Genetic Diversity Among Mycoplasmalike Organisms Associated with Stone Fruit Diseases

U. Ahrens, K.-H. Lorenz, and E. Seemüller

Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Pflanzenschutz im Obstbau, D-69216 Dossenheim, Germany

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Southern blot analysis was employed to examine genetic interrelatedness of mycoplasmalike organisms (MLOs) collected in Germany from peach, apricot, almond, Japanese plum, and flowering cherry trees showing yellowing and decline symptoms. Also, periwinkle-maintained MLOs obtained from stone fruits such as North American western X-disease MLO strains and southern European MLOs transmitted from trees affected by apricot chlorotic leaf roll, plum leptonecrosis, and Molières disease were included in this study. Randomly cloned chromosomal DNA fragments of apple proliferation and vaccinium witches'-broom MLOs and a ribosomal sequence of western X-disease MLO were used as probes. The results show that the German *Prunus* isolates form a homogeneous group that is closely related to apple proliferation MLO but distinctly different from the stone fruit isolates maintained in periwinkle. DNA of the periwinkle-maintained MLOs hybridized with probes from vaccinium witches'-broom MLO. Among these isolates, the X-disease MLOs showed similar patterns, whereas the southern European strains were different from one another and from the X-disease MLOs. The differences among the periwinkle-maintained isolates were confirmed by hybridization results obtained with the ribosomal probe.

Most or all rosaceous fruit tree species are affected by diseases caused by mycoplasmalike organisms (MLOs). Several MLO diseases have been identified in stone fruits. In southern and western European countries, apricot is affected by apricot chlorotic leaf roll (ACLR) (Morvan 1977). A disease of Japanese plum (*Prunus salicina*) occurs in the same area and is called either plum leptonecrosis (PLN) or plum decline (Giunchedi *et al.* 1982). Molières disease (MOL) is a serious disorder of sweet cherry and European plum (*P. domestica*) in an area of southwestern France (Bernhard *et al.* 1977). MLO-induced yellows and decline diseases have also been observed in peach and almond in Europe (Tsalis and Rumbos 1980; Lederer and Seemüller 1992). In North America, the major mycoplasma-associated disorder of stone fruits is X-disease, which affects mainly cherry, peach, and nectarine. MLO infections have also been observed in apricot, almond, and European and Japanese plum in North America (Gilmer and Blodgett 1976; Kirkpatrick *et al.* 1990). Several strains of X-disease MLO are known, such as the western (WX) and eastern X-MLOs. Among the agents occurring in various stone fruits in California, the Green Valley (GVX) strain of cherry buckskin MLO, the peach yellow leaf roll (PYLR) strain of peach X-MLO, and other strains can be distinguished (Purcell *et al.* 1981, 1987).

Until recently, biological tests were the only means to characterize and differentiate the MLOs causing stone fruit diseases. Cross inoculation experiments with European stone fruit MLOs showed that ACLR-MLO induces typical PLN symptoms in Japanese plum and vice versa. Furthermore, MLOs from symptomatic apricots and Japanese plums have been graft-transmitted to peach and almond, where decline symptoms similar to those of naturally infected peach and almond trees were induced (Morvan 1977; Giunchedi *et al.* 1982). Comparable results were obtained with X-disease MLOs from North America (Gilmer and Blodgett 1976). MLOs from stone fruits have also been transmitted to the experimental host *Catharanthus roseus* (periwinkle), in which distinct symptoms were induced by various MLO sources. Two major groups can be distinguished, one being formed by two California WX strains, which do not induce virescence symptoms, and the other by virescence-inducing MLOs transmitted from ACLR-, PLN-, and MOL-infected trees grown in southern Europe (Marwitz 1990; Schneider *et al.* 1993).

Knowledge on interrelatedness of MLOs has considerably increased since serological procedures and the methods of DNA technology were introduced into plant mycoplasmology. Dot hybridization and serological studies showed that X-disease MLOs from various geographic areas of North America are closely interrelated (Jiang *et al.* 1989, Kirkpatrick *et al.* 1990). Southern analysis revealed that eastern and WX-MLOs, together with the clover yellow edge MLO, form a distinct strain cluster (Lee *et al.* 1992). In Southern blot analyses with cloned DNA fragments, cross-hybridization was observed between WX-MLOs and the MLOs associated with walnut bunch, pecan bunch, peach yellows (Kirkpatrick *et al.* 1990), and flavescence dorée of grapevine (Daire *et al.* 1992). However, X-disease MLOs are distinctly different from a great number of MLOs occurring in herbaceous plants (Lee and Davis 1988; Lee *et al.* 1990, 1992; Kuske *et al.* 1991). Restriction site and sequence analyses of MLO 16S rRNA genes showed that European and North American MLOs obtained from diseased stone fruits are genetically diverse and are members of four major taxonomic groups (Schneider *et al.* 1993). The objective of this study was to use Southern blot analysis to differentiate European and North American stone fruit MLO strains maintained in periwinkle

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and field samples from various *Prunus* species collected in Germany. An abstract of this work has been published (Lorenz *et al.* 1993).

