb}	41	35	35	34	34	39	34	34	33	36	34	35	35	35	34		99.7	99.8	98.2	99.5	95.4
X. hyacinthi LMG 739^T	42	36	36	35	35	40	35	36	32	37	35	36	36	36	35	4		99.8	98.1	99.5	95.2
X. theicola LMG 8684^T	42	36	36	35	35	40	35	36	32	37	35	36	36	36	35	3	3		98.1	99.6	95.2
X. sacchari LMG 471^T	24	18	18	17	17	21	19	18	24	19	19	18	18	18	17	27	28	28		98.3	97.0
X. albilineans LMG 494^{Tb}	40	34	34	33	33	38	35	34	32	35	35	34	34	34	33	7	7	6	26		95.3
S. maltophilia LMG 958^{Tb}	48	44	44	43	45	47	45	43	53	46	45	43	43	44	43	69	72	72	45	70	

[a] The values on the upper right are levels of sequence similarity, and the values on the lower left are numbers of nucleotide differences.
[b] Data from reference 38.

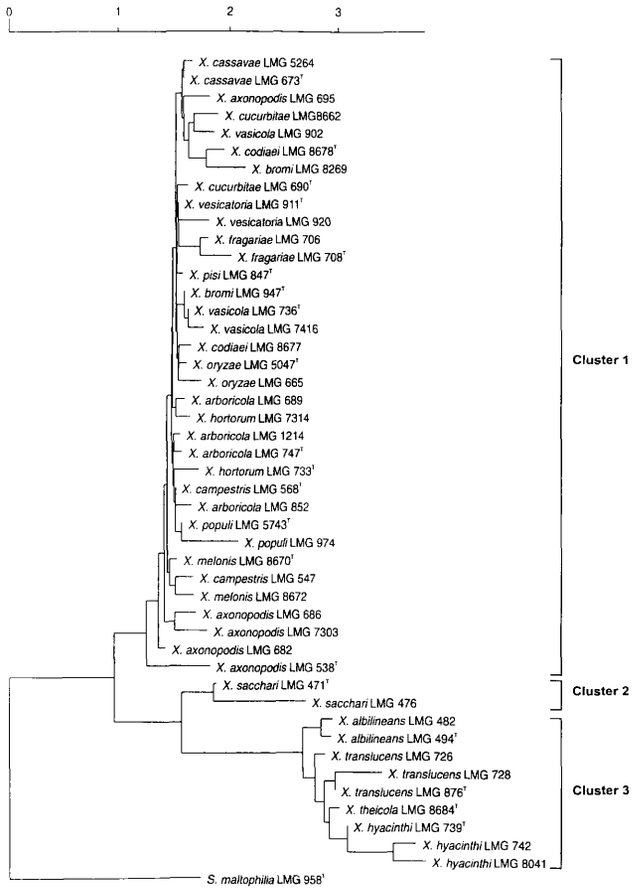

FIG. 1. Neighbor-joining dendrogram depicting estimated phylogenetic relationships based on pairwise comparisons of partial 16S rDNA sequences of all of the *Xanthomonas* strains examined, constructed by neighbor-joining, with *S. maltophilia* LMG 958^T as an outgroup. The distance between two species is obtained by adding the lengths of the connecting horizontal branches, using the scale at the top (percentage of sequence difference). Clusters 1 and 2 represent the *X. campestris* and *X. albilineans* cores, respectively.

an average G+C content of 69 mol%, whereas all of the other xanthomonads have average G+C contents of 65 to 66 mol% (59). Within the *X. albilineans* core, *X. albilineans* itself is an exception to the high G+C contents of the xanthomonads; its G+C content is 64.2 mol%. *X. albilineans*, *X. translucens*, and *X. hyacinthi* are all pathogenic for monocotyledonous plants, but within the *X. albilineans* core, *X. theicola* is an exception. The present data alone, in the absence of clear phenotypic or chemotaxonomic distinctions between the two cores, do not justify splitting the genus *Xanthomonas* into two genera. In addition, based on 16S rDNA sequences, there is an intermediate species, *X. sacchari*, which belongs to neither of the two core groups.

The small degree of divergence in 16S rDNA sequences observed among the xanthomonads contrasts with the very low levels of DNA-DNA homology (as low as 4%) found between some species (58). Strain pairs exhibiting extremely low levels of DNA homology did not necessarily exhibit the lowest levels of 16S rDNA sequence similarity. The lowest level of sequence similarity observed (97.2%) was the level of similarity between *X. fragariae* and *X. theicola* and corresponded to 23% DNA homology. The lowest level of DNA homology (4%) was the level of homology between *X. sacchari* and *X. melonis*, whose level of 16S rDNA sequence similarity is 98.8%. Several strains that exhibited 100% 16S rDNA sequence similarity were found to have DNA binding levels ranging from 20 to 40%.

