

Indicator Technique for Antimetabolic Toxin Production by Phytopathogenic Species of *Pseudomonas*

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A simple bacteriological technique involving inhibition zone production on a lawn of *Escherichia coli* was developed to detect antimetabolite toxin production by phytopathogenic species of *Pseudomonas*. It was established that the mechanism of *E. coli* inhibition paralleled that of phytotoxin-induced chlorosis of plant tissue. Derivatives of *Pseudomonas tabaci* and *Pseudomonas phaseolicola* which did not produce antimetabolite were readily identified by use of this technique. The presence of plasmid DNA in *P. tabaci* strains was demonstrated, but no physical evidence for plasmid involvement in tabtoxin production was found. The role of antimetabolite production in the pathogenicity of *P. phaseolicola* was also investigated. The method was extended to show that strains of *P. maculicola*, *P. syringae*, and *P. coronofaciens* also produce antimetabolites.

The use of wide-host-range plasmids of the P incompatibility group to develop the genetics of previously neglected species of gram-negative bacteria has recently been applied to plant pathogenic pseudomonads (8). Although gene mapping is thus possible (3), there remains a need to use a genetic approach to investigate the pathogenicity of these bacteria. To this end, an indicator technique has been developed to enable one component of plant disease, the production of chlorosis-inducing toxin, to be scored simply within the confines of the laboratory.

Five phytopathogenic species of *Pseudomonas* have been shown to induce chlorosis in plant tissue by means of extracellular toxins (12, 15). *Pseudomonas tabaci* and *Pseudomonas coronofaciens*, the causative agents of wildfire in *Nicotiana tabacum* (tobacco) and halo blight in *Avena sativa* (oats), produce an antimetabolite named tabtoxin. The induction of chlorosis by this toxin is antagonized by L-glutamine, and its mode of action may involve inhibition of the biosynthetic enzyme glutamine synthetase (12, 14). *P. phaseolicola*, which causes halo blight in *Phaseolus vulgaris* (french bean), produces a chlorosis-inducing toxin, phaseotoxin, that inhibits the arginine biosynthetic enzyme ornithine carbamyl transferase (12). *P. tomato* and *P. glycinea*, pathogens of tomatoes and soybeans, produce a toxin which is similar to phaseotoxin (15). In this paper the possibility that another microorganism might be sensitive to these antimetabolites was investigated. This has led to the development of a method to detect

their production without the need for plant infection experiments.

(A preliminary account of this work was presented at the 80th Ordinary Meeting of the Society for General Microbiology [M. J. Gasson, Proc. Soc. Gen. Microbiol. 4:145, 1977].)

MATERIALS AND METHODS

Bacteria. The *Escherichia coli* strains used in this work were ED24, a K-12 strain obtained from N. Willetts (University of Edinburgh, Edinburgh, Scotland), and an *E. coli* B strain provided by C. Clarke (University of East Anglia, Norwich, England).

The isolates of phytopathogenic *Pseudomonas* used are listed in Table 1. The species names are those used by the culture donors. Although these are commonly employed, all the isolates listed would be classified as varieties of *P. syringae* if the eighth edition of *Bergey's Manual of Determinative Bacteriology* is followed.

Plant cultivars. Seeds of the french bean variety Prince and the tobacco variety White Burley are maintained at the John Innes Institute. The bean cultivar Red Mexican U13 was supplied by J. D. Taylor (National Vegetable Research Station, Wellesbourne, Warwickshire, England).

Media. The complete medium used was NBY, consisting of 8 g of Difco nutrient broth per liter, 2 g of Difco yeast extract per liter, 2 g of dipotassium hydrogen orthophosphate per liter, and 0.5 g of potassium dihydrogen orthophosphate per liter. The *Pseudomonas* minimal medium (PMS) consisted of 1 g of ammonium dihydrogen orthophosphate per liter, 0.2 g of potassium chloride per liter, and 0.2 g of magnesium sulfate per liter. The pH was adjusted to 7.0 before autoclaving, and 0.2% glucose was added to provide a carbon source. Solid media contained 1.2% (wt/vol) agar.

