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*Research Articles*

# Ultraviolet Radiation (UVR) Sensitivity Analysis and UVR Survival Strategies of a Bacterial Community from the Phyllosphere of Field-Grown Peanut (*Arachis hypogeae* L.)

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## ABSTRACT

The short-term population dynamics of the culturable bacterial community from field-grown peanut (*Arachis hypogeae* L.) was analyzed over three 2-day periods. As in other phyllosphere studies, significant numbers of pigmented organisms were detected, suggesting the importance of pigmentation in the colonization of this habitat. Isolates were grouped according to pigmentation (orange, pink, yellow, nonpigmented), and the sensitivity of each isolate in the collection ($n = 617$) to ultraviolet radiation (UVR) was determined as the minimal inhibitory dose ($MID_C$) of UVR that resulted in an inhibition of growth compared to an unirradiated control. The majority of isolates recovered (56.1%) had an $MID_C$ equal to or exceeding that of *Pseudomonas syringae* 8B48, a known UV-tolerant strain. Among pigmentation groups, the mean $MID_C$ of pink- and orange-pigmented isolates was significantly greater than that of yellow- or nonpigmented isolates at each sampling time of day. Identification of 213 of the isolates using fatty acid methyl ester analysis indicated that a large proportion of the isolates were gram-positive, with *Bacillus* spp. alone accounting for 35.7% of the total. The genus *Curtobacterium* contained the largest percentage of highly UVR-tolerant strains. Nonpigmented mutants of four *Curtobacterium* strains were selected following ethyl methane sulfonate mutagenesis; these nonpigmented mutants were significantly altered in survival following irradiation with UV-A wavelengths. The strategy of avoidance of UVR through colonization of the abaxial leaf surface was evaluated on three separate occasions by leaf imprint sampling. Only 3 of 120 leaves (2.5%) contained larger bacterial populations on the adaxial surface, indicating that colonization of the abaxial leaf surface is important to phyllosphere survival. Our results indicate that tolerance to UVR is a common phenotype among phyllosphere bacteria, suggesting that solar radiation has a strong influence on the microbial ecology of the phyllosphere.

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## Introduction

Solar radiation is an important component of the physical environment of the plant leaf surface (phyllosphere), since leaves, as the principal photosynthetic organs of plants, are typically positioned for optimal exposure to sunlight. Approximately 3.2% of the total energy of solar radiation is in the ultraviolet (UV) range [5]. Wavelengths in the UV-B range (290 to 320) nm) of sunlight are particularly damaging to living organisms because photons of these wavelengths are of sufficiently high energy to cause direct damage to DNA. This DNA damage is manifested through the formation of DNA photoproducts, of which the cyclobutane pyrimidine dimer (CPD) and the pyrimidine(6-4)pyrimidinone are the most common [10].

Stratospheric ozone depletion is predicted to alter the flux and spectral irradiance of UV-B wavelengths reaching the earth's surface over the next few decades. Much research has been performed in the past decade examining the potential impacts of an increased UV-B environment on biological organisms; the bulk of this work has focused on UV-B effects on plants because of the potential for detrimental effects on agriculture [32]. Although relatively few studies have examined UV-B effects on the microbial flora of plant hosts, such data are also important because of the varied associations of these organisms with plants, including mutualistic and pathogenic associations and a critical role in the nutrient cycling of senescent plant matter. Several studies with filamentous fungi and yeast phyllosphere isolates have demonstrated that UV radiation (UVR) decreased growth, the abundance of specific fungal species, and spore survival, or delayed spore germination [14, 23, 27, 28]. Irradiation with UV-B above ambient levels has also been shown to decrease the rate of decomposition of leaf litter, presumably by fungi, and also to reduce the relative abundance of certain fungal species in litter [11, 24].

Studies of the effects of UVR on phyllosphere bacteria have been directed toward examining mechanisms of UV protection in these organisms. For example, tolerance to UVR in the plant pathogenic bacterium *Pseudomonas syringae* is conferred by the plasmid-encoded *rulAB* DNA-repair operon [34]. The *rulAB* operon is widely distributed among pathovars of *P. syringae,* and possession of *rulAB* enabled strains to maintain significantly higher populations in the bean phyllosphere following irradiation with UV-B wavelengths [34, 35]. The production of an extracellular polysaccharide layer capable of absorbing UVR has also been implicated in conferring tolerance in *Xanthomonas campestris* pv. phaseoli [20]. Pigmentation in phyllosphere bacteria is another mechanism of UV protection, specifically from UV-A wavelengths (320 to 400 nm) which can result in the generation of active oxygen derivatives within cells [8]. Indeed, in several studies of phyllosphere microbial communities, the majority of bacteria isolated produced pigments in culture, suggesting that UVR protection conferred by pigmentation is important to survival in the phyllosphere [1]. Laboratory studies have shown that the yellow membrane-bound pigment xanthomonadin produced by *Xanthomonas campestris* was found to possess antioxidant properties and to protect lipids from peroxidation [26]. Similarly, carotenoid pigments produced by *Erwinia herbicola* (*Pantoea agglomerans*) play an important role in cellular protection from UV-A radiation [2]. Although the role of carotenoid pigments in the protection of cells from UV-B radiation has been unclear, Sandmann et al. [29] recently determined that endogenous levels of neurosporene and β-carotene provided UV-B protection to a heterologous *Escherichia coli* host.

