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The Phloem-Limited Bacterium of Greening Disease of Citrus Is a Member of the α Subdivision of the *Proteobacteria*

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Using the PCR, we amplified the 16S ribosomal DNAs (rDNAs) of an Asian strain and an African strain of the uncultured, gram-negative, walled, phloem-limited bacterium-like organism (BLO) associated with citrus greening disease. We evaded coamplification of chloroplast 16S rDNA by using restriction enzymes; the chloroplast 16S rDNA was sensitive to *BclI* digestion and resistant to *Eco*RI digestion, while the 16S rDNA of the BLO was resistant to *BclI* digestion and sensitive to *Eco*RI digestion. The 16S rDNA of the African BLO strain was amplified from *BclI*-digested DNA extracted from infected periwinkle leaf midribs. The Asian strain was isolated from plant extract by using a specific monoclonal antibody coated onto the surface of a PCR tube. The 16S rDNAs of the two BLO strains were cloned and sequenced. Comparisons with sequences of 16S rDNAs obtained from the GenBank data base revealed that the two citrus greening disease BLOs belong to the alpha subdivision of the class *Proteobacteria*. Even though their closest relatives are members of the alpha-2 subgroup, these BLOs are distinct from this subgroup as we observed only 87.5% homology between the 16S rDNAs examined. Therefore, the two BLOs which we studied probably are members of a new lineage in the α subdivision of the *Proteobacteria*. We propose the trivial name "liberobacter" for this new group of bacteria and will wait until additional characteristics have been determined before we propose a formal name.

The procaryote associated with citrus greening disease was first observed in 1970 by Laflèche and Bové in the phloem of affected sweet orange leaves (17). It was initially thought that the greening organism is a mycoplasma-like organism (MLO), but this organism was soon found to be enclosed by a 25-nm-thick envelope, which was much thicker than the unit membrane envelopes characteristic of MLOs (thickness, 7 to 10 nm) (26). These properties suggested that the greening organism is a walled bacterium and does not resemble mycoplasmas. Organisms similar to the greening agent occur in plants other than citrus and are involved in more than 20 different diseases (3, 4, 13, 15, 16, 23, 25, 28, 29). As far as is known, these organisms are always restricted to the sieve tubes within the phloem tissue. None of them has been obtained in pure culture. By analogy with MLOs, these organisms have been called bacterium-like organisms (BLOs) (9); they have also been inappropriately called rickettsia-like organisms.

Greening is one of the most severe diseases of citrus. It has a large geographic distribution because it is transmitted by two psyllid insect vectors, *Diaphorina citri* in Asia and *Trioza erytreae* in Africa (2, 20). Symptoms of greening in Asia occur even when temperatures are well above 30°C, while in Africa the disease is present only in cool regions. These temperature effects have been reproduced under phytotron conditions (1). In addition, when the greening BLO was experimentally transmitted from citrus to periwinkle plants, the greening reaction was the same as that observed in citrus (8). Therefore, the African BLO is heat sensitive and the Asian BLO is heat tolerant, which is the only known difference between the African and Asian greening diseases.

Characterization has been slow and difficult because the BLOs have not been cultured. Electron microscopy combined with cytochemistry revealed that the greening BLO was surrounded by a peptidoglycan-containing membranous cell wall of the gram-negative type (9). Later, monoclonal antibodies (MAbs) were obtained against two Asian BLO strains, strain Poona from India and strain Fujian from the People's Republic of China, and one African strain, strain Nelspruit from South Africa (10, 11). These MAbs are highly strain specific, and seven different BLO strains have been identified so far by using them (7). Recently, a 2.6-kbp DNA fragment of the Poona BLO genome (fragment In-2.6) has been cloned and sequenced (30, 31). This fragment corresponds to the rather well-conserved *rplKAJL-rpoBC* operon and in particular encodes four ribosomal proteins (proteins L1, L11, L12, and L10). When, for taxonomic purposes, the sequence of the BLO operon was compared with the sequences from other bacteria obtained from the GenBank data base, the greening BLO was unambiguously identified as a member of the *Eubacteria*. However, a comparison of the protein sequences deduced from the genes with their counterparts in other bacterial species failed to reveal a specific relationship between the BLO and any previously described bacterial species.

Southern hybridizations of fragment In-2.6 with DNAs extracted from greening organism-infected citrus plants obtained from various geographic regions (31) revealed that In-2.6 was able to hybridize under high-stringency conditions with all of the Asian strains, but not with the African strain tested. However, at lower stringencies, hybridization was also obtained with the African strain, but Southern hybridization profiles revealed DNA polymorphism (30).

In order to determine the phylogenetic position of the greening BLO and the evolutionary distance between African and Asian BLOs, we PCR amplified, cloned, and sequenced the 16S ribosomal DNAs (rDNAs) of Asian strain Poona and African strain Nelspruit of the greening BLO. Sequence comparisons revealed that the two BLOs are members of the α subdivision of the *Proteobacteria*.

