Temperature-Dependent Growth and Survival of *Xylella fastidiosa* in Vitro and in Potted Grapevines

Helene Feil and **Alexander H. Purcell,** Division of Insect Biology, University of California, Berkeley 94720-3112

ABSTRACT

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Xylella fastidiosa is a xylem-inhabiting bacterium that causes Pierce's disease (PD) of grapevine. Growth rates of *X. fastidiosa* in a rich liquid medium were determined by culturing methods at various temperatures. The slope of the regression line between the points of 18 and 28°C was similar to that reported for *Escherichia coli* between 12 and 30°C and for *Erwinia amylovora* between 9 and 18°C. For three PD strains, two almond strains, and an oleander strain, *X. fastidiosa* grew fastest at 28°C but did not grow at 12°C. Grape seedlings kept at 5, 10, 17, or 25°C for 18 days, beginning 2 weeks postinoculation at 25°C, had 230-fold lower populations of *X. fastidiosa* when kept at 5°C, but populations did not change significantly over time at the other temperatures. In planta populations of *X. fastidiosa* decreased 3 days after placing the seedlings at 5 and 37°C, and subsequent samples yielded no culturable bacteria at 37°C. Based on in vitro and in planta studies, it appears that temperatures between 25 and 32°C may be critical for the epidemiology of Pierce's disease because of its rapid growth rate at these temperatures, whereas temperatures below 12 to 17°C and above 34°C may affect the survival of *X. fastidiosa* in plants.

Additional keywords: Arrhenius plot, *Vitis vinifera*

Xylella fastidiosa (21), the cause of Pierce's disease (PD) of grapevine and other plant diseases (13), is a gram-negative, fastidious, xylem-inhabiting bacterium. *X. fastidiosa* has a wide host range (6,13,14). Purcell (18) provided a list of plant diseases known to be caused by *X. fastidiosa*. Wells et al. (21) compared *X. fastidiosa* 16S ribosomal RNA sequences to other known 16S rRNA of other bacterial species and placed *X. fastidiosa* in the gamma subgroup of the eubacteria (proteobacteria), with *Xanthomonas campestris* and *X. maltophila* as the closest relatives. The bacteria generally occur in geographical areas with mild winter climates (11,16). The incidence of PD increases at the margins of its geographic distribution following warmer than average winters (11). PD symptoms appear earlier in warmer climates (7,9), and our inoculations of grapevines in two cooler coastal regions and two warmer central California regions demonstrated differences in time of appearance and extent of PD symptoms in inoculated plants (H. Feil, *unpublished data*). The optimum growth rate for *X. fastidiosa* in vitro was determined to be approximately 28°C (3), which classifies *X. fastidiosa* as a mesophilic bacterium. We are not aware of other published data on growth response of *X. fastidiosa* at various temperatures. Such data provide a basic understanding for studies of this pathogen's physiology and may yield useful insights into its epidemiology. Our objective was to evaluate the effects of a range of temperatures on the growth and survival of *X. fastidiosa* both in vitro and in planta.

Corresponding author. Alexander H. Purcell
E-mail: purcell@nature.berkeley.edu

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MATERIALS AND METHODS

Bacterial strains and culture media. *X. fastidiosa* strain STL (American Type Culture Collection ATCC 700963) isolated from grapevines with PD in Napa Valley, CA, was used in all experiments. Other strains used in selected experiments were PD grape strain Temecula (ATCC 700964), collected from Temecula, CA; PD grape strain Medeiros (ATCC 700966), collected from Fresno, CA; almond leaf scorch (ALS) strains Dixon (ATCC 700965), collected in Solano County, and Oakley, (ATCC 700967) collected in Contra Costa County, CA; and an oleander leaf scorch (OLS) strain Ann1 (ATCC 700598), collected from Palm Springs, CA. Cells of *X. fastidiosa* were grown on liquid periwinkle wilt medium (PW) (5) or a modified solid version of PW with Gelrite, PWG (10) medium.

