Effects of freshwater sponge Ephydatia fluviatilis on conjugative transfer of antimicrobial resistance in Enterococcus faecalis strains in aquatic environments

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SPECIAL ISSUE ARTICLE

Effects of freshwater sponge *Ephydatia fluviatilis* on conjugative transfer of antimicrobial resistance in *Enterococcus faecalis* strains in aquatic environments

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Significance and Impact of the Study: It has been suggested that antibiotic resistance transfer in the environment takes place in various aquatic systems but direct evidence for this has been difficult to obtain. Sponges survive by filtering particulate material, including bacteria, from the water column. They are extremely efficient and can concentrate the microbes by several orders of magnitude thereby enhancing cell-to-cell contact, a prerequisite for the most efficient gene transfer. We show here that freshwater sponges support antibiotic resistance transfer among vancomycin-resistant enterococci, a significant cause of healthcare-associated infection. Sponges may therefore contribute to the emergence of multidrug-resistant pathogens. Investigating these roles would facilitate a greater understanding of the environmental contribution to antibiotic resistance.

Keywords
biofiltration, conjugation, *Enterococcus faecalis*, *Ephydatia fluviatilis*, horizontal gene transfer.

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Abstract

Filter feeding is a biotic process that brings waterborne bacteria in close contact with each other and may thus support the horizontal transfer of their antimicrobial resistance genes. This laboratory study investigated whether the freshwater sponge *Ephydatia fluviatilis* supported the transfer of vancomycin resistance between two *Enterococcus faecalis* strains that we previously demonstrated to exhibit pheromone responsive plasmid conjugation. Microcosm experiments exposed live and dead colonies of laboratory-grown sponges to a vancomycin-resistant donor strain and a rifampicin-resistant recipient strain of *Ent. faecalis*. Enterococci with both resistance phenotypes were detected on double selection plates. In comparison to controls, abundance of these presumed transconjugants increased significantly in water from sponge microcosms. Homogenized suspensions of sponge cells also yielded presumed transconjugants; however, there was no significant difference between samples from live or dead sponges. Fluorescent *in situ* hybridization analysis of the sponge cell matrix using species-specific probes revealed the presence of enterococci clusters with cells adjacent to each other. The results demonstrated that sponge colonies can support the horizontal transfer of antimicrobial resistance although the mechanism underlying this process, such as binding of the bacteria to the sponge collagen matrix, has yet to be fully elucidated.

Introduction

The emergence of multi-resistant pathogens through the acquisition of mobile antimicrobial resistance genes (ARG) has been designated as a global health threat. The detection of clinically relevant ARG in environmental bacteria has been interpreted as evidence that the increase in antimicrobial resistance (AMR) may, at least in part, be caused by feedback from the natural environment (Cantas *et al.* 2013). Taylor *et al.* (2011) have suggested that

aquatic environments may function as conduits for AMR, because they act as sinks for many different sources of micro-organisms and provide opportunities of close contact between different bacteria, thus facilitating horizontal transfer of resistance genes. First evidence for aquatic biofilms as a hot spot for plasmid transfer in the aquatic environment emerged from work by Fry and Day (1990) on the transfer of heavy metal resistance in the epilithon. This has recently been extended to horizontal ARG transfer, for example, from the identification of an ARG reservoir in river biofilms (Proia *et al.* 2016) or from flow chamber observations of conjugative plasmid transfers in aquatic biofilms (Christensen *et al.* 1998). Other biotic factors and processes for resistance transfer in aquatic environments have largely remained unexplored (Berendonk *et al.* 2015). Lupo *et al.* (2012) have argued that facilitation of AMR transfer by aquatic filter feeders is highly likely, yet this hypothesis has not yet been substantiated by much experimental evidence. Hence, an experimental test of the contribution of filter feeding to antibiotic resistance transfer represents a novel aspect of AMR research.

Enterococci are Gram-positive, facultatively anaerobic bacteria that form an important component of the normal gut microbiota of both man and animals (Byappanahalli *et al.* 2012). They are also opportunistic pathogens known to cause Healthcare Associated Infections on a global scale and the problem is exacerbated by increasing rates of AMR (Guzman Prieto *et al.* 2016). They are widespread in both terrestrial and aquatic ecosystems, largely through contamination with human and animal faeces, (Wade *et al.* 2006). *Enterococcus faecalis*, the most clinically relevant species, transfers vancomycin resistance efficiently in the laboratory (Sterling *et al.* 2020) but very little is known about conjugative gene transfer rates in aquatic environments.