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MATERIALS AND METHODS

Plant material. Periwinkle and citrus plants infected with strain Poona (from India) or strain Nelspruit (from South Africa) of the BLO were obtained as described previously (11). The plants were maintained in a greenhouse at 30°C during the day and at 25°C at night (strain Poona) or at 25°C during the day and at 20°C at night (strain Nelspruit). Healthy periwinkle plants were obtained from seeds and were maintained at 20 to 25°C.

MAb. MAb 2D12 specific for strain Poona BLO was prepared as described previously (11).

DNA extraction and dot blot hybridization. DNA extraction from plant material and dot blotting were carried out as described by Villechanoux et al. (31) by using oligonucleotides labeled with [α-^{32}P]dATP and T4 polynucleotide kinase (Pharmacia). Hybridizations were done in the buffer of Zeff and Geliebter (35).

Immunocapture PCR. For the immunocapture PCR, the organisms in a crude plant extract whose DNAs were to be amplified were first captured by specific antibodies bound to the inside walls of plastic PCR tubes. After this immunocapture step, the plant extract was discarded, and the tubes were carefully washed. A PCR mixture without *Taq* polymerase was added, and the tubes were heated for 30 min at 92°C, a step during which lysis of the organisms occurred and DNA was released. After this pretreatment step, the PCR was started by adding *Taq* DNA polymerase. The plastic tubes (0.5 ml; Eppendorf) were coated with 100 μl of MAb 2D12 immunoglobulin G (20 μg/ml) in 0.1 M carbonate buffer (pH 9.6) overnight at 4°C and washed three times with PBS-600 (0.336 M NaCl, 1.4 mM KH_2PO_4, 8 mM $Na_2HPO_4 \cdot 12H_2O$, 2.6 mM KCl). The tubes were incubated with 250 μl of a bovine serum albumin (BSA) solution (10 mg/ml) for 1 h and washed as described above. A 5-g portion of greening organism-infected periwinkle midribs was disinfected for 5 min with 70% ethanol and for 5 min with 4.8% Chlorox and then rinsed three times with sterile water. The midribs were then finely minced with a razor blade in 10 ml of PBS-600. The liquid phase of the plant homogenate was collected with a syringe and then centrifuged at low speed (1,000 × *g*) for 2 min. The supernatant was centrifuged at high speed (10,000 × *g*) for 15 min, and the resulting pellet was resuspended in 1 ml of PBS-600. A 100-μl portion of plant extract was added to each coated tube, and the preparations were incubated overnight at 4°C. The plant extract was discarded, and the tubes were washed three times with PBS-600.

The 16S rDNA was amplified by the PCR directly in the tubes in 100 μl of PCR mixture containing each of the two universal primers used for amplification of prokaryotic 16S rDNA (32) (forward primer AGAGTTTGATCATGGCT CAG and reverse primer AAGGAGGTGATCCAGCCGC) at a concentration of 1 μM, each of the four deoxynucleoside triphosphates at a concentration of 200 μM, 78 mM Tris HCl (pH 8.8), 2 mM $MgCl_2$, 17 mM $(NH_4)SO_4$, 10 mM β-mercaptoethanol, 0.05% W1 detergent (GIBCO-BRL), and 200 μg of BSA per ml. Oil (100 μl) was added to prevent evaporation. The conditions used for DNA amplification were as follows: 92°C for 30 min (pretreatment); 40 PCR cycles, each consisting of 92°C for 1 min, 47°C for 30 s, and 72°C for 90 s; and 72°C for 10 min for chain elongation. *Taq* polymerase (2.5 U) was added after the pretreatment step.

The amplified DNA was subjected to electrophoresis on 0.7% agarose gels before and after digestion with restriction enzyme *Eco*RI or *Bcl*I and was stained with ethidium bromide.

Cloning. The amplified DNA was digested with *Bcl*I overnight at 50°C and phenol extracted. After precipitation with ethanol (2 volumes), the DNA was phosphorylated with T4 polynucleotide kinase (Boehringer), phenol extracted, and precipitated. Blunt ends were obtained by using the Klenow fragment of DNA polymerase.

Approximately 1 μg of plasmid pUC18 DNA restricted with *Sma*I was dephosphorylated with calf intestinal alkaline phosphatase, phenol extracted, and precipitated. The final volume of each ligation mixture was 10 μl, and the ratio of insert to plasmid was 3/1.

Competent *Escherichia coli* TG1 cells (14) were transformed with 5 μl of ligation mixture; after 1 h in Luria-Bertani medium, the cells were plated onto Luria-Bertani medium containing 50 μg of ampicillin per ml.

Plasmids containing an insert which resisted *Bcl*I digestion but was cut by *Eco*RI were selected and sequenced.

Sequencing method. Plasmids were prepared by using the method of Mierendorf and Pfeffer (21). The inserts were sequenced by the method of Sanger et al. (27), using a T7 polymerase kit (Pharmacia). For each BLO strain, three different clones were sequenced.