Cells of *X. fastidiosa* strain STL grown for 7 days on solid PWG medium were scraped from the medium and suspended in PW broth to a final concentration of approximately 1.5×10^9 CFU/ml determined by dilution plating. The suspension was diluted with PW broth to 1.5×10^3

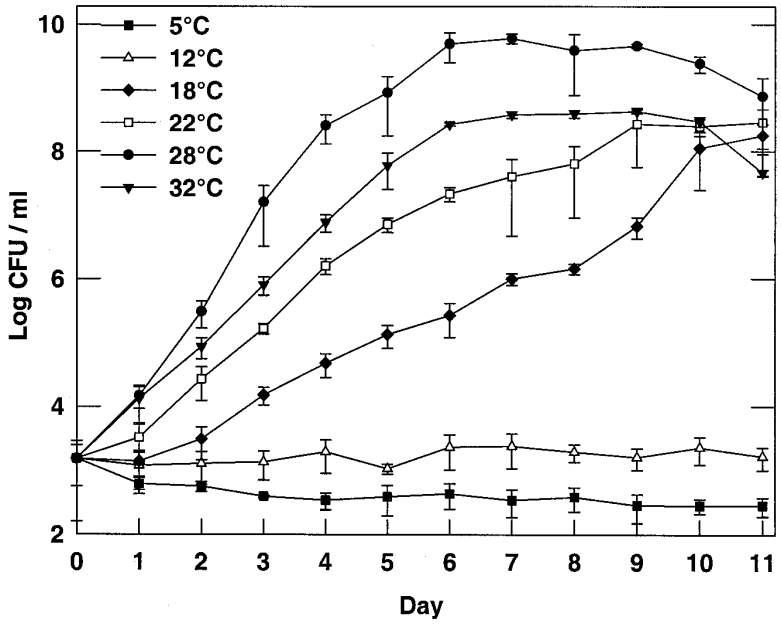

Fig. 1. Growth of *Xylella fastidiosa* strain STL in periwinkle wilt (PW) broth medium at different temperatures over a period of 11 days. Vertical bars represent the standard errors of the mean for two combined experiments.

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CFU/ml, which was similar to the initial concentrations (10^3 to 10^4 bacteria per ml) used in studies by Billing (1) and Ruissen et al. (19). Growth studies were conducted using closed microcentrifuge tubes. One-milliliter aliquots of the inoculum were dispensed in each of 450 microcentrifuge tubes. Glass Erlenmeyer flasks were used as a control to determine if the closed microcentrifuge tubes were oxygen-limiting. Fifty milliliters of inoculum were transferred into each of six 250-ml Erlenmeyer flasks. In two different experiments, 45 microcentrifuge tubes were placed in incubators without shaking at temperatures of 5, 12, 18, 21, 22, 27, 28, 29, 32, and 35°C. Two Erlenmeyer flasks with inoculum were placed on an American Rotator V Model R4140 shaker (American Hospital Supply Corp., Miami, FL) at 100 rpm at 12, 21, and 28°C. Every day for a period of 11 days, three tubes per temperature were selected arbitrarily for sampling. At the same time, 20-µl samples were removed from each of the flasks and plated. The flasks were returned to their respective incubator after each sampling. A dilution series in PBS buffer was prepared for each sample. Dilutions were plated on PWG, incubated for 5 days, and number of colonies determined. This study was performed twice. A separate experiment was conducted to compare the growth of six different strains of *X. fastidiosa* (STL, Temecula, Medeiros, Dixon, Oakley, and Ann1) at 12, 28, 32, and 35°C. Microcentrifuge tubes were prepared as previously described with 1 ml of inoculum per tube. The tubes were sampled every 24 h for 11 days.

We conducted additional experiments to determine if attachment of bacterial cells to the walls of the centrifuge tube caused an underestimation of populations. At each sampling of strain STL, incubated at 12, 22, 28, and 32°C, one microcentrifuge tube was rinsed three times with 1 ml of sterile, deionized water. After the third rinse, 1 ml of deionized water was added to each rinsed tube and sonicated for 5 min at 30 W (Model 375 Vibra Cell sonicator, Sonics and Materials Inc., Danbury, CT), and the final volume was plated on PWG medium to estimate numbers of bacterial cells that may have adhered to the tubes.

Plant material and sampling. Seeds of *Vitis vinifera* 'Cabernet Sauvignon' grapevines collected in a commercial vineyard were sown in March 1999 in U.C. Davis soil mix. The seedlings were grown in an enclosed, heated, air-filtered greenhouse. On 6 June 1999, these seedlings were inoculated by needle puncture with the STL strain of *X. fastidiosa* in the stem above the basal leaf using the method of Hopkins and Adlerz (14).