RESULTS

The two *Hin*dIII fragments from strain AT of apple proliferation (AP)-MLO (AT cocktail probe) hybridized with *Hin*dIII-digested DNA from the homologous organism as well as from diseased flowering cherry, Japanese plum, apricot, and peach trees (Fig. 1). However, the sizes of the two fragments detected in the samples from the diseased *Prunus* spp. were considerably larger than those of the AT fragments used as probe. A hybridization profile similar to that of the diseased apricot, peach, Japanese plum, and flowering cherry trees was observed when DNA from a diseased almond tree was hybridized with the AT cocktail probe (data not shown). An additional fragment with approximately the size of insert IH 184 was detected in the samples from a diseased peach and a diseased apricot tree (data not shown).

The AT cocktail probe did not hybridize to DNA from healthy trees and periwinkles and from periwinkle plants infected with MLO strains ACLR-L, PLN-V6, PLN-V12, MOL, GVX, and PYLR (Fig. 1 and data not shown). Only after long exposure and/or large amounts of sample DNA did a faint band sometimes appear in the samples from strains ACLR-L, PLN-V6, and PLN-V12. This recognized fragment hybridized weakly with probe IH184 and was the size of this probe (data not shown).

Reciprocal results were obtained when the same DNA samples were hybridized with the three *Eco*RI fragments of vaccinium witches'-broom (VAC)-MLO (VAC cocktail probe). This probe hybridized with *Eco*RI-digested DNA of the periwinkle-maintained strains ACLR-L, GVX, MOL, PLN-V6, PLN-V12, and PYLR but not with DNA from AP-infected periwinkles and from diseased almond, apricot, flowering cherry, Japanese plum, and peach trees collected in the field in Germany (Fig. 2A and data not shown). Restriction fragment length polymorphisms (RFLPs) were observed among the periwinkle-maintained stone fruit isolates. Similar but not identical patterns showed the two WX-strains GVX and PYLR. Also, strains PLN-V6 and PLN-V12 were similar but not identical and were different from the WX strains. Strains ACLR-L and MOL were different from one another and from the PLN and WX strains. All periwinkle-maintained stone fruit strains differed in the restriction profile from that of VAC-MLO (data not shown). The differences between the periwinkle-maintained stone fruit strains were also evident when *Eco*RI-digested DNA of these MLOs was hybridized with ribosomal probe PY6. However, the closely related strains of the WX-MLO showed identical patterns with this probe. The same is true for the two PLN strains, while strains ACLR-L and MOL were different from one another and from the other strains (Fig. 2B).

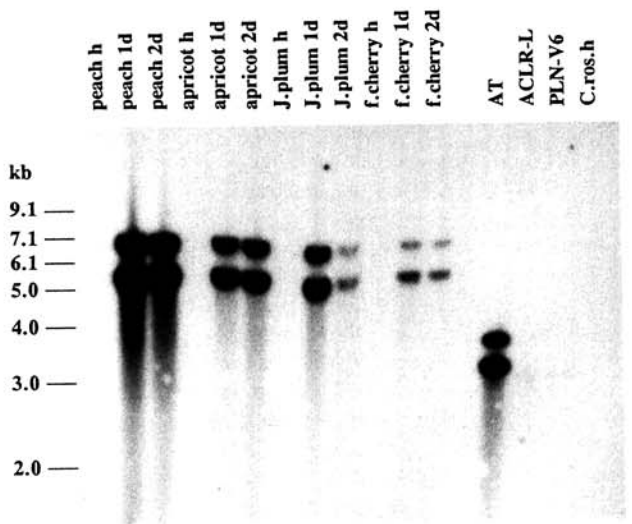

Fig.1. Southern blot hybridization of *Hin*dIII-digested DNA from healthy and MLO-infected fruit trees and periwinkle plants. The blot was hybridized under moderately stringent conditions with two radiolabeled *Hin*dIII fragments of apple proliferation (AP)-MLO strain AT. Samples from peach through flowering cherry (f. cherry) were from 12 different trees (d, diseased; h, healthy). ACLR-L and PLN-V6, periwinkle-maintained strains from diseased apricot and Japanese plum trees, respectively; C.ros.h, healthy periwinkle.

