Bacterial Desiccation: Effects of Temperature, Relative Humidity, and Culture Age on Survival

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ABSTRACT

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Large differences were found in the survival characteristics of bacteria dried and stored for 33 days under varying conditions. *Corynebacterium michiganense* and isolate SC735 (an antagonist to *Pseudomonas glycinea*) were most resistant to drying. Cells of *Xanthomonas phaseoli* were 20-50 times less resistant and *P. glycinea, Erwinia carotovora,* and isolate AN771 (an antagonist to *Bipolaris maydis*) were approximately 1,000 times less resistant. Survival was better at 5 C than at 20 C, and at either temperature it was better at 34% relative humidity (RH) than at 0% or 75% RH. Survival at 20 C was poorest at 75% RH. In general, bacteria from 7- or 14-day-old cultures were more resistant to desiccation than those from 1-, 22-, or 30-day-old cultures. In relation to RH, survival patterns for *P. glycinea* cells both from diseased leaves and from culture were similar.

Even though season-to-season survival of bacterial plant pathogens is essential for species survival and is potentially a weak point in their life cycles, relatively little information is available on the physical, chemical, or microbiological factors that affect survival of bacteria associated with plants (8, 15). With more information it may be possible to destroy plant pathogens during the long-term survival period and in this way to reduce disease. Increased knowledge also may lead to better preservation methods for useful bacteria; e.g., those suitable for biological control of disease.

Hypobiosis, a state of reduced metabolism and decreased sensitivity to environmental variables, commonly is induced in plant pathogenic bacteria by desiccation (8). There appears to have been little systematic work on survival during drying of pathogens and other bacteria associated with plants, particularly under different conditions of relative humidity (5, 6, 14, 23).

Objectives for this investigation were to determine: (i) the effect of temperature, relative humidity (RH), and bacterium (culture) age on survival to desiccation of four plant pathogenic and two nonpathogenic bacteria; and (ii) the effect of lesion age on survival of *Pseudomonas glycinea* to desiccation under different RH regimes.

MATERIALS AND METHODS

Bacterial isolates.—Isolates were *Corynebacterium michiganense* (B. D. Thyr's isolate 15), *Erwinia carotovora* (A. Kelman's isolate 26), *P. glycinea* (our isolate 724), *Xanthomonas phaseoli* (M. L. Schuster's isolate 816), and antagonistic isolates SC735 (9) and AN771 (18). Isolates SC735 and AN771 were antagonistic in vivo to *P. glycinea* and *Bipolaris maydis* (Nisik.) Shoemaker (=*Helminthosporium maydis* Nisikado & Miyake), respectively. Stock cultures were maintained in 15% aqueous glycerol (v/v) at −70 C (3, 4).

Relative humidity control.—Polyethylene freezer containers (10.5 × 10.5 × 6 cm high) were used as individual RH chambers. Anhydrous $CaSO_4$ ("Drierite") was used to obtain the RH that was designated "0%". Saturated solutions of $MgCl_2 \cdot 6H_2O$ and NaCl were used to produce RH levels of approximately 34% and 75%, respectively (24). Bacterial preparations to be dried were placed on glass and suspended on wire racks approximately 3 cm above the drying agent. The RH in the closed containers was maintained within 2-3% of the designated value as measured with an Electric Hygrometer-Indicator, Model No. 15-3001 (Hygrodynamics, Inc., Silver Spring, MD).

Media and cultural conditions.—The three media employed in this study were: NB–nutrient broth (Difco, 8 g/liter); NSA–nutrient sucrose agar (grams/liter: Difco nutrient agar, 23; sucrose, 10); and M71 (7), a selective medium for *P. glycinea*. Cultures were incubated at 24 C.

Preparation of bacteria for survival tests.—Bacterial isolates from stock cultures were transferred to NSA slants and incubated for 24 hours. Cells from these cultures were transferred to 25 ml of NB and incubated for 24 hours on a rotary shaker (150 rpm). One loopful (approximately 8.5 mm in diameter) from the NB cultures was seeded to NSA slants which were incubated for 1, 7,

14, 22, or 30 days. Cells for survival tests were taken from these slants.