Data analysis. The 16S rDNA sequences of the greening BLOs were aligned manually with the sequence of *E. coli* on the basis of bacterial conserved regions and secondary structures. The sequences were then compared and aligned with bacterial 16S rDNA sequences obtained from the GenBank data base by using the retrieve program of the National Center for Biotechnology Information (National Library of Medicine, National Institutes of Health, Bethesda, Md.). The bacterial 16S rDNA sequences used in this study were the sequences of *Afipia clevelandensis* (accession number M69186), *Afipia felis* (accession number M65248), *Agrobacterium tumefaciens* (accession number M11223), *Bacillus subtilis* (accession number X60646), *Bartonella bacilliformis* (accession number X60042), *Brucella abortus* (accession number X13695), *E. coli* (accession number V00348), *Rickettsia rickettsii* (accession number M21293), and *Rochalimaea quintana* (accession number M73228). Multiple alignments were performed by using CLUSTAL software. A distance matrix and phylogenetic trees were constructed by using the PHYLIP 3.2 software package (least-squares method of Fitch and Margoliash [5]).

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in the GenBank data base under accession numbers L22532 (Indian strain Poona) and L22533 (African strain Nelspruit).

RESULTS

Amplification of 16S rDNAs from healthy and Poona BLO-infected periwinkle extracts. Figure 1 shows the results of a PCR performed with universal primers for amplification of prokaryotic 16S rDNAs from healthy and Poona BLO-infected periwinkle extracts. Immunocapture of the BLO with Poona BLO-specific MAb 2D12 was carried out prior to amplification. A DNA band of the expected size (1,500 bp) was observed with both healthy (Fig. 1, track 3) and infected (track 4) extracts. No DNA band was observed in the two control PCR tubes in which the plant extracts were replaced by water (Fig. 1, track 1) or PBS-600 (track 2). The amplified products were analyzed by restriction enzyme digestion, as shown in Fig. 2. The DNA amplified from healthy periwinkle extracts (Fig. 2, track 1) was totally digested into two fragments (1,250 and 250 bp) with *Bcl*I (track 2), but was resistant to digestion with *Eco*RI (track 3). When the DNA amplified from Poona BLO-infected periwinkle extracts (Fig. 2, track 4) was digested with *Bcl*I, three DNA species were obtained, an undigested

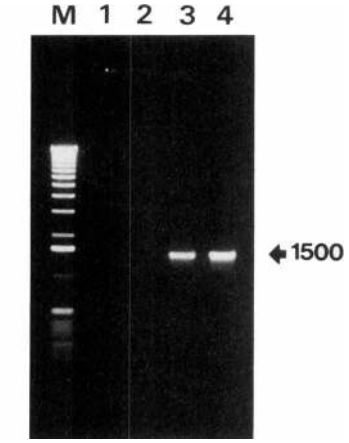


FIG. 1. Agarose gel electrophoresis of DNAs amplified by the PCR by using universal primers for the amplification of prokaryotic 16S rDNAs (30) from water (track 1), PBS-600 (track 2), MAb 2D12-immunocaptured material from healthy periwinkle extract (track 3), and MAb 2D12-immunocaptured material from Poona BLO-infected periwinkle extract (track 4). Track M contained a 1-kb ladder (Gibco BRL).

1,500-bp DNA species and two other DNA fragments (1,250 and 250 bp) (track 5). The signal below the 250-bp fragment in track 5 was due to PCR primers; such primer signals occur occasionally. Digestion with *Eco*RI (Fig. 2, track 6) produced, in addition to an undigested 1,500-bp DNA species, two other fragments (900 and 600 bp). These results showed that there was amplification of plant DNA that was sensitive to *Bcl*I digestion and resistant to *Eco*RI digestion in both healthy and BLO-infected extracts, as well as amplification of DNA resistant to *Bcl*I digestion and sensitive to *Eco*RI digestion in BLO-infected extracts. The 1,500-bp DNA species amplified from infected periwinkle extracts was cloned. As expected, two

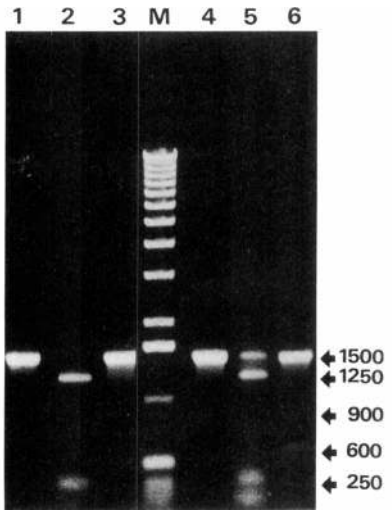


FIG. 2. Agarose gel electrophoresis of DNAs amplified by the PCR by using universal primers for the amplification of prokaryotic 16S rDNAs from MAb 2D12-immunocaptured material from healthy periwinkle extracts (tracks 1 through 3) or Poona BLO-infected periwinkle extracts (tracks 4 through 6) after digestion with *Bcl*I (tracks 2 and 5) or *Eco*RI (tracks 3 and 6). Tracks 1 and 4, no restriction enzyme digestion; track M, 1-kb ladder (Gibco BRL).