These findings resemble, more or less, the situation reported for the interspecific relationships in the genus *Aeromonas*. After the 16S rDNA sequences were analyzed, several inconsistencies were noted regarding relatedness, as obtained from the results of DNA-DNA pairing studies (37). When he examined the *Aeromonas* 16S rDNA sequence data, Sneath (50) found evidence of the occurrence of hybrid events in the form of gene crossovers. A useful criterion for recognizing this phenomenon is that poor ultrametric properties result in low cophenetic correlation on a dendrogram (51). The cophenetic correlation value for unweighted pair group method with arithmetic average clustering of the 16S rDNA sequences used in this study was 0.96. Separate clustering of the left and right subsequences produced cophenetic correlation values of 0.95 and 0.91 respectively. For the genus *Aeromonas* a value of only 0.80 was obtained for the whole sequences, whereas values of 0.95 and 0.87 were obtained for the left and right subsequences, respectively. The high cophenetic correlation values observed for the sequences in this study indicate that the mutation rates within the 16S rDNA genes are relatively constant and spread out homogeneously, suggesting that gene crossovers are not likely to have occurred in the genus *Xanthomonas*.

As pointed out by Stackebrandt and Goebel (53), species possessing 70% or greater DNA similarity usually exhibit more than 97% 16S rDNA sequence similarity. Our sequence data show that for the genus *Xanthomonas* the value is even higher as all of the strains that exhibit 70% or more DNA homology exhibit 100% sequence similarity. The correspondence between the DNA homology data determined by DNA-DNA hybridization (58) and the 16S rDNA sequence similarity data for the genus *Xanthomonas* has been evaluated, and the results are shown in Fig. 2. The percentages of sequence similarity for all of the *Xanthomonas* strains which were completely sequenced are plotted against the DNA-DNA homology values in Fig. 2. Within the genus *Xanthomonas*, there is a correlation between the two sets of data; organisms that exhibit levels of 16S rDNA sequence similarity between 99 and 97% never exhibit levels of DNA homology higher than 40%. This area contains the correlation data derived from pairwise comparisons of the species around the *X. albilineans* core and the *X. campestris* core. Levels of 16S rDNA sequence similarity greater than 99% correspond to DNA-DNA hybridization values between approximately 20 and 100%.

Analyzing the sequences in more detail revealed main variable regions at approximately the following *E. coli* positions: positions 80 to 93 (12 variable positions), located in the V1 region (39); positions 139 and 140, 191 and 192, and 256 to 260

TABLE 4. Numbers of nucleotide differences within and between *Xanthomonas* clusters

Taxon	No. of nucleotide differences		
	X. campestris core (cluster 1)	*X. sacchari* (cluster 2)	*X. albilineans* core (cluster 3)
X. campestris core (cluster 1)	5 ± 4[a]		
X. sacchari (cluster 2)	19 ± 2		
X. albilineans core (cluster 3)	36 ± 2	27 ± 1	5 ± 2

[a] Mean ± standard deviation.

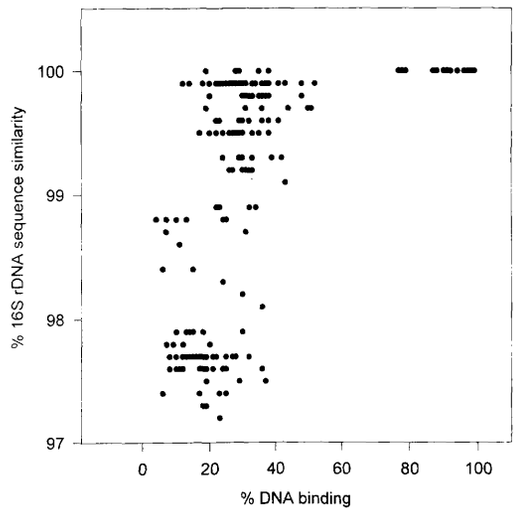

FIG. 2. Correlation plot of the DNA homology data (determined by the spectrophotometric method measuring renaturation [11]) versus levels of 16S rDNA sequence similarity for all *Xanthomonas* type strains.

