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Transposon mutagenesis and tagging of fluorescent *Pseudomonas*: Antimycotic production is necessary for control of Dutch elm disease

(cosmid library/functional complementation/biological control/strain identification by DNA hybridization)

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ABSTRACT **Antimycotic-producing strains of *Pseudomonas syringae* are being tested as Dutch elm disease control agents. We examined the role of antimycotic production in disease control. Transposon Tn*903* was used to mutagenize the antimycotic-producing strain MSU174. Eighty-one mutants that did not inhibit fungal growth were identified among 15,000 Tn*903*-containing derivatives. Linkages between Tn*903* insertions and defects in antimycotic metabolism were established. Three Tn*903*-containing strains (two antimycotic producers and one nonproducer) were individually introduced into American elm seedlings. The seedlings were subsequently challenged with *Ceratocystis ulmi*, the causal agent of Dutch elm disease. Protection of the elms was observed with the two antimycotic-producing strains but not with the nonproducing strain. The introduced strains could be readily recovered from the seedlings after two growing seasons. They were unequivocally identified by the Tn*903* insertions they contain.**

Dutch elm disease (DED) is one of the most destructive plant diseases known to man. The disease was first described in Europe immediately after World War I, and its cause was shown to be the fungal pathogen *Ceratocystis ulmi* [*Ophiostoma ulmi* (Buisman) Nannf.]. Since then it has spread across Europe and North America, killing millions of elm trees. The losses have been in the billions of dollars (1, 2).

Current DED control efforts have centered primarily upon sanitation practices, insect eradication, and the use of systemic fungicides. Recently, however, Myers and Strobel suggested that the introduction of appropriate pseudomonads into an elm tree may confer DED tolerance to the tree (3). Certain fluorescent pseudomonads, including some *Pseudomonas syringae* strains, produce antimycotics that are inhibitory to *C. ulmi* (3–6). Antagonism between bacteria and fungi was observed on agar media made with elm sap or elm wood extracts and in elm seedlings (3). Subsequent field experiments established the potential of fluorescent pseudomonads as biological control agents for DED. In particular, when trees were first inoculated with bacteria and then challenged with *C. ulmi*, they developed significantly fewer symptoms than those not pretreated with bacteria (4–6). An implicit assumption in these studies was that antimycotic production by the introduced *Pseudomonas* in the tree was an important factor in tree protection.

We examined the role of antimycotic production by the *P. syringae* strain MSU174 in DED control. The strategy was to construct isogenic mutant strains of MSU174 that differ only in their ability to produce antimycotics and then to compare their effectiveness in tree-protection experiments. We have shown (7) that the recombinant plasmid pRK2013 (8), which contains the transposon Tn*903* (9–11), could be used to carry out transposon mutagenesis in strain MSU174. In this report we describe (*i*) the isolation and genetic characterization of transposon mutants with altered antimycotic production and (*ii*) the use of such mutants in tree-protection experiments. In addition, we show that the transposon sequences can be used as molecular tags for monitoring the fate of the introduced bacterial strains.

MATERIALS AND METHODS

Microorganisms. The relevant properties and sources of microorganisms used in this study are listed in Table 1.

Bacterial Manipulations. Growth media, conjugations, and transposon mutagenesis were as described (11). Kanamycin was used at a final concentration of 50 μg/ml and tetracycline at 10 μg/ml.

Bioassay for Antimycotic Production and Mutant Isolation. Bacteria to be tested were transferred onto multiple agar plates in an ordered pattern. The plates were incubated until colonies were just becoming visible and then were oversprayed with a suspension of fungal spores. Upon further incubation, fungal growth occurred on the agar plates except where antimycotic-producing bacteria were present, forming zones of clearing. Mutants were recovered from master plates that were not oversprayed. *G. candidum* was used in the initial screens because of its fast growth rate. All mutants were retested and confirmed by overspraying with *C. ulmi*.

DNA Manipulations. These procedures were carried out as described (15, 16). Genomic DNA for library construction was partially digested with *Eco*RI to obtain molecules in the 15- to 30-kb range. The cosmid vector used was pLAFR1 (12).

