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Toxicity of diatom polyunsaturated aldehydes to marine bacterial isolates reveals their mode of action

Milva Pepi [a], Hermann J. Heipieper [b], Cecilia Balestra [a], Marco Borra [a], Elio Biffali [a], Raffaella Casotti [a, *]

[a] Stazione Zoologica A. Dohrn, Villa Comunale, 80121 Napoli, Italy
[b] Helmholtz Centre for Environmental Research – UFZ, Permoserstr. 15, 04318 Leipzig, Germany

HIGHLIGHTS

- Six marine bacterial isolates tolerant to PUAs were characterized by 16S rRNA sequencing.
- Bacteria react to PUAs by changing their membrane properties.
- PUAs cause a toxicity-dependent increase in the degree of saturation of membrane lipid fatty acids.
- PUAs act as contact poisons probably due to their high hydrophobicity and additional chemical toxicity.

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ABSTRACT

Diatoms produce and release polyunsaturated aldehydes (PUAs) during senescence in culture and at the end of blooms in nature and these compounds play different ecological roles, as infochemicals, allelochemicals and pheromones In order to elucidate the toxic effects of PUAs, we isolated six bacterial strains from the Mediterranean Sea during a diatom bloom and tested their tolerance to PUA in terms of growth and cell membrane properties. Based upon 16S rRNA sequencing, these bacteria were assigned to the genera *Pseudomonas*, *Sufflavibacter, Halomonas*, *Vibrio*, *Idiomarina*, and *Labrenzia*. Growth of these strains was reduced by 50% (EC_{50}) at PUA concentrations ranging from 600 to 1700 μM of 2E,4E/Z-heptadienal (HEPTA), 400–800 μM of 2E, 4E/Z-octadienal (OCTA), and 70–400 μM of 2E, 4E/Z-decadienal (DECA). Two of these strains, *Vibrio* sp. and *Halomonas*, sp. were also investigated for membrane fatty acid composition in terms of adaptive modifications of their degree of saturation (ratio between saturated and unsaturated fatty acids) by GC-FID. A direct correlation between hydrophobicity and PUA toxicity was observed, and these bacteria were also found to react to PUAs by increasing the degree of saturation of their membranes fatty acids.

Tested PUAs were 4-fold more toxic than the well-investigated *n*-alkanols, most probably due to their additional chemical aldehyde toxicity to disrupting proteins by the formation of Schiff's bases, and therefore, they act as very toxic and effective poison, probably accumulating in cytoplasmic membranes because of their high hydrophobicity.

1. Introduction

Bacteria are fundamental players in any ecosystem and contribute to key processes and elemental cycles in the marine world, both for their numerical abundance and high functional diversity (Jørgensen, 2006). Diatoms are ubiquitous photosynthetic eukaryotic microorganisms, able to carry out about 20% of global photosynthesis on Earth (Armbrust, 2009), and are at the base of the marine food web. They also release high amounts of Dissolved Organic Carbon (DOC) that is directly degraded and used by heterotrophic bacteria. Diatoms play a key role in the biogeochemical cycles of silica and other elements in the oceans and they significantly contribute to the so-called "biological pump" by withdrawing CO_2 from the atmosphere through photosynthesis. All

Abbreviations: PUA, Polyunsaturated aldehydes; HEPTA, 2E,4E/Z-heptadienal; OCTA, 2E,4E/Z-octadienal; DECA, 2E,4E/Z-decadienal.

* Corresponding author.
E-mail address: raffaella.casotti@szn.it (R. Casotti).

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these processes also affect other surrounding microbial communities, in particular bacterial ones (Armbrust, 2009). Interactions between diatoms and bacteria play a pivotal role in biogeochemical cycling and in nutrient fluxes. In fact, marine bacteria are involved in remineralization of organic matter from the decomposition of dead diatoms, converting it into inorganic constituents including phosphorous, nitrogen, and carbon (Waksman and Butler, 1937). Some bacteria show strict association with growing diatoms establishing specific interactions, and other bacteria colonize sinking diatom, decomposing their released organic matter while they export into the deep ocean (Edwards et al., 2015). Studies on bacterial associations indicate that a small number of genera belonging to two heterotrophic bacterial phyla, *Proteobacteria* and *Bacteriodetes*, are consistently associated with diatoms (Amin et al., 2012), and a mutualistic association closely remembering symbiosis has been recently discovered between a diatom and a *Sulfitobacter* (Amin et al., 2015).

