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Carotenoid Pigmentation in Antarctic Heterotrophic Bacteria as a Strategy to Withstand Environmental Stresses

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Abstract

Bacterial strains isolated from Antarctic environments were used to assess the role of carotenoid pigments as cryo- and solar radiation protectants. Isolates were subjected to one hundred 12-hr freeze-thaw cycles and exposed to ambient simulated solar radiation (300 Wm^{-2}) with growth recovery evaluated after pre-set time intervals. Differences in survival were observed between carotenoid pigmented and non-pigmented strains in response to the different stresses based upon the enumeration of colony forming units. On average carotenoid pigmented strains were more resistant to freeze-thaw cycles as compared to the non-pigmented strains. Survival for non-pigmented strains decreased precipitously from 2×10^7 to 1.5×10^4 cells mL^{-1}, on average, within the first 20 cycles. Similar results were found in the solar radiation experiments. After 2 hrs of solar radiation exposure, 61% of the pigmented organisms survived versus 0.01% for the non-pigmented isolates. We applied an additive mixed model to estimate differences between the carotenoid pigmented and non-pigmented bacterial groups. Modeled results confirmed a positive effect of pigmentation on survivability and provide evidence that carotenoid pigmentation in heterotrophic bacteria isolated from Antarctic environments increases resistance to environmental stressors.

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Introduction

Organisms that exist in icy environments must possess mechanisms to protect themselves from extreme environmental conditions that would otherwise cause severe damage to non-adapted organisms. Cells revived from ice core samples have often endured desiccation, intense solar irradiation while at the surface, freezing, a long period of frozen dormancy, and eventual thawing. Therefore, it is not surprising that a large number of the isolates recovered from icy environments belong to bacterial groups that form spores (e.g., *Bacillus* and *Actinomyces*) or have thick cell walls and polysaccharide capsules (Priscu et al., 2006). These structures can help overcome the stresses associated with water loss, namely increased intracellular solute concentrations, decreased cell size, a weakened cell membrane, and physical cell rupture caused by freezing and thawing. The high frequency of pigment production in recovered isolates from ice cores (Zhang et al., 2008), glaciers (Foght et al., 2004), or marine surface waters (Agogué et al., 2005) suggests that pigmentation plays a role in adaptation to cold environments. Moreover, carotenoid pigments may play a role in the modulation of membrane fluidity in bacteria growing under low temperature conditions (Jagannadham et al., 2000).

Solar radiation is highly variable over a range of scales and wavelengths, and short-wavelength radiation can be more damaging to biological systems than longer wavelengths (Ekelund, 1992; Ross and Vincent, 1998; Cockell and Knowland, 1999). Direct and indirect inhibitory effects of UV-B (280–320 nm) and UV-A (320–400 nm) radiation are well documented (Ekelund, 1992; Häder et al., 1998; Jeffrey et al., 2000; Buma et al., 2001; Agogué et al., 2005; Häder and Sinha, 2005). However, organisms have developed a number of successful strategies to cope with the damage caused by UV radiation. One such approach is known as DNA repair and involves photo-reactivation and dark repair mechanisms (Häder and Sinha, 2005). The second strategy employs UV absorbing or screening compounds such as carotenoid pigments, scytonemin or mycosporine-like amino acids (MAA's) (Cockell and Knowland, 1999). These screening compounds provide a level of protection to phytoplankton (Cockell and Knowland, 1999); and it has been suggested that carotenoid pigments may provide protection in many non-photosynthetic bacteria (Arrage et al. 1993; Zenoff et al., 2006a); however, this has not been empirically tested.

Although adaptation of microbial life to a variety of cold temperature environments has been studied for the past few decades, and detailed information exists about many of their physiological characteristics (Gounot, 1991) and adaptations (Nadeau and Castenholz, 2000; Mueller et al., 2005), our understanding of the effects of environmental stresses on natural bacterial assemblages is still limited. The purpose of this study was to evaluate the role of carotenoid pigments in heterotrophic bacteria as potential cryo- or solar radiation protectants. Carotenoid pigmented and non-pigmented heterotrophic bacterial isolates from Antarctic ecosystems were exposed to freeze-thaw cycles and simulated ambient solar radiation with the effects of these physical stressors examined.

Materials and Methods

SAMPLE COLLECTION AND BACTERIAL ISOLATION

Water samples were collected during the austral summer season 2004/2005 from two Antarctic sites: Pony Lake, which is a shallow, coastal lake located at Cape Royds (77°33′S, 166°00′E)

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TABLE 1

Pigmentation characteristics of Antarctic bacterial isolates associated with growth on R2A media and their closest relatives based on 16S rRNA sequencing.

Isolate	Accession #	Closest relative and accession #	Bacterial group	% ID	Pigment
ANT 11	GU592435	*Flavobacterium segetis* AT1048, AY581115	*Bacteroidetes*	97	orange
ANT 12	GU592436	*Flavobacterium weaverense* AT1042, AY581114	*Bacteroidetes*	99	yellow
ANT 16	GU592437	*Arthrobacter agilis* MB8-13, U85896	*Actinobacteria*	99	dark rose-red
ANT 20	GU592438	*Sphingomonas echinoides* AJ012461	*α-Proteobacteria*	99	yellow
ANT 1	FJ152549	Glacier bacterium FXS9, AY315179	*β-Proteobacteria*	96	none
ANT 3	FJ152551	*Janthinobacterium* sp. Asd M7A2, FM 955878	*β-Proteobacteria*	98	none
ANT 4	FJ152552	*Flavobacterium xinjiangense* AS 1.2749, AF433173	*Bacteroidetes*	99	none

and from a supraglacial fluvial system on Cotton Glacier, which lies in the Transantarctic Mountains (77°07′S, 161°50′E). Both systems experience complete freeze-up during winter and are ice free for several weeks during the summer. Samples were immediately (within 4 hrs) transported back to the Crary Laboratory at McMurdo Station, Antarctica. Bacterial strains were isolated on R2A agar media plates. Briefly, 100 μL of water from each site were streaked onto agar plates and incubated at 4 °C in the dark for 12 days. Single colonies from each site were selected according to morphological characteristics including color, size, and colony shape. These colonies were subsequently subcultured to obtain purified isolates. Isolates were stored in 40% glycerol at −80 °C in the dark. All isolates were tested for catalase activity and Gram stain status.