Most studies of bacterial phyllosphere communities have examined seasonal diversity [e.g., 21, 37]. These studies indicate that phyllosphere communities are characteristically dominated by a relatively small number of taxa when compared to similar communities inhabiting leaf litter, the root zone, or rhizosphere of the same plant species [12, 25]. In some cases, the species diversity isolated was lowest in mid-season compared to early or late in the growing season [37]. The observed reduction in phyllosphere community diversity is usually attributed to the extensive variation in the physical environment of the phyllosphere, with disturbances caused by rapid, extreme fluctuations in parameters such as temperature, relative humidity, and solar radiation being commonplace. Certain aspects of plant host biology, including the availability of nutrients leached onto leaves, leaf surface topography, and competitive interactions among microorganisms, may also play an important role in the modulation of the phyllosphere microbial flora. A number of autecological studies have addressed the ability of phyllosphere bacterial strains to overcome environmental stresses; many of these studies have quite naturally focused on fitness characteristics of plant pathogenic strains [reviewed in 1]. In this study, we selected a single component of the physical environment of the phyllosphere, UVR, and surveyed a bacterial community to determine the range of sensitivity to UVR among the isolates. Our objectives were also to analyze the short-term populations dynamics of the bacterial community to determine the relative distribution of UV-tolerant and UV-sensitive phenotypes as a function of isolation time.

Likewise, we were interested in determining if the ratio of pigmented/nonpigmented strains and the relative abundance of UV-tolerant strains increased during daylight hours as ambient solar radiation increased. Lastly, we studied a subgroup of UV-tolerant isolates of one genus to examine the role of pigmentation in protection from both DNA-damaging UVR and UV-A radiation.

## Materials and Methods

### *Plant Sampling*

On 28 May 1997, peanut (*Arachis hypogeae* cv. 'Florunner') was hand sown at a depth of 2 cm in a field plot established in a sandy loam soil adjacent to the Texas A&M University campus. The plot size was 10 m by 10 m with 1-m row spacings; within rows, seeds were sown at a rate of one seed per 10 cm. Furrow irrigation was provided to the plot as necessary, ensuring that leaf moisture was only provided through natural rainfall and dew formation. Temperature, precipitation, and solar radiation (200SA pyranometer; Licor, Lincoln, NE) were monitored at a site approximately 500 m from the field plot. Data readings were taken every 15 s; hourly averages were recorded automatically using a datalogger (model CR-10; Campbell Scientific, Logan, UT).

### *Short-Term Population Dynamics*

The plants were sampled at 0700, 1100, 1500 and 1900 on 23, 24, 30, and 31 July, and 6 and 7 August 1997. Samples consisted of 20 individual leaves collected at random throughout the plot from the top of the plant canopy. Each leaf was placed individually in a sterile plastic bag and transported to the laboratory on ice. The leaves were weighed and placed in 10 ml prechilled buffer (0.1 M potassium phosphate, pH 7.0, 0.1% peptone), following which bacterial cells were removed by a 7 min sonication treatment in an ultrasonic bath (Model 250T, VWR Scientific; Houston, TX). Samples (0.1 ml) from appropriate dilutions of the sonicate were plated on King's medium B [19], amended with 0.15 mg cycloheximide (KBc)/ml to inhibit fungal growth. Bacterial colonies were counted following 72 hr incubation at 25°C. Individual counts were made for colonies appearing white or cream (nonpigmented) and for those producing yellow, orange, or pink pigments on KBc.

### *Selection of Isolates*

Isolates chosen for further analyses were selected from six leaves chosen randomly from each sampling time. Isolates were recovered from the dilution plates utilized to make the bacterial counts. The method of selecting isolates involved placing the plates on a numbered grid (0–50); three numbers were randomly chosen, and all colonies up to 5 total in the chosen grids were selected for further testing. In this manner, 30 isolates were selected for each sampling time. Isolates were subcultured through two rounds of single colony purification and subsequently stored at −70°C in 15% glycerol. A percentage of the isolates failed to grow during the subculturing process or could not be cultured following storage at −70°C; these isolates were lost for further analysis. A final total of 617 isolates were maintained for further characterization.

### *Ultraviolet Radiation Sensitivity Characterization*

Because of the large number of isolates in the present study, the sensitivity to UVR of each isolate was assayed by determining the minimal inhibitory dose of UV-C (254 nm) radiation ($MID_C$) necessary to inhibit the growth of cells spotted onto KB plates. Cells were prepared by growth in KB broth to a density of $10^8$ cfu/ml. The cells were diluted in sterile saline (0.85% NaCl) and approximately $10^4$ cells were spotted (10 μl) in quadruplicate onto a series of KB plates, following which the cell spots were allowed to air dry in a laminar flow hood. Individual plates were then exposed to UV-C radiation from an XX-15 UV lamp (UVP Products; San Gabriel, CA) placed horizontally at a fixed height above the cell suspensions. The lamp was turned on 15 min prior to use to allow for stabilization of the UV output. The energy output of the lamp was monitored with a UV-X radiometer fitted with a UV-25 sensor (UVP Products) and determined to be 1.5 J $m^{-2}$ $s^{-1}$; UV doses utilized were 50, 100, 150, 200, and 250 J $m^{-2}$. Following the UV dose, the plates were maintained and incubated under dark conditions to minimize photoreactivation. The $MID_C$ was designated as the UV dose that resulted in an inhibition of growth compared to cells spotted onto unirradiated plates. Isolates whose $MID_C$ was initially determined to be 50 J $m^{-2}$ were also subsequently tested at 25 J $m^{-2}$.

Comparisons of the $MID_C$ values with isolate sensitivity to UV-B radiation were done by selecting three strains at random from each $MID_C$ grouping for analysis. Isolates were prepared for UV-B sensitivity assays following growth overnight in KB broth by washing pelleted cells in sterile saline and resuspending the cells in 10 ml saline in a sterile glass petri dish. Cell suspensions were irradiated with UV-B (peak at 302 nm) wavelengths using an XX15-M model UV-B lamp (UVP Products) filtered through polystyrene, a compound that has previously been shown to be an effective screen for wavelengths below 280 nm [42]. The UV-B lamp was turned on 15 min prior to use to allow for stabilization of the UV output. To monitor the energy output of the lamp, the UV-X radiometer was fitted with a UV-31 sensor (UVP Products); biologically-effective doses of UV-B were calculated using the DNA damage action spectrum of Setlow [30]. Under the conditions utilized in this study, the output of the UV-B lamp was 2.8–3.0 J $m^{-2}$ $s^{-1}$. Cell suspensions were continuously mixed while receiving the UV-B dose to eliminate survival due to shading. Following irradiation, appropriate dilutions of surviving cells were plated on KB (conducted under a red safety light), and counts were made after a 72 hr incubation at 25°C.