Under certain physiological conditions such as senescence or nutrient limitation, diatoms are able to produce and release significant amounts of bioactive metabolites involved in activated chemical defense in response to grazing (Ianora et al., 2011). Among these, polyunsaturated aldehydes (PUAs) are produced from fatty acids degradation upon cell breakage, such as during mastication by copepods, and affect copepod reproduction through impairment of their offspring, acting as teratogens (Ianora et al., 2004; Wichard et al., 2007). PUAs are also released independently from grazing and are known to have an effect on surrounding planktonic organisms, including other diatoms, as shown in culture and *in situ* (Casotti et al., 2005; Ribalet et al., 2007, 2008; Balestra et al., 2011). In a previous study, 3 PUAs produced by marine diatoms: 2E, 4E/Z-decadienal (DECA), 2E, 4E/Z-octadienal (OCTA) and 2E, 4E/Z-heptadienal (HEPTA), have been tested on the growth of 33 marine bacterial strains, including 16 strains isolated during a bloom of the diatom *Skeletonema marinoi,* which produces and releases high amounts of PUAs (Ribalet et al., 2014), in the Northern Adriatic Sea, Italy (Ribalet et al., 2008). Concentration-dependent growth reduction was observed for 19 of these bacterial strains at concentrations ranging from 3 to 145 μ mol l^{-1}. The strains isolated during a diatom bloom showed remarkable tolerance to PUA, with only 2 out of 16 strains showing growth inhibition at PUAs concentrations below 106, 130, and 145 μ mol l^{-1} for DECA, 2E,4E/Z-OCTA and HEPTA, respectively. No correlation between taxonomical position of the isolates and sensitivity to PUA was found. Considering that many bacteria thrive in close vicinity of diatom cells, it is likely that these compounds shape the structure of associated bacterial communities inducing selective adaptation. This is even more relevant during the final stages of blooms, when senescence and nutrient limitation increase the potential production and release of aldehydes (Ribalet et al., 2008, 2014) which then invest bacteria thriving on the diatoms cell surface. These data suggest that diatoms-associated bacteria may have evolved tolerance to toxic molecules released by diatoms. The same three PUAs and a mix of HEPTA and DECA were tested on a natural bacterial community from a coastal area of the NW Mediterranean Sea (Blanes Bay, Spain) (Balestra et al., 2011). Total or relative cell abundance or bulk bacterial production were not affected after 6 or 24 h exposure to 7.5 nM of the 3 different PUAs, but the different bacterial phylogenetic groups (*Gammaproteobacteria*, *Bacteroidetes*, *Rhodobacteraceae* and SAR11), were differently affected by the PUAs in terms of metabolic activity, as assessed by Catalysed Reporter Deposition (CARD)-Fluorescence *In Situ* Hybridisation (FISH). These results demonstrate that PUAs have a differential effect on the single-cell activity of distinct bacterial groups also in natural communities and that tolerance or resistance to PUA may confer a competitive advantage to PUA-tolerant groups, with the advantage of allowing them to preferentially use the organic matter released by diatoms, while their competitors are slowed down or eliminated.

Little is known about the cell targets of PUAs on sensitive organisms. The few studies involve metazoans and concentrate on molecular gene targets activated or downregulated (e.g. Lauritano et al., 2012; Varrella et al., 2016).

In general, contact of living cells with toxicants (below a specific threshold) induces changes in cell membrane fluidity, which bacteria counteract by modifying their membrane fatty acid composition so to maintain fluidity at a constant level (Petersen and Klug, 1994). Another mechanism of regulating membrane fluidity against membrane-active substances consists in a modification of the degree of fatty acids saturation (Diefenbach et al., 1992; Heipieper et al., 1994).

The aim of this study was to elucidate and quantify the toxic effects of PUA to six bacterial isolates on growth and cell membrane composition, in terms of adaptive modifications of their degree of saturation of their fatty acids as a mean for membrane rigidity. The strains were isolated during a diatom bloom in the Northern Adriatic Sea (Mediterranean Sea) while dominant diatom species were *Skeletonema marinoi*, *Chaetoceros socialis* and spp., undetermined pennate diatoms.

2. Materials and methods

2.1. Study area, water sampling and bacteria isolation

Water samples were collected from four stations (N2, 44°55′45.581 N, 12°53′35.480E; N3, 44°55′45.581 N, 12°49′37.708E; N4, 44°55′45.581 N, 12°48′17.834E; and N5, 44°55′45.581 N, 12°42′11.887E) in the Northern Adriatic Sea (Italy) (Fig. 1) during a diatom bloom (of *Skeletonema marinoi*, *Chaetoceros socialis* and spp, and of unidentified pennates) in April 2014. 1 ml of seawater from each station was added to 100 ml flasks each containing 20 ml of sterile mineral medium (Mills et al., 1978), in the presence of different concentrations of HEPTA, OCTA, and DECA. The inoculated flasks were incubated statically at 22 °C in the dark. Flasks showing turbidity after two weeks of incubation were subcultured by streaking onto Petri dishes containing the same culture medium with 1.6% of agar (Bacto-Agar, Difco), in the presence of PUAs at the same initial concentrations. Colonies morphologies were observed under a stereomicroscope (Zeiss, V8, Oberkochen, Germany), and those showing differences in shape, color and margins were streak-purified at least three times on the same solid medium, in the presence of the same concentration of PUAs. Isolates were then stored at −80 °C with 30% sterile glycerol (v/v) added until further use.

2.2. Chemicals

2E,4E/Z-heptadienal (HEPTA), 2E, 4E/Z-octadienal (OCTA) and 2E, 4E/Z-decadienal (DECA) were obtained commercially from Sigma-Aldrich Inc. (Milan, Italy). Working solutions were prepared by diluting the stock in absolute methanol (Sigma-Aldrich Inc., Milan, Italy) at room temperature. The effective aldehyde concentration of the working solution was assessed spectrophotometrically by measuring absorption at 274 nm using a specific absorption coefficient (per mole of compound) of 31000 (Pippen and Nonaka, 1958). Toxicity of the methanol solvent was tested for all bacterial strains and resulted to start above 3% of pure methanol per ml of culture (Novak et al., 1985) (data not shown) and therefore the amount of aldehyde solution in each test was kept always below this threshold. Values for the log octanol/water partitioning coefficient (logP) values of the three tested PUAs as an index of