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# Pseudomonas for biological control of Dutch elm disease. I. Labeling, detection and identification of Pseudomonas isolates injected into elms; comparison of various methods

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**Abstract**

To understand the mechanisms involved in biological control of Dutch elm disease by *Pseudomonas*, data were needed on the distribution of the introduced bacteria within elm and on the development of the bacterial population over a period of time.

As traditional biochemical identification techniques are not suitable for distinguishment between individual *Pseudomonas* isolates, three alternative approaches were compared.
1) Chemotaxonomy, using lipopolysaccharide pattern, cell envelope protein pattern or DNA restriction fragment pattern. These techniques were reliable, but tedious.
2) Labeling bacteria with a transposon (Tn903) or a plasmid construct (pMON5003) with a metabolic marker (Lac ZY, coding for β-galactosidase and lactose permease) allowed for a reliable identification of reisolates. However, populations of transposon-labeled bacteria in elms declined much faster than populations of the unlabeled wild type. The plasmid carrying the metabolic marker disappeared from the bacterial populations over time. Apparently both the transposon and the plasmid were a disadvantage to the bacteria compared with the wild type parent strains.
3) Immunoagglutination of representative reisolates with an antiserum against the *P. fluorescens* isolate in use proved to be specific and fast. For routine purposes the immunoagglutination test therefore was the best method of the various ones employed.

Studies on the distribution of a *Pseudomonas* isolate in elm twigs showed that a stable bacterial population developed in the twigs within three months, but that the bacteria in general did not escape from the xylem vessels in which they were introduced.

*Additional keywords: Ophiostoma ulmi*, plasmid construct (pMON5003) metabolic marker, transposon Tn903, immuno- agglutination, lipopolysaccharide pattern, cell envelope protein pattern, DNA restriction fragments.

## Introduction

Dutch elm disease, caused by *Ophiostoma ulmi* (Buisman) Nannf., can be controlled to a certain extent by various means, as recently reviewed by Scheffer and Strobel (1988). Biological methods could potentially improve the possibilities for practical control of the disease because of their proposed longevity, low cost and minimal environmental impact.

*Pseudomonas* spp. applied within the same growing season as the infection by the pathogen is effective as a prophylactic treatment against *O. ulmi* (Scheffer, 1983; Murdoch et al., 1986). To analyze the mechanisms that may explain these disease-controlling effects questions about the spatial distribution and the longevity of the bacteria within the tree have to be addressed. For these studies on population dynamics of bacteria introduced into elm, reliable detection and identification methods are needed. Ultimately, two different markers and several identification procedures were compared.

The possibility of introducing the transposon Tn903, which is identical to Tn601 and Tn55 (Oka et al., 1981), in *Pseudomonas* spp. was shown by Lam et al. (1985).

An alternative for labeling the cells with a transposon, which will be incorporated in the bacterial genome, would be the introduction into *Pseudomonas* spp. of a plasmid-based metabolic marker. The plasmid construct pMON5003 (Drahos et al., 1986), which contains the LacZY genes, provides such a marker system.

Alternatives for specific labeling of the bacteria are various chemotaxonomical techniques that allow for identification of individual isolates of, in our case, mainly the genus *P. fluorescens*. These techniques result in 'fingerprints' of the genome (DNA restriction fragment pattern), or of a large, and therefore highly specific, set of gene products (lipopolysaccharide pattern or cell envelope protein pattern; De Weger et al., 1986, 1987).

In this study, both the transposon and the plasmid-based metabolic marker labeling systems were tested *in planta* and compared with the three chemotaxonomical methods mentioned and with immuno-agglutination, which' specificity depends on the specificity of the antiserum in use.

## Materials and methods

*Micro-organisms.* All micro-organisms used in this study are summarized in Table 1. Fungal cultures were maintained on potato dextrose agar slants. Shake cultures of *O. ulmi* were grown in Tchernoff's medium (Tchernoff, 1965) at 23 °C for 5-7 days. Conidial suspensions of 5 × 106 conidia per ml, as needed for inoculation of elms, were prepared as described earlier (Scheffer, 1983).