Immediately after inoculation, seedlings (n = 7) were placed in growth chambers maintained at 5, 12, 18, 22, 28, and 32°C ± 1°C, respectively. Dilution plating to estimate populations of live *X. fastidiosa* as CFU per gram of plant tissue were made using the method of Hill and Purcell (10). Because we could not detect *X. fastidiosa* in these seedlings after 2 weeks, another group of seedlings was inoculated and maintained in the greenhouse at 25°C for 2 weeks to allow the bacterium to spread systemically throughout the plant. After 2 weeks, the basal petiole for each seedling was sampled and cultured on PWG plates according to the method of Hill and Purcell (10). Plates were incubated for 1 week before quantifying bacterial populations within the petiole, at which time seedlings having populations of approximately 10^8

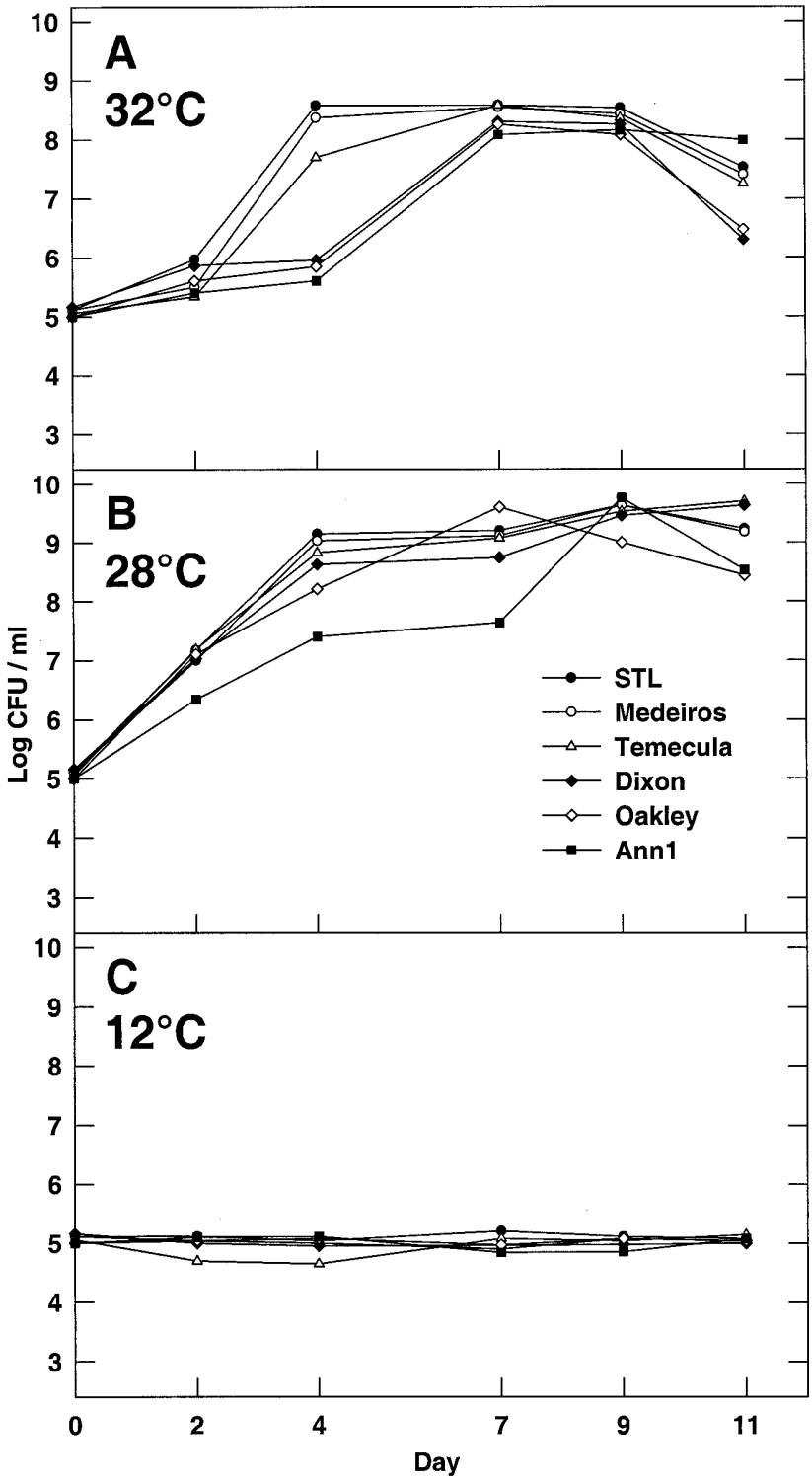

Fig. 2. Growth of *Xylella fastidiosa* strains STL, Temecula, Medeiros, Dixon, Oakley, and Ann1 in periwinkle wilt (PW) broth medium at **A,** 32°C, **B,** 28°C, and **C,** 12°C over a period of 11 days. Each dot represents the mean for two combined experiments.

