Techniques

A Test Tube Assay for Estimating Populations
of *Xanthomonas campestris* pv. *translucens* on Individual Wheat Leaves

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ABSTRACT

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The quantification of bacterial populations associated with individual leaves by most available methods is laborious and expensive. This study was conducted to develop an accurate, inexpensive assay for quantifying a rifampicin-resistant strain (88-14[Rif]) of *Xanthomonas campestris* pv. *translucens*. Nutrient broth amended per liter with 100 mg of cycloheximide, 2 ml of a 0.25% pimaricin suspension, 100 mg of rifampicin, and 10 mg of cephalexin was dispensed aseptically into test tubes that were 16 × 100 mm. Leaves naturally infested with strain 88-14[Rif] were submerged in tubes of amended broth and incubated at 25 C on an orbital shaker at 100 rpm. Tubes were examined 2–4 times per day to establish the time when turbidity resulting from the growth of strain 88-14[Rif] first became visible. The relationship between time until initial turbidity and population size of strain 88-14[Rif] was expressed in the equation $Y = 10.38 - 0.189X + 0.00096X^2$, in which Y = the $\log_{10}$ population size and X = the time of incubation in hours. In addition, the test tube assay determined the population size of strain 88-14[Rif] in artificially infected leaves ($Y = 9.73 - 0.10X$) and the incidence of seed infestation and transmission of seedborne inoculum to seedlings. The test tube assay may be useful for ecological, epidemiological, disease-resistance, or risk-assessment studies requiring detection of and/or estimates of bacterial populations that have selective resistance to antibiotics.

Additional keywords: enrichment culture, epiphytic bacteria, leaf-associated bacteria.

Bacterial streak and black chaff, caused by *Xanthomonas campestris* pv. *translucens*, is widespread on soft red winter wheat (*Triticum aestivum* L.) in Arkansas, Louisiana, and Mississippi and has devastated some fields. The pathogen is known to be seedborne (18), but little is known about the source of initial inoculum or about the epiphytic growth of *X. c. translucens*. Differences in disease incidence and severity, however, have been associated with different seed lots of the same cultivar planted under similar conditions (E. A. Milus, *unpublished data*). These observations suggest that seedborne inoculum may be the most important source of initial inoculum. Disease symptoms rarely are seen on leaves before the heading stage but often are observed first between the heading and flowering stages, after which they develop rapidly on all leaves. Usually, there is no evidence of an upward progression of symptoms on plants (E. A. Milus, *unpublished data*). The latter observations suggest that an epiphytic phase of the pathogen early in the season may be an important source of inoculum for infecting plants later in the season.

Hirano and Upper (7) coined the term "leaf-associated bacteria" to include both bacteria on the leaf surface (epiphytes) and bacteria inside a leaf (endophytes). Populations measured by various techniques, including the proposed test tube assay, are more accurately described as "leaf associated" than "epiphytic." Therefore, we will use the term "leaf associated."

Information on spatial and temporal distributions of leaf-associated bacterial populations has been used in epidemiological studies and for predicting disease development (11,15). Population estimates have been obtained from bulk samples of leaves (1,2,13) or from individual leaves (5,8,10,11,17). Populations of leaf-associated bacteria among leaves of the same age or position in the canopy have approximated lognormal (6) or Weibull (8) distributions. If a population has either a lognormal or Weibull distribution, estimates from bulked samples will overestimate the

mean population size; the magnitude of this overestimation depends on the variance of the population (7). In addition to providing a more accurate estimate of the mean, estimates based on individual leaves allow the variance of the population to be calculated. Using the calculated mean and variance, the distribution of the population can be described.

Dilution plating on semiselective media has been the most common method of estimating populations of leaf-associated bacteria (1,2,8,12,13,17). Because dilution plating is laborious and time consuming, the number of samples that can be analyzed is limited.

Ice nucleation active (INA) populations of leaf-associated bacteria have been estimated by a tube nucleation assay (5). A large number of samples can be processed in 1 day using this technique, and the technique has been used for estimating populations of *Pseudomonas syringae* on bean, oat, tomato, soybean, and corn (5,7). Even though *X. c. translucens* is INA (9), the tube nucleation assay could not be used to estimate populations of this pathogen because *P. syringae* has been isolated from wheat on numerous occasions (E. A. Milus, *unpublished data*).

It has not been possible to quantify wild-type strains of *X. c. translucens* from symptomless plants on available media. The objective of this investigation was to develop an assay that would expedite ecological studies of *X. c. translucens* in which large numbers of individual wheat leaves or seeds could be examined. A preliminary report has been published (14).