Carborundum survival assay.—To rehydrate bacteria (1), H_2O and four sucrose solutions (10, 50, 100, and 200 g/liter) were tested for effect on survival. Bacteria from 8-day-old slant cultures were dried on 22-μm (600-grit) Carborundum, as described below, for 6-9 days at 5 C and approximately 50% RH. Upon rehydration (50-mg samples), the greatest numbers of colony-forming units (CFU) of *E. carotovora, P. glycinea, X. phaseoli,* and AN771 were detected by using the 200 g/liter sucrose solution for rehydration and for making dilutions. Similar numbers of *C. michiganense* and SC735 survivors were detected with all test solutions. To maximize survival, therefore, the 200 g/liter sucrose solution was used.

Bacteria from the different-aged slant cultures were dried on Carborundum at 5 C as follows: (i) Bacteria were suspended in 5 ml of sterile 10% (w/v) powdered milk (Carnation Instant Nonfat Dry Milk) and the initial CFU concentration determined by dilution plating (three replicate plates per dilution). (ii) Two-tenths milliliter of milk suspension was added to 0.5 g of sterile Carborundum, mixed, and spread evenly in a circular area (3 cm in diameter) on a sterile glass surface for drying. (iii) After drying for 3 days at 5 C under the specified RH condition, the treated Carborundum was loosened and mixed with a blunt glass rod (preparations at 75% RH did not appear to be completely dry). (iv) Samples (100 or 200 mg) of the dried Carborundum preparation were rehydrated and assayed for surviving bacteria by dilution plating. (v) Percent survival was determined by comparing the resulting CFU with the initial CFU. Representative colonies were tested for pathogenicity (see below).

Between assays, the treated Carborundum was placed in an open 15- × 45-mm vial and kept at the appropriate RH and temperature.

Glass bead survival assay.—Glass homogenizing beads (VirTis Company, average diameter 0.2 mm) were used to determine the effect of desiccation on survival under the various RH regimes at 5 or 20 C. Procedures were: (i) Bacteria from slant cultures of different ages were suspended in 10% milk. (ii) One-half gram of thoroughly washed sterile glass beads was mixed with 0.2 ml of bacterial suspension and the resulting wet beads were spread on a sterile surface to dry. (iii) After 3 days at the specified conditions, bacteria-bearing beads were scraped from the surface and rubbed lightly with a blunt glass rod to separate individual beads. (iv) Beads were placed in 15- × 45-mm vials and the vial mouth covered with sterile double-layered cheesecloth. (v) To assay, the vial was inverted and approximately 100 beads tapped through the cheesecloth onto the surface of NSA in petri plates. (vi) After 1-5 days, the percentage of beads yielding bacterial growth was computed. Cultures of survivors were tested for pathogenicity as noted below.

Between assays, beads carrying bacteria were stored as noted in the preceding section.

Survival of Pseudomonas glycinea derived from leaf lesions.—The effect of lesion age on survival of *P. glycinea* to desiccation at 0, 34, or 75% RH at 5 C was determined by the glass bead assay. Unifoliate leaves of 12- to 15-day-old soybean [*Glycine max* (L.) Merr. cultivar 'Harosoy 63'] plants were dusted with sterile Carborundum and inoculated by rubbing twice on each side of the midrib with a cotton swab ("Q-tip"; Chesebrough-Ponds, Inc.) dipped into *P. glycinea* inoculum. Splashing of inoculated leaves was avoided. Inoculum was prepared by suspending cells from 48-hour NSA slant cultures in sterile water and adjusting to an absorbance of 0.095 at 630 nm ($1.0\text{-}1.7 \times 10^8$ CFU/ml) in a Spectronic 20 spectrophotometer.

Diseased leaves were collected 7, 15, or 23-29 days after inoculation, and the diseased portions were excised aseptically. Diseased tissue from three leaves (approximately 14 cm^2) was ground with mortar and pestle in 2.5 ml of 10% milk or in water. The resulting suspension was filtered through double-layered cheesecloth, added to beads, and dried. Beads were assayed on selective medium M71.

