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SHORT COMMUNICATION

Use of Amplification Fragment Length Polymorphism to Genotype *Pseudomonas stutzeri* Strains Following Exposure to Ultraviolet Light A

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Abstract

Changes in ultraviolet light radiation can act as a selective force on the genetic and physiological traits of a microbial community. Two strains of the common soil bacterium *Pseudomonas stutzeri*, isolated from aquifer cores and from human spinal fluid were exposed to ultraviolet light. Amplification length polymorphism analysis (AFLP) was used to genotype this bacterial species and evaluate the effect of UVA-exposure on genomic DNA extracted from 18 survival colonies of the two strains compared to unexposed controls. AFLP showed a high discriminatory power, confirming the existence of different genotypes within the species and presence of DNA polymorphisms in UVA-exposed colonies.

Key words: *Pseudomonas stutzeri*, AFLP, polymorphisms, UVA exposure

Microorganisms play a pivotal role in regulating fundamental biogeochemical processes in terrestrial and marine systems. Changes in ultraviolet light (UV) radiation, soil warming and desiccation may be strong selective forces acting on the phylogenetic and physiological composition of microorganisms. We chose to focus on *Pseudomonas stutzeri* since it is a non-fluorescent denitrifying bacterium with a wide distributional range and it occurs as an opportunistic pathogen in humans (Lalucat *et al.*, 2006). *P. stutzeri* is a Gram-negative bacterium known for its high propensity nitrogen fixation and opportunistic pathogenicity. In addition, *P. stutzeri* strains have also been described to exert xenobiotic degradation capacity, mainly naphthalene and other similar aromatic compounds (Brunet-Galmés *et al.*, 2012). In fact, several strains of this species are involved in nitrogen fixation, whilst others participate in the degradation of pollutants or interact with toxic metals, processes that could be influenced by environmental factors (Lalucat *et al.*, 2006). This species was also recently isolated from the oral cavity of asymptomatic health care workers, suggesting the possibility that *P. stutzeri* may behave as a commensal strain occasionally tuning pathogenic in immunocompromised patients (Lima *et al.*, 2015).

In this study, two *P. stutzeri* strains with different origins and optimal growth temperatures were used to assess bacterial viability following exposure to UV radiation.

In addition, we evaluated the applicability of amplification length polymorphism analysis (AFLP) as a tool for genotyping *P. stutzeri* strains.

P. stutzeri DSM 7136, isolated from aquifer cores and with an optimum growth temperature of 30°C, and type strain DSM 5190, isolated from human spinal fluid, with an optimum growth temperature of 37°C were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Germany).

Bacterial cultures were grown in Nutrient Broth (Liofilchem S.r.L., Teramo, Italy) and incubated with shaking at their optimal temperatures until the exponential growth phase was reached ($O.D._{\lambda 600\ nm}$ 0.7). Strains were maintained on Nutrient Agar at 4°C (Liofilchem S.r.L., Teramo, Italy) for the duration of the study.

The effect of ultraviolet light B (UVB) (280–315 nm) and ultraviolet light A (UVA) (315–400 nm) radiation was evaluated by *P. stutzeri* survival following different exposure lengths and periods. The UV-light-generating lamps consisted of two fluorescent narrowband tubes emitting in the 340 nm band (UV-A TLD 15 W,

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Philips, Milano, Italy) or in the 280–315 nm range (UVB TL 20W/01 RS SLV, Philips, Milano, Italy), respectively. We calibrated the UV lamps measuring their potency at a distance of 30 cm from the biological sample (UVA = 11.6 W/m^2; UVB = 5.26 W/m^2) by a radiometric-calibrated detection system consisting of an InstaSpec IV CCD detector head coupled to an MS125 spectrograph (Oriel Instruments, Milano, Italy). Experiments were set up by plating aliquots (100 µl) of bacterial suspensions obtained from cultures of both *P. stutzeri* strains in exponential growth phase, adjusted to a concentration of 2.7×10^8 cell/ml and serially diluted (10^{-1} to 10^{-5}). Plates were immediately exposed for 15, 30, 60 and 90 minutes to UVB light, using an UVB lamp (5.65 W/m^2) positioned at 10 cm distance from the plates. Non-exposed replica plates represented negative control. Following an 18 h incubation at 30°C and 37°C for *P. stutzeri* DSM 7136 and DSM 5190 respectively, CFU counts revealed the expected CFU numbers in the unexposed plates, but no colonies were detectable on the UVB exposed plates, at any of the exposed times examined (data not shown). Previous data published on several Gram-negative bacteria showed a decreasing survival rate following irradiation with UVB and ultraviolet light C (UVC), with the higher survival rates observed post-exposure to UVA (Santos *et al.*, 2013).