MATERIALS AND METHODS

Rifampicin-resistant mutant. To select strains of *X. c. translucens* resistant to rifampicin (Rif), 1-ml aliquots of a cell suspension (about 10^9 colony-forming units [cfu] per milliliter) were plated on Difco nutrient agar (Difco Laboratories, Detroit, MI) amended with 5 g of dextrose (NDA) and 50 or 100 mg of rifampicin per liter. Strains that grew on amended media were transferred to fresh plates of NDA amended per liter with 100

mg of rifampicin and 200 mg of cycloheximide (NDA-RC). Single-colony isolates of resistant strains were checked for stability of rifampicin resistance by making four sequential transfers to NDA, by dilution plating on NDA and NDA-RC, and by comparing the population size recovered from each medium. Stable, resistant strains were tested for pathogenicity on wheat seedlings, and one strain was selected for use. Strains were stored in 15% dimethyl sulfoxide (DMSO) at −80 C.

To evaluate pathogenicity, primary leaves of two 10-day-old wheat seedlings (cv. Florida 302) were infiltrated with deionized water using a 1-ml disposable syringe modified with a short piece of soft-rubber tubing over the tip, and the watersoaked area of the leaves was punctured with a needle carrying cells of each test strain. Water-infiltrated leaves and leaves inoculated with a known pathogenic strain were included as checks. Plants were placed inside a humidity chamber (a translucent-plastic box inverted over a water-filled tray) and were incubated at 25 C with a 12-h photoperiod. Plants were removed from the humidity chamber after 24 h and were incubated for an additional 24 h at 25 C. Strains that caused watersoaking within 48 h were considered pathogenic, and those that did not were considered nonpathogenic.

The selected resistant strain (88-14^{Rif}) and the wild-type strain (88-14) were compared for growth rate by uniformly inoculating separate flasks of Difco nutrient broth and using dilution plating to measure the population at several time intervals. The comparison was carried out twice with four replicate flasks of each strain.

Strain 88-14^{Rif} was tested for stability of antibiotic resistance in planta by infiltrating flag leaves with 1×10^6 cfu per milliliter of suspension, using a syringe as described above. Inoculated plants were incubated for 6 days in a greenhouse at 18–30 C with natural and supplemental light. Comminution of 10 inoculation sites was carried out individually, using a mortar and pestle, and 10-fold serial dilutions were plated on NDA-RC and NDA-C (rifampicin omitted) to determine if populations were similar on media with and without rifampicin. Plates were incubated at 30 C for 4–5 days, and the number of colonies was determined. The experiment was repeated twice.

Strain 88-14^{Rif} was evaluated for stability of antibiotic resistance in the field by comminuting bulk samples of 30 symptomless leaves from a field plot at Hope, AR, in 300 ml of sterile deionized water (sdw) and by plating three replicate dilution series on NDA-RC and NDA-C as described above. Representative single colonies from each medium were tested for pathogenicity. Assays were carried out on 6 March and 3 April 1992, 4–5 mo after strain 88-14^{Rif} was established as a seedborne inoculum.

Establishing a rifampicin-resistant strain in the field. A suspension of strain 88-14^{Rif} was grown in Difco nutrient broth at approximately 28 C on a rotary shaker for 2 days. Cells were collected by centrifugation at 14,000 g for 20 min at 10 C. The pellet was washed in phosphate buffer (pH 7.0) and was resuspended in phosphate buffer. Inoculum was adjusted to 5×10^6 cfu per milliliter of sdw, using a spectrophotometer at 590 nm.

Florida 302 seed was treated using dry heat at 60 C for 10 days to disinfest the seed of naturally occurring *X. c. translucens* (3) and was infested with strain 88-14^{Rif} through vacuum infiltration. Florida 302 seed was placed in a vacuum desiccator, covered with the inoculum suspension, held under a partial vacuum of 600 mm Hg for 15 min, and air-dried on screens in a fume hood. Nontreated, noninfested heat-treated, and infested heat-treated seeds were assayed for the pathogen using the method of Schaad and Forster (16), except that bacteria were extracted by comminuting seed in a blender and NDA-RC was used, in addition to XTS (XTS contained Difco nutrient agar, glucose, cycloheximide, gentamycin, and cephalexin), as an isolation medium.

Infested seed was planted at the Strawberry Substation at Bald Knob, AR, on 29 October 1990, and at the Southwest Research and Extension Center at Hope, AR, on 13 November 1991, within 1 wk of infestation. Plots were approximately 30 × 30 m. Recommended cultural practices were used except at Bald Knob, where

propiconazole fungicide (Ciba-Geigy Corporation) was applied (19.5 g a.i. per hectare) during flag-leaf emergence as well as during flowering, to suppress foliar fungal diseases that might have interfered with the development of strain 88-14^{Rif} populations.

Assays using cultured cells. Difco nutrient broth was amended after autoclaving with 100 mg of rifampicin and 200 mg of cycloheximide per liter (NB-RC). Aliquots were dispensed aseptically into test tubes that were 16 × 100 mm and were refrigerated at 5 C. Tubes were used within 1 wk of preparation. A 10-fold dilution series of strain 88-14^{Rif} from an agar culture was made in sdw, and 0.1-ml aliquots of each dilution were dispensed into each of four tubes (replicates) containing 7 ml of NB-RC. To determine the number of colony-forming units added to the tubes, 0.1-ml aliquots of several of the weaker dilutions were plated on NDA-RC and were incubated as described previously.