CFU/g of tissue were chosen for the study. This was to ensure that the bacteria had spread systemically throughout the plants. At this time (3 weeks postinoculation), the selected seedlings (n = 7) were placed in growth chambers at 5, 12, 18, 22, 28, and 32°C ± 1°C, respectively. After seedlings were placed in the growth chambers (T_0), the seedlings were immediately sampled by arbitrarily sampling one petiole between the basal and eighth node above the point of inoculation. Temperature was monitored in each incubator using Hobo temperature data loggers (Onset Computer Corporation, Pocasset, MA). Four inoculated seedlings for each temperature treatment were sampled every 3 days for 18 days to estimate populations of *X. fastidiosa*. The entire experiment was repeated 1 week later.

Data analysis. For the growth curves presented in Figure 1, the slope and intercept were obtained from the linear part of the exponential phase of the growth curve using the linear regression analysis of SigmaPlot 2000 (SPSS Science, Chicago, IL).

For the growth curves presented in Figure 2, the slope and intercept of *X. fastidiosa* during the exponential phase of the growth curve were determined between day 0 and day 4 for the strains STL, Medeiros, Temecula, Dixon, Oakley, and Ann1 grown at 28°C, between day 2 and day 4 for strains STL, Medeiros, and Temecula grown at 32°C, and between day 4 and day 7 for strains Dixon, Oakley, and Ann1 grown at 32°C, using the linear regression analysis of SigmaPlot 2000 (SPSS Science). Differences between the log CFUs for the grape strains and the log CFUs for the almond and oleander strains of *X. fastidiosa* were determined using the Student's *t* test of SigmaPlot 2000.

An Arrhenius plot (Fig. 3) for *X. fastidiosa* grown in PW broth was made according to the method of Billing (1), with a regression line drawn between the data points of 18 through 28°C. To obtain the extreme ranges for the Arrhenius plot, data for growth were transformed by adding 0.001 so the data for zero observed growth could be included.