(4 variable positions), located in the V2 region; positions 595 and 605, located in the V4 region; positions 652 to 654, 673 to 675 (2 variable positions), 726, 741 to 743 (2 variable positions), and 849, located in the V5 region; position 1144, located in the V7 region; and positions 1253 to 1257, 1272, 1278 to 1282 (4 variable positions), and 1300 and 1301, located in the V8 region. The most hypervariable region of the 16S rDNA sequences of the xanthomonads is located at *E. coli* positions 80 to 93, where 12 variable positions are concentrated within 14 nucleotides. These bases are positioned on helix 6, which is incorporated in the 16S rRNA V1 area (39). The secondary structure of this region is shown in Fig. 3. It is mainly this region that separates the cluster around *X. albilineans* from the *X. campestris* core. Overall, 94.34% of the 16S rDNA sequence of the genus *Xanthomonas* is invariable, 3.99% is variable, and 1.66% comprises ambiguous positions or gaps. The "*Xanthomonas*-constant" 16S rDNA sequence is given in Fig. 4.

Nucleotide sequence comparisons open up the prospect of developing PCR or oligonucleotide probes for diagnostic purposes (12). DeParasis and Roth (13) were the first workers to partially sequence a number of phytopathogenic *Xanthomonas* strains and propose a 27-bp 16S rRNA genus-specific hybridization sequence. These authors sequenced 26 *Xanthomonas* strains from position 457 to position 491 (*E. coli* numbering). Their nucleotide sequence for this region, however, did not correspond completely with our data. In our analysis all of the

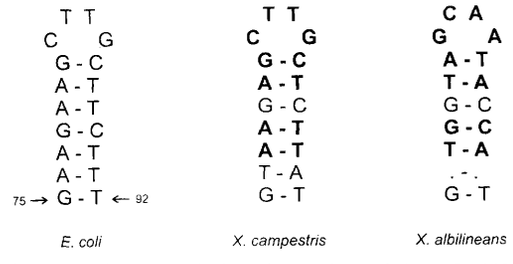

FIG. 3. Diagnostic area in helix 6 (V1 region) of the 16S rDNA which distinguishes the cluster around *X. albilineans* from the *X. campestris* core. Variable positions are shown in boldface type. Dots indicate gaps. *E. coli* 16S rRNA gene sequence numbering was used.

```
   1 AGTGAACGCT G.CGGCAGGC CTAACACATG CAAGTCG..C GGCAGCAC.G ...G.....
  61 ..C...TGGG T..CGAGTGG CGGACGGGTG AGGAATACAT CGGAATCTAC ..TTTCGTGG
 121 GGGATAACGT AGGGAAACTT ACGCTAATAC CGCATACGAC C...GGGTGA AAGCGGAGGA
 181 CCTTCGGGCT TCGCG.G..T ..ATGA.CCG ATGTCGGATT AGCTAGTTGG .GGGGTAAAG
 241 GCCCACCAAG GCGACGATCC GTAGCTGGTC TGAGAGGATG ATCA.CCACA CTGGAACTGA
 301 GACACGGTCC AGACTCCTAC GGGAGGCAGC AGTGGGGAAT ATTGGACAAT GGGCGCAAGC
 361 CTGATCCAGC CATGCCGCGT GG.TGAAGAA GGCCTTCGGG TTGTAAAGCC CTTTTGTTGG
 421 G.AAGAAAA. CA.TCGGTTA ATACCCG.TT GTTCTGACGG TACCCAAAGA ATAAGCACCG
 481 GCTAACTTCG T.CCAGCAG. CGCGGTAATA CGAAGGGTGC AAGCGTTACT CGGAATTACT
 541 GGGCGTAAAG CGTGCGTAGG TGGT..TTTA .GTC.GTTGT GAAAGCCCTG GGCTCAACCT
 601 GGGAATT.CA G.GGATACTG G...ACTAGA GTGTGG.AGA GG.T.GCGGA ATTCCCGG.G
 661 TAGCAGT.AA AT.CGT.GAG ATCGGGGAGGA ACATC.GTGG CGAAGGCGG. .A.CTGGACC
 721 AACACTGACA CTGAGGCACG AAAGCCTGGG GAGCAAACAG GATTAGATAC CCTGGTAGTC
 781 CACGCCCTAA ACGATGCGAA CTGGATGTTG GGTGCAA.TT GGCAC.CAGT ATCGAAGCTA
 841 ACGCGTTAAG TTCGCCGCCT GGGGAGTACG GTCGCAAGAC TGAAACTCAA AGGAATTGAC
 901 GGGGGCCCGC ACAAGCGGTG GAGTATGTGG TTTAATTCGA TGC.ACGCGA AGAACCTTAC
 961 CTGGTCTTGA CATCCACGGA ACTTTCCAGA GATGGAT.GG TG.CTTCGGG AACCGTGAGA
1021 CAGGTGCTGC ATGGCTGTCG TCAGCTCGTG TCGTGAGATG TTGGGTTAAG TCCCGCAACG
1081 AGCGCAACCC TTGTCCTTAG TT.CCAGCAC GT.ATGGTGG G.ACT.TAAG GAGACCGCCG
1141 GTGACAAACC GGAGGAAGGT GGGGATGACG TCAA.TCATC ATGGCCCTTA CGACCAGGGC
1201 TACACACGTA CTACAATGGT ..GGACAGAG GGCTGCAA.C ..GCGA..G. .AGCCAATCC
1261 CAGAAACC.. ATCTCAGTCC GGATTGGAGT CTGCAACTCG ACTCCATGAA GTCGGAATCG
1321 CTAGTAATCG CAGATCAGCA TTGCTGCGGT GAATACGTTC CCGGGCCTTG TACACACCGC
1381 CCGTCACACC ATGGGAGTTT GTTGCACCAG AAGCAGGTAG CTTAACCTTC GGGAGGGCGC
1441 TTG.CACGGT GTGG.CGATG ACTGGGGTGA AGTCGTAA.. .GGTAGCCGT ATC.GAAGGT
1501 GC
```