### *Identification of Isolates*

A total of 213 isolates, representing all of the isolates recovered on 24 July and 7 August 1997 (two sampling dates chosen randomly),

were selected for identification. These isolates were grown on trypticase soy agar (Difco Laboratories; Detroit, MI) prior to analysis. Bacterial identification was done by analysis of fatty acid methyl ester profiles (FAME); extraction and purification of FAMEs and generation of profiles using gas chromatography were done using published methods [33]. Isolate identification was accomplished using the Microbial Identification System software package (Microbial ID, Inc., Newark, DE) and TSBA library version 3.9. The FAME analyses and isolate identification were done by the Texas A&M University Plant Disease Diagnostic Laboratory. Identification at the genus level was accepted if the similarity index was $\geq 0.2$ and the difference from the next match was greater than 0.1.

### *Ultraviolet Radiation Survival Strategies*

We examined two potential UVR survival strategies utilized by members of the bacterial community in the phyllosphere habitat. The first strategy examined was avoidance of incoming solar UVR through colonization of the abaxial surface of leaves. This strategy has been suggested in previous studies [reviewed in 1]; however, there are few quantitative data available showing bacterial population numbers from either leaf surface. We analyzed the adaxial/abaxial bacterial population ratios from peanut leaves that were sampled at 1500 on 25 July and 1 and 25 August 1997. Forty individual leaves were collected at random throughout the plot from the top of the plant canopy and handled as described above. Bacteria present on the adaxial and abaxial surfaces of these leaves were recovered by imprinting leaves on KBc plates for 30 s per surface. During processing, the adaxial surface was imprinted first for 20 of the leaves, and the abaxial surface was imprinted first for the remaining 20 leaves. Bacterial colonies were counted following 72 hr incubation at 25°C.

The second strategy examined was the importance of pigmentation in UVR protection. We selected four pigmented strains, identified by FAME analysis as members of the genus *Curtobacterium,* for characterization. These strains were chosen for examination because they possessed the highest level of UVR tolerance observed among the collection of peanut isolates and because there are relatively fewer data available detailing the role of pigmentation in UVR protection of gram-positive organisms. We utilized a chemical mutagenesis procedure to select nonpigmented mutants of the four *Curtobacterium* strains. Each strain was grown to mid-stationary phase in KB broth, and the cells were washed and resuspended in 3.92 ml Davis minimal medium, pH 7.5 (Difco Laboratories) containing no external carbon source (DMM). Eighty microliters of a solution (100 μg/ml) of ethyl methane sulfonate (EMS; Sigma Chemical Co., St. Louis, MO) dissolved in methylene chloride was added to the cells. This cell suspension was then incubated on a rotary shaker (250 rpm) for 1.5 hr at 25°C. The cells were then pelleted, washed twice in an equal volume of DMM, and resuspended in 0.2 ml DMM. This 0.2 ml cell suspension was then inoculated into 1.8 ml KB broth and grown overnight at 25°C; multiple replicates of cells from appropriate dilutions were then plated on KB. The plates were examined visually for the presence of white, nonpigmented colonies after a 72 hr incubation. The identity of the nonpigmented mutants was confirmed by FAME analysis, the $MID_C$ with UV-C radiation was determined, and a sensitivity analysis to UV-A radiation (365 nm) was done following the methods described above. UV-A radiation was delivered from an XX-15L UV-A lamp (UV Products), monitored with a UVX-36 sensor, and determined to be delivered at 45–48 J $m^{-2}$ $s^{-1}$.

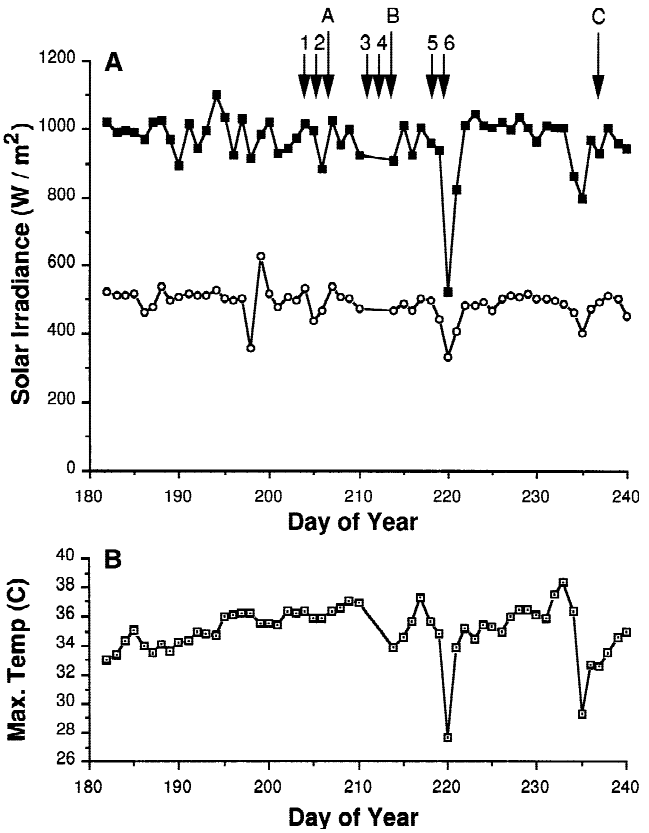


Fig. 1. (A) Total daily (■) and maximum hourly (○) average solar irradiance measured approximately 500 m from the field plot site. The numbered arrows indicate sampling dates for population analyses, and the lettered arrows indicate sampling dates for leaf adaxial/abaxial population ratio analyses. (B) Maximum daily temperature measurements.