DISCUSSION

The MLO isolates from the *Prunus* species examined responded differently when probed with chromosomal fragments from AP- and VAC-MLOs. The MLOs from diseased apricot, peach, almond, Japanese plum, and flowering cherry trees collected in Germany hybridized with the AP cocktail probe but not with the VAC cocktail probe. As the AP agent is only distantly related to all other MLOs examined as yet by dot and Southern hybridization (Bonnet *et al.* 1990a, 1990b; Kollar *et al.* 1990a; Schneider and Seemüller 1993), the isolates from the above *Prunus* species are the only known MLOs that are closely related to AP-MLO. Relatedness of AP-MLO to stone fruit MLOs causing ACLR and premature bud break in plum, respectively, has also been found by Bonnet *et al.* (1990b) in dot hybridization experiments. In this study, the AP agent and the MLOs from *Prunus* showed different RFLP patterns. However, the restriction profiles of the *Prunus* MLOs were similar, thus indicating that these isolates are very closely related. These results are consistent with the MLO classification of Schneider *et al.* (1993) based on restriction site and sequence analysis of the 16S rRNA gene. In that classification, which consists of seven major taxonomic groups (RFLP groups) and three subgroups, the AP isolates represent a major RFLP group (group V). Since the German *Prunus* isolates differ in one *Rsa*I restriction site from the AP isolates, they were considered a subgroup (Va) of AP-MLO. The uniformity of the stone fruit isolates confirms the cross-infectivity of MLOs from various stone fruit species and the induction of similar symptoms by such MLOs in a given stone fruit host as described by Morvan (1977) and Giunchedi *et al.* (1982).

The periwinkle-maintained stone fruit MLOs, which were of California and southern European origin, are only distantly related to the German stone fruit isolates. They did not hybridize or hybridized only weakly with the AT cocktail probe but hybridized with the VAC-MLO fragments. Among the periwinkle-maintained stone fruit MLOs, four major hybridization patterns were observed and were confirmed by hybridization with the ribosomal probe PY6. These hybridiza-

tion results can also be related to the classification of Schneider *et al.* (1993), in which strain MOL is a member of RFLP subgroup Ia, which otherwise consists of stolbur-type MLOs from solanaceous plants. The PLN strains and strain ACLR-L showed hybridization patterns that were different from one another and from that of strain MOL; they form RFLP group II. The strains of that group share a high sequence homology of their 16S rDNA with strains of RFLP group I, including Ia, which predominantly comprises virescence agents of herbaceous plants that are distinctly different from most MLOs associated with tree diseases. The closely related WX strains GVX and PYLR are different from all European *Prunus* MLOs, whether they were transmitted to periwinkle or collected in the field. The WX strains are, like VAC-MLO, members of RFLP group VI (Schneider *et al.* 1993).

This study revealed that the stone fruit MLOs examined can be distinguished and characterized with the probing systems used. It also showed that MLOs of different RFLP groups, including the subgroups, respond differently in Southern blot hybridization. Moreover, MLOs of the same group, such as the PLN strains and strain ACLR-L, could be further differentiated by Southern analysis. The differences between the PLN strains, which were repeatedly found with the VAC cocktail probe but not with the less-specific probe PY6, are most likely due to the transmission of two closely related organisms from a diseased Japanese plum tree.

The periwinkle-maintained stone fruit strains from southern Europe are distinctly different from the homogeneous MLOs found in diseased *Prunus* species in Germany. These differences cannot be explained by the geographic origin. In an ongoing survey to characterize the European stone fruit MLOs, a considerable number of samples from ACLR-diseased apricots and PLN-diseased Japanese plums from southern France, Italy, and Spain responded similarly to the German *Prunus* samples when hybridized with the AT and VAC cocktail probes. MLOs related to the periwinkle-maintained strains were not found in field samples from southern Europe (Lorenz *et al.* 1993). Hence, the organisms detected in *Prunus* spp. in Germany seem to represent the major or only important MLO pathogen of stone fruits in Europe. However, there is indication that the periwinkle-maintained strains also derive from stone fruits. Monoclonal antibodies raised against strain MOL cross-re