Experiments were then repeated using a UVA lamp (11.6 W/m^2), positioned at 10 cm distance from the samples, with the aim to determine the appropriate exposure period resulting in approximately 90–95% killing of bacterial cells (Fig. 1). Plates were incubated overnight at the appropriate temperatures. Colony forming units (CFU) were then counted on each plate.

Surviving bacteria (4–16%) were recovered following a 22-minute exposure, with the environmental strain showing the highest UVA susceptibility ($P < 0.0001$ following unpaired *t*-test, Fig. 1). Further analysis encompassing a wider set of clinical and environmental strains will be necessary to confirm whether environmental isolates are more susceptible to UVA selective pressure.

Nine single colonies and one non-exposed colony (control) per strain were used for genomic DNA extraction and amplification fragment length polymorphism (AFLP) analysis. Genomic DNA was extracted from 5 ml late exponential-phase cultures of *P. stutzeri* as described by Grüntzig *et al.* (2001), with minor modifications. Genomic DNA was extracted from lysed cells following three phenol chloroform-isoamyl alcohol treatments. An equal volume of the chloroform-isoamyl alcohol mixture was added to the recovered aqueous layer (24:1, vol/vol). Nucleic acids were then precipitated with 1 ml isopropanol and 0.3 M ammonium acetate. Following a 30 min incubation at room temperature, precipitated DNA was recovered by centrifugation. The DNA pellet was washed once with 70% ethanol and suspended in 50 µl of TE, pH 8.

The presence of genomic DNA modifications in *P. stutzeri* strains following UVA exposure was evaluated by AFLP analysis. This technique is a high-resolution genotyping method that allows evaluation of strains relatedness as well as strain replacement/maintenance and the extent of micro-evolutionary events (Tavanti *et al.*, 2007; Xiang *et al.*, 2010; Vos *et al.*, 1995). However, to the best of our knowledge, this molecular method has not yet been applied to characterize *P. stutzeri* strains. Preliminary *in silico* analysis of AFLP applied to the genome sequence of *P. stutzeri*

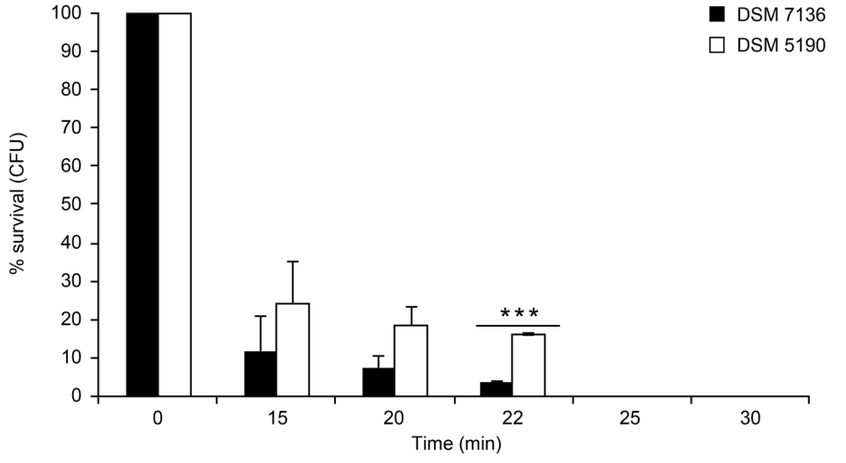

Fig 1. *P. stutzeri* survival following different exposure times to a UVA lamp (11.6 W/m^2), positioned at 10 cm distance from Petri dishes. Two different strains of *P. stutzeri* DSM 5190 (isolated from human spinal fluid) and DSM 7136 (isolated from aquifer cores) were tested. Data are expressed as mean ± standard error of mean of 4 independent experiments. ***$P < 0.0001$.