We have recently shown that filter feeding zooplankton from the genus *Daphnia* facilitate transfer of vancomycin resistance between *Ent. faecalis* strains (Olanrewaju *et al.* 2019). It is therefore likely that other filter feeders may also support this transfer process. Freshwater sponge populations can be sufficiently large to achieve extensive coverage of lake bottom area (e.g. 44% in Lake Baikal, Pile *et al.* 1997) and can filter a water quantity equivalent to their own biovolume within 24 s (Patterson *et al.* 1997). Thus, a finger-sized 25 cm^3 sponge colony would filter 90 l per day, with efficient removal of waterborne bacteria (Frost 1978). Among faecal bacteria discharged into surface waters, some strains of enterococci are well adapted for survival and contain both virulence and antibiotic resistance genes on mobile genetic elements (van Tyne and Gilmore 2014). These bacteria are likely to be encountered by sponges in the aquatic environment. Therefore, this study investigated the potential of the freshwater sponge species *Ephydatia fluviatilis* to contribute to the transfer of vancomycin resistance between two *Ent. faecalis* strains that had been recovered from a river catchment and previously demonstrated to exhibit pheromone responsive plasmid conjugation of vancomycin resistance (Conwell *et al.* 2017).

Results and discussion

We developed a simple *in vitro* cultivation system for *E. fluviatilis* by successfully hatching gemmules under aseptic conditions (Fig. 1 supplementary). This allowed us to challenge the sponges with different ratios of donor and recipient *Ent. faecalis* strains and to recover vancomycin-resistant transconjugants by antibiotic double selection. In comparison to controls, the abundance of enterococci with phenotypic resistance against both rifampicin and

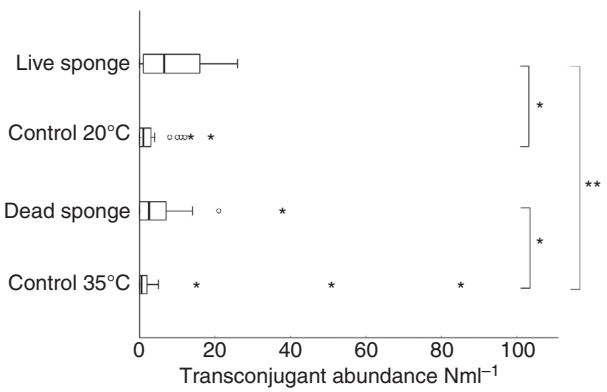

Figure 1 Abundance of presumed *Enterococcus faecalis* transconjugants as colony forming units from water in sponge microcosms and controls (20°C) for live sponges and controls (35°C) for dead sponges. *Ephydatia fluviatilis* gemmules (Cavan River, Republic of Ireland) were hatched at 20°C in Petri dishes with 10 ml UV treated mineral water after 10 min treatment with 1% H_2O_2 and subsequent storage in autoclaved deionized water at 4°C. 'Dead sponges' had been hatched 2 days earlier and been inactivated by 48 h exposure to 35°C. Overnight cultures of *Ent. faecalis* strains MF06036^{Van} and MW01105^{Rif} in tryptone soya broth (TSB, Oxoid), 10-fold diluted and grown in fresh TSB for 90 min were mixed with a 1 : 14 donor to recipient ratio after another 10-fold dilution in UV-treated mineral water. Twenty millilitre aliquots of bacterial suspension replaced water from each microcosm and control for a 24 h incubation at 20°C. 1 ml aliquots of water from microcosms was spread on double selection plates of tryptone soya agar (Oxoid) with 100 μg ml^{-1} rifampicin and 10 μg ml^{-1} vancomycin (both from Sigma) and incubated for 48 h incubation at 37°C. Colonies with both resistance phenotypes were counted as presumed transconjugants. Differences between treatments were analysed with Kruskal-Wallis tests and Mann-Whitney *U* tests for pairwise comparisons; the significance threshold was $P < 0{\cdot}05$. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$; all other differences were not significant.

