Journal of Experimental Marine Biology and Ecology 479 (2016) 106–113

Contents lists available at ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

Enzymes manage biofilms on crab surfaces aiding in feeding and antifouling

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ARTICLE INFO

Article history:
Received 27 May 2015
Received in revised form 16 March 2016
Accepted 17 March 2016
Available online 31 March 2016

Keywords:
Biofilm
Microfouling
Decapod crustaceans
Enzymes
Chemical ecology
Fouling management

ABSTRACT

Biofilms are sticky exopolymer matrices with embedded microorganisms that form on virtually all submerged surfaces. Depending on the context, biofilms are beneficial or detrimental to macroorganisms. Two groups of decapod crustaceans, sand fiddler crabs (*Uca pugilator*) and blue crabs (*Callinectes sapidus*), were investigated to test whether externally secreted enzymes were present and if they were used to remove or prevent biofilm formation in the environment and on their surfaces. Three hydrolytic enzymes were of interest because of their ability to degrade biofilm components: amylase, trypsin and lysozyme. The role of these enzymes was investigated in two contexts, removal of biofilms from the environment for food detection and processing and prevention of biofilm formation on the macroorganism surface itself. To test this, enzyme activity was measured on feeding appendages of fiddler crabs, which feed on biofilms, and on egg masses externally brooded by blue crabs. All three enzyme activities were found on feeding appendages of fiddler crabs and in egg masses of blue crabs. For the context of removal of biofilms from the environment, the combined data of the enzymes present in fiddler crab saliva and the high diatom extraction efficiency (60–90% removal in less than 9 s), suggests a role in separation of organic content from sand particles. Secondly, the data of enzymes present on fiddler crab legs (the main location of their chemoreceptors) and that leg exposure to a human amylase inhibitor significantly decreased fiddler crab feeding responses, suggests a role of the enzymes on fiddler crab legs in generating phagostimulants. For the context of prevention of microfouling, it was found that the same enzymes are present in blue crab egg mass fluids and that the activity levels increase as the eggs mature, particularly amylase, which increased dramatically in clutches with late stage embryos. These findings of enzymes present in egg mass fluid throughout maturation inform a large body of work on pheromones and hatching cues for blue crabs. Detachment of brooded eggs and incubation with enzymes at levels found in egg masses resulted in survival and hatching while detachment without addition resulted in death. These findings suggest that the same suite of enzymes is important to biochemically manage biofilms in two ecologically relevant contexts. Biochemical management of biofilms may be more prevalent in macroorganisms than previously thought.

1. Introduction

In aqueous environments, biofilms are ubiquitous on all inert surfaces. Microfouling biofilms are composed of a consortia of microorganisms embedded in an exopolymer matrix (Reviews Costerton et al., 1987, 1995; Whitfield, 1988; Wingender et al., 1999; Review Sutherland, 2001; Review Flemming and Wingender, 2010). Understanding mechanisms used by marine organisms to remove biofilms from surfaces and prevent microfouling is of great interest to understand animal–microbe interactions at the interfaces of which they meet (Wahl, 1989, 2008; Dale and Moran, 2006; Wahl et al., 2012; Mcfall-Ngai et al., 2013), and for bioinspired environmental and medical applications (Essock-Burns & Rittschof, in press).

The present study examines macrofouler use of biochemical mechanisms to manage biofilms in two contexts, direct removal from another surface for feeding and preventative measures on the macroorganism itself to protect egg masses. To do this, fiddler crabs *Uca pugilator* were used to assess biochemical measures employed to detect and remove biofilms from sand particles and blue crabs *Callinectes sapidus* were used to assess biochemical measures to manage biofilms on brooded egg masses.