PHYLOGENETIC ANALYSIS

Bacterial isolates were sent to Laragen (http://www.laragen.com) for 16S rRNA sequence analysis using an Applied Biosystems ABI3730 (http://www.appliedbiosystems.com) automated sequencer. Nearly full length 16S sequences were obtained with multiple reads. Nucleotide sequences were assembled and aligned in BioEdit (Hall, 1999) using the ClustalX function with data obtained from GenBank using the BLAST search tool (BLASTN 2.2.21, ncbi.nlm.nih.gov/BLAST/, Zhang et al., 2000).

PIGMENT EXTRACTION

The steps for pigment extraction preparation were carried out rapidly on ice under minimal light conditions to avoid degradation of pigments. Bacterial isolates from glycerol stocks were incubated on R2A plates at 6 °C in the dark. Cell cultures were harvested and transferred to 1.5 mL 95% methanol (HPLC grade) in sterile Eppendorf tubes. Cell suspensions were gently sonicated in an ice water bath (two 30 sec bursts at 17 W). Subsequently, pigments were allowed to extract for 12 hrs at −20 °C. After extraction the supernatant was collected by centrifugation (5000 rpm for 5 min at 4 °C). If the sample still appeared to be cloudy the centrifugation step was repeated. Absorbance scans (220 to 750 nm) were measured using a spectrophotometer (Nanodrop ND 1000) with a UV-VIS absorbance module. Additionally, bacterial isolates were sent to Horn Point Laboratory (http://www.hpl.umces.edu) for HLPC pigment analysis. HPLC analysis was performed following the protocol and standards outlined in Van Heukelem and Thomas (2001) using a C8 column (Eclipse XDB C8, 4.6 mm × 150 mm, Agilent Technologies), a reverse-phase gradient system with solvent A as 70:30 methanol, 28 mm aqueous tetrabutyl ammonium acetate and solvent B as 100% methanol.

FREEZE-THAW EXPERIMENTS

Bacterial isolates used in this analysis were selected solely on the presence or absence of carotenoid pigmentation. Carotenoid pigmented and non-pigmented isolates (Table 1) from glycerol stocks were enriched in R2A broth media at 6 °C for 5 days at log phase. After enrichment a concentrated bacterial stock solution for each organism was prepared. Five hundred μL of bacterial stock solutions were added to Falcon tubes containing 9.5 mL of R2A broth media, to exclude the stress factor of nutrient limitation while exposed to freeze-thaw cycles. Triplicate broth cultures of each bacterial strain were prepared for each sampling point and subjected to a series of standardized 12-hr freeze-thaw cycles (0, 1, 2, 6, 10, 15, 20, 30, 40, 50, 70, and 100 cycles). Samples were rotated between a −20 °C freezer and a 6 °C dark incubator every 6 hrs. At the end of each thawing period the samples were gently inverted to counteract sedimentation of the bacteria. Triplicate control samples were incubated at 6 °C in darkness. At each sampling point serial dilutions were made in 1× PBS buffer for each cell suspension. One hundred μL of each sample dilution were spread plated onto R2A agar plates. Colony forming units (CFUs) were enumerated, as a measure of the quantity of bacteria that survived the treatment. Based upon individual grow characteristics, plates were incubated at 6 °C in the dark for 3 days for ANT 1, ANT 4; 4 days for ANT 3; 5 days for ANT 11, ANT 12; and 8 days for ANT 16, ANT 20 in order to allow for optimal growth of the organisms.

SOLAR RADIATION EXPERIMENTS

The same bacterial strains used in the freeze-thaw experiments were also used in the solar radiation experiments. Triplicate 10 mL enrichment cultures (9.5 mL broth media plus 0.5 mL stock solution) were prepared as above. Samples were then transferred to 15 mL quartz tubes to ensure full-scale light penetration and sealed with parafilm. The samples were exposed to simulated solar radiation in an environmental solar simulator (Russells Technical Products, Inc., model WMD-230-5-5), using a metal halide (MHG) lamp (K. H. Steuernagel) to simulate solar radiation between 280 and 3000 nm. This lamp produced a dense multiline spectrum of the rare earth, which is comparable to a continuous spectrum of light (http://www.khslight.com). Samples were gently agitated using an orbital shaker. A temperature probe was placed

inside one quartz tube to record the temperature (~4 °C) throughout the experiment. To determine the potential protective role of pigmentation as solar radiation protectants, isolates were exposed to a simulated solar radiation dose of 300 W m^{-2} for 12 hrs. The spectrum can be broken into its individual constituents (UV-B [0.9 W m^{-2}], UV-A [19.9 W m^{-2}], PAR [125.2 W m^{-2}], NIR [110.1 W m^{-2}], and IR-B [43.9 W m^{-2}]). A dosage of 300 W m^{-2} was chosen to reflect ambient Antarctic conditions measured in the nearby McMurdo Dry Valleys (Dana et al., 1998). Samples were analyzed at the beginning of the experiment and after 2, 6, and 12 hrs. Similar to the freeze-thaw experiments, dilutions from triplicate samples were made. One hundred μL of sample was spread plated on agar media plates, incubated, and CFUs were enumerated.

In order to determine potential dark repair mechanisms present in the strains in response to solar radiation damage, irradiated cells were subjected to a longer incubation period of 5 days after the initial count of CFUs. After the 5 additional days, the number of CFUs was enumerated again.

STATISTICAL ANALYSES

For both the freeze-thaw and the solar radiation experiments, the experimental design involved repeated measurements on each strain across experiments (cycles or solar radiation exposure time) and triplicate measurements of each observation. To address this design a mixed model structure, as in Pinheiro and Bates (2004), was used incorporating a random intercept for each bacterial strain in each group (pigmented and non-pigmented, treatment and control groups). This framework allowed us to account for the random variation in the initial CFUs of each bacterial strain (trial) and for comparing different fixed effects (pigmentation and control group vs. treatment, and cycles or exposure time). The random intercept component of the model also induced a correlation structure similar to compound symmetry for the repeated measurements within each group. Compound symmetric correlation is often used in repeated measures models to model all the measurements on a bacterial strain with an equal positive correlation. Additionally, the changes in $\log_{10}$ counts over time were observed to be relatively smooth within each group (pigmented, non-pigmented, controls), which led to the use of a nonparametric trend (Wood, 2006) to capture the change in mean CFUs for each group in the mixed model structure. Penalized cubic regressions splines were used to estimate these smooth effects.