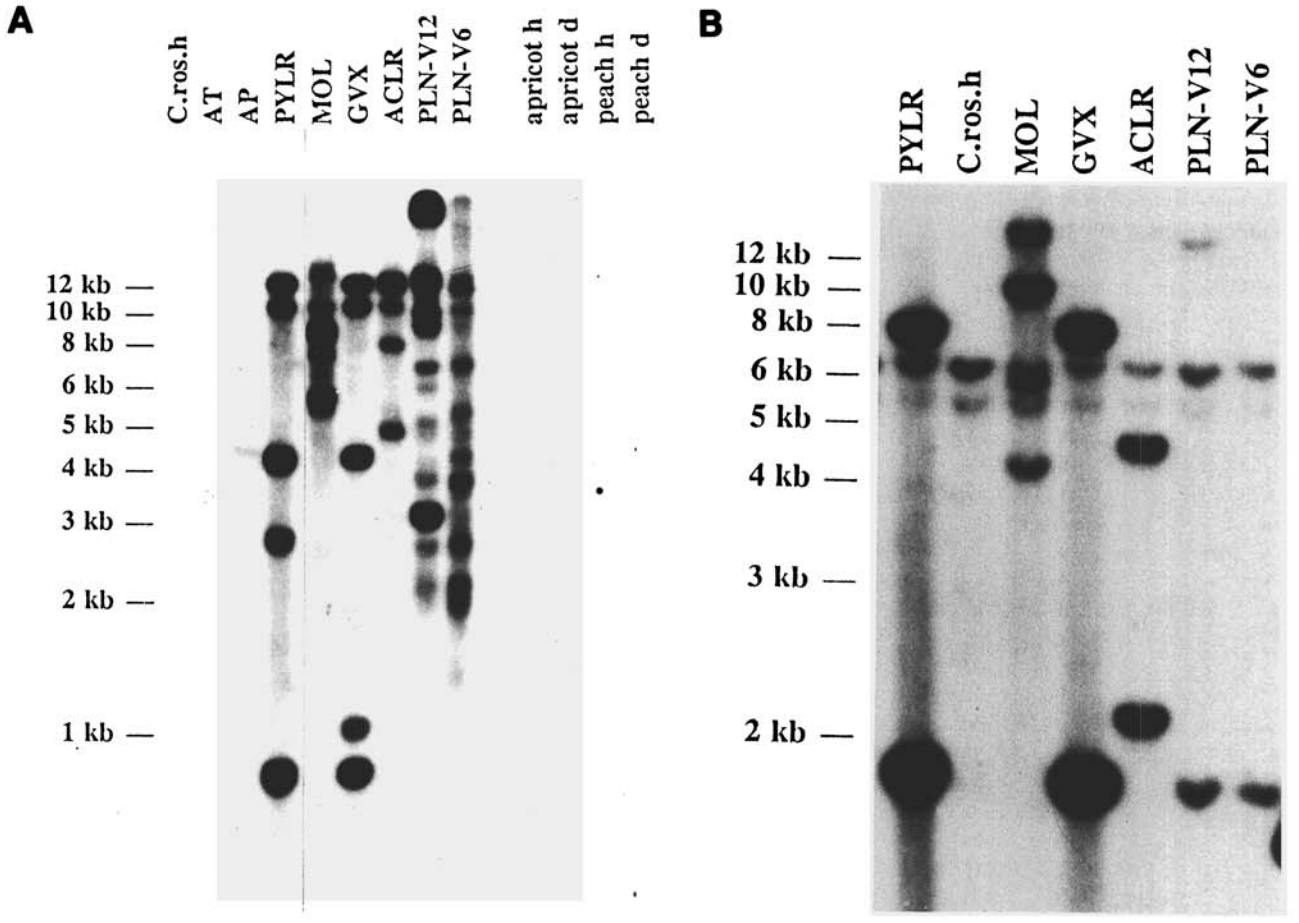

Fig. 2. Southern blot hybridization of *Eco*RI-digested DNA from healthy and MLO-infected fruit trees and periwinkle plants. The blots were hybridized under moderately stringent conditions with either three radiolabeled *Eco*RI fragments of vaccinium witches'-broom MLO (**A**) or a ribosomal *Eco*RI/*Hin*dIII fragment of western X (WX)-MLO (**B**). AP, strain AP-15 of apple proliferation (AP)-MLO; PYLR, strain of WX-MLO; MOL, MLO from cherry affected by Molières disease; GVX, Green Valley strain of WX-MLO; ACLR, strain ACLR-L from diseased apricot tree; PLN-V6 and V12, MLOs from the same diseased Japanese plum tree. See Figure 1 for explanation of other abbreviations. Note the different patterns of strains PLN-V6 and V12 in A; in B, the band of PLN-V6 at approximately 13 kb is not visible because of low amounts of sample DNA.