Test tubes were incubated inside a dark growth chamber (model E-7, Conviron Products of America, Pembina, ND) at 25 C on a VWR table-top orbital shaker (VWR Scientific, Dallas, TX) at 100 rpm. Tubes were examined 2–4 times per day to establish the time when turbidity resulting from the growth of strain 88-14^{Rif} first became visible. Holding the tubes in front of a small fluorescent lamp and gently shaking facilitated the observation of the onset of turbidity. The test was repeated three times. Regression analysis was used to determine the relationship between the time from inoculation until initial turbidity and the initial population of strain 88-14^{Rif} in a test tube.

Assays using inoculated wheat leaves. Previous experiments using leaves were confounded by growth of fungal and bacterial contaminants in some tubes of NB-RC, as a result the medium was modified. For more effective control of fungi, 2 ml of pimaricin (0.25% aqueous suspension) was added per liter, and cycloheximide was reduced to 100 mg per liter. Cephalexin at 10 mg per liter was added to suppress bacterial contaminants. This modified medium (NB-RCPC) was used in all experiments involving leaves and seeds.

Flag leaves of soft red winter wheat cvs. Florida 302, Stoneville FFR 525W, Keiser, and Terral 101 were inoculated by infiltration with strain 88-14^{Rif} (1×10^6 cfu per milliliter) at four sites per leaf as described previously. Plants were incubated in a growth chamber at 25 C with a 12-h photoperiod. Inoculation sites were sampled 0.5, 24, 48, 96, and 144 h after inoculation to determine the population size of strain 88-14^{Rif}; one flag leaf from each of two plants was sampled each time. The four inoculation sites on each leaf were treated as two paired samples. One site from each pair was selected at random, was comminuted using a mortar and pestle, and was dilution plated on NDA-RC as described previously. The other site was sliced to allow bacteria to diffuse, using a tool holding 11 scalpel blades 1.2-mm apart, was submerged in a test tube containing NB-RCPC, and time until initial turbidity was determined as described previously. The population size of strain 88-14^{Rif}, estimated by dilution plating one inoculation site per pair, was plotted versus time until initial turbidity with the other inoculation site of the pair. The experiment was repeated twice, and a linear regression equation was fitted to the points.

Assays using field-grown wheat leaves. Tillers with symptomless leaves were selected at random from each of four quadrants of the experimental plot at Hope, AR, on 6 March and 3 April 1992. Samples were shipped on ice to the laboratory by overnight delivery and were processed within 24 h of removal from the field. To determine whether populations of strain 88-14^{Rif} were equally distributed on each half of a leaf, the uppermost, fully expanded leaf on each tiller was cut in half longitudinally with a sterile scalpel. Each half was comminuted individually in 5 ml of sdw, using a sterile mortar and pestle, and was dilution plated (10^0–10^{-3} dilutions) on NDA-RC. Plates were incubated, and populations were determined as described previously. Population size on the right half of each leaf was plotted versus the population size on the left half, and a linear regression equation was fitted to the points. The experiment was repeated twice, utilizing 60

leaves each time.

Additional tillers with symptomless leaves were taken from the plot at Hope, on 9 and 15 April 1992 to determine the relationship between population size of strain 88-14[Rif] associated with leaves and time until initial turbidity. Leaves were cut in half longitudinally, and one half, selected at random, was submerged in a test tube containing NB-RCPC. The other half was comminuted and dilution plated (10^0–10^{-3} dilutions). To confirm the presence of strain 88-14[Rif], a loopful of suspension from tubes that became turbid was streaked on NDA-RC, and a sample of these isolates was tested for pathogenicity. Sampling, incubation, and data recording were carried out as described above. Population size on one half of each leaf, estimated by dilution plating, was plotted versus time until initial turbidity for the other half, and linear and quadratic regression equations were fitted to the points. The experiment was repeated twice, utilizing 120 leaves each time.

Assay using individual wheat seeds. Seeds were harvested from the plot at Bald Knob, AR, on 6 June 1991 and were stored at room temperature. Within 3 mo of harvesting, seeds were assayed for the presence of strain 88-14[Rif] associated with seeds; three methods were used. In the first method (shake), individual seeds were placed into tubes containing 3 ml of phosphate-buffered saline (PBS; 6.8 g of KH_2PO_4, 1.16 g of NaOH, and 8.5 g of NaCl in 1 L of deionized water; pH 7.0) and were incubated at 4 C on an orbital shaker at 200 rpm for 24 h. Serial dilutions (10^{-1} and 10^{-2}) from each tube were plated on NDA-RC and were incubated as described previously. In the second method (crush/shake), individual seeds were wrapped in a piece of weighing paper and were crushed with a pestle before being placed in tubes containing PBS as described above. In the third method (tube), individual seeds were placed in tubes containing 3 ml of NB-RCPC and were incubated at 25 C on an orbital shaker at 100 rpm. Tubes were examined daily for evidence of turbidity. A loopful of suspension from tubes that became turbid was streaked on NDA-RC, and a sample of isolates was tested for pathogenicity to confirm the presence of strain 88-14[Rif]. The experiment was repeated twice, using 120–180 seeds for the shake and crush/shake methods and the tube method.