After fitting an initial model for the freeze-thaw cycle experiment, residual diagnostics suggested that the ANT 11 strain is an outlier relative to the other pigmented, treatment observations. Instead of completely removing this unusual observation, we accommodated ANT 11 by placing ANT 11 in its own group (initially we worked with two categories: pigmentation present or absent). Based on the low pigment concentration detected in ANT 11 compared to the other Antarctic bacterial strains (see HPLC analysis) and the categorization of ANT 11 as an outlier in its response to freeze-thaw cycles, we are able to justify our regrouping of strains. Nonetheless, since the other three bacterial strains in each group (pigmented, non-pigmented) showed a similar response to the freeze-thaw cycle regime, we focus on the interpretation of the results on the pigmented group that did not include ANT 11, by estimating a contrast between the treatment response of the pigmented and non-pigmented groups.

For the solar radiation exposure experiment, non-constant variance was detected in the initial model diagnostics. In order to accommodate this additional aspect of the data set, a different variance coefficient was estimated for each of the four different exposure levels and incorporated in the mixed model framework.

Other models, including more typical repeated measures models, were considered as well. However, model selection using Akaike's Information Criterion (AIC) (Akaike, 1973) led to final models which incorporated additive effects and differences based on pigmentation. Further details on the models considered and the statistical methods employed are available in the online Appendix (http://instaar.colorado.edu/AAAR/full_text_access/index.php). All models were estimated using the statistical software package R (R Development Core Team, 2009) and either the nlme package (Pinheiro et al., 2009) or the mgcv package (Wood, 2006).

Results

PHYLOGENY OF BACTERIAL ISOLATES

The phylogenetic characterization of selected bacterial isolates from Antarctic environments is summarized in Table 1. The closest identified relatives were reported as being psychrotrophic strains. Isolates ANT 11 and ANT 12 were most closely related to *Flavobacterium segetis* AT1048 and *Flavobacterium weaverense* AT1042, respectively (Yi and Chun, 2006). *Flavobacterium segetis* AT1048 produced an orange pigmentation and *Flavobacterium weaverense* AT1042 appeared yellow when growing on R2A agar (Yi and Chun, 2006). ANT 16 was most closely related to *Arthrobacter agilis* MB8-13, a dark rose-red pigmented bacterium (Fong et al., 2001). ANT 20 showed highest similarity with the yellow pigmented bacterial strain of *Sphingomonas echinoides* (Denner et al., 1999). Non-pigmented ANT 1 (Foreman et al., in review) was related to Glacier bacterium FXS9 (Foght et al., 2004), ANT 3 (Foreman et al., in review) was taxonomically affiliated with *Janthinobacterium* sp. Asd M7A2 (Vardhan Reddy et al., 2009) and ANT 4 (Foreman et al., in review) were most closely related to *Flavobacterium xinjiangense* AS 1.2749 (Zhu et al., 2003). The isolates used in this study shared similar characteristics (e.g. colony coloration) with their nearest relatives. Furthermore, all isolates were catalase positive and, with the exception of ANT 16, Gram negative.

CHARACTERISTICS OF EXTRACTED PIGMENTS

The UV-VIS absorption spectra of methanol extracts from the Antarctic isolates were measured to determine the presence of pigments. All pigmented strains showed multiple absorption peaks between 400 and 550 nm (Fig. 1), which are characteristic of carotenoid pigments (Chauhan and Shivaji, 1994; Mueller et al., 2005; Du et al. 2006). Maximum absorption peaks for *Flavobacterium segetis* AT1048 and *Flavobacterium weaverense* AT1042, the closest relatives to ANT 11 and ANT 12, were reported at 452 nm and 472 nm and at 451 nm and 479 nm, respectively (Yi and Chun, 2006), thus closely resembling the spectra from our isolates. ANT 16 showed similar absorption spectra as reported for *Arthrobacter agilis* MB8-13 with double *cis*-absorption peaks that are typical of an acyclic or monocyclic chromophore (Britton, 1995a; Fong et al., 2001). ANT 16 also exhibited an additional peak at 320 nm. This peak at 320 nm and similar absorption spectra were found to be carotenoid pigments in *Micrococcus roseus*, a psychrotrophic Antarctic bacterium (Chattopadhyay et al., 1997). It has been reported that members of the *Arthrobacter* and *Micrococcus* lineages are closely related and phylogenetically intermixed (Koch et al., 1994, 1995). The absorption spectra of ANT 20 resembled the ones reported for *Pseudomonas echinoides* (Czygan and

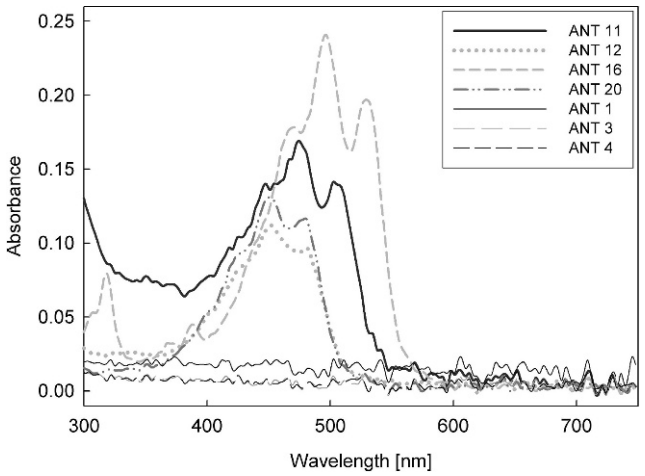

FIGURE 1. Absorption spectra of selected pigmented and non-pigmented heterotrophic Antarctic bacterial isolates.