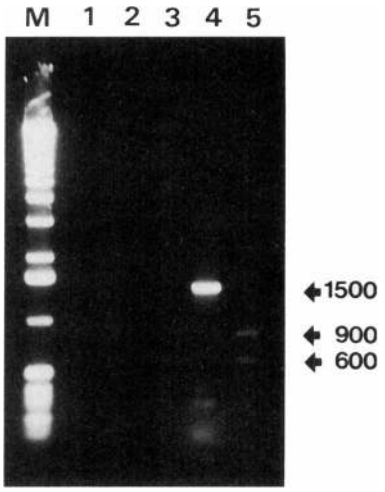
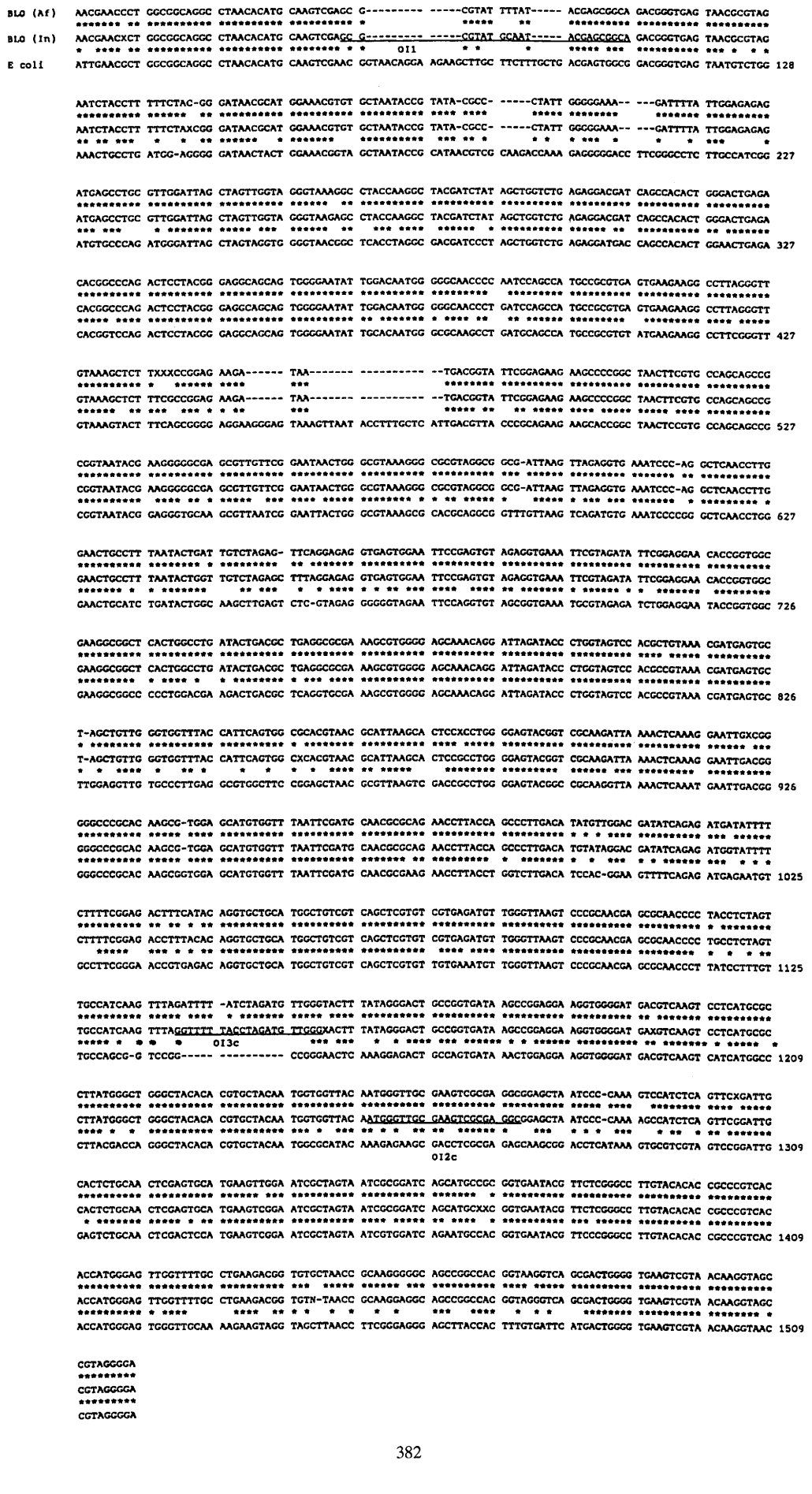


FIG. 3. Agarose gel electrophoresis of DNAs amplified by the PCR by using universal primers for the amplification of prokaryotic 16S rDNAs from water (track 1), PBS-600 (track 2), *Bcl*I-digested DNA extracted from healthy periwinkle midribs (track 3), *Bcl*I-digested DNA extracted from Nelspruit BLO-infected periwinkle midribs (track 4), and *Eco*RI-digested DNA from track 4 (track 5). Track M contained a 1-kb ladder (Gibco BRL).

types of inserts were obtained. One was sensitive to *Bcl*I digestion and resistant to *Eco*RI digestion, while the other was resistant to *Bcl*I digestion and sensitive to *Eco*RI digestion. Partial sequencing of the DNA sensitive to *Bcl*I digestion and present in both healthy and infected extracts revealed that this DNA corresponded to chloroplast 16S rDNA (data not shown). The DNA resistant to *Bcl*I digestion and sensitive to *Eco*RI digestion, which was amplified only from Poona BLO-infected periwinkle extracts, was considered to be the 16S rDNA of strain Poona BLO. Proof of this is given below.