acted with an MLO in a diseased peach tree from France (Kenyon and Clark 1993). Also, the close relationship between the PLN strains from Italy and strain ACLR-L from Spain, which are the only members of RFLP group II, may indicate a specificity for colonizing stone fruits. It is possible that the organisms transmitted to periwinkle occur in trees infected with the predominant MLO that hybridizes with the AT cocktail probe. This MLO appears to be pathogenic because its presence in trees is closely correlated with disease. In contrast, the role of the MLOs transmitted to periwinkle is unclear. They may be present in low numbers and therefore be undetectable with the probes used. However, these MLOs may be more readily transmitted to periwinkle with dodder than the AP-related MLOs.

There is another example of differences between a periwinkle-transmitted MLO and the MLOs detectable in a naturally infected host. From grapevine showing typical symptoms of grapevine yellows, an MLO of RFLP group I was transmitted to periwinkle. However, in several diseased grapevines, only MLOs of subgroup Ia could be detected using a polymerase chain reaction procedure with various primers (Maixner *et al.* 1994). This example and the southern European stone fruit strains discussed above indicate that the identity of periwinkle-transmitted MLOs must be carefully examined before they are used in taxonomic studies or as sources for preparing molecular detection tools. However, strains AT and VAC, from which the probes used in this study were developed, are authentic because similar MLOs were regularly detected in naturally infected apple trees and blueberry plants, respectively (Kison *et al.* 1994 and unpublished results). Also, strains GVX and PYLR are authentic WX-MLOs (Neimark and Kirkpatrick 1993).

MATERIALS AND METHODS

Sources of MLOs.

The MLOs included in this study were obtained from experimentally infected periwinkle or from their natural host plants. The following strains were maintained in periwinkle and were transmitted to this host by the researchers given in parentheses: ACLR-L, MLO from apricot chlorotic leaf roll-diseased apricot from Spain (G. Llacer, IVIA, Moncada-Valencia, Spain); AP-15, AP-MLO from Italy (Carraro *et al.* 1988); AT, AP-MLO from Germany (Marwitz *et al.* 1974); GVX, Green Valley strain of X-disease MLO from California (D. D. Jensen, University of California, Berkeley); MOL, MLO from cherry affected by Molières disease from France (F. Dosba, INRA, Bordeaux); PLN-V6 and PLN-V12, MLOs from leptonecrosis-affected Japanese plum from Italy (subclones obtained by grafting from the same dodder [*Cuscuta*] transmission) (L. Carraro, Universita degli Study, Udine, Italy); PYLR, obtained as peach yellow leaf roll MLO, but according to B. C. Kirkpatrick (personal communication), a typical strain of WX-MLO (D. D. Jensen, University of California, Berkeley); and VAC, vaccinium witches'-broom MLO (Marwitz et al. 1987). Field samples from three diseased peach, three diseased apricot, one diseased almond, three diseased Japanese plum, and four diseased flowering cherry (*P. serrulata*) trees as well as one or two healthy trees from each of these species were collected in southwestern Germany.

DNA isolation.

A modification of an MLO-enrichment procedure described by Kirkpatrick *et al.* (1987) was used to extract DNA from healthy and MLO-infected periwinkle plants. Shoots were ground in ice-cold buffer consisting of 125 mM potassium phosphate, 10% sucrose, 0.15% bovine serum albumin (BSA), 2% PVP-15, and 30 mM ascorbic acid, pH 7.6. The homogenate was centrifuged at 4° C for 4 min at 700 *g*. The supernatant was decanted and recentrifuged at 4° C for 25 min at 15,000 *g*. The MLO-enriched pellet was resuspended in a small volume of a buffer consisting of 50 mM Tris-HCl, 10 mM ethylenediamine tetraacetic acid (EDTA), and 0.1% mercaptoethanol, pH 8.0, to which sodium dodecyl sulfate was added to a final concentration of 1.0%. After incubation for 5 min at room temperature, one third volume of 5.0 M potassium acetate was added, and the mixture was incubated for 30 min on ice. Precipitated carbohydrates were removed by centrifugation at 13,000 rpm for 5 min in a microfuge. One tenth volume of 3.0 M sodium acetate was added to the supernatant, and the DNA was precipitated with one volume of isopropanol. After centrifugation, the DNA pellet was washed with 70% ethanol, dried under vacuum, and disolved in a small volume of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