Pathogenicity tests.—Progeny from surviving plant pathogenic bacteria CFU, obtained directly from assay plates (5-10 days old), were tested for pathogenicity as follows: (i) Tomato [*Lycopersicon esculentum* (L.) Merr. cultivar 'Heinz 1350'] cotyledons of 9- to 13-day-old plants were dusted with 22-μm (600-grit) Carborundum and inoculated by rubbing with a moist inoculum-laden Q-tip (Q-tip method). Pathogenicity of *C. michiganense* was based on development of small, white spots on the cotyledons (20). (ii) Unifoliate leaves of soybean (8- to 14-day-old plants) or bean (*Phaseolus vulgaris* L. cultivar 'Red Kidney'; 6- to 10-day-old plants) were dusted with Carborundum and inoculated by the Q-tip method. Development of characteristic water-soaked lesions determined pathogenicity of *P. glycinea* and *X. phaseoli.* (iii) *Erwinia carotovora* was tested by production of soft rot symptoms on potato (*Solanum tuberosum* L.) tuber slices. Surface-sterilized slices were inoculated with a loopful of bacteria and incubated under moist conditions at 24 C.

RESULTS

Survival of bacteria at 5 C—Carborundum method.—Of the two survival assay methods, the Carborundum technique was the more precise. Dried bacteria were assayed for survival in three tests at different times after 3 or 33 days of storage on Carborundum at 0, 34, or 75% RH. Data for the 33-day assay (Table 1) showed large differences in the relative sensitivity to desiccation among species. *Corynebacterium michiganense* and isolate SC735 were the most resistant. *Xanthomonas phaseoli* was 20-50 times less resistant than these organisms, whereas *P. glycinea, E. carotovora,* and isolate AN771 were approximately 1,000 times less resistant. Maximum survival of all species was at 34% RH and, in general, was obtained with bacteria from 7- or 14-day-old cultures. Surviving plant pathogens were pathogenic, except that loss of pathogenicity was noted for some of the *P. glycinea* survivors from older cultures (14, 22, and 30 days).

To establish a base line for survival after 33 days, the isolates also were assayed at 3 days in three tests, as mentioned above. Generally, there was increased survival with increasing RH, in contrast to the 33-day data. This was probably because drying at the 75% RH level was not

completed by the time the 3-day assay was made. For comparison with the 33-day data (Table 1), percent survival after 3 days from 7-day-old cultures at 0, 34, and 75% RH was, respectively: *C. michiganense* = 2.8, 55.8, and 106.2; SC735 = 9.4, 22.9, and 37.5; *X. phaseoli* = 0.41, 3.63, and 3.98; *P. glycinea* = 0.09, 4.12, and 13.82; *E. carotovora* = 0.02, 0.62, and 1.09; AN771 = 0.04, 0.51, and 0.12.

Survival of bacteria at 5 or 20 C–Glass bead assay.—The order of species' sensitivity to desiccation was the same, whether determined by the Carborundum or bead method (Table 2). Nevertheless, the bead method was less precise than the Carborundum technique. To illustrate: survival of *C. michiganense* and SC735 at 5 C, as detected by the Carborundum method (Table 1), was much greater at 34% RH than at 0 or 75% RH; however, the bead assay was not sufficiently sensitive to show comparable differences at 5 C (Table 2).

Survival of the six isolates was compared at 5 or 20 C after 3 or 33 days of storage at the three RH levels. Data for the 3-day assay are not given in detail, because no differences were noted in survival of *C. michiganense,* SC735, or *X. phaseoli* at the two temperatures; however, survival was lower at 20 C for the desiccation-sensitive *P. glycinea, E. carotovora,* and AN771. The 33-day data in Table 2 show that the trend of lower survival at the high temperature was observed for all bacteria. At 20 C, survival after 33 days generally was best at 34% RH and in all cases was poorest at 75% RH. Where significant differences occurred in relation to culture age, 7- or 14-day-old cultures usually had the higher rates of survival. Plant pathogens surviving after 3 or 33 days were pathogenic.