The *Pseudomonas* isolates with WCS codes were used in extensive studies aiming at biocontrol of plant diseases and improving crop yield, as recently reviewed by Schippers et al. (1987). All *Pseudomonas* isolates were maintained on 'King B' (medium B of King et al., 1954) agar slants. Cultures in King B broth showed a tendency to form a white deposit, probably because of the formation of $MgHPO_4$. Several variations in pH and composition resulted in a simple modification of the medium: $K_2HPO_4$ (1.5 g $l^{-1}$) was replaced by $KH_2PO_4$ (1.2 g $l^{-1}$ to maintain the molarity of King B). As a result of this, the pH of the medium shifted from 7.4 to 6.6, no deposit was formed and the growth rate of *P. fluorescens* WCS374 and *P. syringae* M27+ remained exactly the same as in King B. This modified medium is referred to as 'modified King B'. *Pseudomonas* cultures used for the inoculation of trees were grown in 250 ml modified King B in 1 l Erlenmeyer flasks, shaken vigorously on a reciprocating shaker at 24 °C for 40 h. These cultures were inoculated with 2.5 - 3 ml of a one-day-old pre-culture grown under identical conditions. Foam plastic plugs (Scientific Industries International, Loughborough, UK) proved to be important for optimal growth and intensive fluorescence of *Pseudomonas*.

Table 1. Micro-organisms used in this study.

| Isolate code | Relevant description | Reference/source |
|---|---|---|
| *Ophiostoma ulmi* | | |
| H6 | aggressive (NAN) | Gibbs et al., 1975 |
| H106 | aggressive (NAN) | obtained from Dr C.M. Brasier, UK |
| *Pseudomonas fluorescens* | | |
| WCS374 | wild type; from potato soil | Geels and Schippers, 1983a, b |
| WCS374 RJS101 | adapted to low water potential | Scheffer et al., 1989 |
| WCS374 RJS112 | contains transposon Tn903 | this study |
| WCS374 RJS122 | contains plasmid pMON(5003) | this study |
| *Escherichia coli* | | |
| HB101 (pRK2013) | | Figurski & Helinski, 1979 |
| M182 (pMON5003) | | B. Hemming |

*E. coli* cultures were maintained on nutrient agar (Difco) with 5 g $l^{-1}$ NaCl.

For laboratory studies, bacteria were grown in 3 ml nutrient broth (Difco) with 5 g $l^{-1}$ NaCl or modified King B at 200 rpm and 25 °C. For studies on the cell envelope, protein and lipopolysaccharide patterns, stationary phase cultures were diluted 100-fold into 25 ml fresh King B broth. These cultures were grown for 20 h under vigorous aeration at 28 °C.

*Labeling of P. fluorescens isolates.* Two labeling procedures were attempted: insertion of the transposon Tn903 into the genome of *P. fluorescens* WCS374 and the introduction of a plasmid (pMON5003) containing the simply recognizable Lac Y and Lac Z genes into *P. fluorescens*. The suicide vector pRK2013 (Figurski and Helinski, 1979) which contains the kanamycin-resistant transposon Tn903 was introduced into *P. fluorescens* WCS374 by conjugation of the multiple auxotrophic *Escherichia coli* HB101 (pRK2013) with *P. fluorescens* cells. Late-log phase shake cultures of donor and recipient were grown in nutrient broth and centrifuged in a bench top centrifuge. Pellets were resuspended in 0.1 % proteose peptone and dilution series of donor, recipient and a 1 : 1 mixture were plated out on nutrient agar plates. After 16 h at 25 °C replicates were made on Dye's medium (55 mM glucose, 10 mM $(NH_4)_2HPO_4$, 5 mM ornithine, 3.5 mM KCl, 1 mM $MgSO_4$) with 50 mg $l^{-1}$ kanamycin. Kanamycin-resistant mutants were collected and normal appearance and growth was checked. Insertion of the transposon was made plausible using the Southern blot technique (Lam et al., 1985; Maniatis et al., 1982). The frequency of kanamycin-resistant derivatives was $1.2 \times 10^{-5}$ for *P. fluorescens* WCS374 plus HB101(pRK2013) and $\leq 10^{-9}$ for donor or recipient alone.