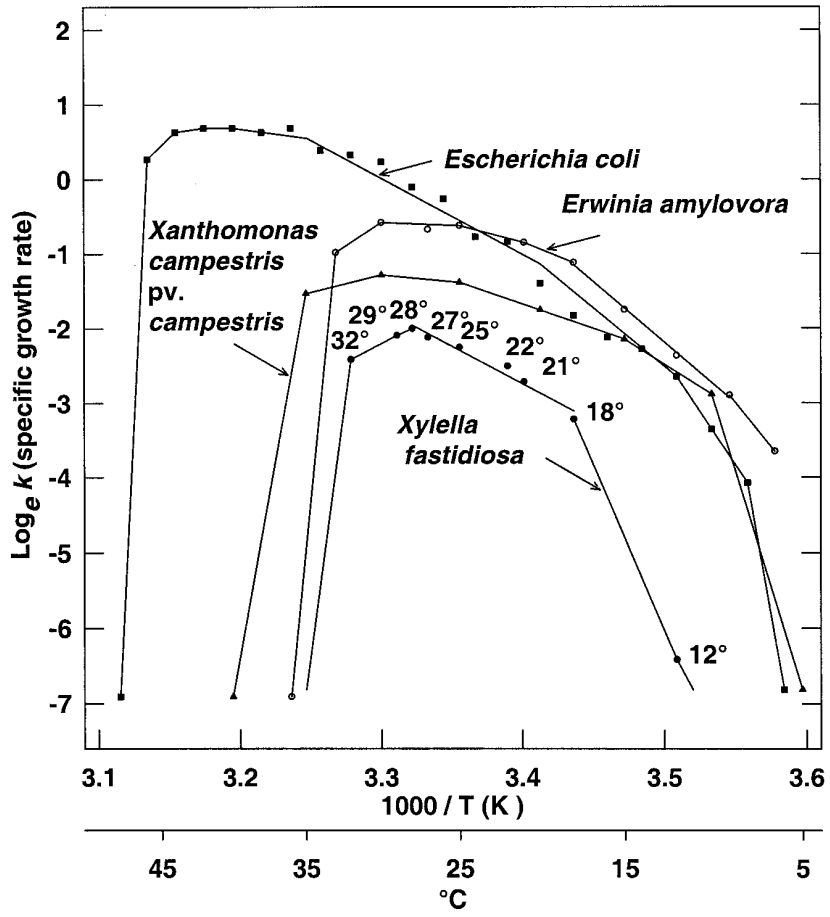

Fig. 3. Arrhenius plot of growth rates of *Xylella fastidiosa* strain STL (closed circles) compared with three other bacteria. *Escherichia coli* specific growth rate data (closed squares) as reported by Ingraham (15), *Erwinia amylovora* growth rate data (open circles) as reported by Billing (1), and *Xanthomonas campestris* pv. *campestris* growth rate data (closed triangles) from Ruissen et al. (19).

RESULTS

Populations of *X. fastidiosa* grown for up to 4 days in flasks on a shaker at 28 and 21°C were on average 26% greater than those grown in plastic at the same temperatures. This difference decreased to almost zero after 8 days when the cells reached the stationary phase. However, we observed no growth for either method at 12°C (data not presented). Also, when we sonicated the microfuge tubes used in the experiment after rinsing and filling them with deionized water, we could not detect any bacteria in the sonicated rinse water, indicating that bacterial adhesion to the microfuge tubes over the 18 days of the experiment did not affect our bacterial counts. However, it could be that rinsing and sonication is not sufficient to dislodge all attached bacteria.

Figure 1 represents the growth curves for the STL strain at 5, 12, 18, 22, 28, and 32°C. For each curve, the doubling time (*d*) was determined by calculating the amount of time (*t*) required by the cells to increase by a factor of 2 using the equation $\log y = a\,t + b$, where the ordinate *y* is the number of cells per milliliter (log CFU/ml), *a* is the slope, and *b* the intercept of the regression line. *X. fastidiosa* populations peaked in PW broth after 6 days incubation at 28°C. After 3 days, populations of *X. fastidiosa* were about 20 times less at 32°C than at 28°C. At 28°C, populations of *X. fastidiosa* were 5 times less after 10 days than after 7 days. By 3 days, populations of *X. fastidiosa* were 100 and 1,000 times higher at 28°C than at 22 and 18°C, respectively. *X. fastidiosa* populations remained almost constant at 12°C and declined about fivefold after 3 days at 5°C. The specific growth rates for in vitro growth of *X. fastidiosa* for each temperature are presented in Table 1.

Figure 2 depicts the in vitro growth curves for *X. fastidiosa* strains STL, Temecula, Medeiros, Dixon, Oakley, and

Table 1. Specific growth rate (*k*) and doubling time for *Xylella fastidiosa* strain STL grown at different temperatures in a rich medium

	Temperature (°C)										
	5	**12**	**18**	**21**	**22**	**25**	**27**	**28**	**29**	**32**	**35**
k (h^{-1})	–[a]	0.0016	0.0400	0.0660	0.0816	0.1050	0.1203	0.1350	0.1233	0.0894	–
Doubling time (h)	–	–	17.33	10.5	8.49	6.60	5.76	5.13	5.62	7.75	–

[a] – = no bacterial growth.

Ann1 at 32, 28, and 12°C, respectively. The strains Dixon, Oakley, and Ann1 grew more slowly than the PD strains STL, Temecula, and Medeiros at 32°C during the exponential phase. At this temperature, CFU densities after mid-logistic phase (4 days) for the almond and oleander strains Dixon, Oakley, and Ann1 were 1.7 to 3 log lower than for the grape strains STL, Temecula, and Medeiros (Student's t test = 8.49, P = 0.001, df = 4). Likewise, the grape strains grew faster during early exponential phase (days 0 to 4) at 28°C, but one almond strain (Dixon) grew almost as fast (Fig. 2B). All strains reached approximately the same maximum population density for each temperature tested and declined thereafter. No growth was detected after incubation for 11 days at 12°C. After 3 days incubation at 37 or 40°C, no viable cells of any strain of *X. fastidiosa* were detected.