## Results

### *Short-Term Population Dynamics of Phyllosphere Bacterial Populations from Peanut*

The climatic conditions observed during the 89-day growth period of the peanut plants were typical for this region of Texas. During a 56-day period (days 182–237) encompassing all of the sampling dates, solar radiation was consistently high with an average daily irradiance (400 to 700 nm) recorded of 476.9 W $m^{-2}$ during a typical 15-hr day (Fig. 1A). The maximum hourly average solar irradiance observed each

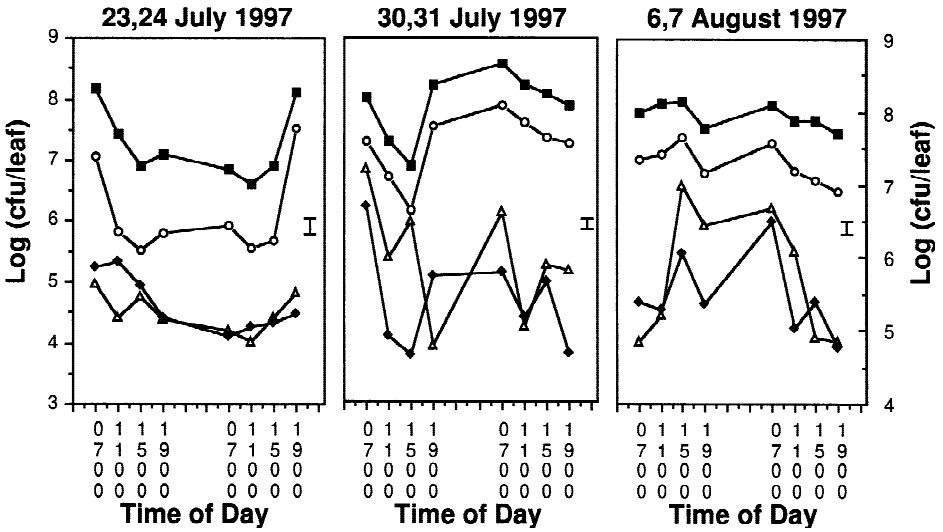


Fig. 2. Short-term population dynamics of total (■), yellow-pigmented (○), orange-pigmented (△), and pink-pigmented (♦) bacteria recovered from the phyllosphere of field-grown peanut. Each sampling point represents a population mean from 20 individual leaf samples. For clarity, the error bars in each panel show the minimum standard error of the means observed.

day was consistently greater than 1,000 W $m^{-2}$ (Fig. 1A). The average daily irradiance fell below 400 W $m^{-2}$ on only 2 days during this period. During days 182–237, the average daily maximum and minimum air temperatures were 35.1°C and 24.3°C, respectively (Fig. 1B). Only three rain events were recorded during this period: 1.0 mm on day 210, 8.6 mm on day 219, and 11.4 mm on day 220. Thus, our sampling of the phyllosphere bacterial community was expected to yield bacteria that were adapted to the heat and dry conditions, and most especially to the prevailing solar radiation in this environment.

Twenty individual leaves from the top of the canopy of peanut located in a field plot at College Station, TX, were sampled at 0700, 1100, 1500, and 1900 on 2 consecutive days at weekly intervals over a 3-week period. Relatively large ($10^7$ to $5 \times 10^8$ cfu/g) populations of bacteria were recovered from peanut leaves at each sampling time (Fig. 2). Examination of bacterial counts taken at 0700 each day showed that total population counts and population counts of the yellow, orange, or pink pigmented bacteria could vary as much as 10- to 50-fold on a day-to-day basis (Fig. 2). The use of frequent samplings during particular 24-hr periods enabled us to detect large population increases or declines (>10-fold) over relatively short (4-hr) sampling intervals. The total bacterial populations did not always decline during daylight hours; in some cases, rapid population bursts were observed, even between samples collected at 1100 and 1500 on the same day (Fig. 2).

### *Ultraviolet Radiation Sensitivity Characterization of Isolates*

A total of 30 isolates were randomly selected at each sampling time for further analysis. These colonies were grown and cycled through two single colony purifications on KB and then stored at −70°C in 15% glycerol. During the purification steps and following storage of the isolates, it was found that a total of 103 isolates (14.3%) were not able to be subsequently cultured; these isolates were dropped from the study. Ultraviolet radiation sensitivity analyses were performed on the remaining 617 isolates. The UV analyses indicated that the majority of strains had an $MID_C$ of 50 or 100 J $m^{-2}$ (31.1% and 28.8%, respectively) (Fig. 3A). For comparison purposes, we determined that the $MID_C$ of a known UV-sensitive organism, *Pseudomonas aeruginosa* PAO1 [18], was 5 J $m^{-2}$, and the $MID_C$ of a known UV-tolerant organism, *P. syringae* 8B48 [35], was 100 J $m^{-2}$.

The percent recovery of isolates with each $MID_C$ was then collated and analyzed as a function of isolation time (Fig. 3B). Approximately 35–40% of strains isolated at 0700 and 1100 exhibited UV $MID_C$s of 50 J $m^{-2}$, while the largest $MID_C$ grouping (approximately 35%) from the 1500 and 1900 samples was at the 100 J $m^{-2}$ level (Fig. 3B). Likewise, the percentage of strains recovered with an $MID_C$ of 150 J $m^{-2}$ was larger at the 1500 and 1900 samples (Fig. 3B). A chi-square analysis was used to determine if the frequencies of bacteria in particular UV-$MID_C$ groups differed among the 0700, 1100, 1500, and 1900 samples. The frequencies were found to significantly vary by time of day ($\chi^2 = 27.662$, $df = 15$, $P = 0.026$), with the percentage of bacteria with an $MID_C$ of $\geq$100 J $m^{-2}$ increasing at each sampling time (Fig. 3B). Thus, a shift in the proportion of isolates with a particular UV $MID_C$ was observed dependent upon the time of isolation. UV-C radiation (254 nm) was chosen for the $MID_C$ analysis of the isolate collection because of the large number of isolates examined. We also examined the sensi-