The metabolic marker system used in this study consisted of a plasmid construct (pMON5003) based on the vector pKT230 (Bagdarisan et al., 1981) in which a 7.7 kb BamHI fragment purified from pMC903 (Casadaban et al., 1980) containing the lacZY genes, coding for β-galactosidase and lactose permease, was inserted (Drahos et al.,

1986). *Pseudomonas* spp. usually lack β-galactosidase and the marker therefore can be detected using the chromogenic substrate X-gal (5-chloro-4-bromo-3-indolyl-β-D-galactopyranoside) which incorporated in solid media gives rise to blue-green colonies (Drahos et al., 1986). The plasmid construct pMON(5003) was purified from *E. coli* M182 (pMON5003) according to the procedure developed by Birnboim and Doly (1979). Competent *P. fluorescens* WCS374 cells were prepared as follows: 20 ml liquid culture of early log phase was incubated on ice for 30 min, the cells were precipitated by centrifugation (5000 *g* for 5 min) and the pellet resuspended in 5 ml ice-cold 150 mM $MgCl_2$. After incubation on ice for 20 min the cells were precipitated as above and the pellet resuspended in 100 µl ice-cold 150 mM $MgCl_2$. Transformation was carried out by mixing 50 µl of the freshly prepared competent cells with ca. 200 ng of pure plasmid. After incubation on ice for 10 min the mixture was heat-shocked (37 °C for 5 min) and again incubated on ice for 12 min. After addition of 0.7 ml modified King B the mixture was incubated for 2.5 h at 27 °C, followed by plating on nutrient agar amended with 50 mg $l^{-1}$ kanamycin plus 40 mg $l^{-1}$ X-gal. Transformants showed as blue colonies.

*Bacterial treatments of elms.* Gouge pistols (Scheffer, 1983) were used for treating trees with bacteria for all experiments except those where twigs were inoculated for quantitative reisolation of bacteria.

Trees were injected at a height of 1.2 - 1.4 m every 5 cm of circumference with 1 ml of shake culture. For every treatment, 10 to 15 trees were used, selected at random within plots of approximately 15-year-old trees. The trees were *Ulmus* × *hollandica* 'Commelin' and 'Belgica', both susceptible to the aggressive strain of *O. ulmi*. They were incinerated upon termination of the experiment.

For the experiments aiming at quantitative reisolation of bacteria, twigs of young trees were inoculated near their base with a 3 mm-wide chisel. Approximately 100 twigs of both the clone *U.* × *hollandica* 'Belgica' and 390 were inoculated with bacteria and an equal number served as controls. Three times 20 µl of a shake culture of WCS374 or WCS374 RJS122 was applied per twig. Controls received modified King B.

*Inoculation with O. ulmi.* Inoculations with *O. ulmi* were performed with a 1 : 1 mixture of conidial suspensions of the aggressive isolates H6 and H106; 5 × 106 conidia $ml^{-1}$. Stems were inoculated with two times 50 µl at opposite sites (Scheffer, 1983); twigs similarly at their base using a 3 mm wide chisel.

*Quantitative reisolation.* Quantitative reisolation of *P. fluorescens* was performed by homogenizing twig pieces (twig experiments) or pieces of wood taken from root or stem by saw and chisel.

In the twig experiments, 10 twigs per group were periodically sampled over a period of 67 weeks upon inoculation. These twigs were surface-sterilized with ethanol, the bark was peeled back and a piece taken at 3.5 to 6.5 cm above the site of inoculation and clipped into chips. The chips were homogenized with an Ultra-Turrax homogenizer for 30 s in 10 ml of a 0.1 % proteose peptone solution (Straka and Stokes, 1957). A second sample with the same length was taken at ⅔ of the length of the twig. The homogenate was aseptically filtered through nylon gauze and plated in a dilution series on modified King B plates for assessing the *Pseudomonas* density. Material from twigs

inoculated with WCS374 RJS122 was also plated on modified King B amended with 50 mg $l^{-1}$ kanamycin, 25 mg $l^{-1}$ streptomycin and 40 mg $l^{-1}$ X-gal. For the trunk inoculation experiments, big wedges were taken from the tree. After surface sterilization with ethanol the bark was removed, smaller pieces of wood comprising the current and last year's growth ring were collected and c. 1 g was clipped into chips. The chips were treated as those from twigs.