Heumann, 1967). The yellow pigment in *Pseudomonas echinoides* (reclassified as *Sphingomonas echinoides*; Denner et al., 1999) has been identified as the carotenoid nostoxanthin (Jenkins et al., 1979). The three non-pigmented isolates (ANT 1, ANT 3, and ANT 4) did not have any absorption peaks between 220 and 750 nm. None of the isolates yielded spectral evidence for bacteriochlorophyll, which was also confirmed by HPLC analysis. HPLC analysis further revealed that no pigments were present in ANT 1, ANT 3, and ANT 4. ANT 11 and ANT 12 contained zeaxanthin, and other unknown carotenoids and zeaxanthin-like degradation products, respectively. ANT 16 showed several small carotenoid peaks, all of similar spectra, and ANT 20 had large amounts of an unknown carotenoid similar in spectral signature to zeaxanthin.

FREEZE-THAW CYCLES

Carotenoid pigmented and non-pigmented isolates were exposed to one hundred 12-hr freeze-thaw cycles (Fig. 2). On average, the carotenoid pigmented isolates experienced a slight decrease in viability in response to temperature fluctuations (−20 and 6 °C). In contrast, the viability of non-pigmented bacteria was significantly decreased by the freeze-thaw regime during the first 20 cycles. All three non-pigmented strains responded similarly with a drop in CFUs from 2×10^7 to 1.5×10^4 cells, on average. Over the remaining 80 cycles, viability in the non-pigmented isolates leveled off. Within the carotenoid pigmented group each bacterial strain displayed a unique response to the repeated freeze-thaw cycles. ANT 12 increased in CFUs during the first 50 cycles. CFUs for ANT 16 increased during the entire experiment, whereas CFUs for ANT 20 decreased almost linearly by three orders of magnitude over the time course of the experiment. An exception within the carotenoid pigmented group was ANT 11, and it is unclear why ANT 11 behaved differently from the other pigmented isolates. During the first 6 cycles CFUs for ANT 11 increased by one order of magnitude, but after 40 cycles this strain was no longer culturable. The behavior of ANT 11 was also distinctly different from the other pigmented and non-pigmented isolates in the control group. Whereas all the other bacterial strains increased in CFUs during the 50-day incubation period at 6 °C, ANT 11 remained constant during incubation and dropped approximately two orders of magnitude between 35 and 50 days.

The response of the pigmented isolate ANT 11 to repeated freeze-thaw cycles was not only different within its initially assigned group, but also in comparison to the non-pigmented strains. Since the closest relative to ANT 11 has been designated as a psychrophilic strain with a theoretically minimum growth temperature of −29.7 °C (Yi and Chun, 2006), its low tolerance to the freeze-thaw regime was unexpected. Instead, a comparative study revealed that ANT 11 resembled the decreasing pattern in CFUs of *Escherichia coli* K12 in response to the freeze-thaw cycle regime (data not shown). Consequently, due to its unusual response to freeze-thaw cycles ANT 11 was defined as an outlier and treated as a separate, pigmented 'group' in our analysis.

SOLAR RADIATION EXPOSURE

Exposure of the isolates to ambient levels of Antarctic solar radiation clearly showed that a protective function was conferred on the organisms that possessed carotenoid pigments (Fig. 3). The viability of carotenoid pigmented isolates was higher, on average, when compared to the non-pigmented bacteria after 12 hrs of exposure to 300 W m^{-2} at ~4 °C. After 6 hrs of exposure non-pigmented isolates decreased CFUs from 1×10^7 to 8×10^1. Furthermore, after 12 hrs of exposure the non-pigmented bacteria were no longer culturable. In contrast, the carotenoid pigmented strains showed only a slightly negative response to solar radiation during the first 6 hrs of exposure. ANT 11 and ANT 12 were unaffected by solar radiation exposure after 6 hrs and did not show a significant change in viability. The culturability of ANT 16 and ANT 20 decreased two and four orders of magnitude, respectively, over the same time interval. Although long-term exposure (12 hrs) markedly decreased the viability of the pigmented strains, the organisms were still viable, with CFUs of 2×10^1–4.5×10^3 cells mL^{-1}. Analogous to the freeze-thaw cycle experiments, the non-pigmented isolates all showed a similar negative response to solar radiation, whereas representatives within the pigmented group showed a range of responses. This range of responses within the pigmented isolates supports the idea that carotenoid pigments may function differently in cell membranes. Although 6 hrs of exposure caused damage in two of the carotenoid pigmented strains, the presence of these pigments still conferred an enhanced level of protection from solar radiation when compared to the non-pigmented strains.

After CFUs were enumerated for the final time point, the bacterial isolates were incubated in the dark for an additional period of five days to determine if these organisms could recover from the damage caused by solar radiation. Although damage was observed in both bacterial groups after a 12 hr period of exposure, the carotenoid pigmented isolates achieved higher recovery, on average, compared to the non-pigmented group. The pigmented bacterial strains increased by approximately one order of magnitude over the additional 5 days of dark incubation. With the exception of ANT 11, none of the isolates recovered to their initial numbers at the beginning of the experiments. ANT 11 increased CFUs by four orders of magnitude and achieved almost full recovery to pre-exposure numbers. Conversely, the non-pigmented strains showed almost no evidence of dark repair mechanisms (Table 2).

STATISTICAL ANALYSIS

To address differences between the carotenoid pigmented and the non-pigmented group we applied an additive mixed model using nonparametric fixed effects along with random effects to