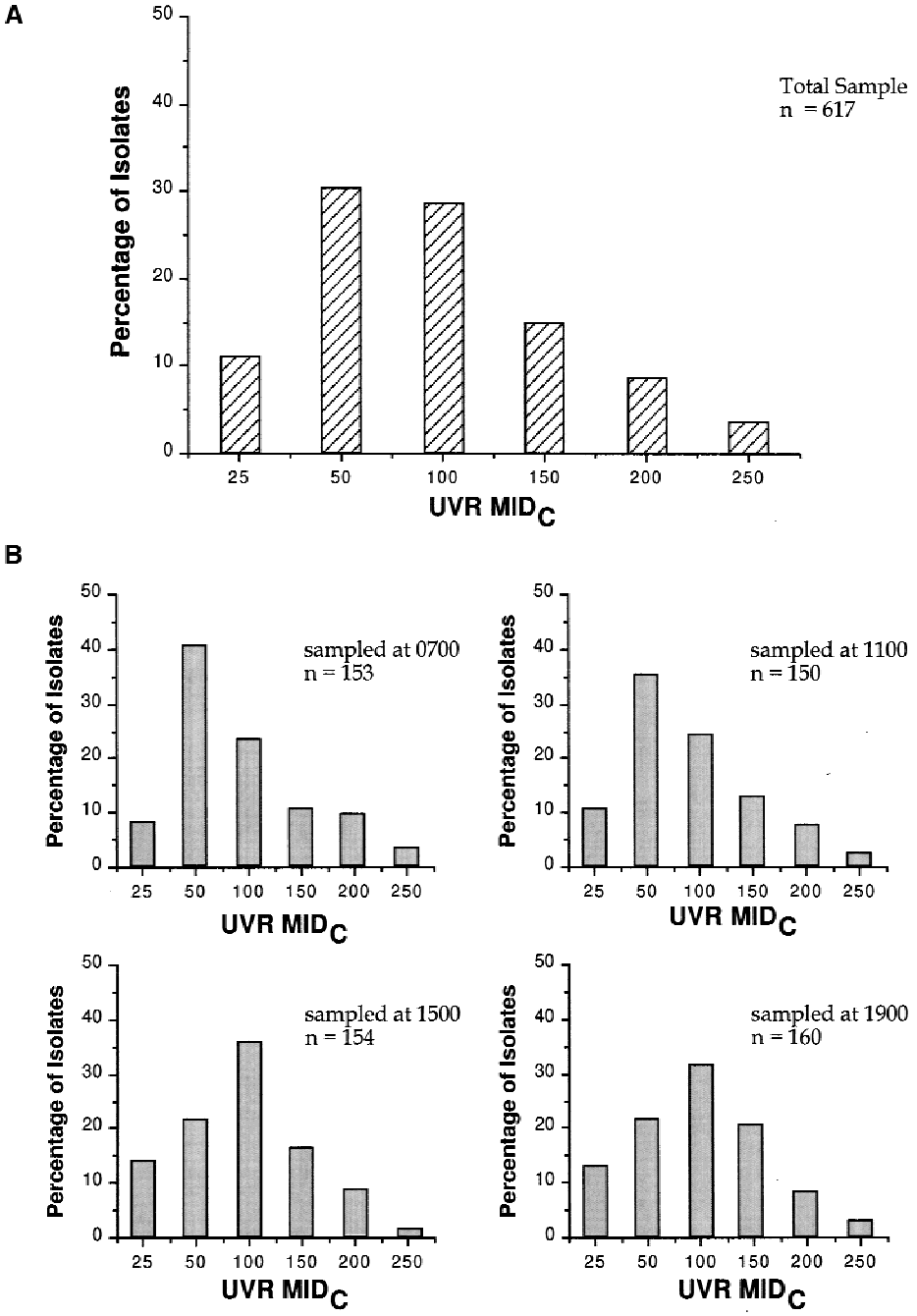


Fig. 3. Analysis of the sensitivity to UVR of bacterial isolates from the peanut phyllosphere. (A) Percentage of isolates from the total collection; (B) percentage from individual collections sampled at 0700, 1100, 1500, and 1900 on six sampling dates with the corresponding minimal inhibitory dose of UV-C (254 nm) radiation ($MID_C$).

tivity of a subgroup of strains from each $MID_C$ grouping to UV-B radiation (302 nm). We found that the sensitivity level of strains to UV-B radiation paralleled their sensitivity to UV-C (data not shown). Similar comparative results have been observed previously with studies of *P. aeruginosa* and *P. syringae* [18, 35] and are predicted because the biological effects of both UV-C and UV-B radiation are mainly due to direct DNA damage [10].

The $MID_C$ data for the 617 isolate collection was also analyzed in terms of the pigmentation of the isolates. When pigmentation was included, it was apparent that the total collection of pigmented isolates (yellow, orange, and pink) contained a larger proportion of isolates with higher range $MID_C$s ($\geq$100 J $m^{-2}$) when compared to nonpigmented isolates (Fig. 4A). A breakdown of the $MID_C$s into individual pigment groups determined that the orange and pink pigmented isolates were responsible, as these isolates comprised the largest groups with $MID_C$s of $\geq$200 J $m^{-2}$ (Fig. 4B). The mean $MID_C$ of the collection of isolates (grouped by pigmentation) was then evaluated using a one-way analysis of

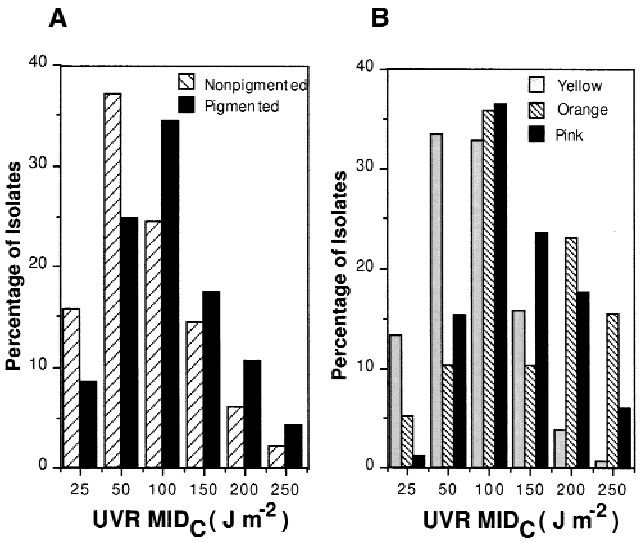


Fig. 4. Analysis of the sensitivity to UVR of bacterial isolates from the peanut phyllosphere as related to the pigmentation of the isolates. (A) Percentage of nonpigmented and pigmented isolates with the corresponding minimal inhibitory dose of UV-C (254 nm) radiation ($MID_C$); (B) percentage of the total groups of yellow, orange, and pink pigmented isolates with the corresponding $MID_C$.