Sand fiddler crabs, *U. pugilator*, are semi-terrestrial deposit feeders, removing organic content from sand particles. *U. pugilator* rely on chemoreceptors on their chelae, or claws, and dactyls, or legs to detect phagostimulants, or food cues, in their habitat (Robertson et al., 1981; Rittschof and Buswell, 1989; Reinsel and Rittschof, 1995). Phagostimulants include diatoms, benthic algae, blue-green algae, ciliates and bacteria (Robertson et al., 1981) and carbohydrate components and amino acids of diatoms (Robertson et al., 1981; Rittschof and Buswell, 1989; Weissburg and Zimmer-Faust,

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http://dx.doi.org/10.1016/j.jembe.2016.03.009
0022-0981/

1991), stimulating a feeding response in *Uca* species. Robertson et al. (1981) suggested that continued feeding after initial chemoreception may require additional input from the buccal region.

Once stimulated to feed, fiddler crabs are proficient at separating organic content from sand (Miller, 1961; Robertson et al., 1980; Reinsel and Rittschof, 1995). *U. pugilator* feed by using their minor cheale, to deliver pinches of damp sand to their buccal cavity, removing biofilms containing microorganisms and proteinaceous matter and leaving behind balls of sand (Miller, 1961; Reinsel and Rittschof, 1995). *Uca* feeding efficiency is attributed to their specialized appendages used for mechanical removal of organic material (Miller, 1961; Robertson and Newell, 1982; Lim, 2004) and biochemical contributions to feeding have been unexplored. The present study investigates the enzymology of buccal and dactyl secretions through saliva samples and leg washings to address the role of exoenzymes in *U. pugilator* food detection and processing.

In addition to the role of biochemical mechanisms to remove biofilms from separate surfaces, the role of hydrolytic enzymes in prevention of microfouling on a macroorganism surface is of interest. How do crustaceans keep themselves clean where they cannot scratch? To investigate this, ovigerous female blue crabs were used with attention to their externally brooded egg masses. As in most crustaceans, blue crabs (*C. sapidus*) molt frequently, groom appendages and bury, all mechanisms to control microfouling. The egg mass of an ovigerous female is a large area too sensitive to use mechanical means of biofilm removal. Each egg mass averages 2 million to over 6 million eggs of approximately 250–275 μm diameter (Dickinson et al., 2006; Darnell et al., 2009).

Brooded crustacean eggs are glued in strings to abdominal pleopod filaments (Rogers-Talbert, 1948; Saigusa, 1994). Embryo development is generally synchronous and can be tracked by observing color changes and embryo details in the egg clutch, starting with yellow eggs, transitioning to orange and brown, and finally black, prior to larval release (DeVries et al., 1983). For embryo survival, eggs must be minimally fouled to facilitate oxygen delivery to embryos and waste removal (Crisp, 1959; Baeza and Fernández, 2002; Fernandez and Brante, 2003; Reinsel et al., 2014). If crustacean embryos become detached from the female, they become fouled with biofilm and die (Fisher, 1976, 1983; Fisher et al., 1976; Forward and Lohmann, 1983; Saigusa, 1992, 1993). Due to the detrimental outcomes of such microfouling, other crustaceans have even developed symbiotic relationships with bacteria to protect egg masses from biofilms leading to fungal infections for shrimp (Gil-Turnes et al., 1989) and lobster (Gil-Turnes and Fenical, 1992). The present study examined the enzymology of blue crab egg masses to gain insight into biochemical mechanisms to prevent biofilm formation on eggs.

Enzymology on externally brooded egg masses is also relevant to understanding the role of chemical cues during development that elicit various behaviors of the female. Decapod crustaceans are well studied with respect to chemical and physical cues and synchrony of hatching (Forward, 1987; Review Rittschof, 1993; Tankersley et al., 2002; Review Rittschof and Cohen, 2004; Reinsel et al., 2014). Specific chemical cues trigger larval release behavior (DeVries et al., 1989), in which females physically assist in egg hatching and propel the embryos into the water column (Forward and Lohmann, 1983; Rittschof et al., 1985; Forward, 1987; Saigusa, 1992). Due to the types of peptide pheromones detected from ovigerous females, trypsin-like proteases are implicated in pheromone production and egg hatching (Rittschof et al., 1985; Forward, 1987; DeVries and Forward, 1991; Saigusa, 1992; Darnell and Rittschof, 2010).