Amplification of 16S rDNAs from DNAs extracted from healthy and Nelspruit BLO-infected periwinkle plants. In the experiments performed with strain Nelspruit, immunocapture of the BLO was omitted. Instead, DNA extracted from Nelspruit BLO-infected periwinkle midribs was treated with *Bcl*I to digest chloroplast 16S rDNA prior to amplification with the same universal primers used in the experiments described above. Control amplification reactions were performed with water or PBS-600 and with *Bcl*I-digested DNA extracted from healthy periwinkle midribs. Figure 3 shows that no DNA was amplified in the tubes containing water (Fig. 3, track 1), PBS-600 (track 2), or *Bcl*I-digested DNA extracted from healthy periwinkle midribs (track 3). Amplification of a 1,500-bp DNA was obtained with *Bcl*I-digested DNA extracted from infected periwinkle midribs (Fig. 3, track 4). This DNA was totally digested with *Eco*RI (Fig. 3, track 5) and was assumed to be the 16S rDNA of strain Nelspruit BLO. The bands at the bottom of track 4 in Fig. 3 were due to PCR primers (lowest band) and minor PCR side reactions (the two bands above the primer signal). These bands were very weak compared with the 1,500-bp DNA band.

Sequences of the *Eco*RI-sensitive DNAs amplified from Poona and Nelspruit BLO-infected extracts. Cloning and sequencing of the amplified DNAs were carried out as described in Materials and Methods. Figure 4 shows the alignment of the sequences with the 16S rDNA sequence of *E. coli*. The levels of homology of the sequences with the *E. coli* 16S rDNA sequence were close to 70%, indicating that the se-



BLO (Af)
BLO (In)
E coli
OI1
OI3c
OI2c

FIG. 4. Sequence alignment of 16S rDNAs amplified from Poona BLO- and Nelspruit BLO-infected periwinkle extracts with *E. coli* 16S rDNA. The numbering system used was the *E. coli* system (33). The asterisks indicate identical nucleotides; the dashes indicate deletions; the positions of OI1, OI2c, and OI3c are indicated. BLO (Af), African BLO strain Nelspruit; BLO (In), Indian BLO strain Poona.

quenced DNAs were indeed 16S rDNAs. The level of homology between the Poona and Nelspruit sequences was 97.7%.

A comparison of the BLO sequences with the 16S rDNA sequences of other bacteria allowed us to define three oligonucleotides (oligonucleotides OI1, OI2c, and OI3c) that were specific for the amplified DNAs. These oligonucleotides are underlined in Fig. 4. OI2c and OI3c corresponded to the sequence of the complementary strand.

Proof that the sequenced DNAs represent the 16S rDNAs of strain Poona and Nelspruit greening BLOs. Oligonucleotide OI3c (which was identified at a time when only the Poona sequence had been determined) was used in dot blot hybridization experiments with DNA extracted from healthy or Poona BLO-infected periwinkle and citrus midribs. This oligonucleotide hybridized specifically with DNAs extracted from infected plants whether they were periwinkle plants (Fig. 5, track 2) or citrus plants (track 4). No hybridization was observed with the DNA extracted from healthy periwinkle plants (Fig. 5, track 1) or citrus plants (track 3). In addition, the intensity of the hybridization signal was lower with citrus DNA than with periwinkle DNA. This result was expected as previous experiments had shown that the levels of BLOs are much lower in citrus plants than in periwinkle plants (31).

In a second experiment we used OI1 (forward primer) and OI2c (reverse primer) for PCR amplification of 16S rDNAs. The DNAs subjected to the PCR were extracted from the following materials: periwinkle plants infected with one of several BLO strains or MLOs (6, 12), *E. coli*, and *Xylella fastidiosa*, the xylem-restricted bacterium of citrus variegated chlorosis (24). The results are shown in Fig. 6. We found that when OI1 and OI2c were used, DNA of the expected size (1,160 bp) was amplified in periwinkle plants infected with any of the five BLO strains tested, including strain Poona from India (Fig. 6, track 3), strain Nelspruit from Africa (track 4), strain Fujian from the People's Republic of China (track 5), strain Lipa city from The Philippines (track 6), and strain Nakhom Pathom from Thailand (track 7). In contrast, no amplification was obtained when the DNA came from *E. coli* (Fig. 6, track 9), *X. fastidiosa* (track 15), or periwinkle plants infected with the stolbur MLO or the MLO of witches' broom disease of lime (tracks 11 and 13, respectively), although these DNAs could be amplified with universal primers (tracks 8, 10, 12, and 14). These results confirmed that the sequenced DNAs were indeed the 16S rDNAs of strain Poona and Nelspruit BLOs and not the DNAs of contaminating organisms.