A1501 assisted us in the selection of the appropriate set of restriction enzymes, adaptors and primers available. Among those, HindIII-0 (5'-GACTGCGTAC CAGCTT-3') and Cy5-labelled EcoRI-0 (5'-GACT GCGTACCAATTC-3') primers were chosen to perform AFLP. Genotyping by AFLP was performed on 60 ng of genomic DNA as previously described (Tavanti *et al.*, 2007). The restriction enzyme combination EcoRI/HindIII was used in the first restriction/ligation step, with a HindIII (5'-AGCTGGTACGCAGTC-3') and EcoRI (5'-CTCGTAGACTGCGTACC-3') adaptor concentration of 0.45 μM. Pre-selective, selective amplifications and gel electrophoresis conditions were performed as previously described, using Eco0/HindIII0 primer combination (Hensgens *et al.*, 2009). AFLP profiles, ranging from 100 to 900 bases, were exported as a TIFF file and analyzed with the TotalLab TL120 software package (Nonlinear Dynamics Ltd, UK). DNA bands obtained for each isolate were size-matched. Each AFLP fragment was analysed and labelled by time and by the surface of the fluorescent peak it formed. Background fluorescence was subtracted from each lane and the surface of each peak was determined to better quantify each AFLP fragment. The sum of all peak surfaces for each AFLP profile was defined as "lane volume" and set as 100% and peak surfaces were expressed as a percentage of the "lane volume". Only those fragments, which were at least represented as 0.5% of the lane volume, in at least one of the isolates, were included in the analysis (Hensgens *et al.*, 2009). Consequently, bands with a relative intensity of less than 0.03% of the lane volume were not included in the analysis.

Computerised analysis of the profiles obtained clearly indicated that the two isolates have completely different AFLP-genotypes, demonstrating that this technique can be successfully applied to molecular typing of this bacterial species. This finding is in agreement with previous data obtained using different methods, demonstrating the existence of several genomovars within the *P. stutzeri* species (Sikorski *et al.*, 1999; Sikorski *et al.*, 2005). In particular, strain *P. stutzeri* DSM 5190 is described as belonging to genomovar 1, while *P. stutzeri* DSM 7136 was assigned to genomovar 9 (Sepúlveda-Torres *et al.*, 2001). Indeed, the two isolates shared only few bands (n = 8) out of the approximately 100 fragments obtained for each strain (Fig. 2). Such a low number of fragments shared by the two *P. stutzeri* patterns could be statistically expected for unrelated species (Krauss,

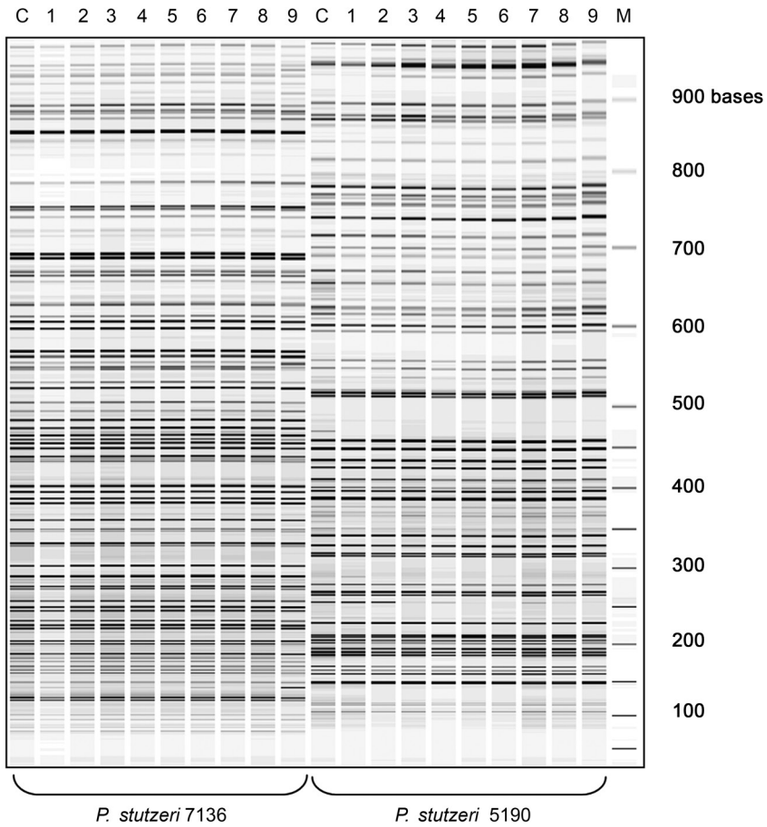

Fig 2. AFLP profiles of *P. stutzeri* DSM 7136 and DSM 5190 strains.
Lanes 1–9, patterns obtained from UVA-exposed independent colonies; Lane C, unexposed control colonies.
M – molecular weight marker.