Seeds from the plot at Bald Knob, were planted in flats filled with potting mix (6:4:3:3:2, peat moss/vermiculite/loam soil/ sand/perlite) and were incubated in a growth chamber at 20 C with ambient relative humidity (30–40%) during a 12-h photoperiod. The temperature was raised to 25 C after seedlings emerged. The first true leaf from each of 300 14-day-old seedlings
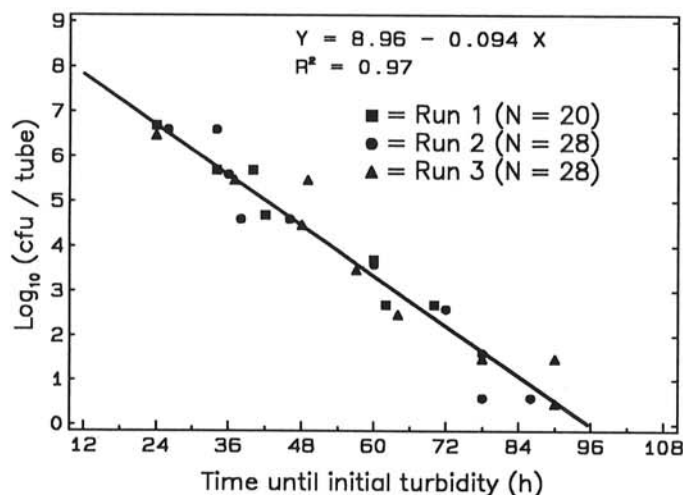
was placed in separate tubes containing 7 ml of NB-RCPC and was incubated at 25 C on an orbital shaker. Test tubes were examined daily for turbidity and were assayed to confirm the presence of strain 88-14[Rif] as described previously. The experiment was repeated twice.

Data analysis. All population sizes were transformed to $\log_{10}$ before analysis. Regression analyses were carried out using PROC GLM (SAS Institute, Inc., Cary, NC). For each experiment, plots of residuals for individual runs and data combined over runs were examined to determine whether there were systematic patterns indicative of lack of fit to each model.

RESULTS

Rifampicin-resistant mutant. Only one of 57 wild-type strains of *X. c. translucens* tested produced stable mutants resistant to 100 mg of rifampicin per liter. One single-colony isolate (strain 88-14[Rif]) of the stable mutant was selected. Strains 88-14 and 88-14[Rif] had similar growth rates in liquid culture and produced similar levels of disease on seedlings of several wheat cultivars. Population sizes of strain 88-14[Rif] reisolated from flag leaves inoculated in the greenhouse or symptomless leaves from the plot at Hope, AR, were similar on NDA-RC and NDA-C (data not shown), indicating the rifampicin resistance was stable under these conditions.

Establishing a rifampicin-resistant strain in the field. *X. c. translucens* was not detected in the nontreated or noninfested heat-treated seed on XTS or NDA-RC. However, numerous bacterial contaminants on XTS may have obscured the pathogen in the nontreated seed. Only a few contaminants were recovered from the noninfested heat-treated seed. Heat-treated seeds artificially infested with strain 88-14[Rif] averaged 7.2×10^3 cfu per seed about 1 wk after infestation, which was approximately five times the highest level of natural seedborne inoculum previously detected in Arkansas (E. A. Milus, *unpublished data*).

Assay with culture-grown cells. Inoculated test tubes became turbid 24–90 h after inoculation (Fig. 1). Initial turbidity was evidenced by a pale-yellow cloudiness in the previously clear medium. We sought to estimate the time from initiation of the experiment until the earliest evidence of turbidity. If the medium was obviously very turbid when examined, the time midway between the present and previous observation times was recorded as the best approximation of the time until initial turbidity. There
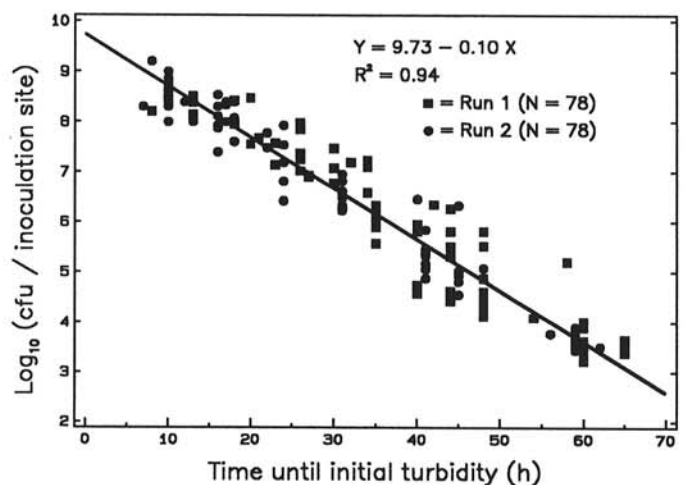
Fig. 1. Relationship between initial population size of *Xanthomonas campestris* pv. *translucens* strain 88-14[Rif] from agar culture and time until initial turbidity. Population size was determined by dilution plating on NDA-RC selective medium, and time until initial turbidity was determined by the test tube assay in nutrient broth amended with 100 mg of rifampicin and 200 mg of cycloheximide per liter and incubated at 25 C on an orbital shaker at 100 rpm. The solid line represents the regression line.

