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Survival of *Pseudomonas aeruginosa* exposed to sunlight resembles the phenom of persistence

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ABSTRACT

During exposure of *Pseudomonas aeruginosa* stationary phase cells to natural solar radiation, a reduction in the rate of loss of bacterial viability was observed when survival fractions were lower than 1/10,000. This reduction was independent of the growth medium used and of the initial bacterial concentration, and was also observed when irradiation was performed with artificial UVA radiation (365 nm, 47 W m^{-2}). These results indicate the presence of a small bacterial subpopulation with increased tolerance to radiation. Such a tolerance is non-heritable, since survival curves comparable to those of the parental strain were obtained from survivors to long-term exposure to radiation. The radiation response described here resembles the phenomenon called persistence, which consists of the presence of a small subpopulation of slow-growing cells which are able to survive antibiotic treatment within a susceptible bacterial population. The condition of persister cells is acquired via a reversible switch and involves active defense systems towards oxidative stress. Persistence is probably responsible for biphasic responses of bacteria to several stress conditions, one of which may be exposure to sunlight. The models currently used to analyze the lethal action of sunlight overestimate the effect of high-dose irradiation. These models could be improved by including the potential formation of persister cells.

1. Introduction

Modeling survival of bacteria exposed to radiation allows the use of numerical parameters to describe, and to some extent predict, the response of these organisms. This approach could help to evaluate the effectiveness of disinfection treatments based on the action of radiation, and therefore improve the models used is of interest. In preliminary experiments, performed to evaluate the effect of solar radiation on the ability of *Pseudomonas* sp. to form biofilms, we found that the models currently used to analyze the bactericidal effect of sunlight failed to predict this effect when the expected surviving fractions were near the detection limit of the counting method used. This observation led us to consider whether these models were suitable for analyzing our results.

Most of the bactericidal effect of solar radiation is produced by its UVA component [1,2]. The mechanism responsible for the lethal action of UVA on bacteria remains unclear to date [3], and a precise model that describes this action is unavailable. In practice, several mechanistic and empirical models have been applied in studies concerning the effect of sunlight on bacteria. The equations corresponding to several of them are shown in Table 1. Models based on the target theory for the biological action of radiations [4], have been used to describe exponential survival curves, as well as curves with an initial portion exhibiting unchanged viability (called shoulder) followed by exponential decay [5,6]. The target theory assumes that a cell contains one or more sensitive volumes, called targets, and loses its ability to divide due to events of energy deposition, called hits, occurring at this target (or targets). No assumption is made about the chemical structure or biochemical function of the target. An exponential survival curve is expected if cell death is produced by a single hit, and this case is described by the "single-hit single-target" model (Eq. (1)), where the parameter q is inverse to the dose required for $1/e$ (37%) surviving fraction. The "single-hit multiple-target" model (Eq. (2)) predicts survival curves with exponential decays preceded by shoulders, and assumes that cells survive until a certain number of hits are accumulated, and die when this number is exceeded. The parameter n is proportional to this number of hits, and k is inverse to the dose required for $1/e$ surviving fraction, during the exponential decay beyond the shoulder. A combination of the above models, designated "target theory" model (Eq. (3)) in the present study, assumes that in an irradiated bacterial population, some cells lose viability with kinetics similar to those predicted by the

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Table 1
Equations corresponding to the models used.

Model	Equation[a]	Eq. num.	Reference
Single hit-single target	$\log(N/N_0) = \log(e^{-qt})$	(1)	[4]
Single hit-multiple target	$\log(N/N_0) = \log(1-(1-e^{-kt})^n)$	(2)	[4]
Target theory	$\log(N/N_0) = \log(e^{-qt}) + \log(1-(1-e^{-k})^n)$	(3)	[4]
Linear-quadratic	$\log(N/N_0) = -a\,(b\,t + c\,t^2)\log(e)$	(4)	[4]
Log-linear with shoulder	$\log(N/N_0) = \log(e^{-kt}((1+d)/(1+d\,e^{-k})))$	(5)	[10]
Weibull type	$\log(N/N_0) = -(t/m)^p$	(6)	[11]

[a] In these expressions N/N_0 is the survival fraction after an irradiation time t.