Indicator technique. Freshly grown cultures of *E. coli* were washed once and resuspended in PMS. They were added to molten PMS agar (10^7 cells per ml) and

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TABLE 1. *Strains used in this study*

| Species | Strain no. | Source ^a |
|-------------------------|--|-----------------------|
| <i>P. phaseolicola</i> | 380, 604, 605, 607, 608, 1057, 1098, 1099, 1100, 1101, 1102, 1103, 1321, 1341, 2765 | NCPPB |
| | 31A, 710A, 716B, 777A, 882 | |
| | 2706, 2730 | |
| | 3A | |
| | | |
| <i>P. tabaci</i> | | J. D. Taylor, NVRs |
| <i>P. lachrymans</i> | 1401, 1736 | NCPPB |
| | | |
| <i>P. coronofaciens</i> | 876, 1253, 1356, 1357 | NCPPB |
| <i>P. pisi</i> | 297, 1364, 1366, 2222 | NCPPB |
| <i>P. maculicola</i> | 1766, 2038, 2039 | NCPPB |
| <i>P. mors-prunorum</i> | NA3, 107, 170, 408, 405, D5, P23 | P. Matthews, JII |
| | | |
| <i>P. syringae</i> | G11, G4, G5, G2b, W10, W176, 1071, 186, M59, G8, C109 | P. Matthews, JII |
| | | |
| | | |

^a Isolates of phytopathogenic species of *Pseudomonas*. NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, Hertfordshire, England; NVRs, National Vegetable Research Station, Wellesbourne, Warwickshire, England; EMRS, East Malling Research Station, East Malling, Kent, England; JII, John Innes Institute, Norwich, England.

poured to provide test plates. *Pseudomonas* isolates were stabbed into these plates and incubated either at 27°C for 24 h or at 20°C for 48 h. When L-amino acids were tested for their ability to antagonize the toxins, stabs were made into unseeded plates and, after incubation, the *E. coli* indicator was overlaid in 0.7% water agar containing 0.8 mg of the amino acid under test. A further period of 24 h of incubation at 37°C was necessary to allow indicator growth. Isoleucine and valine were added together because individually they inhibited growth of the indicator.

Chlorosis of plant tissue. To test isolates for their ability to cause chlorosis of host plant tissue, cultures were grown in PMS and centrifuged, and the supernatants were sterilized by filtration. These cell-free supernatants were spotted onto the leaves of bean or tobacco plants, and the plant tissues were gently pricked through the spots. Chlorosis was scored after growth of the plants in conditions of high humidity.

Bean pod inoculation. To determine the pathogenicity of *P. phaseolicola* isolates, freshly picked bean pods of cultivars Prince and Red Mexican U13 were infiltrated with freshly grown suspensions of the pathogen as described by Taylor (16). Bean pods were kept in conditions of high humidity and scored every 24 h for response to infection.

Isolation of plasmid DNA. The method used to extract plasmid deoxyribonucleic acid (DNA) was that described by Guerry et al. (5). Cultures (100 ml) were harvested, resuspended in 4 ml of lysis buffer (25% sucrose, 50 mM tris(hydroxymethyl)aminomethane [Tris], pH 8) and 0.8 ml of a 5-mg/ml solution of lysozyme in 0.25 M Tris (pH 8) was added. After 5 min on ice, 1.6 ml of 0.25 M ethylenediaminetetraacetic acid (EDTA), pH 8, was added, followed by sodium lauryl sulfate to a final concentration of 1%. The bacterial suspensions lysed readily, and 2 ml of 5 M sodium chloride was added with gentle mixing. Lysates were held at 4°C for 2 h and cleared by centrifugation

at 17,000 × *g* for 30 min. Polyethylene glycol 6000 was added to the supernatant, giving a final concentration of 10% (wt/vol). The mixture was stored overnight at 4°C, and the resulting DNA-polyethylene glycol precipitate (7) was harvested by gentle centrifugation at 4,000 rpm for 4 min in a Beckmann JA14 rotor. The DNA was redissolved in TES buffer (0.05 M Tris, 0.005 M EDTA, 0.05 M NaCl, pH 8), ethidium bromide was added to 500 µg/ml, and cesium chloride was added to a refractive index of 1.3925. Cesium chloride gradients were prepared by centrifugation in a Beckmann 40 rotor for 60 h at 36,000 rpm. The satellite band of plasmid DNA was removed from the gradient by horizontal insertion of a syringe, ethidium bromide was extracted with ice-cold isoamyl alcohol, and cesium chloride was extracted by dialysis against TE (0.05 M Tris, 0.005 M EDTA, pH 8.0) buffer.

Agarose gel electrophoresis. Vertical agarose gels were prepared with 0.7% agarose (Sigma, type II) in 40 mM Tris-acetate (pH 7.9), 20 mM sodium acetate, and 1 mM EDTA, and the same solution was used as running buffer. Electrophoresis was for 2 h at 100 V, and gels, which contained 0.5 µg of ethidium bromide per ml to stain the DNA, were photographed under a 365-nm ultraviolet lamp with a red filter.