Fig. 2. Relationship between population size of *Xanthomonas campestris* pv. *translucens* strain 88-14[Rif] in one of a pair of inoculation sites and time until initial turbidity of the other inoculation site. Population size was determined by dilution plating on NDA-RC selective medium, and time until initial turbidity was determined by the test tube assay in nutrient broth amended per liter with 100 mg of rifampicin, 100 mg of cycloheximide, 2 ml of a 0.25% pimaricin suspension, and 10 mg of cephalexin and incubated at 25 C on an orbital shaker at 100 rpm. The solid line represents the regression line.

was little variation in time until initial turbidity among the four replicate tubes. Tubes with estimated initial populations of 1–9 cfu became turbid 84–90 h after inoculation. Results of the three runs were similar and were combined for analysis. An examination of the plots of residuals versus time for the individual runs and the combined data revealed no systematic pattern indicative of lack of fit. There was a highly significant ($P \leq 0.0001$) log-linear relationship between the initial population size of culture-grown strain 88-14[Rif] cells and the time until the assay tubes first showed turbidity. The regression equation (equation 1) for predicting the initial population was $Y = 8.96 - 0.094X$, in which $Y =$ the initial $\log_{10}$ population of strain 88-14[Rif] estimated by dilution plating on NDA-RC selective medium and $X =$ the incubation time in hours until the tube first became visibly turbid; $R^2 = 0.97$.

Assay using inoculated wheat leaves. Test tubes containing leaf sections with sliced inoculation sites became turbid 8–65 h after initiation of the experiment (Fig. 2). There was no observed interference from fungal or bacterial contaminants. Results of both runs were similar and were combined. An examination of the plots of residuals versus time for the individual runs and the combined data revealed no systematic pattern indicative of lack of fit. There was a highly significant ($P \leq 0.0001$) log-linear relationship between the population size of strain 88-14[Rif], determined by dilution plating cells from one of a pair of inoculation sites, and time until initial turbidity of the other inoculation site incubated in a tube. The regression equation (equation 2) for predicting the population size in inoculation sites was $Y = 9.73 - 0.10X$, in which $Y =$ the $\log_{10}$ population size of strain 88-14[Rif] associated with an inoculation site at the time of sampling and $X =$ the incubation time in hours until the tube first became visibly turbid; $R^2 = 0.94$.

Assays using field-grown wheat leaves. Population size of strain 88-14[Rif] was similar on right and left halves of comminuted and dilution-plated wheat leaves (Fig. 3). Although population sizes generally were larger in the second run, the results of both runs were similar and were combined. The regression equation fitted to all the data (dashed line in Fig. 3) did not fit points well for values of $X > 3$. The slope of this equation was 0.85, and
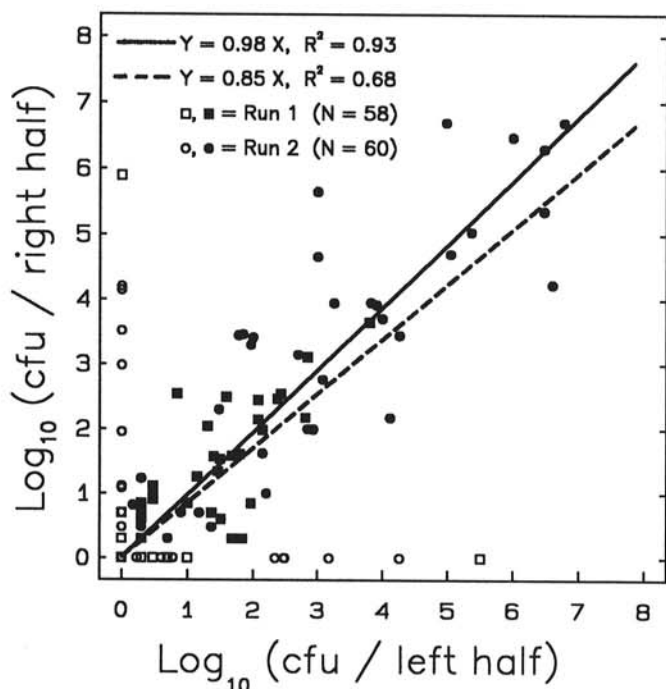
it differed significantly from 1 ($P = 0.51$). Seven leaves had more than 1×10^3 cfu on one half of the leaf and no detectable colony-forming units of strain 88-14[Rif] on the other half. The slope of the regression equation fitted only to points that had no detectable colony-forming units of strain 88-14[Rif] on right and/or left halves of the leaves was 0.0 (regression line not shown), including these points biased the estimate of the slope.