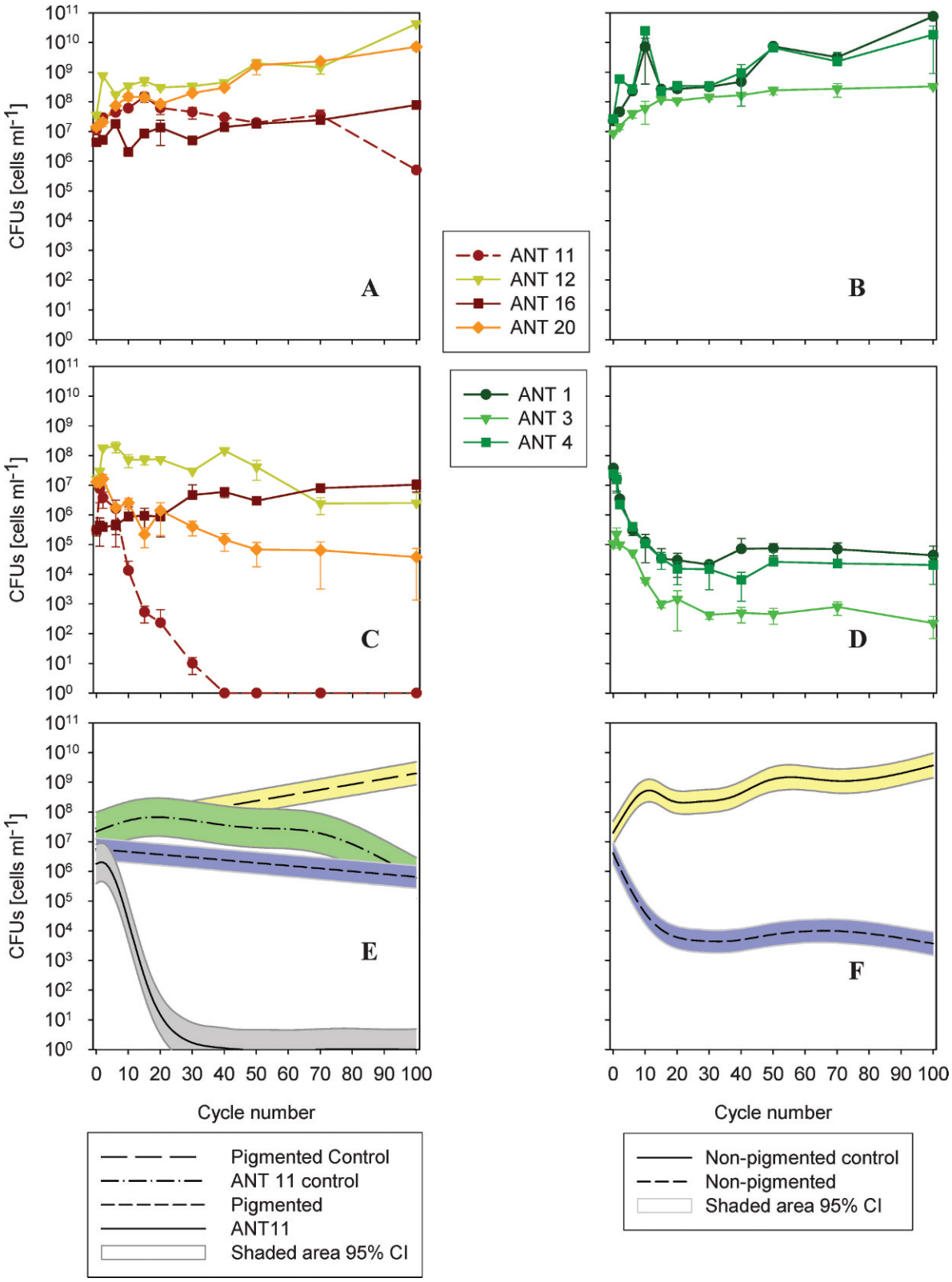

FIGURE 2. Response of bacterial isolates to freeze-thaw cycles. (A and B) Control group. Pigmented (ANT 11, 12, 16, 20) and non-pigmented (ANT 1, 3, 4) isolates incubated for 50 days at 6 °C. (C and D) Treatment effect on pigmented and non-pigmented bacteria strains. (E and F) Additive mixed model results showing the estimated means for the pigmented group and non-pigmented groups. Shaded area shows the approximate 95% confidence intervals (CI) for the estimated mean values obtained from the additive mixed model. Error bars show one standard deviation from the mean.

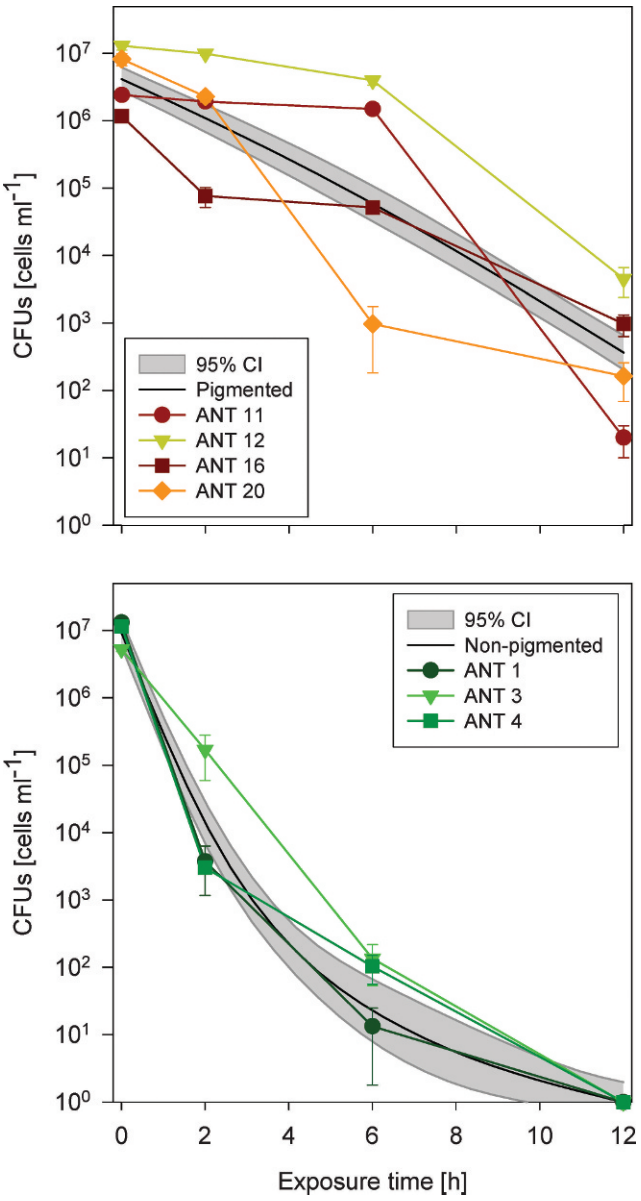

FIGURE 3. Response of bacterial isolates to solar radiation exposure (300 W m^{-2}). Top: Pigmented isolates. Bottom: Non-pigmented isolates. Error bars show one standard deviation from the mean. Shaded area shows the approximate 95% confidence intervals (CI) for the estimated mean values obtained from the additive mixed model.

describe the repeated measurement structure of the experimental design. The favored models evaluated by AIC model selection criterion demonstrate a positive carotenoid pigment effect and provide supportive evidence for the increasing resistance of pigmented bacteria to environmental stressors such as temperature fluctuations and solar radiation. After adjusting for different, random starting points for each bacterial strain, for each experiment the modeled, estimated mean and the approximate 95% confidence intervals are displayed in Figures 2E, 2F, and 3. More details on these models and results are available in the online Appendix (http://instaar.colorado.edu/AAAR/full_text_access/index.php). The different responses of the carotenoid pigmented and non-pigmented groups are illustrated by the estimated effects for each group in each graph. The carotenoid pigmented group displayed a linear trajectory with a slightly negative slope in the

TABLE 2

Response of Antarctic bacterial isolates to exposure to ambient, simulated solar radiation as demonstrated by colony forming units (CFUs), and evidence for dark repair mechanisms as shown by recovery of the isolates after a 5-day incubation period following exposure. CFUs represent counts after 12 hrs of exposure to solar radiation.