FIG. 4. Conserved positions within the 16S rDNA sequences of *Xanthomonas*. Positions where variability, ambiguity, or gaps occur are indicated by dots.

Xanthomonas type strains had TGTTC at positions 478 to 482, whereas DeParasis and Roth (13) found CCTTA. It should be mentioned that DeParasis and Roth (13) sequenced an rRNA template with reverse transcriptase, whereas PCR-amplified rDNAs were sequenced in this study. Clayton et al. (9) have recently pointed out that there are unexpectedly high levels of variation in small-subunit rRNA sequences of duplicate strains deposited in the GenBank database. The possibility of variation should be taken into account, especially when sequence data are used for probe development. The sequence data of DeParasis and Roth (13) were used by Maes (32) to develop a 17-mer PCR oligonucleotide probe for the genus *Xanthomonas*. Two nucleotides, which were not complementary with our sequence data, were located at positions 14 and 15 near the 5′ end of this primer. Positions 16 and 17 did complement our sequence data. As the first 13 nucleotides at the 3′ end were specific for the genus *Xanthomonas*, Maes (32) described this primer as genus specific.

The partial sequences of one or more additional strains of each species were used to analyze the intraspecific variability of the 16S rDNA sequences of the *Xanthomonas* species, as well as to confirm the observed interspecific differences. The numbers of nucleotide differences between the type strains and the additional strains of each species are shown in Table 1. These values ranged from 0 to 8 (as much as 0.5% of the total 16S rDNA). The number of nucleotide differences within *X. axonopodis* ranged from 7 to 8, values which are relatively high compared to the values obtained for the other *Xanthomonas* species, which had between 0 and 6 internal nucleotide differences (approximately 0.4% of the total 16S rDNA). The heterogeneity within *X. axonopodis* reflects the results of the DNA-DNA hybridization studies, in which *X. axonopodis* exhibited a mean binding value of 77% internally, the lowest value in the genus. Both data sets demonstrate that *X. axonopodis* is the least homogeneous species of the genus.

The type strains of *X. vasicola* and *X. bromi* exhibited no nucleotide differences, based on nearly complete sequences, yet the 16S rDNAs of the additional strains of these species had one to four nucleotide differences, compared with the sequences of the respective type strains. These minimal differences probably reflect random mutations under no (or limited) evolutionary pressure and, once more, point out the homogeneity of the 16S rDNA sequences of the *X. campestris* core. For this reason, it may not be possible to define sequence signatures that reliably differentiate the species within the genus *Xanthomonas*. Some nucleotide positions or regions that are

constant within one species but different from the positions or regions of other species could be detected, but any 16S rRNA gene probes or primers based on these positions would need to be tested with many reference strains before we can be sure about their specificity. Because of the greater sequence variability in the 16S-23S rRNA internal transcribed spacers and the 23S rRNA, sequencing these regions may prove to be more useful for developing probes or primers. Maes et al. (33) developed an internal transcribed spacer oligonucleotide primer specific for *X. translucens*.

In conclusion, based on the results of this study, 16S rDNA sequence analyses provide a new picture of the genus *Xanthomonas*, which appears to be composed of two very homogeneous cores and a separate branch comprising the single species *X. sacchari*. These relationships were not discerned previously by DNA hybridization or any other technique except PCR restriction fragment length polymorphism of 16S rRNA (41). This observation, together with the fact that many strain pairs with identical or nearly identical sequences have very low levels of DNA homology indicates that 16S rRNA (or rDNA) sequencing has, in terms of taxonomic resolution, no overlap with DNA hybridization. The conclusion of Stackebrandt and Goebel (53) that sequence analysis of 16S rRNA should not replace DNA-DNA hybridization for delineation of species is supported by the results of this study.

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