Complementation and Colony Spot Matings. Fresh cultures of each donor pool and the mobilization helper HB101(pRK-2013) were mixed and spread on selective plates [PM agar (11) containing $FeCl_3$ and tetracycline]. Overnight cultures of individual mutant recipients were diluted (200- to 500-fold) and 10-μl aliquots were spotted on the spread plates. After incubation, the plates were oversprayed with a fungal spore suspension and later scored for zones of clearing (see Fig. 3 *Left*). Complementing clones were then isolated from identified pools in a colony spot mating procedure. Fresh cultures of individual mutant recipients and HB101(pRK2013) were mixed and 5-μl aliquots were spotted in an ordered pattern onto selective plates. Fresh individual colonies from an identified pool were picked and spotted onto master plates (nutrient agar containing tetracycline) according to the above pattern and then were smeared onto the corresponding spots on the selective plates prepared above. The plates were

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Abbreviation: DED, Dutch elm disease.

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Table 1. Relevant properties and sources of microorganisms

Strain	Relevant properties	Source*
Escherichia coli		
HB101(pRK2013)	Transposon donor and mobilization helper	7
HB101(pLAFR1)	Wide host range, mobilizable cosmid vector	12
BHB2688	Phage λ lysogen used to prepare packaging extracts	13
BHB2690	Phage λ lysogen used to prepare packaging extracts	13
HB101	Host for genomic library	14
P. syringae		
MSU174	Wild type; shows fungal inhibition	11
MSU174-R1†	Shows fungal inhibition	TS
MSU174-206†	Shows fungal inhibition	TS
MSU174-13†	No fungal inhibition	TS
MSU174-88†	No fungal inhibition	TS
C. ulmi CU-5F	DED-causing fungus; antimycotic sensitive	3
Geotrichum candidum	Test fungus; antimycotic sensitive	‡

*Numbers refer to references; TS, this study.
†Tn*903*-containing derivatives of MSU174.
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oversprayed after incubation, and clones that yielded spots with zones of fungal inhibition were recovered from the master plates.

Treatment of Trees. One-year-old seedling elms were planted in sandy loam soil in 22-cm diameter plastic pots and maintained at 20–30°C under greenhouse conditions and natural lighting. At the time of bacterial inoculation, the height of the seedlings was ≈100 cm. Bacterial cultures were injected into trees under the inner bark, using a hypodermic syringe with a no. 25 needle, at nine locations in a spiral pattern in a 2- to 3-cm band beginning at 5 cm from ground level. Approximately 0.1 ml, containing 10^8 cells, was applied. In the controls, only water was applied. Treatments were made at a time early in the season when the plants were in the process of leaf development. Two weeks after the application of bacteria, a suspension (0.01 ml) of *C. ulmi* conidia (*ca.* 1000) was placed at the base of each plant about 3 cm from ground level with a hypodermic syringe. Each group challenged with *C. ulmi* included 12 elms, and 3–6 elms not challenged with *C. ulmi* were left as controls.

Sampling and Recovery of Microorganisms. At the end of the first and second growing seasons, the basal area of each elm near the place of the original *C. ulmi* inoculation was surface-sterilized with 95% ethanol. The outer bark was removed. A small wedge of wood (25–30 mg) was removed with a razor blade. The wedge was cut into smaller pieces and placed on the *C. ulmi* selective medium of Miller *et al.* (17). Wood wedges (25–30 mg) were also taken near the locations where the *P. syringae* strains had been injected. Sampling was done at mid- to late-season in both years. Each wedge was placed in 1.0 ml of sterile distilled water and incubated for 24 hr at 23°C. Aliquots and appropriate dilutions were plated on a *Pseudomonas* selective medium [the BCBRVB medium of Sands (18) minus vancomycin and benomyl] with and without kanamycin.

RESULTS

Isolation of Mutants with Altered Antimycotic Metabolism. The antimycotic-producing activity of strain MSU174 can be monitored in an agar plate bioassay as a zone of fungal growth inhibition. When 2500 individual colonies of MSU174 were examined in the bioassay, all showed fungal growth inhibition. Tn*903*-containing derivatives of MSU174 were generated by conjugation, with the recombinant plasmid pRK2013 as donor (11). Such derivatives were obtained at a frequency of 2×10^{-6} per parental bacterium (11). Among 15,000 derivatives screened in the bioassay, 81 did not produce any zone of fungal growth inhibition regardless of the growth medium (Fig. 1). Other mutant phenotypes were also observed, including ones that produced larger zones of inhibition and ones that produced zones of inhibition only on certain growth media (Fig. 1). For the purpose of this study, we have concentrated on the first group of mutants.

