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Increased production of outer membrane vesicles by cultured freshwater bacteria in response to ultraviolet radiation

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ABSTRACT

Secretion of membrane vesicles is an important biological process of both eukaryotic and prokaryotic cells. This process has been characterized in pathogenic bacteria, but is less clear in non-pathogenic bacteria from aquatic ecosystems. Here, we investigated, for the first time, the process of formation of outer membranes vesicles (OMVs), nanoscale vesicles extruded from the outer membrane (OM) of gram-negative bacteria, in cultures of freshwater bacteria after exposure or not to ultraviolet radiation (UVR) as an environmental stressor. Non-axenic cultures of freshwater bacteria isolated from a Brazilian aquatic ecosystem (Funil reservoir) were exposed or not to UVR (UVA + UVB) over a 3 h period, during which cell density, viability and ultrastructure were analyzed. First, we showed that UVR induce bacterial death. UVR triggered significant negative effect on cell density after 3 h of UVR treatment. This decrease was directly associated with cell death as revealed by a cell viability fluorescent probe that enables the distinction of live/dead bacteria. Transmission electron microscopy (TEM) revealed changes indicative of cell death after 3 h of UVR exposure, with significant increase of damaged cells compared to the control group. Second, we demonstrated that gram-negative bacteria release OMVs during normal growth and after UVR exposure. OMVs were clearly identified as round, membrane-bound vesicles budding off from the bacterial OM as isolated or clustered vesicles or free in the extracellular medium. Remarkably, quantitative TEM analyses showed that bacteria respond to UVR with increased formation of OMVs. Moreover, while OMVs numbers per intact or damaged cell did not differ in the untreated group, UVR led to a higher vesiculation by bacteria in process of death. This means that degenerating bacteria release OMVs before lysis and that this secretion might be an adaptive/protective response to rapid changes in environmental conditions such as UV radiation.

1. Introduction

In recent years, the extracellular release of membrane-bound vesicles by prokaryotic cells has become the subject of great interest. These nanoscale vesicles (20–300 nm) are extruded from the outer membrane (OM) of gram-negative bacteria and have been associated with varied biological processes that resemble those used by higher organisms, such as pathogenesis (Kolling and Matthews, 1999; Rivera et al., 2010), cellular defense (Manning and Kuehn, 2011; Baumgarten et al., 2012), cell communication (Mashburn and Whiteley, 2005) and DNA transfer (Rumbo et al., 2011; Pérez-Cruz et al., 2013). It is now recognized that outer membrane vesicles (OMVs) can store and transport a broad repertoire of cargo derived from bacterial periplasm and cytoplasm. Thus, vesicular transport represents a relevant signal trafficking system in prokaryotes (reviewed in Kulp and Kuehn, 2010; Haurat et al., 2015).

The current knowledge on the biogenesis of OMVs and their proposed roles in bacteria are mostly based on different models of pathogenic species (reviewed in Kulp and Kuehn, 2010; Haurat et al., 2015). Much less known are the ability of bacteria from aquatic ecosystems to secrete OMVs, their abundance and potential functions in aquatic biology. In marine microbial communities, bacteria-derived vesicles were reported to be abundant in coastal and open-ocean seawater samples and implicated in marine carbon flux (Biller et al., 2014). Recently, we have provided the first evidence that bacteria from freshwater ecosystems produce OMVs

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in their natural environment (Silva et al., 2014). In studying the ultrastructure of aquatic bacteria from water samples collected from varied tropical freshwater sites, we identified typical bilayer membrane vesicles budding off from the bacteria OM, a morphological indicative of a distinct secretory process into the environment (Silva et al., 2014).

Here, we investigated the process of OMV formation in cultures of freshwater bacteria after exposure to the ultraviolet radiation (UV) as an environmental stressor (Häder and Sinha, 2005; Kultz, 2005). It has been demonstrated that UV induces adverse effects on the dynamics of aquatic bacteria (Paul et al., 2012; Hunting et al., 2013; Garcia-Corral et al., 2014) and lead to damages on their cellular structures and DNA (Santos et al., 2013; Strickler et al., 2015). The UVA and UVB radiation spectra (UVR) are the most significant to the bacterial alterations on Earth, since UVC spectrum is totally absorbed by ozone layer on atmosphere (Diffey, 1991). Recently, the UV effects on aquatic ecosystems have been considered more prominent due to the ozone depletion and other factors associated with climate changes, such as increasing temperatures and precipitation (reviewed in Häder et al., 2011; Häder et al., 2015).

By using transmission electron microscopy (TEM), including quantitative TEM and approaches for cell density and viability evaluations, we demonstrated, for the first time, that OMVs are naturally secreted by freshwater bacteria into the culture medium during normal growth and that the release rate of OMVs significantly increased in response to cell viability changes induced by UVR. The amplified bacterial ability to release OMVs may be associated with adaptive responses to rapid changes in environmental conditions.