*Cell envelope protein pattern and lipopolysaccharide pattern.* Cell envelopes were isolated by differential centrifugation after disruption of the cells by ultrasonic treatment (Lugtenberg et al., 1975). Samples were solubilized by incubation for 15 min at 95 °C in the standard sample mixture (Lugtenberg et al., 1975). For separation of the membrane proteins by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, samples (15 $\mu$l) containing approximately 1 mg of cell envelope protein per ml were applied to 11 % polyacrylamide gels and after electrophoresis gels were stained using Fast Green FCF (de Weger et al., 1986). For analysis of lipopolysaccharides, solubilized cell envelopes (1 mg cell envelope per ml) were incubated with proteinase K (50 $\mu$g $ml^{-1}$) at 56 °C for 1 h. Fifteen microliters of the 10-fold diluted samples were applied to 13 % polyacrylamide gels (De Weger et al., 1987). Gels were stained by a silver staining procedure (Tsai and Frasch, 1982).

*Total bacterial DNA pattern.* Bacterial shake cultures (100 ml) were washed twice with TES buffer (50 mM TRIS, 50 mM EDTA, 50 mM NaCl; pH 8.5) and the cells were lysed by addition of 2 ml lysing solution (0.6 M sucrose, 50 mM TRIS, 50 mM NaCl, 5 mM $Na_2EDTA$, 0.2 mg $ml^{-1}$ RNAse, 2 mg $ml^{-1}$ lysozyme, pH 8.5) by incubation at 37 °C for 10 min, followed by incubation on ice for 20 min. After addition of an equal volume of 2 % sarkosyl (N-laurolysarcosine sodium salt) the mixture was incubated at 65 °C for 20 min. After addition of 6 ml of TES buffer the mixture was kept at 65 °C for another 10 min (Joan Henson, pers. comm.). The DNA was purified by centrifugation in a cesium chloride gradient followed by ethanol precipitation (Maniatis et al., 1982). Digests and agarose gel electrophoresis were according to standard procedures (Maniatis et al., 1982).

*Immuno-agglutination.* Bacterial suspensions of pure cultures of isolates in PBS (phosphate-buffered saline: 0.15 M NaCl + 0.02 M sodium phosphate buffer, pH 7.2) were mixed with a 1 : 400 dilution in PBS of a rabbit anti-WCS374 serum (10 $\mu$l + 10 $\mu$l) on glass multitest slides (Flow Laboratories, Irvine, Ayrshire, UK). The anti-WCS374 serum showed a high specificity for WCS374 cells in the agglutination tests (P.A.H.M. Bakker, L.A. de Weger, J.W.L. van Vuurde and B. Schippers, pers. comm.).

## Results

*Comparison of immuno-agglutination with chemotaxonomical identification techniques.* Bacteria were isolated from stems of 'Commelin' and 'Vegeta' elms three months after inoculation; in total 137 isolates were tested. Agglutination tests with anti-WCS374 serum were positive for WCS374 for 80 - 90 % of the samples of the *Pseudomonas* isolates. Total DNA restriction patterns (Fig. 1), cell envelope protein patterns and lipopolysaccharide patterns (Fig. 2) consistently confirmed the agglutination tests