Sample ID	CFUs (mL^{-1}) before exposure	CFUs (mL^{-1}) after exposure	CFUs (mL^{-1}) after 5 additional days
ANT 11	2.42×10^6	20	3.26×10^5
ANT 12	1.30×10^7	4517	4.93×10^4
ANT 16	1.17×10^6	975	6100
ANT 20	8.23×10^6	163	280
ANT 1	1.32×10^7	0	10
ANT 3	5.30×10^6	0	0
ANT 4	1.15×10^7	0	20

freeze-thaw cycle experiment (Fig. 2E), whereas the non-pigmented group dropped off precipitously within the first 20 cycles before leveling off (Fig. 2F). Figure 2E also clearly highlights the distinctly different response of ANT 11 to the freeze-thaw cycle regime. In contrast to the slightly linear decrease in the estimated mean for the pigmented group with higher numbers of freeze-thaw cycles, ANT 11 decreased in CFUs exponentially and become undetectable after 30 cycles.

A positive carotenoid effect can also be seen in the model-based estimated means for the two groups (pigmented, non-pigmented) in response to solar radiation exposure. The carotenoid pigmented group in the solar radiation experiment followed a convex path whereas the non-pigmented group showed a pronounced concave curve reaching undetectable levels after 12 hrs of exposure (Fig. 3).

The estimated contrasts between the pigmented (excluding ANT 11) and non-pigmented group in their response to either freeze-thaw cycles or solar radiation exposure are displayed in Figure 4. These contrasts explicitly point towards a positive pigment effect in heterotrophic bacteria by displaying how much larger the CFUs in the pigmented group were compared to the non-pigmented group. With the exception of the starting point, when no treatment was applied, at no point during either treatment did the CFUs of the non-pigmented group exceed the counts of the pigmented group, signifying a remarkably higher level of resistance among the pigmented strains.

Discussion

FREEZE-THAW CYCLES

Temperature and the availability of liquid water are critical parameters for life and set the limits for life processes. Only within a small range, ~5 to 50 °C, have most organisms found their niches (Bölter, 2004). These two factors become even more important when environmental conditions exist at the far extremes. Freezing and thawing, along with changes in nutrient availability and drought, are common processes in cold regions on Earth, including the polar and high-altitude alpine regions. Temperature fluctuations of 30 °C and more may occur within one day in coastal continental Antarctica (Bölter, 2004). Temperature is a physical factor that immediately affects a cell. Thermal stresses have strong impacts on the membrane lipids of organisms that influence membrane structure and function (Hazel, 1995). The transition from the fluid to the gel phase reduces the activity of membrane-bound enzymes, slows the rate of lateral protein

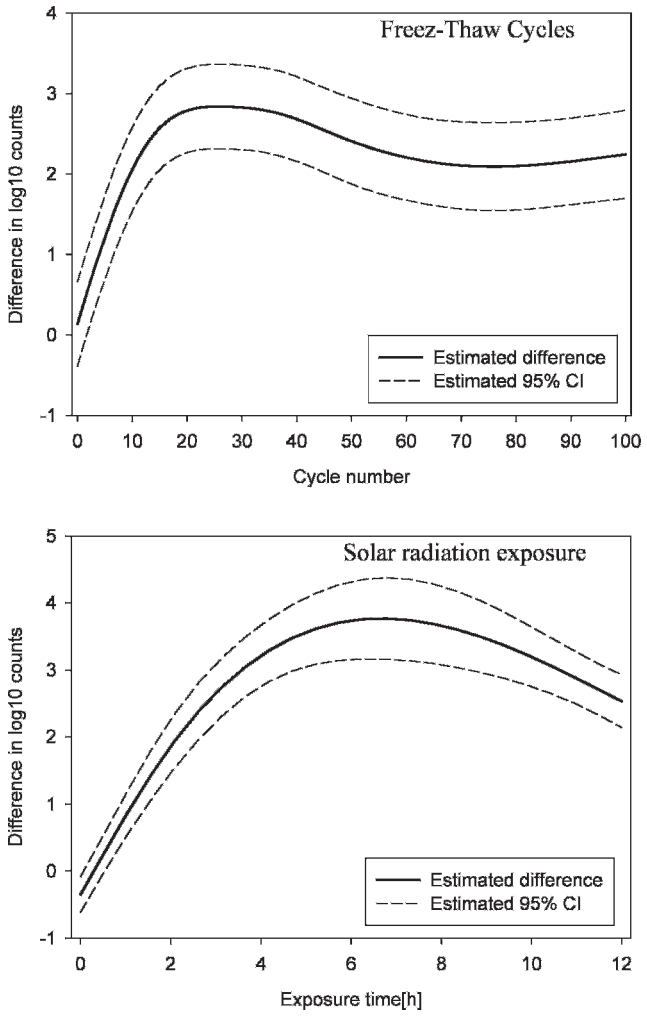

FIGURE 4. Estimated contrast in CFUs between the pigmented and non-pigmented group in their response to the treatments and the estimated 95% confident intervals (CI). Top: Freeze-thaw cycle regime (100 cycles = 50 days). Bottom: Solar radiation exposure experiment (12 hrs). Both models display a positive effect of carotenoid pigmentation in heterotrophic bacteria to increase the resistance to environmental stresses such as freeze-thaw cycles and solar radiation.