RESULTS

E. coli was chosen as an indicator for detection of antimetabolite toxin production for a number of reasons. It is sufficiently distinct taxonomically to eliminate interference from lysogenic bacteriophages, bacteriocins, or naturally occurring phytotoxin resistance, and it grows well on the minimal salts medium routinely used in studies of plant pathogenic pseudomonads. Its higher optimum growth temperature facilitates the preferential growth of test organisms or indicator when both are present on the same plate, and the availability of sophisticated genetic technology could be of value in future development of the work.

Inhibition zone production by *P. tabaci* and *P. phaseolicola* was detected when isolates of these phytopathogens were stabbed into PMS agar that was seeded with *E. coli* cells. After incubation at 27°C for 24 h, clear zones of inhibition were present around *P. tabaci* stabs (Fig. 1) but not around *P. phaseolicola* stabs. When tests were repeated at 20°C for 48 h, positive results were also obtained with isolates of *P. phaseolicola* (Fig. 2). This dependence on low incubation temperature for inhibitor production by *P. phaseolicola* parallels observations of temperature effects on toxin-induced chlorosis of infected plants (10). Cell-free filtrates of both phytopathogens also produced inhibition zones when spotted onto *E. coli* lawns and caused chlorosis when applied to the leaves of host plants. By varying the time and temperature of incubation, the stab tests could be used to produce inhibition zones which virtually filled a

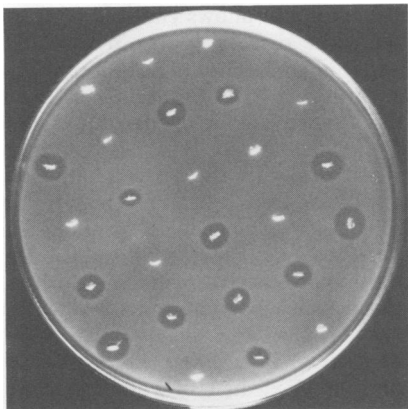


FIG. 1. The inhibition zones are caused by anti-metabolite-producing clones of *P. tabaci* NCPPB 2706, whereas nonproducing clones show no inhibition of the *E. coli* lawn.

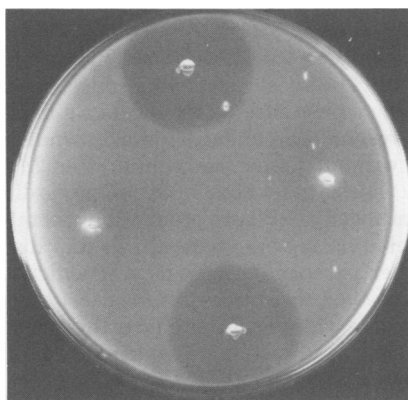


FIG. 2. The inhibition zones are caused by anti-metabolite-producing clones of *P. phaseolicola* NCPPB 1103, whereas nonproducing clones show no inhibition of the *E. coli* lawn.

petri dish or which remained small, as in Fig. 1.

Twenty L-amino acids were tested individually for their ability to protect *E. coli* from inhibition by *P. tabaci* and *P. phaseolicola*. In the case of *P. tabaci*, the inhibition zone was greatly reduced when L-glutamine was incorporated in the test medium. No other amino acid had any effect, including L-methionine, which has also been implicated as an antagonist of tabtoxin (2, 12). Hence, the inhibition zone produced on an *E. coli* lawn appears to be the result of a block in glutamine biosynthesis at the enzyme glutamine synthetase. For *P. phaseolicola*, only L-arginine protected *E. coli* from inhibition. The arginine precursors L-ornithine and L-citrulline were also tested. The latter prevented inhibition, but the former did not, suggesting that the *P. phaseolicola* antimetabolite blocks

arginine biosynthesis at the enzyme ornithine carbamyl transferase. These sites of action are in accordance with the known properties of the chlorosis-inducing toxins produced in plants by *P. tabaci* and *P. phaseolicola* (12, 14). Production of inhibition zones with these specificities was demonstrated for three strains of *P. tabaci* and 20 strains of *P. phaseolicola* that were isolated from many parts of the world.