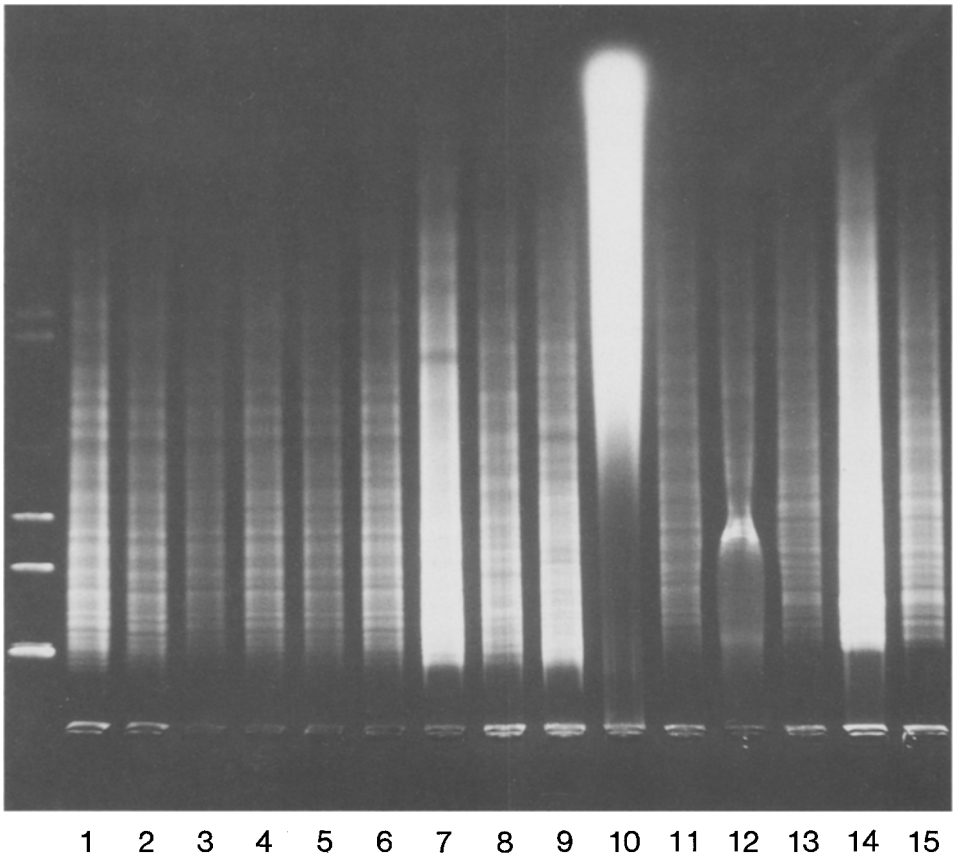


Fig. 1. Total DNA EcoRI digests of pure cultures of bacteria reisolated from elm. Lane 1, 2: *P. fluorescens* WCS374. Lane 3-4: marked WCS374 substrains similar to the one in lane 5. Lane 5: WCS374 RJS122. Lane 6: WCS374 RJS101 (described in part 2 of this study; Scheffer et al., 1989). Lane 9, 10, 13-15: isolates from a 'Groeneveld' elm, inoculated with WCS374 three growing seasons before. Lane 7, 8, 11, 12: isolates from 'Commelin', inoculated with WCS374 three months before (in Fig. 2 resp. lane 13, 14, 12, 16). The patterns in lane 1-6 and 11 are identical to WCS374; those in lane 7-9 and 13-15 are not. Lane 10 and 12 did not show proper banding in this gel, precluding further analysis. The results confirm immuno-agglutination tests with anti-WCS374 serum, which were positive for the bacteria analyzed in lane 1-6 and 11.

except for two isolates from one 'Vegeta' elm that were positive in the agglutination tests but not completely identical to WCS374 regarding their cell envelope protein and/or lipopolysaccharide pattern (Fig. 2, lanes 10 and 11).

*Population dynamics of wild type versus transposon or plasmid-labeled P. fluorescens.* The bacterial population in elm twigs was monitored in two experiments; for one the wild type *P. fluorescens* WCS374 was used as an inoculum, for the other WCS374 RJS122, a derivative of WCS374 containing the plasmid pMON5003. In both elm clones tested, the population of WCS374 cells decreased c. 100 times in the first month upon