variance based on time of day of isolation, and differences among the mean $MID_C$ values were assessed using the Student–Newman–Keuls test. At each time of day, the mean $MID_C$ of the pink and orange-pigmented isolates was significantly greater than that of the other groups (Table 1). The mean $MID_C$ of the orange-pigmented isolates fluctuated depending upon the time of day of isolation, and thus was significantly lower than that of the pink-pigmented isolates at 0700 and 1500 (Table 1). The mean $MID_C$ of the yellow and nonpigmented isolate groups was only significantly different at the 0700 and 1500 sampling times (Table 1).

### *Identification and UV Sensitivity Analysis of Two Subsets of Strains*

Two sampling dates (24 July and 6 August 1997) were randomly chosen and all of the isolates (213) recovered on those dates were subjected to FAME analysis for identification purposes. Members of 20 named genera were identified (Table 2). Only 5.2% of samples did not match any entries in the MIS Aerobic Bacteria library; these bacteria were unnamed and were subsequently listed as "No match" (Table 2). Data regarding time of day of isolation and $MID_C$ were also tabulated for each bacterium subjected to FAME analysis. The majority of strains identified were gram positive,

**Table 1.** Comparison of the $MID_C$ of four pigmentation groups of bacterial isolates from the peanut phyllosphere at four sampling times of day[a]

| Pigmentation group | Mean $MID_C^b$ per sampling time | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0700 | | 1100 | | 1500 | | 1900 | |
| Pink | 142.3 | a | 145.0 | a | 153.1 | a | 143.2 | a |
| Orange | 117.5 | b | 143.3 | a | 114.7 | b | 134.4 | a |
| Yellow | 88.6 | c | 74.5 | b | 97.9 | c | 93.1 | b |
| Nonpigmented | 76.0 | d | 89.2 | b | 86.3 | d | 90.8 | b |

[a] Isolates were recovered on six sampling dates; samples were taken at 0700, 1100, 1500, and 1900 on each sampling day.
[b] Within a column, means not followed by the same letter are significantly different at $P = 0.05$, following an analysis of variance and the Student–Newman–Keuls test.

with *Bacillus* spp. alone accounting for 35.7% of the total (Table 2). It is possible that the relatively high percentage of *Bacillus* spp. may have been affected by the sampling of endospores that germinated after sampling. However, 32.9% of the *Bacillus* spp. assayed had $MID_C$s $\geq$100 J m$^{-2}$, indicative of UV tolerance. The majority of strains within a genus had similar $MID_C$s, and the mean $MID_C$ of the gram-positive strains (112.5 J m$^{-2}$) was much larger than that of the gram-negative strains (77.7 J m$^{-2}$) (Table 2). Pigmented strains of the gram-positive genus *Curtobacterium* contained the largest percentage of representatives with $MID_C$s of 150 J m$^{-2}$ or above. Four of the *Curtobacterium* strains with $MID_C$s of 250 J m$^{-2}$ were selected for further characterization to examine the role of pigmentation in cellular UVR protection (see below).

### *Ultraviolet Radiation Survival Strategies*

Populations of bacteria were recovered on three separate occasions from both the adaxial and abaxial surfaces of 40 individual leaves by leaf printing. The leaves were sampled from the tops of the plant canopy at 1500 on each sampling date. The ratio of the cell counts was determined for each leaf and plotted. Larger bacterial populations on the adaxial leaf surface were recovered from only 2.5% of leaves (3/120), and only one of those leaves harbored greater than twofold more bacteria (Fig. 5). On 13.3% of leaves (16/120), the populations recovered from the adaxial leaf surface were lower by at least one order of magnitude. Visual examination of the plates did not suggest that the relative recovery of pigmented/nonpigmented isolates differed depending on the leaf surface (data not shown).

**Table 2.** The number of bacterial isolates per genus, and the frequency distributions of $MID_C$ and sampling recovery time for these isolates[a]

| Strain ID | No. | UV minimal inhibitory dose | | | | | | No. isolates recovered at each time of day | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 25 | 50 | 100 | 150 | 200 | 250 | 0700 | 1100 | 1500 | 1900 |
| Gram negative | | | | | | | | | | | |
| *Actinobacillus* | 5 | — | 5 | — | — | — | — | 2 | 3 | — | — |
| *Enterobacter* | 4 | — | 2 | 2 | — | — | — | — | 1 | 1 | 2 |
| *Escherichia* | 5 | — | 5 | — | — | — | — | 4 | — | — | 1 |
| *Pantoea* | 13 | 2 | 1 | 9 | 1 | — | — | — | 3 | 7 | 3 |
| *Pseudomonas* | 5 | 1 | 2 | 2 | — | — | — | — | 3 | 2 | — |
| *Rhodobacter* | 4 | — | — | 3 | — | — | 1 | 3 | — | 1 | — |
| *Xanthomonas* | 6 | 4 | 1 | — | 1 | — | — | — | 1 | — | 5 |
| Others[b] | 4 | — | — | 3 | 1 | — | — | 2 | — | 1 | 1 |
| Gram positive | | | | | | | | | | | |
| *Arthrobacter* | 3 | — | — | 1 | 2 | — | — | 1 | — | 1 | 1 |
| *Aureobacterium* | 3 | — | 2 | — | 1 | — | — | — | — | 2 | 1 |
| *Bacillus* | 76 | 6 | 45 | 9 | 5 | 11 | — | 20 | 22 | 16 | 18 |
| *Brevibacterium* | 13 | — | 7 | 5 | 1 | — | — | 2 | — | 10 | 1 |
| *Clavibacter* | 19 | — | — | 10 | 4 | 3 | 2 | 3 | 1 | 9 | 6 |
| *Curtobacterium* | 31 | — | — | 4 | 14 | 7 | 6 | 5 | 11 | 4 | 11 |
| *Kurthia* | 5 | 1 | 3 | 1 | — | — | — | 2 | — | 1 | 2 |
| *Micrococcus* | 4 | — | 2 | 2 | — | — | — | 1 | 1 | 2 | — |
| *Staphylococcus* | 2 | 1 | 1 | — | — | — | — | — | — | — | 2 |
| No match | 11 | — | 1 | 7 | 3 | — | — | 5 | 1 | 2 | 3 |