Southern Blot Analysis of Mutants. *Eco*RI and *Sal* I (enzymes that do not cut within Tn*903*) digests of each mutant were examined with radioactively labeled pRK2013 as probe (Fig. 2). Each mutant yielded only one hybridized band with either enzyme, suggesting that there was only one Tn*903* insertion in each mutant genome. The positions of the hybridized bands in many mutants were different. In several cases, mutants which gave different hybridized fragments with one restriction enzyme yielded fragments of the same size with the other enzyme. Assuming that the mutant phenotype was the direct consequence of Tn*903* insertions, we infer from the above data that genes on many restriction fragments (more than 8 for *Eco*RI) are involved in antimycotic metabolism and some of these fragments are adjacent to each other.

Isolation of Wild-Type Sequences Capable of Restoring Fungal Growth Inhibition. A genomic library of the wild-type strain MSU174 was constructed by using the wide-host-range cosmid vector pLAFR1 (12). This vector can be mobilized into MSU174 and stably replicates in that strain (unpublished results). The library was maintained as pools, each of which contained approximately 200 independent clones. Such pools were conjugated into individual MSU174-derived mutants by using pRK2013 as mobilization helper (19) and selecting for tetracycline resistance, which is carried on the cosmid vector. Tetracycline-resistant transconjugants, obtained at a frequency higher than 1 of 100 parental bacteria, were examined for complementation of the mutant phenotype (defined here as restoration of the ability to inhibit fungal growth; Fig. 3 *Left*). A complemented transconjugant presumably has received wild-type sequences corresponding to the site of the mutation. Unfortunately, because of the antagonism between bacteria and fungi, viable transconjugants were not always recoverable from the bioassay and necessitated a second round of conjugation (colony spot

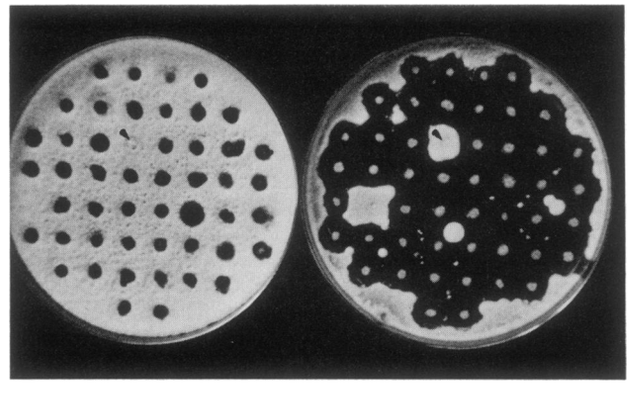

FIG. 1. Bioassay for antimycotic production and mutant isolation. Replica of bacterial colonies on *Pseudomonas* F agar (*Left*) and PM agar (11) with $FeCl_3$ (*Right*). The plates were oversprayed with *G. candidum*. MSU174 produces larger zones of fungal growth inhibition on PM than on *Pseudomonas* F agar; here the zones overlapped such that one sees a continuous clear background except where a nonproducing mutant is located (arrows).

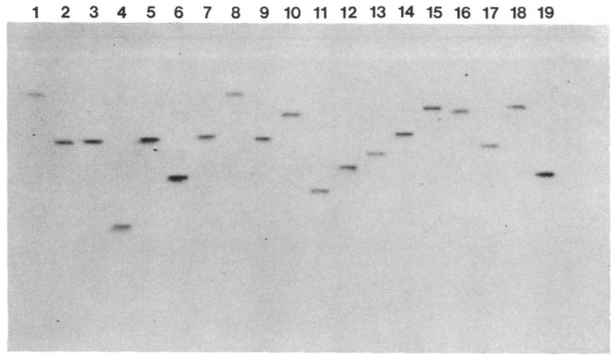

FIG. 2. Southern blot analysis of mutants. Each lane contains *Eco*RI-digested DNA from a different mutant. The probe was radioactively labeled pRK2013. The Tn*903* insertions are in many different restriction fragments.

matings, see *Materials and Methods*) and transconjugant testing (Fig. 3 *Right*). Each identified clone was conjugated into the entire set of mutants to determine which ones it could complement. Five different clones have been isolated which together complemented 46 of the 81 mutants.