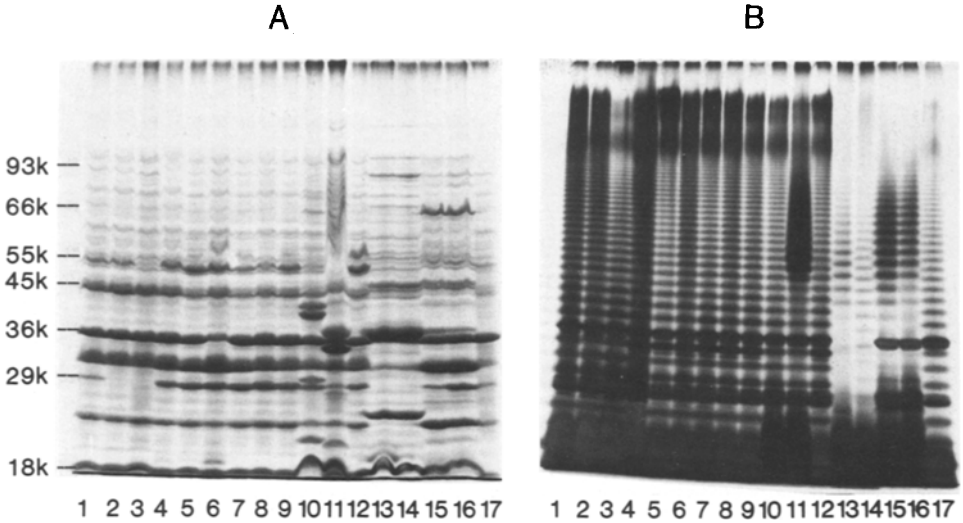


Fig. 2. Cell envelope protein patterns (A) and lipopolysaccharide patterns (B) of pure cultures of *Pseudomonas* spp. isolated from elms. Lane 1-4: isolates from twigs, inoculated with WCS374 RJS122 three months before, that were positive for the lacZY genes (X-gal metabolism) and resistant to kanamycin and streptomycin. Lane 5-8: isolates from the same experiment that did not react with X-gal and were sensitive to the antibiotics. Lane 9-11: isolates from 'Vegeta' elms inoculated with WCS374 three months before. Lane 12-16: isolates from 'Commelin' elms inoculated with WCS374 three months before. Lane 17: WCS374. The patterns in lane 1-9 and 12 are identical to WCS374, lane 17; the patterns in lane 13-16 are not. For lane 10 and 11 the cell envelope protein pattern (A) is distinct from WCS374, but the lipopolysaccharide pattern (B) closely resembles that of WCS374. The results confirm agglutination tests with anti- WCS374 serum which were positive for the isolates of lane 1-12 and 17, except for the two isolates analyzed in lane 10 and 11. These two isolates, from one 'Vegeta' elm, were the only exceptions where the various identification techniques did not concord.

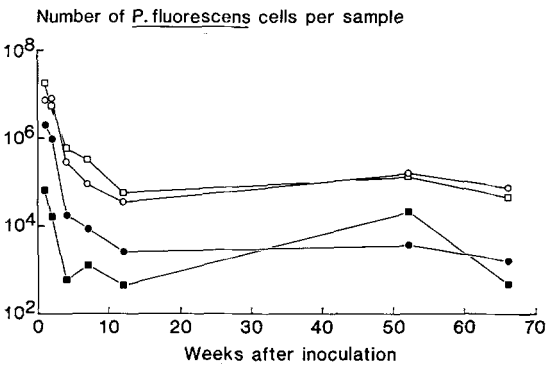


Fig. 3. *P. fluorescens* WCS374 in twig samples of 3 cm in length of the susceptible elm clone 'Belgica' and the moderately resistant clone 390 during a 67 week period. Each datum point is the average of 10 twigs per experiment and also the average of both experiments. Samples were taken at the base of the twigs, just above the site of inoculation ((○) 'Belgica'; (□) clone 390) and at ⅔ of the twig length, i.e. at ⅓ from the top ((●) 'Belgica'; (■) clone 390).

inoculation and stabilized after two months, see Fig. 3. The concentration was consistently c. 35 times higher near the place of inoculation (c. $7 \times 10^4$ per twig sample) than higher up in the twigs (c. $2 \times 10^3$ per twig sample). No major differences were observed between the susceptible clone 'Belgica' and the moderately resistant 390, except that the bacterial concentration high up in twigs of clone 390 was lower initially than in 'Belgica'.