To complement the in vitro experiments, populations studies of *X. fastidiosa* were also performed in grape seedlings at 5, 10, 17, 25, 34, and 37°C using the grape strain STL. For this study, we determined that the in planta detection threshold for leaf petioles was 10^3 CFU/g according to the method of Hill and Purcell (10). Assuming the plated bacteria are randomly distributed (Poisson distribution), we would expect (95% confidence interval) to detect one or more CFU per 20 µl of samples that had at least 200 cells per ml. In a preliminary experiment, *X. fastidiosa* populations in grape petioles were below our detection threshold for 10 days following inoculation of grapevines that were kept at 5, 12, or 32°C. After 7 days, we detected *X. fastidiosa* in one of seven plants at 18°C, one of seven plants at 22°C, and three of seven plants at 28°C. We detected *X. fastidiosa* in all seven plants sampled at 18, 22, and 28°C after 10 days. Therefore, in order to measure the effect of temperatures on established *X. fastidiosa* populations, we incubated the inoculated seedlings for 2 weeks in a greenhouse heated to 25°C prior to being incubated at the various regulated temperatures. After plants were incubated at the various temperatures, sampling was conducted at 3-day intervals. Before transfer to various temperatures, the inoculated plants harbored populations ranging between 4 to 7×10^8 CFU/g of petiole tissue (Fig. 4). Populations of *X. fastidiosa* remained constant in plants at 17°C and increased fivefold at 25°C over 6 days. *X. fastidiosa* populations declined slightly at 10°C and decreased approximately 20- and 230-fold at 34 and 5°C, respectively, over 18 days. The number of CFU per gram of grape petiole tissue declined 350-fold from the initial concentration after 3 days at 37°C, and the plants died in 3 to 6 days at this constant high temperature. In contrast to the in planta population of *X. fastidiosa,* which remained constant at 17°C, the population of *X. fastidiosa* increased at 18°C in PW medium. Populations of *X. fastidiosa* increased in vitro at 18, 22, 28, and 32°C, whereas populations of *X. fastidiosa* in planta increased at 25°C but not at 18 or 22°C. At 5°C, populations of *X. fastidiosa* declined both in vitro and in planta.

The specific growth rate constant (k) was calculated from the exponential phase of the growth curves of *X. fastidiosa* (Table 1). For this plot, the specific growth rate k was determined as: $k = (\log_e 2)/d$, where d is the mean doubling time during the exponential growth. The Arrhenius plots for three other purple proteobacteria, *Escherichia coli*, *Erwinia amylovora*, and *Xanthomonas campestris* pv. *campestris*, based on the data of Ingraham (15), Billing (1), and Ruissen et al. (19), respectively, are also given in Figure 3 for comparison. The temperature characteristic (µ) for *X. fastidiosa* was calculated from the slope of the Arrhenius plot (slope = $-\mu/R$ where R is the universal gas constant) between 21 and 25°C, where the range of temperature is normal and the relationship between growth rate and temperature is linear (1). From 21 to 25°C, the relationship between growth rate and temperature is linear, and from the slope of this line, the temperature characteristic (µ) for *X. fastidiosa* was calculated as 2.4×10^4 cal mol^{-1}. The Arrhenius plot for *X. fastidiosa* showed a narrower range of growth temperatures than *E. coli*, *Erwinia amylovora,* and *X. campestris* pv. *campestris*. The overall shape of the Arrhenius plot for *X. fastidiosa* resembles the one obtained for *Erwinia amylovora*. However, *X. campestris*, the nearest relative of *X. fastidiosa* in this study, is more tolerant of cold (below 12°C) or warm (above 32°C) temperatures. In general, even at optimal temperatures, the growth rate of *X. fastidiosa* is much slower than that of other typical gamma-subdivision Proteobacteria. The temperature characteristic ($\mu = 2.4 \times 10^4$ cal mol^{-1}) calculated between 21 and 25°C, the normal range of temperature for the growth of *X. fastidiosa*, was similar to that of *Erwinia amylovora* between 9 and 18°C (1) or *E. coli* between 12 and 30°C (8,15).

DISCUSSION

The temperature characteristic of an organism is useful for comparative purposes because it varies little over a broad range of medium richness, which can also influence growth rates (19). The number of culturable bacteria in plants declined 160-fold after 15 days at 5°C. This suggests that populations of *X. fastidiosa* inside grape xylem vessels decline when temperatures within xylem vessels are below 5°C. We can expect that temperature in the xylem vessels of grape foliage, where *X. fastidiosa* resides, does not differ greatly from the surrounding air temperature, since it was found that temperature inside grape leaves does not differ