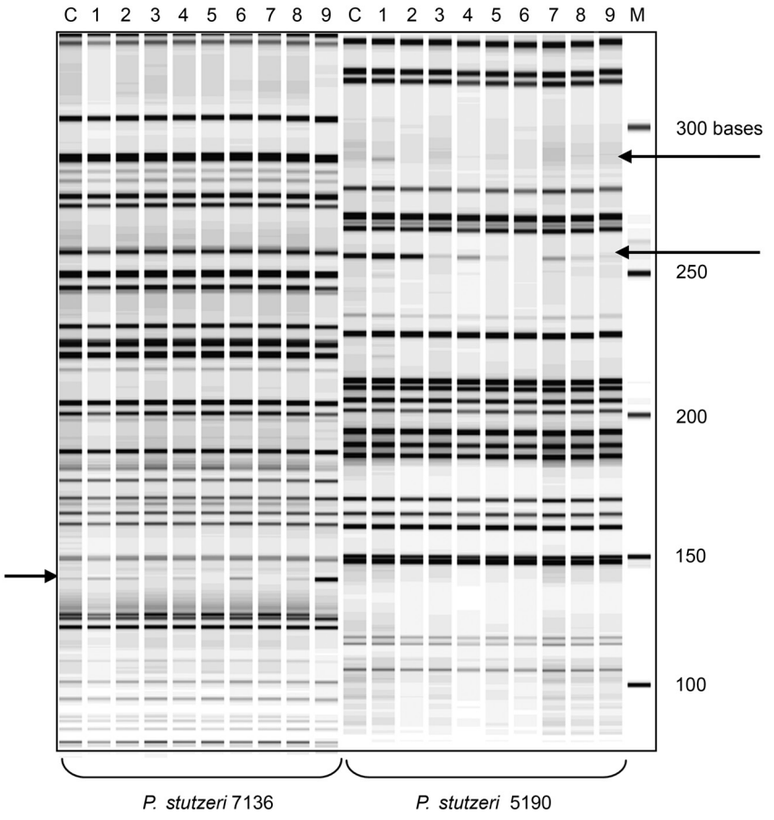

Fig 3. AFLP profiles within the range of 100–300 bases, showing minor changes (indicated by black arrows) in DNA patterns obtained from independent UVA-exposed (lanes 1–9) and unexposed control colonies (lane C) for both *P. stutzeri* isolates. M – molecular weight marker.

2000). Therefore, AFLP profiles indicates a high degree of genetic diversity, which could be in agreement with the definition of "genomovar", a term coined to describe different genomic species lacking of discriminative phenotypes (Lalucat *et al.*, 2006). However, more strains belonging to different genomovars should be included in the analysis to confirm this hypothesis.

As expected, AFLP profiles were highly similar in all selected UVA-exposed colonies and control unexposed colony within each strain. However, for both strains minor differences could be observed in the AFLP profiles following UVA exposure (Fig. 3). In addition, the two different *P. stutzeri* isolates showed different polymorphic sites in their respective AFLP profiles, which were not shared by all colonies analysed per strain. This could confirm the genetic diversity of the two isolates used in this study, although we cannot exclude the possibility that these polymorphisms may have also arisen naturally by culturing the bacteria over time and in the absence of UV-induced stress.

The results presented here confirm that AFLP is a robust typing technique, which can be successfully applied for the molecular characterization of *P. stutzeri* strains. In fact, different genomovars gave distinctive AFLP patterns. In addition, AFLP profiles obtained from UVA-exposed colonies of both *P. stutzeri* strains showed the presence of polymorphisms, which could be related to UVA exposure. However, due to the limited number of colonies screened it is not possible to ascertain the origin of these polymorphisms.

Given the involvement of both of UVA and UVB in bacterial inactivation, it will be very interesting to determine the nature of the observed polymorphisms. Future studies will be performed to investigate further the molecular mechanisms by which polymorphisms in this species arise, in order to better understand the effect of natural light on bacterial populations.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AT and LL designed the study. RS and CR processed the data, MZ and CGE completed data analysis. AT wrote the manuscript. All authors read and approved the final manuscript.

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