[a] The isolates were recovered from the peanut phyllosphere on 24 July and 7 August 1997.
[b] Includes one strain each of *Methylobacterium, Salmonella, Vibrio,* and *Xanthobacter*.

### *Characterization of Nonpigmented* Curtobacterium *Mutants*

White, nonpigmented mutants were selected from four *Curtobacterium* strains following mutagenesis with EMS. The mutation frequency was approximately $1 \times 10^{-6}$. The nonpigmented mutants selected for analysis had similar growth rates and identical FAME profiles with the corresponding wild-type strains. The sensitivity of each mutant/wild-type strain combination to UV-A radiation and their $MID_C$ was determined. No differences in $MID_C$ were observed with any of the mutant/wild-type strain combinations. However, from the four mutant/wild-type strain combinations analyzed, two patterns of increased UV-A sensitivity of the mutant strains were observed. In the first pattern, exemplified by strain F13, large differences, as much as $1 \times 10^6$ at higher doses, in UV-A sensitivity of mutant and wild-type strains were observed, with the wild-type strain being relatively insensitive to UV-A radiation doses $\leq$250 kJ $m^{-2}$ (Fig. 6A). The second pattern (strains F8, G17, and U20) was similar to the first, except the differences in percent survival following doses larger than 200 kJ $m^{-2}$ were smaller (10- to 50-fold) than those observed for strain F13 (Fig. 6B).

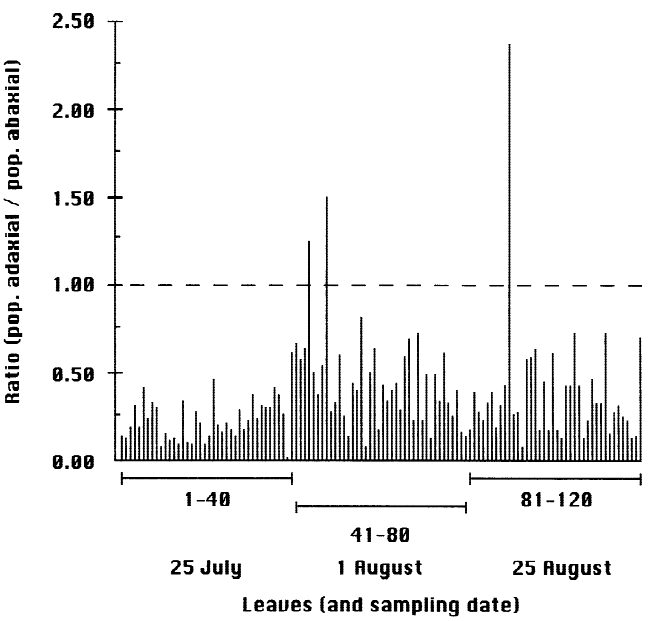


Fig. 5. Ratios of populations recovered from the adaxial and abaxial surfaces of 40 peanut leaves from 3 sampling dates. The dashed line indicates an equivalent population ratio from each surface.

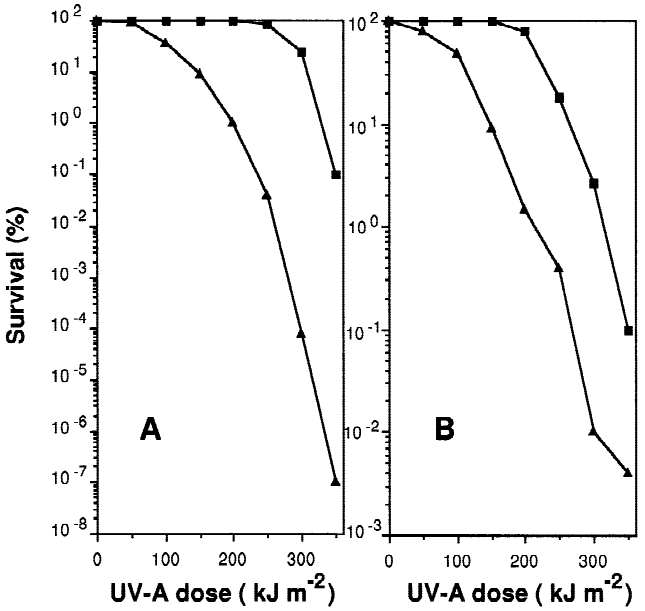


Fig. 6. Survival following irradiation with UV-A radiation of wild-type *Curtobacterium* strains and pigmentation-deficient mutants generated with ethyl methane sulfonate. Strains are (A) *Curtobacterium* F13 (■) and EMS mutant F13-np1 (▲) and (B) *Curtobacterium* F8 (■) and EMS mutant F8-np1 (▲).