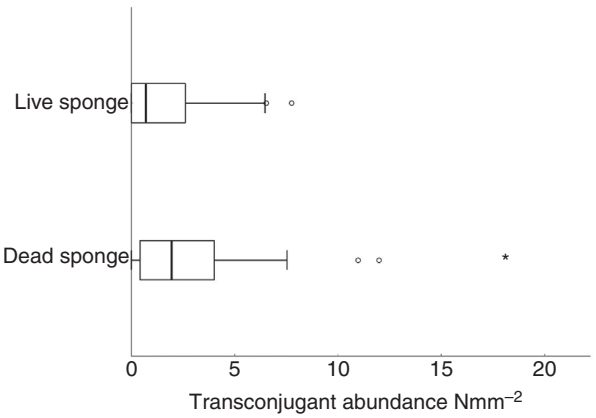

Figure 2 Transconjugant abundance in live and dead sponges as colony forming units per unit surface area covered by the sponge, which had been calculated from sponge colony diameter in image processing software cellSens 1·3 (Olympus) under an Olympus SZX16 microscope with camera. Eppendorf tubes with sponges and 200 µl of autoclaved water had been vortexed for two minutes and 100 µl sponge cell suspension was plated onto a double selection plate for incubation and counting as above. The difference of transconjugant numbers between live and dead sponges was not significant.

vancomycin was significantly higher in water from microcosms with sponges (Fig. 1). Molecular biological characterization of the investigated *Ent. faecalis* strains by Conwell *et al*. (2017) has provided strong evidence for plasmid mediated conjugative transfer of vancomycin resistance between donor and recipient. Therefore, in this study, bacterial colonies with phenotypic resistance against both antibiotics on double selection plates were presumed to be transconjugants and no double-resistant enterococci were ever recovered from untreated sponge controls. While the presence of sponges clearly increased the bacterial conjugation frequency surprisingly, there was no significant difference in abundance between microcosm water samples from live or dead sponges. Furthermore, cell suspension samples of live and dead sponges were not significantly different in regard to the population density of transconjugants per unit of surface area covered by sponge colonies (Fig. 2). Elevated numbers of transconjugants in water from sponge microcosms consistently indicated that the presence of freshwater sponges supported horizontal resistance gene transfer. However, the lack of significant differences between microcosms of live and dead sponges in microbial analysis results from water and sponge cell suspensions made it evident that active filtration was not a significant contributing factor and suggested that more sensitive visualization methods would be required to investigate what was occurring in these microcosms.

Image analysis of fluorescent *in situ* hybridization micrographs (Fig. 3) showed a presence of *Ent. faecalis* associated with sponge mesohyl and spicule surfaces. The observed distribution pattern was clustered, and with many *Enterococcus* cells in close spatial proximity to each other suggestive of the characteristic clumping previously observed in established conjugation systems with these strains (Conwell *et al*. 2018; Olanrewaju *et al*. 2019). This species-specific assay cannot discriminate between donor and recipient strains, but no staining was observed in sponges without exposure to the laboratory strains, thus

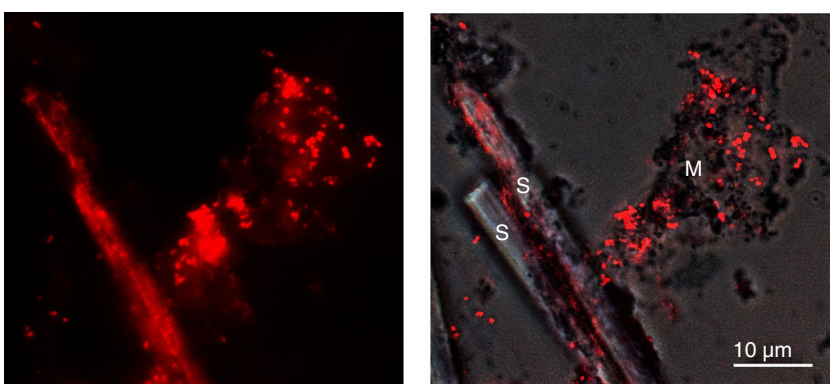

Figure 3 Left: Micrograph of the fluorescence signal after fluorescent *in situ* hybridization of mesohyl microsections of freshwater sponge *Ephydatia fluviatilis*, which had been exposed to *Enterococcus faecalis* strains in microcosms. After fixation in 50% ethanol PBS for 24 h at −20°C cryosectioning involved cutting 10 micron sections through whole sponges and deposition on 0·75% gelatine coated slides. An *Ent. faecalis* probe with a 5′ texas red fluorophore (IDT) was hybridized to sponge sections for 90 min at 10 ng µl^{-1} (Waar *et al*. 2005) and slides were mounted with ProLong gold antifade. Slides were imaged through a 100x objective on a Nikon eclipse E400 microscope with a Nikon DS-fi1c using a G2-A and UV filter set and images were captured with software NIS-Elements (Nikon) and ImageJ (open source). Right: The fluorescence signal has been overlaid by a phase contrast micrograph displaying spicules (S) and sponge mesohyl cells (M), while clusters of *Ent. faecalis* cells remain visible in red (Image: M. Conwell).

indicating that indigenous enterococci were not present in the microcosm and would therefore not be able to contribute resistance genes to the organisms in the conjugation assay.