Phylogenetic analysis. The 16S rDNA sequences of the BLOs were used for a phylogenetic analysis. The greening BLOs clustered in the alpha subdivision of the class *Proteobacteria*. Table 1 shows the evolutionary distances between the BLOs and bacteria belonging to the alpha subdivision. The closest BLO relatives were members of the alpha-2 subgroup; however, the level of 16S rDNA sequence homology was only 87.5%. We also looked for the presence on the BLO 16S rDNA of sequence signatures (bases and oligonucleotides) that distinguished the alpha subdivision from the beta and gamma subdivisions, as well as signatures that distinguished subunits of the alpha subdivision (33, 34).

The 16S rDNA sequences of the greening BLOs possessed all of the base signatures except one (position 1116) and six oligonucleotide signatures characteristic of the alpha subdivision (AUAAUG, positions 451 to 481; CUAACCG, positions 1443 to 1450; CUCACUG, positions 735 to 741; AAAUUCG,

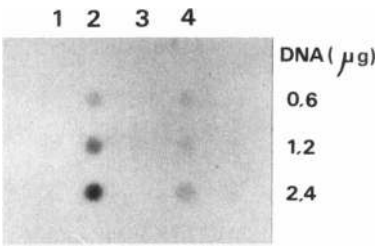


FIG. 5. Dot blot hybridization of various amounts of DNA extracted from healthy (tracks 1 and 3) or Poona BLO-infected (tracks 2 and 4) periwinkle midribs (tracks 1 and 2) or citrus midribs (tracks 3 and 4) with oligonucleotide OI3c.

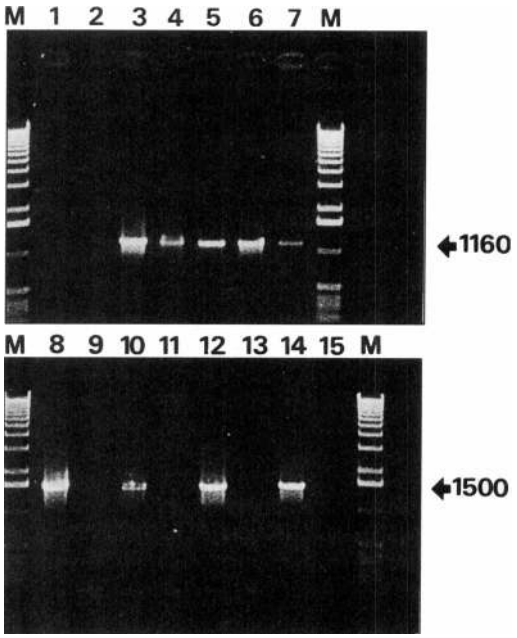


FIG. 6. Agarose gel electrophoresis of DNAs amplified by the PCR by using primers OI1 and OI2c. Track 1, water; track 2, PBS-600; tracks 3 through 7, DNAs extracted from periwinkle midribs infected with greening BLOs from India (strain Poona), South Africa (strain Nelspruit), the People's Republic of China (strain Fujian), The Philippines (strain Lipa City), and Thailand (strain Nakhom Pathom), respectively; tracks 11 and 13, MLOs of tomato stolbur and witches' broom disease of lime, respectively; track 9, *E. coli* DNA; track 15, *X. fastidiosa* DNA. Tracks 8, 10, 12, and 14 contained the amplification products obtained with universal primers for amplification of 16S rDNA and DNAs extracted from *E. coli*, periwinkle plants infected with the MLOs of tomato stolbur and witches' broom disease of lime, and *X. fastidiosa*, respectively. Tracks M contained a 1-kb ladder (Gibco BRL).

TABLE 1. Evolutionary distances derived from a comparison of 1,253 bases of the 16S rDNA sequences of various bacterial species in the α subdivision of the *Proteobacteria* and two outgroup bacteria, *E. coli* and *Bacillus subtilis* (a low-G+C-content, gram-positive bacterium)

Taxon	Subdivision of the *Proteobacteria*	Subgroup	Evolutionary distance from:									
			Strain Poona	*Afipia clevelandensis*	*Bartonella bacilliformis*	*Brucella abortus*	*Agrobacterium tumefaciens*	*Afipia felis*	*Rochalimaea quintana*	*Rickettsia rickettsii*	*Escherichia coli*	*Bacillus subtilis*
African BLO (strain Nelspruit)			0.0166	0.1388	0.1198	0.1269	0.1273	0.1389	0.1149	0.1838	0.2489	0.2461
Indian BLO (strain Poona)				0.1374	0.1207	0.1264	0.1248	0.1364	0.1167	0.1834	0.2474	0.2407
Afipia clevelandensis	α	α2			0.1324	0.1038	0.1222	0.0217	0.1246	0.1933	0.2581	0.2472
Bartonella bacilliformis	α	α2				0.0712	0.0682	0.1263	0.023	0.1707	0.2617	0.2403
Brucella abortus	α	α2					0.0636	0.0988	0.0648	0.1746	0.2519	0.2284
Agrobacterium tumefaciens	α	α2						0.1191	0.0691	0.1694	0.2627	0.2328
Afipia felis	α	α2							0.1185	0.1970	0.2494	0.2381
Rochalimaea quintana	α	α2								0.1657	0.2572	0.2318
Rickettsia rickettsii	α										0.3102	0.2605
Escherichia coli	γ											0.2734