diffusion within the plane of the membrane bilayer, and induces cluster formation of integral membrane proteins (Hazel, 1995). Living organisms typically encounter temperature changes throughout their life cycle and most microorganisms can tolerate a variety of changing conditions and stresses in their surrounding environment. Several defense mechanisms exist against temperature stresses, such as the production of cold and heat shock proteins and the alteration of membrane fatty acids (Gounot, 1991). These strategies allow nutrient exchange and enzyme activity to occur, and most importantly prevent intracellular ice nucleation. Recent studies have suggested that pigments such as carotenoids may play a role in the modulation of membrane fluidity in bacteria (Jagannadham et al., 2000). Jagannadham et al. (2000) speculated that when cells grow at low temperatures (5 °C), the unsaturated and branched-chain fatty acids increase the fluidity of the membrane, whereas polar carotenoids counterbalance this effect by stabilizing the membrane. Thus, polar carotenoids confer more rigidity to lipid membranes as compared to non-polar carotenoids. Differences in polarity, configuration, position, or orientation of the pigment that have substantial effects on the properties of the membrane (Britton, 1995b) may contribute to the reduced resistance of ANT 11 to the freeze-thaw cycles compared to the other pigmented isolates. Another possible explanation for the anomalous behavior of ANT 11 may be provided by the HPLC analyses. These analyses showed that ANT 12 contained six times more zeaxanthin pigment than ANT 11, and the unknown peak in ANT 20 was approximately six times larger than even this zeaxanthin peak in ANT 12.

For the closest relatives to the pigmented (ANT 11, ANT 12, and ANT 20) and non-pigmented (ANT 3 and ANT 4) isolates, abundant monounsaturated and branched fatty acids in the membranes have been documented (Denner et al., 1999; Zhu et al., 2003; Yi and Chun, 2006; Vardhan Reddy et al., 2009). Since these membrane modulations were reported for close relatives in both groups, one can speculate that additional factors contributed to the different response to freeze-thaw cycles. It has been demonstrated that lower cultivation temperatures promoted an increased carotenoid production in psychrotolerant bacteria (Jagannadham et al., 2000). Similarly, Yokoyama et al. (1996) reported that bacteria growing under extreme conditions (e.g. low temperatures) have adopted carotenoids suitable for membrane stabilization. Since the bacterial cytoplasmatic membrane must always be maintained at a critical level of fluidity (Chauhan and Shivaji, 1994), the pigments appear to contribute to membrane stabilization by modulating membrane fluidity (Chattopadhyay et al., 1997; Jagannadham et al., 2000; Fong et al., 2001). Furthermore, it has been shown that carotenoid production was greater in cells growing at 5 °C than at 25 °C (Chattopadhyay et al., 1997; Fong et al., 2001). Although the exact function of carotenoids in membranes is still unknown, it has been demonstrated that bacterioruberin binds to membrane vesicles and increases the rigidity of the membrane (Strand et al., 1997). Bacterioruberin is a long chain (C-50) carotenoid and its presence has been identified in *Arthrobacter agilis* MB8-13 (Fong et al., 2001), the closest relative to ANT 16. To date, bacterioruberin has only been isolated from extremophilic bacteria and several archaea (Fong et al., 2001). Our data, and the additive mixed model, contribute to the functional understanding of carotenoids; carotenoid pigmented heterotrophic bacteria tolerate low temperatures and episodic freezing more successfully than non-pigmented bacteria. These findings strongly suggest that carotenoids may act in the modulation of membrane fluidity and contribute to membrane stability, a necessary adaptation to withstand the physiological stress incurred under low or freezing temperatures.

SOLAR RADIATION EXPOSURE

The impact of solar radiation on microorganisms depends upon the spectral composition of the light, the time pattern of exposure, and the presence of protective mechanisms (e.g. UV absorbing compounds, active or passive migration) (Ekelund, 1992; Häder et al., 1998, 2003; Ross and Vincent, 1998; Cockell and Knowland, 1999; Buma et al., 2001; Häder and Sinha, 2005; Agogué et al., 2005). Considering that bacteria may account for up to 90% of the cellular DNA in aquatic environments (Joux et al., 1999) and that these organisms constitute a fundamental link in carbon flow, especially in microbially dominated systems, understanding the influence of solar radiation on bacteria is essential. Results from field studies on marine bacteria indicated that exposure to natural solar UV radiation resulted in a decrease in total cell abundance, a reduction in amino acid uptake, a depression of the activity of enzymes, and a significant inhibition of protein and DNA synthesis (Jeffrey et al., 2000).

Zenoff et al. (2006a) exposed bacterial isolates from high-altitude wetland waters from the Andean region, Argentina, to 4.49 W m^{-2} UV-B irradiance over 36 hrs. A general resistance to UV-B radiation was found among the majority of the culturable community. Most of these bacterial isolates were Gram-positive, spore-forming, and/or showed pigmentation; characteristics that are expected to provide increased adaptation and resistance to UV radiation. With the exception of ANT 16, all of the Antarctic isolates in this study were Gram negative. Further, closest relatives assigned to our Antarctic isolates were non-spore-forming organisms (Koch et al., 1995; Denner et al., 1999; Yi and Chun, 2006), highlighting the important role of pigmentation as solar radiation screening compounds. The large proportion of pigmented bacteria, primarily reds and yellows, found in oceanic surface layers (Tsyban, 1971; Hermansson et al., 1987; Du et al., 2006), may provide indirect evidence of resistance to intense solar radiation, but this protective effect of pigments has never been experimentally demonstrated. In contrast, Agogué et al. (2005) isolated 90 bacterial strains from sea surface layers and underlying waters of two sites in the northwestern Mediterranean Sea, but their results show no correlation between pigmentation, solar radiation levels, and the occurrence of bacteria in the surface microlayer. They argued that pigmentation may only have an indirect effect on the resistance of bacterial cells to solar radiation.

Zenoff et al. (2006b) found a correlation between UV-B resistance and the environment from which the bacteria were isolated. Organisms isolated from environments exposed to high levels of solar radiation showed high UV-B resistance and/or repair abilities compared to those isolated from less irradiated environments. Nevertheless, after a 20 min exposure to UV-B light (3.3 W m^{-2}) only 12–48% survival was observed. Similar results were found by Joux et al. (1999) after exposure of marine bacterial isolates to UV-B light (2.3 W m^{-2}).