The regression equation fitted only to points that had nonzero values for population size on both right and left halves of leaves (solid line in Fig. 3) was the best estimate of the relationship between population sizes on each half of a leaf. An examination of the plots of residuals for the individual runs and the combined data revealed no systematic pattern indicative of lack of fit. This regression equation (equation 3; $P \leq 0.0001$) was $Y = 0.98X$, in which $Y =$ the $\log_{10}$ population size on the right half and $X =$ the $\log_{10}$ population size on the left half. The slope was not significantly different from 1 ($P = 0.005$); $R^2 = 0.93$.

Assay tubes containing half a leaf became turbid as a result of the growth of strain 88-14[Rif] after 15–108 h of incubation (Fig. 4). Results of both runs were similar and were combined. An examination of the plots of residuals versus time for the individual runs and the combined data revealed no systematic pattern indicative of lack of fit. The best-fitting regression equation (equation 4; $P \leq 0.0001$) for predicting the population size associated with leaves from the field was $Y = 10.38 - 0.189X + 0.00096X^2$, in which $Y =$ the $\log_{10}$ population size determined
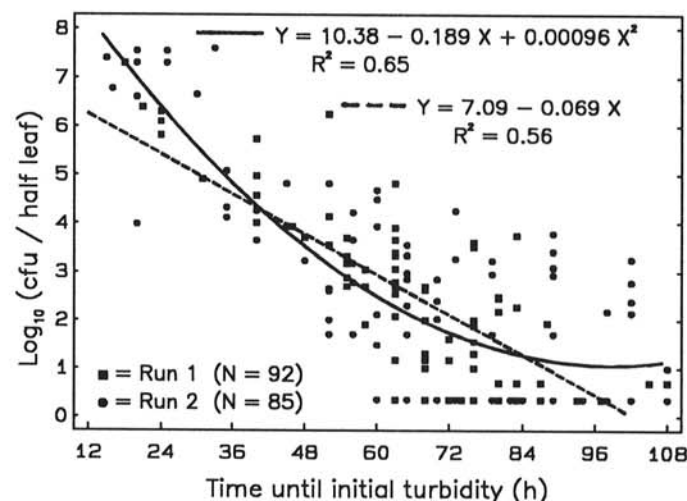

Fig. 4. Relationship between population size of *Xanthomonas campestris* pv. *translucens* strain 88-14[Rif] on one half of a field-grown wheat leaf and time until initial turbidity on the other half of the leaf. Population size was determined by dilution plating on NDA-RC selective medium, and time until initial turbidity was determined in tubes containing nutrient broth amended per liter with 100 mg of rifampicin, 100 mg of cycloheximide, 2 ml of a 0.25% pimaricin suspension, and 10 mg of cephalexin and incubated at 25 C on an orbital shaker at 100 rpm. The solid line represents the quadratic regression, and the dashed line represents the linear regression.

Fig. 3. Relationship between population size of *Xanthomonas campestris* pv. *translucens* strain 88-14[Rif] on right and left halves of longitudinally cut, field-grown wheat leaves. Population size was determined by dilution plating on NDA-RC selective medium. The dashed regression line was fitted to all data points, and the solid regression line was fitted only to points with nonzero values (solid squares and circles).

TABLE 1. Evaluation of assay methods for detecting the incidence of *Xanthomonas campestris* pv. *translucens* strain 88-14[Rif] on wheat seeds and seedlings

	Seed assay[x]			Seedling assay[y]
Run	Shake	Crush/ shake	Tube	Tube
1	1.7[z]	15.0	41.1	29.3
2	0.8	17.5	34.4	19.7

[x] Incidence was determined using 120–180 seeds for the shake and crush/ shake methods and tube method.

[y] Incidence was determined using primary leaves from 300 seedlings grown from naturally infested seed.

[z] The numbers represent the percentage of seeds or seedlings with strain 88-14[Rif].

by dilution plating half a leaf and X = time until initial turbidity after the other half was incubated in a tube containing NB-RCPC; $R^2 = 0.65$. A linear regression equation did not fit the points well for population sizes >10^5 cfu per half leaf.

Of the 177 data points used to determine the regression equation, 128 points had nonzero values for both population size and time until initial turbidity, and 49 points had nondetectable dilution-plated populations and nonzero values for time until initial turbidity. A population size (0.35 log cfu) midway between the minimum detectable population and 1 cfu per half leaf was assigned to these 49 points. Of the 63 data points not included in the regression analysis, 43 points were not usable because there was no population detectable by dilution plating or by test tube assay; 17 points were eliminated because tubes were contaminated with other bacteria (most contained both strain 88-14^{Rif} and a contaminant); and three points were eliminated because strain 88-14^{Rif} was detected (200 or fewer colony-forming units per half leaf) by dilution plating, but the corresponding tubes did not become turbid.