Conjugation between Gram-positive bacteria requires close cell to cell contact, which is established and maintained through the involvement of cell surface proteins (Werner *et al.* 2013). These aggregation or adhesion processes are essential first steps in achieving spatial proximity (Jett *et al.* 1994). For Gram-positive bacteria, whose conjugation rates in liquids with widely dispersed cells can be exceedingly low, *in vitro* conjugation studies with filter assays have often achieved a much higher conjugation success rate than other solid surfaces. The filter systems enforce the required spatial aggregation of cells by retaining and concentrating them around filter pores (Lorenzo-Dias and Espinosa 2009). Sasaki *et al.* (1988) observed that the physical structure of the filter material has a large impact on mating success, with spongy materials achieving substantial elevation of transfer rates so it was important to further investigate this aspect in the current study.

Analogous to technical filter systems, filter feeding sponges would also be expected to retain and concentrate formerly waterborne bacteria in spatial proximity, thereby increasing the chances for conjugation between them. The absence of supporting experimental results from analysis of water and sponge cell suspensions in this study suggests that the sponges' filtration activity was neither the cause for releasing a significant number of waterborne transconjugants into the ambient water, nor was it the main cause for transconjugant presence or retention within sponge colonies. While ingestion and digestion by sponges in this experiment could have inactivated any filtered bacteria before a resistance transfer, the increased abundance of transconjugants in samples of ambient water from all sponge microcosms may have been caused by the release of transconjugants from exterior sponge surfaces after these had provided an adhesion substrate for conjugation. The observed spatial clustering of enterococci in the sponge cell matrix (Fig. 3) provided evidence of close contact between individual *Ent. faecalis* cells. Thus, an essential precondition for conjugation was fulfilled. The observation that isolates formed enhanced biofilm on collagen coated substrate and gelatine media supports the assumption that parent strains of transconjugants in the experiments are likely to be capable of producing the collagen binding adhesins such as *ace*, a surface exposed protein, as well as enzymes for collagen hydrolysis. Production of *ace* appears to be ubiquitous among *Ent. faecalis* (Chowdhury *et al.* 2014) and gelatinase activity is generally widespread among enterococci (Thurlow *et al.* 2010). Both of these properties have previously been demonstrated in our test strains (Daniels 2011). These proteins would facilitate adhesion to the sponge, invasion of its cell matrix and feeding on collagen, which they encounter as a tightly meshed layer immediately below the sponge pinacoderm in the genus *Ephydatia* (Leys *et al.* 2009). Collagen is an important component of the mesohyl, particularly when it is newly formed (Alexander *et al.* 2015) as in the newly hatched sponges of the current study.

Conjugative horizontal gene transfer can also be enhanced as a stress response (Beaber *et al.* 2004), and the presence of bioactive compounds has been verified for many sponge microbe associations (Thomas *et al.* 2010). Our study differs from the majority of reports in the literature in that we have focussed on laboratory hatched material that was effectively disinfected prior to hatching. As such we do not expect the sponges to have a microbiome equivalent to, for example, what has been reported by Keller-Costa *et al.* (2014) who demonstrated that *E. fluviatilis* from natural environments harbours *Pseudomonas* species with antimicrobial activities. We did not detect any endogenous bacteria in our hatched material by cultivation on general purpose growth media. While it cannot be ruled out that some bioactive compounds, such as those described by Costa *et al.* (2013), may also have been released by *E. fluviatilis*, thus contributing to the observed presence of transconjugants, it would appear more likely that the newly hatched sponges lacked a normal microbiome and hence were unable to defend effectively against the *de facto* infection of their mesohyl by *Ent. faecalis*.