2. Materials and Methods

2.1. Aquatic bacteria cultures

Cultures of aquatic bacteria were established from water samples collected directly from the subsurface (0.5 m; pH 7.4; 28 °C) of the *Funil* reservoir, an aquatic ecosystem located in southwest of Brazil (Supplementary Fig. 1). Funil Reservoir is a hydropower reservoir 22° 30′ S, 44° 45′ W, altitude 440 m, Cwa climate (i.e. warm temperature, dry winter, hot summer in the Köppen climate classification system) with 16 800 km^2 of catchment area, a surface area of 40 km^2, mean and maximum depths of 22 and 70 m (total depth at the sampling station 50 m), respectively, a total volume of 890×10^6 m^3 and a retention time of 25 to 80 d. Funil Reservoir was constructed at the end of the 1960s by damming the *Paraíba do Sul* River (Soares et al., 2009). No specific permissions were required for the water samples collected from this reservoir. Our field studies did not involve endangered or protected species.

Briefly, serially diluted (1:10, 1:100, 1:1000, 1:10000) water samples were spread onto two different non-selective solid culture media: Reasoner's 2A agar (R2A, Himedia®, Mumbai, India) and trypticase soy agar (TSA, BD, Sparks, MD), and incubated at 28 °C. This temperature was chosen based on the temperature assayed at the sampling point. R2A is a nutrient-poor medium, whereas TSA is a nutrient-rich medium. The use of culture media with different nutrients concentration enables the recovery of a broad heterotrophic bacteria variety from aquatic environments (Rice et al., 2012). After visible growth, individual colonies morphotypes were then harvested from plates and transferred onto a new plate. This process was repeated two to three times in order to isolate individual bacterial colonies (Scott et al., 2012). Bacterial isolates from both culture media were transferred to trypticase soy broth media (TSB, BD, Sparks, MD) and incubated at 28 °C. After incubation (24 h), aliquots (1.5 mL) from growth cultures were taken and mixed in glycerol (final concentration of 30%) to constitute stock bacterial isolates that were stored at −80° C for further use in the experiments as below.

2.2. UV exposure

Prior to experiments, bacterial frozen isolates were thawed and streaked onto a plate containing TSA. Solid cultures were grown at 28° C until observation of visible growth. After, colonies were extracted from plates and grown in TSB liquid media overnight at 28 °C with 225 rpm shaking. For all experiments the cultures were re-suspended in 40 mL of TSB medium at an initial concentration of 10^6 cells/mL. The UV exposure was performed using the same intensity and procedures as previous work (Noyma et al., 2015). Briefly, cultures in borosilicate glass Erlenmeyers were submitted to artificial UVR (UVA + UVB, 280–400 nm) supplied by UVA (TL 40/05; Philips; emission peak at 365 nm) and UVB (TL 20/01; Philips; emission peak at 312 nm) lamps. The UV intensities used in experiments were 11.8 W m^{-2} (UVA) and 0.54 W m^{-2} (UVB). The UV intensity used was based on natural solar radiation measurements taken during May, 2009 in Juiz de Fora City (21°45′51″ S), in southeast, Brazil (Noyma et al., 2015). UVR-treatment was performed during 3 h and control cultures were maintained in the dark, both at 28 °C. Experiments were performed in triplicates and all samples were carefully homogenized prior to analysis.

2.3. Bacteria quantification

For cell quantification, bacteria were stained with 4′,6′-diamidino-2-phenylindole (DAPI); 0.01 μg mL^{-1} final concentration (Porter and Feig, 1980). Analyses were performed during different time points of UV exposure (0, 1, 2 and 3 h). Samples were fixed with free-particle 37% formaldehyde (0.2 μm filtered) to a final concentration of 2%. After, samples (1 mL) plus 10 μL of DAPI were placed in megafunnels (Shandon Mega funnel, Thermo, UK) for immediate centrifugation in a cytocentrifuge (Shandon Cytospin 4, Thermo, United Kingdom), at 452 g and high acceleration for 10 min (Silva et al., 2014). Acceleration and speed were established as the procedures for medical microbiology provided by the Cytospin manufacturer manual. Cytocentrifugation was done by using regular slides without any coating. Analyses were performed on a fluorescence microscope (BX-60, Olympus, Melville, NY, USA) and U-MWU2 filter (330–385 nm excitation wavelengths). Bacteria were directly counted in 10 random fields at 1000x magnification using an ocular graticule grid.

2.4. Bacteria viability

Cell membrane viability was investigated using the LIVE/DEAD® BacLight™ kit (Molecular Probes Eugene, inc, ThermoFisher Scientific, OR, USA). This kit contains a mixture of fluorescent stains (SYTO 9 and propidium iodide) that differ both in their ability to penetrate healthy bacterial cell membranes and allows differentiation between bacteria with intact and damaged cell membranes (Boulos et al., 1999; Freese et al., 2006). Cells with intact membranes (viable cells) stain green and those with damaged membranes (non-viable) stain red. Bacteria were stained by 1 mL of each sample to 3 μL of *BacLight* and slides (n = 9) for each time point (0, 1, 2 and 3 h) were prepared in a citocentrifuge (Shandon Cytospin 4, Thermo Electron Corporation, Madison, WI, USA) as previously described (Silva et al., 2014). Analyses were performed under a fluorescence microscope (BX-60, Olympus, Melville, NY, USA) at 450–480 nm excitation wavelengths, which enable simultaneous visualization of *BacLight* stains. Bacteria were directly counted in 10 random fields (at an ocular graticule grid) at 1,000 x