The earliest evidence of turbidity was observed as a thin wisp of pale-yellow cloudiness originating from a portion of a leaf and dispersing into otherwise clear medium when the tube was shaken gently in front of a fluorescent lamp. If the medium was obviously very turbid when examined, the time midway between the present and previous observation times was recorded as the best approximation of the time until initial turbidity.

Although fungal contaminants were observed in some tubes, they did not interfere with the observation of turbidity because mycelial masses grew slowly and did not disperse into the medium. It was important to submerge leaves completely in the medium to suppress fungal growth. Various bacterial contaminants did cause turbidity in some tubes. With experience, many of these contaminants could be differentiated by color and appearance from strain 88-14^{Rif} in the assay tube. All isolates morphologically similar to strain 88-14^{Rif}, obtained by streaking from turbid tubes and tested for pathogenicity, were pathogenic.

Assays using individual wheat seeds. Test tubes with seeds turned turbid as a result of the growth of strain 88-14^{Rif} 48–120 h after initiation of the experiment. The tube method was more effective than the shake or crush/shake methods for detecting strain 88-14^{Rif} associated with naturally infested wheat seed (Table 1). The tube method detected 30 and 2.3 times more seed infestation than the shake and crush/shake methods, respectively. As determined by the test tube assay, the seed lot averaged 37.8% seed infestation and 24.5% transmission of strain 88-14^{Rif} from seed to seedlings' primary leaves. All isolates morphologically similar to strain 88-14^{Rif}, obtained by streaking from turbid tubes and tested for pathogenicity, were pathogenic.

DISCUSSION

The test tube assay was useful for estimating population sizes of a rifampicin-resistant strain of *X. c. translucens* associated with field-grown wheat leaves and individual inoculation sites. The assay also was useful for detecting transmission of seedborne inoculum on individual seedlings and was more sensitive than dilution plating for detecting the incidence of infestation on individual seeds and leaves from the field. With less labor and expense, a greater number of individual leaves, inoculation sites, and seeds could be assayed in test tubes than could be assayed by dilution plating.

The approximate cost of ingredients for NB-RCPC and NDA-RC is two cents per tube and sixteen cents per plate, respectively. Because it takes at least four serial dilutions to cover the range of population sizes likely to be recovered, at least four plates of NDA-RC are needed for each sample. Therefore, on a per-sample basis, the cost of ingredients is approximately two and sixty-four cents for the tube and dilution-plating methods, respectively. Other costs are difficult to quantify and compare; however, pipettes, water blanks, and extraction of bacteria are needed for dilution plating but not for the test tube assay.

Bacteria associated with leaves tend to have highly skewed distributions and are often modeled by a lognormal (6) or Weibull (8) distribution. Thus, accurate estimation of means and variances of bacterial populations associated with leaves requires population estimates from numerous individual leaves per sample. Research on the ecology of leaf-associated bacteria has been hindered because obtaining these estimates by traditional dilution plating is costly in terms of labor and supplies. Utilization of a test tube assay has the potential to enhance the quality and reduce the cost of research in which accurate estimation of bacterial numbers is required and for which an appropriate selective medium is available.

When used for detecting or estimating bacterial populations, the test tube assay is well suited to a wide range of purposes, in addition to ecological or epidemiological studies of foliar plant diseases. Levels of disease resistance expressed as reduced bacterial populations could be quantified. Risk-assessment studies with genetically engineered bacterial strains could be simplified. Bacteria could be selected or characterized for resistance to various antibiotics by incubation in test tubes containing amended media.

It was necessary to examine the tubes several times over a 108-h period to determine the time until initial turbidity. Data became more continuous, and smaller differences in population size could be detected as the time interval between examinations became shorter. Based on observations of increasing turbidity over time after a tube first becomes turbid, it was determined that a time midway between the previous and present examination times was the best approximation of time until initial turbidity for tubes that were obviously very turbid when examined. Using this approximation allowed smaller differences in population size to be determined and made the data more continuous.

For example, using cells from culture (Fig. 1) or leaf sections with sliced inoculation sites (Fig. 2), the number of colony-forming units increased by approximately 2.4 log units per 24 h of incubation. If tubes were examined at 12-h intervals, then population differences of 1.2 log units could be detected. Data collected in such a manner tend to be discontinuous (i.e., data points accumulate at values 1.2 log units apart). If very turbid tubes were estimated to have first become turbid midway between examinations, then differences of 0.6 log units could be detected. Incubation temperature could be manipulated to shorten or lengthen the time during which the tubes become turbid.

Variation in population size of strain 88-14^{Rif} between halves of individual leaves (Fig. 3) probably increased the variance surrounding the regression line in Figure 4 because the tube assay and dilution plating were done on different halves of the same leaf. This compounded variation is reflected in the moderate coefficient of determination for equation 4. Variability probably would be less if there was a method to estimate population size and time until initial turbidity on the same leaf.