DNA from healthy and MLO-infected stone fruit trees was extracted as described by Kison *et al.* (1994) or by using a modification of the urea-phosphate-hydroxyapatite procedure described by Kollar *et al.* (1990b). Using the latter method, 10 g of midribs was ground in liquid nitrogen, and urea buffer (8.0 M urea, 2.5% SDS, 0.1% mercaptoethanol, and 100 mM sodium phosphate, pH 6.8) was added to the ground tissue when still frozen. After thawing, the homogenate was incubated at 55° C for 30 min. Proteins were extracted by adding first one half volume of Tris-saturated phenol (Maniatis *et al.* 1982) and then, after mixing, another half of the original volume of chloroform/isoamyl alcohol (24:1). After centrifugation, the aqueous phase was transferred to a test tube containing 1 g of hydroxyapatite (Bio-Gel HT, Bio-Rad) equilibrated with urea buffer without SDS. The suspension was gently shaken at 4° C for 30 min and centrifuged for 1 min at 200 rpm. The pellet was resuspended in fresh urea buffer (without SDS) and recentrifuged. This procedure was repeated twice, and then three more times with 100 mM sodium phosphate buffer. DNA was recovered from hydroxyapatite by gentle shaking in 500 mM sodium phosphate buffer for 30 min at room temperature. After centrifugation, the supernatant was treated with proteinase K (50 μg/ml) at 37° C for 30 min followed by one extraction with chloroform/isoamyl alcohol. The DNA was further purified by gel filtration on a Sepharose CL-4B (Pharmacia) column and recovered by centrifugation of the DNA peak at 4° C for 24 hr at 140,000 *g*.

Probe preparation and Southern blot hybridizations.

Recombinant plasmids containing inserts of chromosomal DNA from strains AT and VAC and the WX-MLO were selected for this study and were isolated using the method of Birnboim and Doly (1979). The inserts were excised with the appropriate restriction endonuclease, purified by electrophoresis in 1% agarose gel, and eluted from the gel using the Geneclean kit (Bio-101). The inserts were labeled with ^{32}P-dATP using the Multiprime labeling kit (Amersham).

From strain AT, two *Hin*dIII fragments, cloned by Bonnet *et al.* (1990a) and designated IH184 and IH196, were employed; they were 3.1 and 3.7 kb in size, respectively. From strain VAC, three *Eco*RI fragments cloned by Schneider and Seemüller (1993) were used. They were designated VAC13.3, VAC11.2, and VAC13.4, and were 1.6, 2.4, and 4.0 kb, respectively, in size. The AT and VAC fragments, whose chromosomal nature has been proven as described by Schneider and Seemüller (1993), were used as mixed cocktail probes. From WX-MLO, a 1.9-kb *Eco*RI/*Hin*dIII fragment was used, which was designated PY6 and consisted of the 5′ portion of the 16S rRNA gene and an upstream region of approximately 1.3 kb in size (Kirkpatrick *et al.* 1990).

For Southern blot hybridization, sample DNA was digested with either *Hin*dIII or *Eco*RI restriction endonucleases and separated by electrophoresis in horizontal, 1.0% agarose gels using TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 3 V/cm. After alkali denaturation, the DNA was transferred to a nylon membrane (Hybond N, Amersham) following the manufacturer's instructions. The blots were prehybridized in solutions containing 6× SSC (1× SSC = 0.15 M NaCl and 15 mM sodium citrate, pH 7.0), 0.5% SDS, 5× Denhardt's solution (0.1% Ficoll, 0.1% PVP, 0.1% BSA), and 100 µg/ml herring sperm DNA at 55° C for 2 hr. Hybridization with the [^{32}P]dATP-labeled probes was performed for 16 hr at 55°C in the presence of 6× SSC, 0.5% SDS, and 100 µg/ ml herring sperm DNA. After hybridization, the membranes were washed twice in 2× SSC and 0.1% SDS at room temperature for 15 min, followed by two washes with 0.2× SSC and 0.1% SDS at 55° C for 30 min. The membranes were exposed to X-ray film (Fuji RX) at –80° C using intensifier screens. All hybridization experiments were repeated at least twice with independently prepared sample DNA.

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