Although previous experiments have mainly focused on the inhibitory effect of different fractions of the radiation spectrum, we exposed bacterial strains to simulated solar radiation between 280 and 3000 nm. We assume that complex interactions in the spectral composition may induce cell damage. Hernandez et al. (2004) reported that mortality values resulting from PAR + UV-A + UV-B treatments were higher than those observed under strictly PAR + UV-A treatments. Helbling et al. (1995) demonstrated that the impact of UV-A on the viability of bacterioplankton from Antarctic waters was considerably greater than the impact of UV-B radiation. However, because UV-B wavelengths overlap the absorption spectra of DNA it is expected that UV-B is more responsible for DNA damage. It is therefore assumed that the loss of viability in the UV-A range is due to photodynamic reactions involving reactive oxygen species (Helbling et al., 1995). Our results suggest that carotenoid pigmentation provides a natural sunscreen for bacteria allowing them to tolerate increased solar radiation exposure. Furthermore, carotenoid pigmentation may protect the organism against reactive oxygen species like superoxide and free radicals. Reactive oxygen species (ROS) generated from photochemical reactions have been shown to severely damage cellular structures and the physiology of aquatic microorganisms (Fridovich, 1986). Against these toxic oxygen species, microorganisms possess various defense mechanisms such as antioxidants, quenchers, and scavengers including carotenoids and enzymes (Chow and Chow, 1988). All pigmented and non-pigmented isolates in this study were found to be catalase positive. Catalase is essential for the decomposition of hydrogen peroxide. Kuznetsov et al. (1979) reported an increase in cell lysis of catalase negative isolates, which they attributed to oxygen toxicity. Although both groups, pigmented and non-pigmented isolates, showed catalase activity, they responded differently during the solar radiation exposure experiments. Therefore, we assume that other cell mechanisms, independent from enzyme production, may contribute to additional resistance in the pigmented group. Mikell et al. (1986) found accelerated cell lysis in a carotenoid-negative mutant compared to its pigmented counterpart. They argued that cell lysis resulted from membrane disruption by autocatalytic lipid peroxidation independent of superoxide dismutase activity and catalase production, and that carotenoids in the pigmented isolate quenched intermediates of this process. Although ROS production was not measured during the solar radiation experiments, carotenoids may have positively counteracted cellular damage by toxic oxygen species as previously suggested (Mikell et al., 1986; Clocksin et al., 2007). Hernandez et al. (2002) detected no effect of hydrogen peroxide on the yellow pigmented strain *Arthrobacter* UVvi. Similarly, Arrage et al. (1993) found a higher resistance of pigmented bacterial isolates to reactive oxygen species when compared to non-pigmented strains. Our study shows that after an initial short-term exposure (2 hrs) to a full-scale solar radiation flux of ~300 W m^{-2}, 61% of the carotenoid pigmented isolates survived, as compared to only 0.01% of the non-pigmented isolates. Between 6 and 12 hrs of exposure, non-pigmented bacteria experienced a complete loss of culturability, whereas pigmented isolates were able to tolerate these levels of solar radiation. The additive mixed model confirmed these findings and the positive effect of carotenoid pigmentation. The modeled means of each group showed an inverse shape of the graph, indicating a protective role of carotenoids against solar radiation exposure.

It is well known that microorganisms can enter a viable but non-culturable state (Roszak and Colwell, 1987). Therefore, the loss of culturability of non-pigmented isolates would not necessarily imply mortality. The loss of culturability only implies that an organism is damaged, whereas respiratory chain reactions and morphological integrity may remain almost unchanged (Muela et al., 2000). As a result, bacteria have developed several repair mechanisms to overcome damage. In response to UV radiation, repair mechanisms are usually classified into dark repair and photoreactivation, with recovery strategies differing widely between different bacterial strains (Häder and Sinha, 2005; Zenoff et al., 2006a). Kashimada et al. (1996) proposed an asymptotic model assuming that the reactivation phenomenon (dark repair and/or photoreactivation) follows a saturation first-order reaction and that the inactivation and reactivation curve is a function of time. Agogué et al. (2005) reported that bacterial strains that were highly resistant to solar radiation exposure had a different response with respect to the lag time of the growth curve. After 5 hrs of exposure the lag time could last 24 hrs longer as compared to normal growth.

If organisms have developed mechanisms to compensate for DNA damaging effects of solar radiation it is more likely that the DNA damage in the three non-pigmented strains was accumulated over the time interval of the experiment and that the repair mechanisms were insufficient to undo the damage. Furthermore, enzyme based repair mechanisms, like DNA repair, are generally temperature dependent and colder temperatures decrease the efficiency of these repair mechanisms (Ross and Vincent, 1998; Buma et al., 2001). Nevertheless, under cold incubation conditions (~6 °C) carotenoid pigmented isolates in our experiment recovered more effectively than the non-pigmented isolates. Consequently, our data suggest that pigmentation attenuated the intensity of DNA damage, although, with the exception of ANT 11, none of the isolates were able to reach their initial abundance.

Summary

In this study we demonstrate that carotenoid-pigmented, heterotrophic bacteria, isolated from Antarctic lake and river systems, are more resilient to environmental stressors than are non-pigmented bacteria. We applied common physical stressors such as freeze-thaw cycles and solar radiation. In each treatment the culturability of non-pigmented bacterial strains was more affected than of the pigmented group. These distinctly different behavioral patterns and the positive pigmentation effect were clearly highlighted by the applied additive mixed model. Although the bacterial isolates, especially within the non-pigmented group, exhibited a similar response to the treatments, generalizations should be handled with care, since the sample size in each group (pigmented and non-pigmented) was small. The unique behavior of the bacterial isolate ANT 11 during the freeze-thaw regime emphasizes the need for caution. Although pigmented, the response of this bacterial strain resembled neither the general response pattern detected in the pigmented group, nor the one in the non-pigmented group and eventually became unculturable after 30 cycles. Nonetheless, when exposed to ambient simulated solar radiation, ANT 11 showed a similar resistance to radiation as detected for the other pigmented strains.

Our results indicate that carotenoid pigmentation increases the resistance of heterotrophic bacteria to environmental stressors. Differences in the response to the physical stressors and the varying extent of the treatment effects on pigmented strains suggest that numerous factors such as pigment structure, their location within the membrane, and pigment concentration may play a role in protecting cells against these stresses.

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