Linkages Between Tn*903* Insertions and Mutant Phenotypes. The complementing clones were used as probes in Southern blot analyses of the mutants. An example is shown in Fig. 4. The clone used here complemented mutant isolate 88 (lane 2) but not mutant isolate 13 (lane 3). The wild type isolate (lane 1) and isolate 13 yielded identical hybridization patterns; isolate 88, on the other hand, yielded one larger fragment. When this blot was reprobed with pRK2013, isolate 13 yielded one new hybridized fragment, but isolate 88 did not (data not shown). These results suggest that the Tn*903* insertion in isolate 88 resides within the DNA sequence corresponding to that contained in the complementing clone.

Role of Antimycotic Production in DED Control. Neither a growing colony nor a sonicated lysate of mutant isolate 88 produced any zone of fungal growth inhibition in the agar plate bioassay. Thus, it appears that isolate 88 is defective in the production as well as transport of the antimycotic. This mutant isolate and two other Tn*903*-containing derivatives of MSU174, isolates R1 and 206, were compared for their effectiveness in elm tree-protection experiments. Isolate R1 produces an antifungal zone indistinguishable from that produced by the wild-type parent and contains one Tn*903* insertion at an uncharacterized place in the genome. Isolate 206 was identified by the larger antifungal zone it consistently

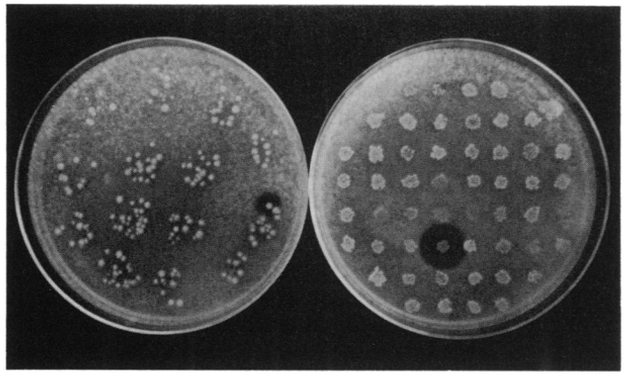

FIG. 3. Isolation of complementing clones. (*Left*) A pool of ≈200 different genomic clones was mobilized into a mutant recipient strain. The transconjugants were examined for fungal growth inhibition. (*Right*) Individual clones from a positive pool were mobilized into the mutant recipient in a colony spot mating procedure, and the transconjugants were tested for fungal growth inhibition.

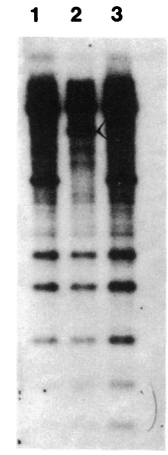

FIG. 4. Southern blot analysis showing linkage between Tn*903* insertion and mutant phenotype. A clone which complemented the mutant isolate 88 (lane 2) but not mutant isolate 13 (lane 3) was radioactively labeled and used as the probe. Each lane contains *Eco*RI-digested DNA from a bacterial strain. The wild-type isolate (lane 1) and isolate 13 yielded identical patterns; isolate 88 yielded one larger fragment (arrowhead).

produced in the bioassay. This phenotype is probably caused by the Tn*903* insertion it contains, since three other independently derived mutants showing the same phenotype also contain Tn*903* insertions in the same restriction fragment (unpublished results).

The three isolates were separately introduced into American elm seedlings. None of the isolates produced any visible symptoms in the inoculated elms throughout the experiment. A subgroup of the elms was later challenged with *C. ulmi*. Within a few weeks after fungal inoculation, wilting and chlorosis began to appear on the elms (Fig. 5). Significantly ($P < 0.05$) more elms developed symptoms in the groups treated with water alone or isolate 88 than those treated with isolate R1 or 206 (Table 2). At the end of the first growing season, *C. ulmi* was recovered from significantly more elms in the first two groups than in the last two. During the second season, no apparent progression in symptoms was observed in any of the elms and the ratio of elms with symptoms to those without remained the same. *C. ulmi* was recovered from at least one tree in each group at the end of the second season but there was no significant difference between groups.