"single-target single-hit" model and, in a simultaneous process, damage accumulates, killing other cells with a kinetics comparable to those predicted by the "multi-target model" model. The "linear-quadratic" model (Eq. (4)) assumes that DNA is the target for radiation and the occurrence of a double strand break is the event responsible for the loss of viability. The parameter a is a biological effectiveness factor for double strand breaks. The other parameters lump together several constants related with the probability of double strand breaks produced by a single energy deposition event (b), or by single strand breaks associated in both space and time (c). This model has not been used in the analysis of the effects of sunlight on bacteria, but the predicted survival curves seem compatible with the experimental results. On the other hand, empirical models developed to describe the effects of mild heat treatments on bacteria were also used to analyse the bactericidal action of solar radiation [7–9]. These models make no assumptions about the action mechanism of the radiation or the physical meaning of the parameters involved. In the case of the model designated "log-linear with shoulder" (Eq. (5)), an adjustment function based on the Michaelis–Menten kinetics is introduced to account for the shoulder preceding the log-linear portion of the curves [10], and the model is compatible with the assumptions of the target theory, as well as with alternative interpretations like the presence of an intracellular or extracellular protector compound which is destroyed during irradiation. The shape usually observed in the survival curves obtained for bacteria exposed to sunlight suggests that another empirical model, originated from the Weibull frequency distribution and proposed by Mafart and coworkers [11], could be used. This model was designated "Weibull type" model (Eq. (6)) in this study. The reader is referred to citations in Table 1 for details about the derivation of the equations shown there, and the physical meaning of the parameters involved.

The application of the models included in Table 1 for the analysis of the responses of *Pseudomonas aeruginosa* to solar and UVA radiation revealed that, at high doses, the real effect of the radiation deviates significantly from the predictions made by all of these models. The present paper describes this observation, proposes an interpretation based on recent findings in the field of bacterial physiology, and suggests its potential implications for the use of solar radiation in order to improve the microbiological quality of water.

2. Materials and methods

2.1. Bacterial strains and growth conditions

P. aeruginosa ATCC27853 was used throughout. Bacteria from stock cultures (kept on nutrient agar plates at 7 °C) were loop inoculated in M9 minimal medium (1 g NH_4Cl, 0.5 g NaCl, 3 g KH_2PO_4, 6 g Na_2HPO_4, 0.2 g $MgSO_4{\cdot}7H2O$, per liter) with 0.015 M DL-sodium succinate as the carbon source, or in Nutrient broth (8 g Difco nutrient broth, 5 g NaCl, per liter), and incubated for 24 h at 37 °C with shaking in the dark. An aliquot of the stationary phase culture obtained in this way was diluted in 500 volumes of the same medium and incubated for 22 h under the same conditions. Cells in stationary phase were harvested by centrifugation (8000 g, 8 min, 20 °C), washed twice with M9 without a carbon source, and suspended in the same medium. The bacterial concentration was approximately 4×10^8 colony forming units ml^{-1} (optical density at 650 nm 0.1), except in a few assays carried out with suspensions diluted to 1/10 of this initial concentration. Suspensions obtained in this way were maintained in the dark for an hour at room temperature, and then used in the sunlight and UVA response assays.

2.2. Sunlight irradiation

The irradiation device and the protocol used, as well as the procedures for irradiance and sample temperature measurements, have been described previously [6]. Bacteria suspended in M9 minimal medium without a carbon source were transferred to the irradiation device and bubbled with air in the dark for 15 min before the start of irradiation. Then they were exposed to sunlight in 1 cm path quartz spectrophotometer cells ($1 \times 1 \times 4.5$ cm) placed in an aluminum holder which covered their top, bottom, and sides but left their front and back faces free. The temperature of the samples was controlled by water circulation through the holder, which was equipped with an equatorial mount in order to maintain the front faces of the quartz cells perpendicular to the direction of the sunlight during irradiations. The assays were performed on the roof of the laboratory (34°34′S 58°30′W) on cloudless days at noon.

2.3. Artificial UVA irradiation

The radiation source was a bench with 6 Philips TDL 18W/08 tubes located 0.5 cm above the bacterial suspension. At the level of the free surface of the bacterial suspensions, the fluence rate was 47.5 W m^{-2} as measured with a 9811-58 Cole-Parmer radiometer (Cole-Parmer Instruments, Chicago, IL, USA). According to the information provided by the manufacturer, over 95% of the emissions from Philips TDL 18W/08 tubes are at 365 nm. Thirty ml of bacterial suspension, prepared as described in Section 2.1, were transferred to a disposable Petri dish (9 cm diameter) which was placed in the irradiation device using a stainless steel holder. The holder was cooled by water circulation from a thermostatic bath maintaining the temperature of the sample at 22 °C. During the irradiations, the suspension was aerated by slow magnetic stirring, which also ensured a homogeneous distribution of the cells in the irradiated volume. Another portion of the suspension prepared for irradiation was kept under the same conditions except for the radiation exposure and used as dark control from irradiation.

2.4. Enumeration procedure and data analysis

At the onset of the experiments and at regular intervals during irradiations, samples were removed and diluted in decimal steps with M9 minimal medium without a carbon source. Aliquots of