It was reported as early as 1937 (1) that strains of *P. tabaci* tend to lose their ability to induce chlorosis in their host plant after prolonged maintenance on laboratory media. The indicator technique described above was used to investigate this phenomenon. The *P. tabaci* strains, received as lyophilized cultures, were plated for single colonies, and 100 from each strain were tested for their ability to produce inhibition zones on *E. coli* lawns. Forty-eight colonies from strain NCPPB 2706, 29 colonies from strain NCPPB 2730, and 57 colonies from strain 3A gave negative results (Fig. 1). Ten colonies from each strain, five which produced inhibition zones and five which did not, were used to prepare cell-free filtrates, and these were tested for chlorosis-inducing ability on tobacco leaves. A complete correlation between production of *E. coli* inhibition zones and the induction of chlorosis in plant tissue was found. Purified toxin-producing single colonies were used to make overnight broth cultures, and these were screened in a similar manner for the presence of cells which no longer produced an inhibition zone. It was repeatedly observed that such cells arose at a frequency in the order of 1% and that storage of the broth cultures led to further reduction in the proportion of toxin-producing bacteria (Table 2). Hence, it appears that deterioration in the chlorosis-inducing ability of *P. tabaci* during laboratory maintenance is due to the spontaneous loss of toxin genes.

A similar loss of chlorosis-inducing ability in *P. phaseolicola* has not been reported, but an ultraviolet-induced derivative which did not produce toxin has been isolated (13). The indicator

TABLE 2. Reduction in proportion of toxin-producing bacteria after storage

| Strain | % of nonproducers at day: ^a | | | |
|-----------------------------|--|---|----|----|
| | 0 | 7 | 14 | 21 |
| <i>P. tabaci</i> 2706 | 1 | 5 | 18 | 23 |
| <i>P. tabaci</i> 2730 | 1 | 7 | 6 | 11 |
| <i>P. phaseolicola</i> 1103 | 0.04 | 1 | 1 | 2 |
| <i>P. phaseolicola</i> 710 | 0.04 | 1 | 1 | 1 |

^a The percentage of cells failing to produce toxin is shown after storage of an overnight culture grown from a pure toxin-producing single colony.

technique was used to investigate the stability of antimetabolite production by laboratory-maintained cultures of this organism. Each of the cultures obtained for this study was plated for single colonies, and 100 of these were screened for their ability to produce inhibition zones on an *E. coli* lawn. Ten isolates contained 1 or 2% of cells which failed to produce antimetabolite, showing that spontaneous loss of toxin-producing ability also occurs in *P. phaseolicola* (Fig. 2). The presence of such clones in overnight broth cultures grown from purified toxin-producing single colonies was also detected, but at a much lower frequency than in *P. tabaci* (Table 2). Again, further loss was noted upon storage.

The frequent loss of antimetabolite production by these species suggests that the character may be controlled by plasmid-encoded genes. This possibility was investigated for the more unstable species, *P. tabaci*, by physical isolation of plasmid DNA from both toxin-producing clones and derivatives which failed to produce toxin. In strains NCPPB 2706 and NCPPB 2730, plasmid DNA was isolated from both types of culture and subjected to agarose gel electrophoresis. Although plasmid DNA was detected, no differences in the plasmid complements of the two types of strains was revealed (Fig. 3).

The ready isolation of derivatives not produc-

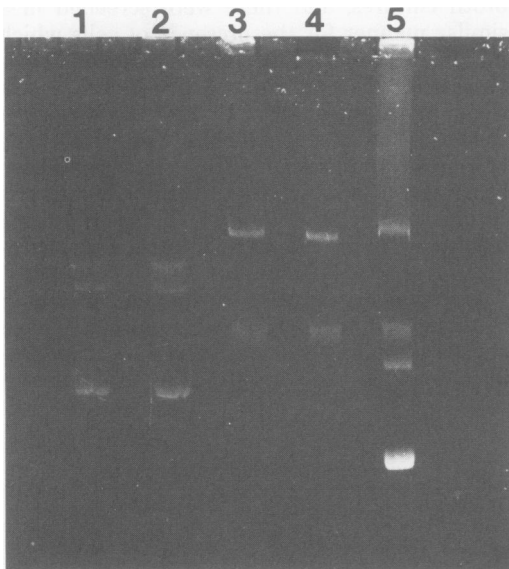


FIG. 3. Agarose gel electrophoresis of plasmid DNA from toxin-producing and nonproducing derivatives of *P. tabaci* strains NCPPB 2706 (tracks 3 and 4) and NCPPB 2730 (tracks 1 and 2) are shown. Track 5 contains plasmids RP1 (34 megadaltons, top band) and pCR11 (6.1 megadaltons, bottom band) to provide an indication of molecular weight.