Comparison of regression lines for equations 1, 2, and 4 (Fig. 5) suggests that a linear regression would be sufficient to estimate population sizes from data in Figure 4. However, the linear regression (Fig. 4) does not fit the data points and greatly underestimates population sizes for populations >10^5 cfu per leaf. Gbur (4) showed that constraints on a response variable can cause linear relationships to be nonlinear and cause slopes to be underestimated. The limit of detection for dilution plating was 5 cfu per half leaf, and the limit of detection for the test tube assay appeared to be 1 cfu per half leaf. Forty-nine tubes with half leaves turned turbid after 60–108 h of incubation, but the corresponding dilution-plated half leaves had no detectable population of strain 88-14^{Rif}. Because the test tube assay was more sensitive than dilution plating, population estimates based on dilution plating were constrained. The simplest solution was to fit a quadratic equation to the data.

Seedborne populations of *X. c. translucens* appear to be below the seed surface and cannot be removed easily because of the 13-fold difference in incidence of detectable strain 88-14^{Rif} between the shake and crush/shake methods of extraction (Table 1). The data support previous experiments in which *X. c. translucens* was extracted from seed lots by shaking, as described by Schaad and Forster (16), and was extracted again by comminuting in
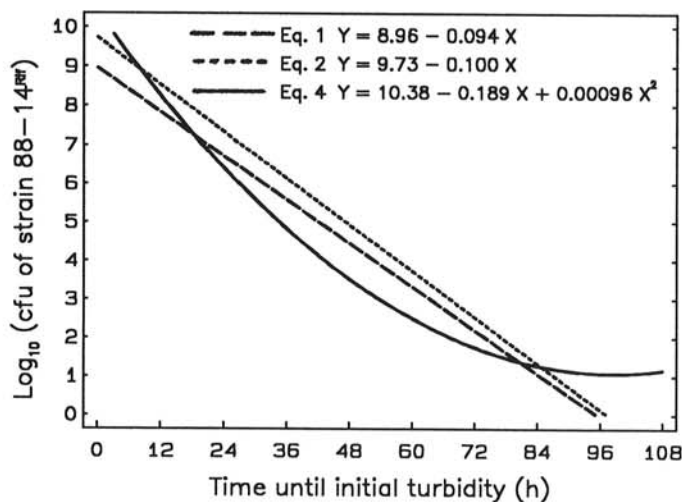
Fig. 5. Comparison of regression curves for equations 1, 2, and 4 showing the relationship between population sizes of *Xanthomonas campestris* pv. *translucens* and time until initial turbidity. Equation 1 used cells from agar culture, equation 2 used infiltrated leaves, and equation 4 used leaves from the field.

fresh buffer. The comminuted samples generally had counts that were 10-fold higher than counts from the first extraction by shaking (E. A. Milus, *unpublished data*). Tubes with seeds turned turbid as a result of growth of strain 88-14^{Rif} over a period 12 h longer than in experiments with leaves. This longer period may be caused by a lag time associated with cells of *X. c. translucens* below the seed surface coming into contact with the amended broth and then dispersing into the medium. No attempt was made to quantify seedborne inoculum because of probable complications resulting from lag time.

The test tube assay proposed here may be more widely applicable than the tube nucleation assay. A relatively small portion of leaf-associated bacteria is INA (ice nucleation active). This is especially true among plant pathogens. Even though *X. c. translucens* is INA (9), the ice nucleation assay could not be used to estimate its population on leaves because of the probable association of other INA bacteria with wheat leaves. The test tube assay is based on growth rate and provides a more direct estimate of a bacterial population than the tube nucleation test, which is based on ice nucleation frequency.

Strain 88-14^{Rif} was well suited to ecological studies in the field and to test tube assays. It was not affected by 100 mg of rifampicin per liter in agar or broth media, and in addition, this concentration inhibited most of the background bacteria. Rifampicin resistance appeared to be a good specific marker for tracking introduced inoculum because only one of 57 wild-type strains produced stable mutants. However, there appears to be a low probability of finding strains as well suited as strain 88-14^{Rif}.

Fungal contamination in the tubes was not a problem because cycloheximide and pimaricin suppressed growth until near the end of the examination period. Mycelial growth did not make the medium turbid. However, bacteria other than strain 88-14^{Rif} occasionally caused turbidity in tubes. Using cephalexin at 10 mg per liter in combination with rifampicin made the tube assay more selective. Preliminary experiments showed that cephalexin could be used at 20 mg per liter without affecting the growth

of strain 88-14^{Rif} (E. A. Milus, *unpublished data*), and use of this higher concentration may have been better. Gentamycin at 5–8 mg per liter was useful in a semiselective medium (XTS) for wild-type *X. c. translucens* (16). However, strain 88-14^{Rif} did not grow in broth tubes amended with as little as 0.08 mg of gentamycin per liter.

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