## Discussion

As an initial foray into an examination of the role of UVR in the modulation of bacterial community dynamics in the phyllosphere, we examined the UVR sensitivity of 617 isolates recovered from a field planting of peanut. The majority of isolates recovered (56.1%) survived UV doses at levels equal to or exceeding that of *P. syringae* 8B48, a known UV-tolerant strain. In previous studies, *P. syringae* 8B48 was able to maintain similar population levels on UV-irradiated and nonirradiated bean leaves [35]. In contrast, populations of UV-sensitive *P. syringae* strains were reduced to significantly lower levels on UV-irradiated bean leaves [35]. Thus, our results indicate that most phyllosphere bacterial isolates from peanut posses a level of UVR tolerance that might significantly enhance their fitness in the peanut leaf environment. Ecological studies from other environments have indicated that solar radiation may strongly affect bacterial population dynamics. For example, in marine environments, effects of solar radiation on bacterioplankton include decreases in total cell density, increases in measurable DNA photoproducts, and significant inhibitions of protein and DNA synthesis [17, 22]. Tong and Lighthart [38], in studies of atmospheric bacterial isolates, found that solar radiation selected for pigmented bacteria with higher levels of UV tolerance. In marine environments, the negative effects of solar radiation are attenuated with depth, coinciding with a decrease in irradiance with depth. The phyllosphere environment would differ from the marine example in that leaf sites located close spatially might be exposed to drastically differing levels of irradiance [13] depending upon aspects such as leaf surface (adaxial vs abaxial), shading, and canopy position. The possession of UV tolerance may enable bacteria to exploit areas of the leaf surface under conditions of solar irradiance that would be inhibitory or lethal to corresponding UV-sensitive isolates.

Our examination of the short-term population dynamics of phyllosphere bacteria from peanut showed mostly small variations in population size during the course of individual days. However, there were cases in which population increases or decreases of greater than 10-fold were observed within a 4-hr period. The observation of sudden shifts in population sizes is not uncommon; studies that have utilized frequent sampling techniques have detected population surges of greater than 10-fold with similar regularity [15, 36]. Examinations of short-term dynamics in the phyllosphere have also shown that bacterial populations typically experience diel fluctuations, decreasing during daylight hours and increasing at night [15, 36]. Although we observed similar results for most of the sampling dates, there were two occasions where large population increases were observed between samples taken at 1500 and 1900. Since the majority of isolates recovered were tolerant to UVR, the increase in incoming UVR during daylight hours may not have adversely affected this bacterial community. Population trends of pigmented bacteria generally mimicked those of the total bacterial populations, although the magnitude of the changes differed on some occasions.

Bacterial identification of the isolates recovered on two of the sampling days indicated that the community we were analyzing largely comprised gram-positive strains, and that these gram-positive strains on average possessed a higher $MID_C$ than the gram-negative strains. Gram-positive organisms have been isolated in abundance from the phyllosphere of different host plants [3, 4, 9, 25], representing up to 76% of the total bacteria isolated in one study [31]. The main difference in community makeup that we observed in our study compared to many others was the relative absence of *Pseudomonas* spp., which are common phyllosphere residents [16]. This may be due to the relatively high average

temperatures and solar radiation intensity and overall dry conditions at the sampling location. Reductions in populations of *P. syringae* during summer months have been noted in previous studies [37, 41]. Another study has also shown that the specific growth rate at 40°C of *Bacillus* spp. isolated from the oak phyllosphere was significantly higher than that of *Pseudomonas* spp. collected from the same habitat [25].

Bacteria that were clearly sensitive to UVR ($MID_C$ of 25 J $m^{-2}$) comprised a small proportion of the community isolated at each sampling time in our study. These organisms may survive in leaf sites, such as substomatal chambers, that are not exposed to UV, or as aggregates that would allow shading of some of the cells. Alternatively, UV-sensitive bacteria may be limited to colonization of adaxial leaf surfaces or the surfaces of shaded leaves lower in the plant canopy. The leaf epidermis is an effective attenuator of incoming UV radiation [6]; thus, bacteria present on the undersurface of a leaf may be protected from solar radiation. Indeed, our analysis of 40 individual leaves sampled on each of three occasions indicated that only three of 120 leaves (2.5%) harbored larger bacterial populations on the adaxial surface as detected by leaf imprinting. While population size on individual leaf surfaces may be affected by topography (stomatal number or trichome frequency, for example) and other environmental factors, solar radiation also appears to have an important effect on populations. We are currently assessing whether UV-sensitive strains are impaired in the leaf surface area available for colonization, depending upon whether or not a particular surface is exposed to solar radiation.

Pigmentation appears to be an important adaptation of bacteria for UVR protection. In our study, organisms producing orange or pink pigments in culture had significantly higher levels of UV tolerance (measured as mean $MID_C$) when compared to nonpigmented or yellow-pigmented organisms. The contribution of pigmentation to UV-C or UV-B radiation tolerance is unclear, however. Although UV-B shielding pigments such as mycosporine and scytonemin have been characterized in cyanobacteria [7], these pigments are typically brown. We did not detect any brown-pigmented bacteria in this study. Instead, the pigments observed in this study more closely resemble carotenoids, pigments that play an important role in protection from UV-A radiation damage [39, 40]. Our examination of four pigment-deficient mutants of *Curtobacterium* indicated that the mutant strains were significantly more sensitive to UV-A radiation, but had an equivalent $MID_C$ when their sensitivity to UV-C radiation was determined. Two patterns of UV-A sensitivity were observed; the nonpigmented mutant of strain F13 was reduced by as much as $1 \times 10^6$ when compared to the wild-type strain (Fig. 6B). This mutant may have a mutation in a regulatory locus that controls pigment production and possibly other determinants involved in protection from damage induced by UV-A radiation. Smaller differences in percent survival of the other three nonpigmented strains suggest that these strains may be mutated in a structural pigment-encoding gene(s).

Direct testing of the role of UVR in modulating phyllosphere bacterial communities would involve examining population dynamics on plants grown under UVR-transmitting or UVR-nontransmitting shields. Such experiments are underway in our laboratory. Our current results show that UVR-tolerant bacteria comprise a large proportion of the phyllosphere community from peanut. We are also examining whether tolerance to UVR increases the available sites of colonization on leaves and how UVR-tolerant and UVR-sensitive bacteria compete in shaded sites on leaves.

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