In the twig experiment with WCS374 RJS122 the *Pseudomonas* concentrations detected on selective medium (modified King B agar with kanamycin and streptomycin) in the second year after inoculation were around the lower detection limit and generally less than 1 % of the *Pseudomonas* concentrations found on modified King B agar without antibiotics. No such discrepancy was found during the first year. As loss of the marker plasmid would explain these results, further identification of bacterial samples was performed. Agglutination tests with anti-WCS374 serum were consistently positive for WCS374 for samples of the *Pseudomonas* isolates that prospered only on modified King B agar, but not on the selective medium. Indeed, such isolates also showed both a cell envelope protein pattern and a lipopolysaccharide pattern identical to WCS374, confirming the identity of the isolates with WCS374 by two independent techniques (Fig 2).

In an experiment comprising 47 different elm clones, samples were taken from the base of two-year-old plants and from the current year's shoot four months after inoculation with *P. fluorescens* WCS374 (data not shown). The *P. fluorescens* concentrations close to the site of inoculation varied from $3 \times 10^4$ to $3 \times 10^6$ per 3 cm sample. However, no significant differences were observed that might have indicated that within certain elms establishment of a *Pseudomonas* population was especially promoted, or, that specific elms did not allow for establishment of a *Pseudomonas* population. In this experiment no, or hardly any bacteria were isolated from 88 % of the samples of the new year's shoots. If *Pseudomonas* spp. were isolated, the concentration was always lower than 1 % of that near the point of inoculation.

In 'Commelin' elms *P. fluorescens* WCS374 was compared with the transposon-labeled derivative WCS374 RJS112 and the plasmid-labeled derivative WCS374 RJS122. The bacterial treatments were followed 10 days later by an inoculation with *O. ulmi*. Only the wild type WCS374 was still present in the trunks of the trees in detectable concentrations 13 weeks after the bacterial treatments: the average concentration of WCS374 was $4.8 \times 10^4$ g$^{-1}$ wood. Both the labeled WCS374 derivatives could not be recovered from any tree.

## Discussion

A reliable identification procedure for the bacterium strain in use is essential for studies on its spatial distribution and longevity, where classical taxonomy, for characterization of *Pseudomonas* spp. according to Doudoroff and Palleroni (1974), is insufficient. In this study, three major approaches were compared: 1) chemotaxonomy, based on lipopolysaccharide pattern, cell envelope protein pattern or DNA restriction pattern, 2) labeling the bacteria with a transposon or a plasmid-based metabolic marker, and 3) indirect identification with a specific rabbit antiserum against the *Pseudomonas* isolate in use.

The combination of lipopolysaccharide pattern and cell envelope protein pattern

proved to be a powerful tool for identification of individual bacterial isolates (De Weger et al., 1986; 1987). In this study these techniques indeed clearly distinguished *P. fluorescens* WCS374 from other fluorescent *Pseudomonas* isolates. Total DNA restriction patterns, although less extensively tested, are most probably a reliable basis for distinguishing isolates from each other too. However, the current procedure, which involved a centrifugation step in a cesium chloride gradient, is too tedious to make it attractive.

Marking the bacteria with a transposon or a plasmid seemed to be attractive because identification of such a labeled reisolate is straightforward and, especially for the plasmid-based metabolic lacZY marker, very reliable. A fundamental drawback of the introduction of a transposon in a genome is the difficulty to prove that the transposon is not interfering with expression of a host gene. Furthermore, the randomness of a transposon insert and its stability in a bacterial genome under selection pressure is sometimes questioned, for instance of Tn7 in *P. syringae* and *Xanthomonas campestris* (Mills, 1985), Tn5 in *P. syringae* (Anderson and Mills, 1985) and recently of Tn903, also in *Pseudomonas* spp. (Lam et al., 1987).

A plasmid-based metabolic marker would not have these drawbacks, as such a marker would not interfere with the host genome. Beside possible problems with the durable maintenance of the plasmid, again a fundamental problem exists: if host bacterial cells that are cured of the plasmid arise, these cells would have an energetic advantage that might result in elimination of the plasmid-bearing population.

Although Lam et al. (1987) showed the feasibility of using transposon-marked *Pseudomonas* derivatives, it was found in this study that populations of a transposon-marked WCS374 derivative (WCS374 RJS112) in elms declined much faster than comparable wild type populations. The transposon may have interfered with expression of a bacterial gene facilitating survival in the tree, although the derivative was indistinguishable in vitro from the wild type.