Recovery of Introduced Bacterial Strains from Elms. Kanamycin-resistant, antimycotic-producing pseudomonads were easily recovered at the end of each growing season from most of the trees inoculated with either isolate R1 or 206 (Table 2). Kanamycin-resistant pseudomonads were also easily recoverable from trees inoculated with isolate 88, but none of them produced the antimycotic. No kanamycin-resistant, antimycotic-producing pseudomonads could be recovered from trees not inoculated with bacteria. The same results were obtained whether the trees were subsequently challenged with *C. ulmi* or not.

Random representatives of the recovered pseudomonads were examined for their Tn*903* contents by Southern blot hybridization. Genomic DNA from each strain was digested with *Eco*RI and probed with pRK2013 (Fig. 6 *Upper*). Three kanamycin-resistant *Pseudomonas* strains recovered from each of five trees inoculated with isolate 88 were examined. All showed the same restriction enzyme digestion patterns (data not shown) and hybridization patterns as isolate 88 (lane 1). Strains recovered from the other groups of inoculated trees yielded similar results, with the following exceptions. (*i*) Several kanamycin-resistant, antimycotic-negative strains

FIG. 5. Elms inoculated with mutant isolates of MSU174. (*Left*) Isolate 206 (antimycotic producer). (*Right*) Isolate 88 (antimycotic nonproducer). Both elms were subsequently challenged with *C. ulmi*. Symptoms developed in the antimycotic nonproducer (*Right*) in 3–4 weeks after fungal inoculation and no symptoms developed in the antimycotic producer (*Left*). The symptoms were characterized as yellowing, severe wilting, curling, and drying of the leaves.

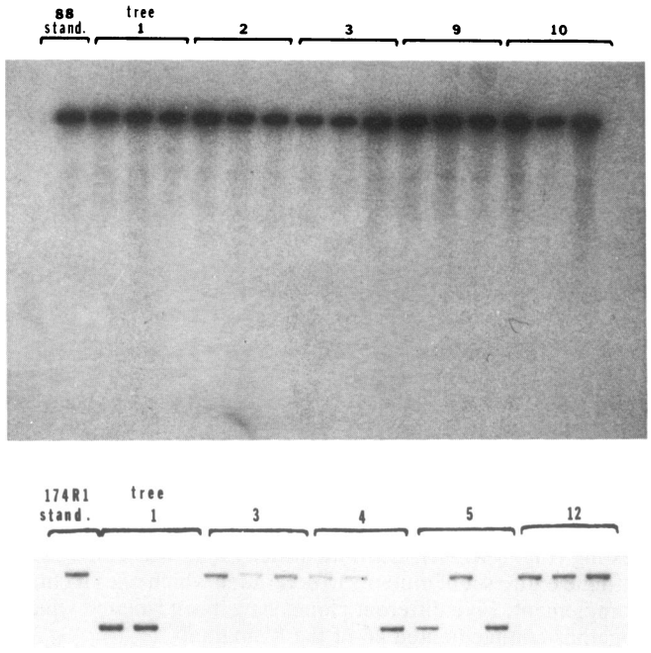

FIG. 6. Southern blot analysis of bacterial strains recovered from elms. DNA from each strain was digested with *Eco*RI and probed with pRK2013. Lanes under each bracket contain DNA from bacterial strains isolated from one tree. (*Upper*) Strains recovered from trees that were inoculated with isolate 88 and subsequently challenged with *C. ulmi*. All showed the same pattern as that of authentic isolate 88 (lane 1). (*Lower*) Strains recovered from trees that were inoculated with isolate R1 and subsequently challenged with *C. ulmi*. Several strains showed a pattern different from that of authentic isolate R1 (lane 1).

recovered from R1- or 206-inoculated trees were examined. They showed different restriction patterns and yielded no hybridization. We believe they are naturally occurring kanamycin-resistant *Pseudomonas* strains unrelated to MSU174. (*ii*) While strains recovered from R1-inoculated trees that were not challenged with *C. ulmi* yielded a hybridization pattern identical to that of isolate R1, several strains recovered from trees that were inoculated at the same time with the same culture of isolate R1 and were later challenged with *C. ulmi* yielded a different hybridization pattern (Fig. 6 *Lower*).

DISCUSSION

Most (>90%), if not all, of the antimycotic-negative derivatives isolated after transposon mutagenesis are expected to be caused by Tn*903*, since the spontaneous frequency was relatively low (<1 in 2500). In particular, linkage between the Tn*903* insertion and the mutant phenotype in isolate 88 was demonstrated. This mutant provided no protection against DED. We conclude that antimycotic production is necessary for disease control.