ing antimetabolite allows the role of this character in plant disease to be investigated. To illustrate this, the ability of antimetabolite-non-producing derivatives of *P. phaseolicola* to induce disease symptoms on their host plant was investigated. Isolates of *P. phaseolicola* are differentiated into two races by the response to infection of the french bean cultivar Red Mexican UI3. Race 2 pathogens produce disease symptoms on this cultivar, whereas race 1 strains induce a hypersensitive response. Both races 1 and 2 cause disease symptoms on other bean cultivars (11, 16). Bean pods of the race 1-susceptible cultivar Prince and the race 1-resistant cultivar Red Mexican UI3 were therefore infiltrated with washed suspensions of antimetabolite-producing and non-antimetabolite-producing variants of three race 1 strains (NCPBP 607, NCPBP 1103, and 710A) and three race 2 strains (NCPBP 1341, NCPBP 2765, and 716B). All race 2 strains produced a pathogenic response, causing a dark green, water-soaked lesion in which white spots of bacterial growth later developed. The race 1 strains produced a similar pathogenic response on Prince, but a hypersensitive response involving a light brown discoloration without tissue damage on "Red Mexican UI3". These responses occurred regardless of the pathogen's ability to produce antimetabolite, thus eliminating an essential role for this toxin in either pathogenicity or host specificity. The *P. phaseolicola* isolates could also be used to investigate a more subtle role for the antimetabolite, such as in systemic spread of the pathogen through a whole plant (13).

Additional species of phytopathogenic pseudomonads were screened for antimetabolite-producing ability. None was detected for strains of *P. lachrymans*, *P. mors-prunorum* or *P. pisi*. Of four tested isolates of *P. coronofaciens*, two (NCPBP 1357, NCPBP 1356) produced inhibition zones that were antagonized by L-glutamine but by no other amino acid. This is consistent with reports that the pathogen produces a chlorosis-inducing toxin closely related to tabtoxin (12, 15). Inhibition zones were also produced by 11 *P. syringae* strains examined and by three *P. maculicola* strains. In tests for amino acid antagonism of these zones, *P. syringae* strains isolated from citrus (G8), lilac (1071), apricot (M59), and pea (W176) together with 3 *P. maculicola* strains from *Brassica* species were shown to inhibit arginine biosynthesis. Unlike *P. phaseolicola* toxin, it was found that all of these antimetabolites were neutralized by addition of either L-ornithine or L-citrulline. It appears that in these isolates an antimetabolite is produced which inhibits arginine biosynthesis at an earlier stage than the phaseotoxin target en-

zyme ornithine carbamyl transferase. The inhibitors produced by the remaining *P. syringae* strains have yet to be fully characterized, but they include antimetabolites with different specificities.

DISCUSSION

The technique described was developed to extend a genetic study of phytopathogenic pseudomonads to include at least one aspect of the diseases that they cause. Its value for this purpose is in facilitating the rapid scoring of large numbers of colonies for their ability to produce a toxin, without the constant need for plant infection experiments. A number of interested researchers (J. D. Taylor and N. Panopoulos, personal communications) have used the technique, and it should find application in further investigation of pathogenicity in these bacteria.

The frequent failure of *P. tabaci* and *P. phaseolicola* to produce antimetabolite suggests that genes essential for toxin production are being lost and that this may be caused by spontaneous curing of plasmid DNA. For *P. tabaci*, the presence of plasmid DNA has been detected in two isolates, but the same plasmid complement was also found in derivatives which did not produce tabtoxin. Despite this observation, it is still possible that a toxin plasmid is present, but not detected by the purification procedure employed. This situation has been encountered with catabolic plasmids in other species of *Pseudomonas* (9). More convincingly, physical data supporting the possibility that toxin production in *P. phaseolicola* is plasmid controlled have recently been reported (4). The indicator technique offers an opportunity to extend such studies.

The ready isolation of *P. phaseolicola* derivatives which fail to produce antimetabolite enabled the role of this toxin in phytopathogenicity to be investigated. Although the bean pod inoculation technique provided only a qualitative assessment of pathogenicity and host specificity, it was clearly shown that antimetabolite production was not required for the expression of either property.

In a brief survey of phytopathogenic pseudomonads, further instances of antimetabolite production were discovered, showing that the phenomenon is not restricted to those species which

cause chlorosis in plant tissue. In this regard, it is of interest that an analogy has been drawn between these antimetabolites and the microcin antibiotics produced by some gram-negative bacteria isolated from a medical environment (6).

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