positions 694 to 700; AUAUUCG, positions 704 to 710; AAAUCCCAG, positions 608 to 617 [*E. coli* numbering]). In addition, as shown in Fig. 7, the BLO 16S rDNA sequences had, between positions 180 and 220 (*E. coli* numbering), the secondary loop structure characteristic of the alpha subdivision of the *Proteobacteria* (33). Even though the closest bacterial relatives of the BLOs are members of the alpha-2 subgroup of the *Proteobacteria*, only one oligonucleotide signature (CAAUACG, positions 90 to 101) characteristic of the alpha-2 subgroup was found. This signature was present in the Indian BLO sequence but not in the African BLO sequence, in which the two first bases of this signature were modified from CA to TT. In addition, three oligonucleotide signatures characteristic of the alpha-1 subgroup (ACAAG, positions 935 to 939; CACUCCG, positions 875 to 881; UCAcACCAUG, positions 1406 to 1415) and two signatures characteristic of the alpha-3 subgroup (AUUAAG, positions 592 to 597; UAAUACCG, positions 171 to 178) were present in both BLO sequences.

Figure 8 shows a phylogenetic tree constructed by using the distance matrix method. This tree shows that the Indian and African BLOs clustered together, but that these organisms were distinct from members of the alpha-2 subgroup.

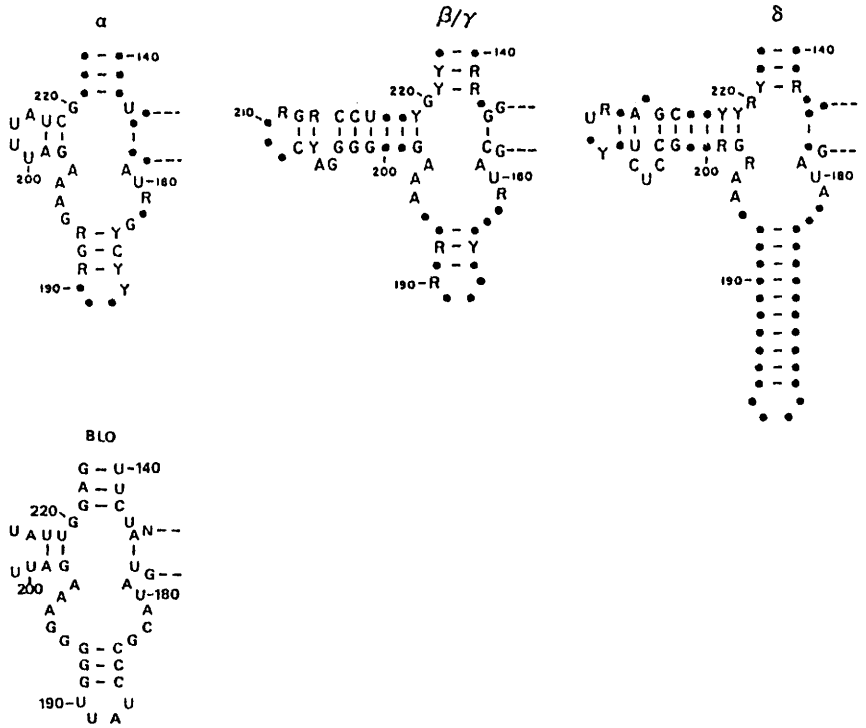


FIG. 7. 16S rDNA secondary loop structures between nucleotides 180 and 220 of the greening BLO and bacteria belonging to the alpha, beta, and gamma subdivisions of the *Proteobacteria* (32).

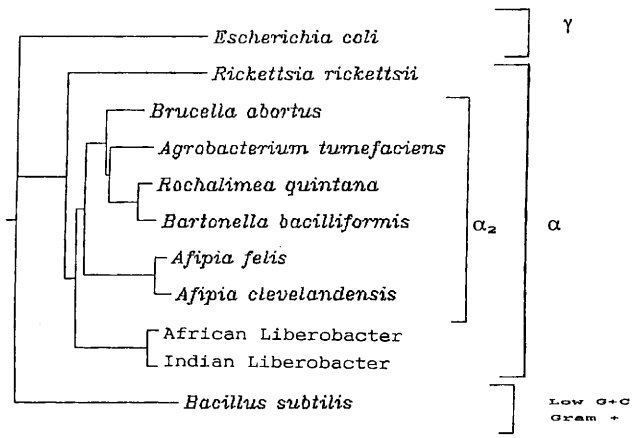


FIG. 8. Phylogenetic tree showing the relationships of the African and Indian BLOs and representatives of the α subdivision of the *Proteobacteria*. *Bacillus subtilis* was used as an outgroup.