Survival of Pseudomonas glycinea derived from

TABLE 1. Survival of bacteria after 33 days at 5 C with respect to species, age of culture, and relative humidity (RH). Carborundum method[a]

Bacterium	Culture age (days)	Survival of bacteria (%) at: 0% RH	34% RH	75% RH	LSD (P=0.05) all combinations[g]
Corynebacterium michiganense[b]	1	1.3	29.5	6.6	
	7	2.0	68.6	66.5	
	14	1.9	56.4	10.6	11.5
	22	0.9	42.3	9.0	
	30	0.4	24.3	11.9	
SC735[c]	1	0.4	6.6	2.5	
	7	3.4	19.6	7.5	
	14	3.0	17.5	2.2	2.9
	22	3.0	13.1	0.9	
	30	1.7	11.3	0.2	
Xanthomonas phaseoli[b]	1	A[d]	0.266	A	
	7	0.001	0.770	0.053	
	14	0.003	1.199	0.059	0.179
	22	0.001	0.023	A	
	30	0[e]	0	0	
Pseudomonas glycinea[b]	1	0	A	0	
	7	A	0.019	A	
	14	0	0.034	A	0.029
	22	0	0.050	0	
	30	A	0.088	0	
Erwinia carotovora[b]	1	A	A	0	
	7	0.001	0.024	A	
	14	0.003	0.044	A	0.010
	22	0.001	0.025	A	
	30	0.001	0.021	A	
AN771[f]	1	A	0.001	0	
	7	A	0.004	0	
	14	A	A	A	0.001
	22	0	A	0	
	30	0	0	0	

[a]Bacterial suspensions prepared in 10% milk and applied to sterile Carborundum. Preparations dried under specified conditions and assayed by dilution plating. Survival based on bacteria initially applied to Carborundum. Means of three experiments at different times.

[b]Plant pathogenic isolate.

[c]Isolate antagonistic to *P. glycinea* (9).

[d]"A" indicated that colony-forming units (CFU) were detected, but were <0.001% of the number applied.

[e]"0" indicates no CFU detected.

[f]Isolate antagonistic to *Bipolaris maydis* (18).

[g]The interaction (culture age × RH) was significant for each species.

diseased host leaves.—*Pseudomonas glycinea* survives in nature in association with plant tissue. There are periods, however, when the pathogen is subjected to desiccation due to evaporation of dew or rain water. Thus, in our studies, we chose to test the sensitivity (survival rates) of *P. glycinea* from lesions to drying after being suspended in both water (unprotected) or milk (protected). The bead test method was used.

As noted for *P. glycinea* derived from culture, survival of *P. glycinea* derived from lesions was greatest at 34% RH and least at 75% RH after 33 days (Table 3). Additionally, *P. glycinea* from 15-day-old lesions proved most resistant to drying. Data for *P. glycinea* dried after suspending in water are not given in detail, since, with two exceptions, *P. glycinea* survived on less than 2% of the beads. The exceptions were at 34% RH in which *P. glycinea* from 15- and from 23- to 29-day-old lesions were detected from 37% and 10% of the beads, respectively. In these tests, <1% of the beads yielded bacteria other than *P. glycinea* on M71 medium. Surviving *P. glycinea* were pathogenic.

DISCUSSION

In nature, pathogenic bacteria probably survive best in close association with plant tissue in "protected positions" (8). Therefore, during these experiments, bacteria were provided some degree of protection by suspension in milk prior to drying.

The four pathogens and two nonpathogens generally responded similarly to the treatments, although large differences were noted in the overall sensitivity to desiccation. In agreement with other investigators (2, 10, 13, 21), survival of the dried bacteria was better at 5 C than at 20 C. Survival at 20 C was poorer at 75% RH than at 0 or 34% RH, a finding of general interest and perhaps of some utility. Survival was best at 34% RH at both temperatures. Other workers have reported on the detrimental effect of high temperature (20-40 C) and high

TABLE 2. Survival of bacteria after 33 days with respect to species, age of culture, relative humidity (RH), and temperature. Glass bead method[a]