The metabolic marker, contained in a plasmid, was stable in rich media. Apparently the plasmid was lost from the *Pseudomonas* population under selection pressure in the tree since mainly non-marked wildtype bacteria were recovered after one year.

The third approach, indirect identification by means of immuno-agglutination with a specific rabbit antiserum against a *Pseudomonas* isolate was extensively tested.

In this study only two false positive identifications were recorded, from one 'Vegeta' elm: they were identified as WCS374 by the agglutination test, but showed different cell envelope protein patterns. The lipopolysaccharide pattern of one isolate (lane 10 in Fig. 2) resembled that of WCS374 and the other showed an identical base pattern; therefore the possibility that the antiserum reacted with these lipopolysaccharides probably explains the positive agglutination tests.

For routine purposes indirect identification with a specific rabbit antiserum against the *Pseudomonas* isolate in use clearly proved the best combination of reliability and speed.

*Pseudomonas* populations in elm twigs declined steeply upon inoculation of the twigs initially, but apparently became rather stable within three months, up till the end of the second growing season when the experiments were terminated. The much lower

bacterial populations higher up in the twigs, compared with the samples taken close to the base and thus close to the site of inoculation, probably reflects an impossibility for *Pseudomonas* cells to escape from the xylem vessels into which they were introduced.

In the experiment with 47 different elm clones no, or hardly any bacteria were isolated from 88 % of the samples of the new year's shoots. This too may show that *Pseudomonas* cells probably cannot escape from the vessels in which they were introduced. Within these vessels apparently stable populations develop.

Prophylaxis against Dutch elm disease may last as long as a *Pseudomonas* population is maintained at a sufficiently high level within the metabolically active part of the tree, for elm mainly the two outer growth rings. The foregoing may imply that it is unlikely that the period during which the prophylactic effect lasts will be much longer than the two years during which *P. fluorescens* populations in twigs were monitored.

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**Samenvatting**

*Biologische bestrijding van de iepeziekte met Pseudomonas. I. Het merken, detecteren en identificeren van in de iep geïnjecteerde Pseudomonas isolaten; een vergelijking van verscheidene technieken*

Voor een beter begrip van de mechanismen die ten grondslag liggen aan de biologische bestrijding van de iepeziekte door *Pseudomonas* spp. zijn gegevens over de verspreiding van de bacteriën binnen de iep en over het verloop van de bacteriedichtheid in de tijd nodig.

Omdat klassieke biochemisch-taxonomische technieken niet geschikt zijn voor het identificeren van individuele *Pseudomonas*-isolaten zijn drie alternatieve benaderingen vergeleken. Chemotaxonomie gebaseerd op lipopolysaccharidepatronen, celenvelop eiwitpatronen of DNA-restrictiepatronen bleek betrouwbaar, maar arbeidsintensief. Merken van bacteriën met een transposon (Tn903) of een zogenaamde 'metabolic marker' (het LacZY gen, dat codeert voor $\beta$-galactosidase en lactose permease) maakte een betrouwbare identificatie van herisolaten mogelijk. Het bleek echter dat de populatiedichtheid van transposon-gemerkte bacteriën in de iep sneller afnam dan de dichtheid van wildtype populaties. Ook bleek het plasmide met het LacZY gen uit de bacterie-populaties te verdwijnen. Blijkbaar had zowel het transposon als het plasmide een negatief effekt op de bacteriën, wat deze methode onbetrouwbaar maakt omdat

de verkregen gegevens niet geëxtrapoleerd mogen worden naar het bijbehorende wildtype. Identificatie met immuno-agglutinatie met een antiserum bereid tegen het betreffende *Pseudomonas*-isolaat bleek specifiek en snel. Immuno-agglutinatie bleek daarom de beste methode voor routinewerk.

Studie naar het verloop van de *Pseudomonas* populatie in twijgen van iepen liet zien dat zich binnen drie maanden een stabiele bacteriepopulatie instelde (circa $7 \times 10^4$ bacteriën per twijgmonster), maar dat de bacteriën zich mogelijk niet naar hoger gelegen xyleemelementen konden verspreiden vanuit de houtvaten waar zij bij inoculatie waren ingebracht.

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