DISCUSSION

For the first time the 16S rDNAs of two strains of the greening BLO were obtained by PCR amplification by using universal primers for the amplification of prokaryotic 16S rDNA. In the case of BLO strain Poona, both BLO 16S rDNA and chloroplast 16S rDNA were amplified as a 1,500-bp DNA despite an immunocapture step meant to retain the BLO in the absence of host plant material. However, the chloroplast 16S rDNA was hydrolyzed specifically with *Bcl*I, and this treatment prevented it from being amplified. In the case of BLO strain Nelspruit, the immunocapture step was omitted, and the DNA subjected to amplification was extracted from BLO-infected periwinkle plants and digested with *Bcl*I before it was used for the PCR.

In this way, the 16S rDNA of an uncultured, phytopathogenic, phloem-restricted bacterium (i.e., a BLO) was isolated, cloned, and sequenced. Hybridization and PCR experiments performed with oligonucleotides specific for the amplified sequence revealed that the DNAs obtained and sequenced were the 16S rDNAs of the greening BLOs and not the DNA of a contaminating organism.

As determined by our phylogenetic analysis, the greening BLO belongs to the α subdivision of the *Proteobacteria*. The α subdivision of the *Proteobacteria* is a diverse group of microbes that includes both plant pathogens or symbionts with some distinctive properties (*Agrobacterium tumefaciens*, *Bradyrhizobium* spp.) and human pathogens (*Rochalimea* spp., *Bartonella baciliformis*, *Brucella abortus*, *Afipia* spp., etc). The organisms in this group live in intimate association with eucaryotic cells and, in many cases, have acquired the ability to survive and grow within an arthropod vector. The greening organism fits this description quite nicely. Indeed, it grows in a specialized niche in its eukaryotic plant host, phloem sieve tubes, and it is transmitted by two arthropod vectors, the psyllids *T. erytreae* and *D. citri*, in which it multiplies both in the hemolymph and within the cells of the salivary glands.

Previously, we have shown that greening BLO strains from Africa could be distinguished from greening BLO strains from Asia on the basis of temperature sensitivity (1), serology (7), and genomic properties (30, 31). A comparison of the 16S rDNAs of the Asian BLO strain Poona and the African BLO strain Nelspruit showed that they are 97.7% homologous. The close phylogenetic relationship between Indian and African greening BLOs is not surprising as these organisms cannot be distinguished morphologically and they induce similar symptoms and disease in citrus and periwinkle plants. While in nature Asian BLOs are transmitted by *D. citri* and African BLOs are transmitted by *T. erytreae* because of the geographic distribution of these psyllids, experimentally both psyllid vectors can transmit both BLOs (18, 19). However, we recently cloned and sequenced the rather well-conserved *rplKAJL-rpoBC* operon of BLO strain Nelspruit strain and observed only 70% homology between this organism and the Indian BLO strain (unpublished data). This finding and the 16S rDNA results suggest that these two strains might be members of two different species of the same genus. On the basis of our results, it is clear that the greening BLO is a member of the α subdivision of the *Proteobacteria* and that its closest relatives are members of the alpha-2 subgroup. However, the presence of only one oligonucleotide signature of the alpha-2 subgroup and the presence of signatures characteristic of the alpha-1 and alpha-3 subgroups indicate that the greening BLO does not belong to the alpha-2 subgroup, a finding also shown by the phylogenetic tree. Consequently, the greening BLO might represent descendants of an early offshoot and the first member of a new subgroup in the alpha subdivision.

Our data revealed that the greening organism is undoubtedly a bacterium; hence, the designations BLO and rickettsia-like organism should be abandoned. We propose that organisms belonging to this new group in the α subdivision of the *Proteobacteria* should be referred to by the trivial name "liberobacter" (from the Latin *liber* [bark] and *bacter* [bacteria]) until it is possible to characterize them for a formal name. Recently, Murray and Schleifer made a proposal for recording the properties of putative taxa of procaryotes (22). Following this proposal, the greening liberobacter from India should be described as follows: "*Candidatus* Liberobacter asiaticum" [(α-*Proteobacteria*) NC; G-F; NAS (GenBank number L22532), oligonucleotide sequence complementary to unique region of 16S rRNA 5′-GCGCGTATGCAATACGAGCG GCA-3′, S (*Citrus*, phloem; *Diaphorina citri* (Psyllidae), hemolymph, salivary glands); M;]. And the greening liberobacter from South Africa should be described in the following way: "*Candidatus* Liberobacter africanum" [(α-*Proteobacteria*) NC; G-F; NAS (GenBank number L22533), oligonucleotide sequence complementary to unique region of 16S rRNA 5′-GCGCGTATTTTATACGAGCGGCA-3′, S (*Citrus*, phloem; *Trioza erytreae* (Psyllidae), hemolymph, salivary glands); M;].

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