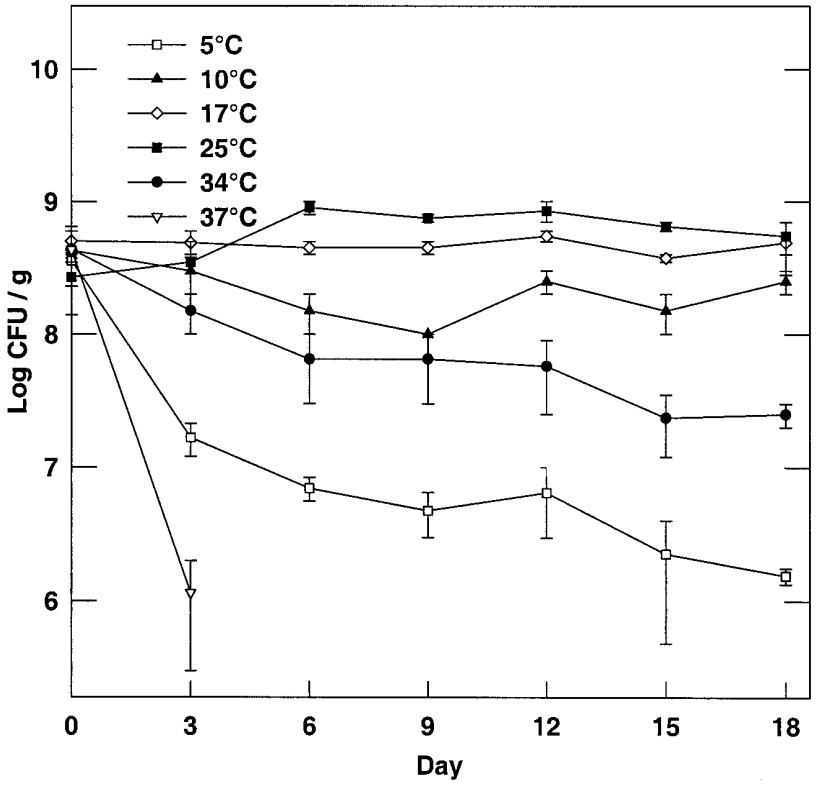

Fig. 4. Survival of *Xylella fastidiosa* in *Vitis vinifera* cv. Cabernet Sauvignon seedlings over a period of 18 days beginning 2 weeks after inoculation. Vertical bars represent the standard errors of the mean for two combined experiments.

from the air temperature by more than 1 or 2°C (20).

Our studies on the effects of temperature on *X. fastidiosa* population changes in vitro used a uniform medium. However, *X. fastidiosa* growth in planta depends on many other factors besides temperature, for example, nutrition or possible growth stimulants or inhibitors (2,5,12). Chang and Donaldson (2) showed that deletion of one amino acid reduced *X. fastidiosa* growth by 75% in their defined medium. Davis et al. (4) reported that hemin-chloride enhanced in vitro growth of *X. fastidiosa*. Hopkins (12) showed that foliar applications of indoleacetic acid, a plant growth regulator, lowered populations of *X. fastidiosa* in grapevines and prevented symptom development. Even if bacterial growth within plants cannot be modeled with temperature alone, knowing temperature effects may be helpful in understanding how temperatures can limit the growth of *X. fastidiosa* in the field. Our studies showed that populations of *X. fastidiosa* decline at cool but above-freezing temperatures. We determined the minimum threshold temperature for growth of *X. fastidiosa* in plants to be between 17 and 25°C. In vitro populations grew at 18°C but not 12°C. The minimum threshold temperature for growth can have important epidemiological consequences. It is possible that in field conditions *X. fastidiosa* does not multiply during cool nights or at temperatures below the lower threshold temperature. It should be noted that in this study we did not measure the effect of temperature or diurnal temperatures on inducing a lag phase of growth of *X. fastidiosa*. Regions with warm day and night summer temperatures (e.g., Texas, Florida) should expect less interruption to exponential phase growth and consequently much faster growth of populations of *X. fastidiosa* in planta compared with regions with similarly warm days but much cooler nights, such as California. A lag phase of growth in *X. fastidiosa* could significantly retard bacterial growth even beyond periods of permissive growth temperatures if cool nights can induce a lag phase. Vector transmission of *X. fastidiosa* to grape during early spring months is thought to be most important in establishing chronic infections (17). The very small numbers of *X. fastidiosa* cells introduced into plants by vector transmission might decrease the likelihood of successful infection for vector inoculations that are followed by prolonged cool weather during early spring.

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