Fouling management is defined by any biochemical or physical measures that are preventative or responses to microfouling (Essock-Burns & Rittschof, in press). The present study aims to understand biochemical measures, specifically hydrolytic enzymes, to prevent and remove products of microfouling. The context for studying preventative measures of microfouling was blue crab egg masses and the context for response to microfouling was fiddler crab feeding. Fiddler crab feeding exemplifies responses to microfouling in its habitat, rather than on the macroorganism itself. The hydrolytic enzymes examined were amylase, trypsin and lysozyme as they hydrolyze chemical bonds in carbohydrates, proteins and bacterial cell walls, respectively. These three enzymes were hypothesized to have roles in degrading biofilm components including the extracellular matrix made up of glycoproteins and proteoglycans, as well as bacterial cell walls and cells embedded in the matrix. The data support the roles of enzymes in both contexts through the removal of biofilms and provide insight into biochemical measures to manage fouling.

2. Methods

2.1. Crab collection

Sand fiddler crabs (*U. pugilator*) were collected by hand at night in the Rachel Carson National Estuarine Research Reserve near the Duke University Marine Laboratory in Beaufort, North Carolina (34°42.65′ N, 76°40.40′ W). Large crabs were used to facilitate the collection of test fluids. Fiddler crabs used in feeding experiments were maintained in groups of 100–200 in a large water table (1.5 m diameter) maintained at ambient temperature with constant dripping seawater and sand for burrowing. Crabs were exposed to the natural diurnal light cycle and fed pulverized dry "Ocean Flavored" Friskies cat food. Ambient temperature was 25 °C and animals used for collection of fluids for studies were kept individually in 40 mL glass finger bowls with approximately 0.5 cm seawater and fasted overnight prior to feeding experiments. Sexes were tested separately and each test group ranged from 10–30 crabs.

Blue crabs (*C. sapidus*) were collected in the Rachel Carson Research Reserve on foot using dipnets at night. Ovigerous females with egg clutches at each stage of development as indicated by clutch color (Rogers-Talbert, 1948) were used. Ovigerous females in each stage were as follows: 5 yellow, 11 orange, 8 brown, and 12 black. Crabs were kept in tanks with flowing seawater at 33 to 36 salinity and ambient temperature. For experiments, crab fluids were sampled 1–3 h after placement in a damp bucket at 25 °C.

2.2. Fluid sample collection

Fiddler crab saliva and leg washings were collected from the crabs using a 200 μL mechanical micropipette. Saliva was extracted from the buccal cavity by gentle pipetting of the fluid at the entrance to the mouth cavity. Leg washings were obtained by repeatedly pipetting 50–80 μL aliquots of 100,000 kDa-filtered aged seawater over all 10 legs in a 1.5 mL Eppendorf tube. Samples were kept on ice and used in assays immediately following collection.

Blue crab egg masses were sampled by pipetting 100 μL of fluid from each of the 36 egg clutches. Control samples were taken from the tanks in which the crabs were kept. The color of the egg mass was recorded for each crab at the time of sampling.

2.3. Enzyme activity measures

2.3.1. Overview

Enzyme activities were measured using enzyme specific substrates for all sample types including: *U. pugilator* saliva and leg washings, and *C. sapidus* egg mass fluid. The substrates become absorptive or fluorescent when cleaved by an amylase, trypsin or lysozyme. Enzyme activity was quantified as a function of change in absorbance or fluorescence over time, using a spectrophotometer Spectramax M2 Microplate Reader (Molecular Devices). The substrates and enzymes for standards are described below in detail. Using the unit definition for each enzyme the optical density readings from the spectrophotometer were converted into moles of product produced per milliliter fluid per unit time.