The frequency (0.54%) at which we obtained antimycotic-negative mutants among the general insertion-mutant population suggests that a substantial number of genetic loci are involved in the metabolism of the antimycotic. This possibility is further supported by the Southern hybridization data (Fig. 2), which indicate that the mutants are located on many restriction fragments. The mutations are somewhat clustered, as five cosmid clones together complemented over half of the mutants. The structural relationships among these cloned fragments have not yet been defined.

We monitored the persistence of introduced bacterial strains in elm trees through two growing seasons. The criteria we used for strain identification included the ability to grow on selective media, antimycotic production, and, ultimately, the presence and location of Tn*903* (by Southern hybridization). We feel that the last test provides unequivocal identification of any Tn*903*-tagged *Pseudomonas* strain because (*i*) we have not observed any *Pseudomonas* isolates that naturally harbor Tn*903*, and (*ii*) even if a natural isolate indeed contains Tn*903*, it is extremely unlikely that the insertion

Table 2. Effect of MSU174 derivatives on control of DED and recovery of microorganisms from trees

Treatment	Elms with symptoms	Recovery of *C. ulmi*		Recovery of kanamycin-resistant antimycotic-producing *Pseudomonas*	
		Season 1	Season 2	Season 1	Season 2
No bacteria	11/12 (A)	10/12 (A)	4/12 (A)	0/12 (A)	0/12 (A)
MSU174-88	11/12 (A)	12/12 (A)	3/12 (A)	0/12 (A)	0/12 (A)
MSU174-RI	6/12 (B)	3/12 (B)	1/12 (A)	9/12 (B)	8/11 (B)
MSU174-206	6/12 (B)	3/12 (B)	1/12 (A)	8/12 (B)	6/12 (B)

Numbers followed by the same letter are not significantly different at $P < 0.05$ according to a 2-way χ^2 contingency table test.

would be in a restriction fragment of the same molecular weight. Drug resistance (which provides a positive selection) and transposon-tagging coupled with identification by DNA hybridization could be a generally applicable monitoring method for bacterial environmental studies (Table 2 and Fig. 6). If the transposon can be shown not to exist naturally within the environment being studied, a simplified DNA dot-blot procedure, which would allow numbers of strains to be sampled, may suffice.

Our data indicate that derivatives of strain MSU174 can persist in elm trees at least through two growing seasons (Table 2 and Fig. 6). Since we did not attempt to monitor the distribution of the bacteria throughout the elm trees but only examined the presence of the bacteria at a particular point in each tree, we could have underestimated the fraction of trees still harboring the bacteria.

Previous reports have demonstrated the potential of *Pseudomonas* as biological control agents for DED (3–6). The tree-protection experiments described here were designed to examine the role of antimycotic production in disease control. Therefore, we placed the bacterium and fungus in close juxtaposition to maximize the effects of their interactions. In extensive field experiments under more "natural" conditions, Scheffer (4, 5) found that the bacteria offered protection against DED whether the fungus was introduced in the upper part of the stem or in the twigs. The introduced bacterial population underwent an initial drop and then stabilized after 1 month. This may reflect an intrinsic "holding capacity" of elms for these bacteria. Bacteria resembling those introduced were recovered at the end of the experiments near the sites of inoculation (in the trunk) but not in the twigs, suggesting a limited systemic spread. With the transposon-tagging methodology, a more rigorous description of the population dynamics and ecology of the introduced bacteria in elms should be possible.

The antimycotic produced by MSU174 is not yet purified. Many questions remain about its production and distribution within the tree. Such investigations must await the elucidation of the structure of the compound.

The DNA hybridization results obtained from trees inoculated with isolate R1 were unexpected (Fig. 6 *Lower*). The trees were inoculated and handled identically and were not subdivided (randomly) into two groups until the time of fungal challenge. The different hybridization pattern observed *only* in strains recovered from challenged trees is therefore likely to be the result of events that occurred after fungal introduction. Although we do not know the cause of such events, the fungus itself has to be considered a likely candidate. The nature of the events has not been defined.

Several limited control measures for DED are available at this time. However, they are costly and less than totally effective. A relatively inexpensive biological control measure would offer an alternative. Hopefully, our findings that *P. syringae* can persist in elm trees over long periods of time and that antimycotic production is necessary for disease control answer some of the questions concerning the prospect of such an approach.

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