Bacterium	Culture age (days)	Glass beads (%) yielding bacteria at: 0% RH 5 C	0% RH 20 C	34% RH 5 C	34% RH 20 C	75% RH 5 C	75% RH 20 C	LSD (P=0.05) all combinations[e]
Corynebacterium michigananse[b]	1	99	99	100	100	98	30	
	7	100	100	100	100	100	99	
	14	100	100	100	100	100	94	8
	22	100	100	100	100	99	73	
	30	100	100	100	100	100	60	
SC735[c]	1	100	76	100	100	98	7	
	7	100	100	100	100	99	51	
	14	100	100	100	100	94	6	12
	22	100	100	100	100	99	4	
	30	35	21	97	95	53	0	
Xanthomonas phaseoli[b]	1	34	11	100	99	80	26	
	7	87	47	100	100	99	0.4	
	14	83	20	100	100	99	0.4	12
	22	2	0	84	40	11	0	
	30	0	0	0	0	0	0	
Pseudomonas glycinea[b]	1	11	0	85	6	20	0	
	7	15	0	100	18	28	0	
	14	24	0	100	6	28	0	9
	22	13	0	100	2	0.5	0	
	30	6	0.2	99	4	0.6	0	
Erwinia carotovora[b]	1	17	0	13	0	0	0	
	7	39	3	36	5	7	0	
	14	44	6	82	3	4	0	8
	22	25	1	91	0.6	0	0	
	30	2	0	69	0	0.3	0	
AN771[d]	1	3	0.5	11	0.4	0	0	
	7	22	4	17	0.8	0	0	
	14	11	0.4	4	0.9	0	0	5
	22	5	0	3	0	0	0	
	30	0.4	0	0.6	0	0	0	

[a]Bacterial suspensions prepared in 10% milk and applied to sterile glass beads. Beads dried under specified conditions and assayed by sprinkling onto the surface of agar medium. Means of two experiments at different times.
[b]Plant pathogenic isolate.
[c]Isolate antagonistic to *P. glycinea* (9).
[d]Isolate antagonistic to *Bipolaris maydis* (18).
[e]The interaction (culture age, RH, and temperature) was significant for each species.

TABLE 3. Survival of *Pseudomonas glycinea* derived from diseased host leaves and dried at 5 C after 33 days with respect to lesion age and relative humidity (RH). Glass bead method[a]

Lesion age (days)	Glass beads (%) yielding bacteria at:		
	0% RH	34% RH	75% RH
7	18	93	0.3
15	55	100	14
23-29	32	98	0.4

[a]Diseased leaf pieces ground in 10% milk and applied to sterile glass beads. Beads dried under specified RH conditions and assayed by sprinkling onto the surface of agar medium. Means of two experiments conducted at different times.

RH (45-100%) on the viability of dried plant pathogenic (5, 6, 14, 23) and/or other bacteria (11, 12, 22). Wilson et al. (23) suggested that retention of sufficient moisture in the hygroscopic exudate of *X. phaseoli* at high RH would allow depletion of reserve nutrients through metabolic activity. Thus, the reduced survival observed at 75% RH and 20 C in our study may have resulted from diminished nutrient reserves.

Culture age affected survival. In general, bacteria from 7- or 14-day-old cultures were most resistant to desiccation, a fact observed previously with *Pseudomonas aeruginosa* (17). Increased survival from older cultures suggests that such bacteria may be in a hypobiotic state. Sherman and Albus (16) have described "physiological youth" in *Bacterium coli (Escherichia coli)* and *Proteus vulgaris*. They found bacteria from young cultures were more sensitive to exposure to cold, heat, phenol, and NaCl than were bacteria from older cultures. Likewise, aging bacterial cells have been reported to be more resistant to adverse environmental conditions than young cells (19). These results and those from our studies indicate that for survival studies in which bacteria are subjected to stress conditions, it would be important to use bacteria of maximum resistance, such as those obtained from 7- or 14-day-old cultures.

Survival of *P. glycinea* cells from culture and from diseased host material was similar. It would be useful to determine if other pathogens behave similarly.

With more study it may be possible to make practical use of some of the findings of the present work. For example, could plant pathogenic bacteria associated with seed be eliminated by storing seed for a period of time under conditions of high temperature and RH without harm to the seed? Or, could survival of a beneficial bacterium, such as an antagonist used for seed treatment [e.g., SC735 (9)], be increased by using older cultures, low temperatures, and 34% RH?

The survival assay techniques developed in this work should prove useful in future studies. The Carborundum assay is more precise, but requires more materials and time to accomplish than the glass bead assay. Although simpler and faster in operation, the bead assay is less accurate and is only useful for detecting large differences between treatments.

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