This study has provided evidence for a facilitation of AMR transfer in enterococci by the cell matrix of *E. fluviatilis*. However, while it was not designed to identify the mechanisms in operation, it did confirm that direct sponge filtration activity was, at best, a minor contributing factor. This may be due to the experimental focus on nascent sponge colonies in our *in vitro* cultivation system that put the potential contribution from the sponge microbiome or other environmental factors under tight constraints. Marine sponges are well known to bioaccumulate trace elements and pollutants and they have been proposed as useful biomonitoring tools (Orani *et al.* 2018). Freshwater sponges are ideally placed to function as biomonitors in river catchments and, combined with their potential to concentrate bacteria, antibiotics and bioactive agents, could function as reservoirs for generation of novel resistant organisms. While our results represent a first step towards an assessment of sponge associated AMR transfers at the ecosystem level, it therefore remains to be seen whether such processes universally apply to this and other species of freshwater sponges. Further investigation warrants experiments with

increased biological complexity and molecular characterization of sponge populations in situ in sites that have been well characterized for anthropogenic input.

Materials and methods

After collection from the Cavan River, Republic of Ireland, *E. fluviatilis* gemmules were subjected to a 10-min treatment with 1% H_2O_2 and subsequent storage in autoclaved deionized water at 4°C. For the experiments 'live sponges' were hatched at 20°C in Petri dish microcosms with 10 ml UV treated mineral water. Viability was confirmed by measuring an increase in sponge size over time and by demonstrating active filtration of ink by newly hatched sponges age-matched with those employed in the conjugation experiments (Fig. S1). 'Dead sponges' had been hatched two days earlier and had subsequently been inactivated by 48 h exposure to 35°C as this killed the sponge without degrading the tissue or causing it to shrink. Dead sponges were used as a control to test the impact of sponge filtration on conjugal transfer as they provided the sponge structure and texture without the filtering ability. Sponges were 7 days post-hatch when they were used for the experiment as this early colony age allowed for the development of a miniature sponge with ostia and oscula to enable filtering. Controls contained only water and were exposed to the same temperature regime as their treatment group with sponges. All control and treatment groups had thirty replicates. Bacterial cultivation and conjugation conditions were adapted from Conwell *et al*. (2017). Overnight cultures of *Ent. faecalis* strains $MF06036^{Van}$ (donor) and $MW01105^{Rif}$ (recipient) in tryptone soya broth (TSB; Oxoid, Basingstoke, UK, CM0129), were diluted 1 : 10 and grown in fresh TSB for 90 min. The strains were mixed with a 1 : 14 donor to recipient ratio after another tenfold dilution in UV treated mineral water. A 1 : 14 donor to recipient ratio produced maximal conjugal transfer in preliminary experiments (data not shown). 20 ml aliquots bacteria suspension replaced water from each microcosm and control for a 24 h incubation at 20°C. For counts of waterborne bacteria 1 ml aliquots of water from microcosms were spread on a double selection plate of tryptone soya agar (TSA; Oxoid, CM0131) with 100 μg ml^{-1} rifampicin and 10 μg ml^{-1} vancomycin (both from Sigma, Gillingham, UK) and incubated for 48 h incubation at 37°C. In each experiment the parent strains were also tested on antibiotic double-selection TSA plates to confirm that the observed transconjugants were due to the experimental procedure and not prior mutations in the parents. Colonies with both resistance phenotypes were counted as presumed transconjugants. Differences between treatments were analysed with Kruskal-Wallis tests and Mann-Whitney *U* tests for pairwise comparisons; the significance threshold was $P < 0{\cdot}05$.

For the preparation of sponge cell suspensions individual sponges were vortexed in 200 μl of autoclaved water and 100 μl suspension aliquots were plated onto double selection plates for incubation and counting as above. Transconjugant abundance in live and dead sponges was calculated as the number of colony-forming units per unit surface area covered by the sponge, which had been determined through measurements of sponge colony dimensions in image processing software cellSens 1·3 (Olympus, Southend-on-Sea, UK) under an Olympus SZX16 microscope with camera DP72.

Subsequent to a 24 h exposure to *Ent. faecalis* strains in microcosms at 20°C freshwater sponge colonies of *E. fluviatilis* were prepared for mesohyl microsections through fixation in 50% ethanol PBS for 24 h at −20°C. Cryosectioning involved cutting through whole sponges and depositing sections with a thickness of 10 μm on 0·75% gelatine coated slides. For fluorescent *in situ* hybridization an *Ent. faecalis* probe with a 5′ texas red fluorophore (IDT) was hybridized to sponge sections for 90 min at 10 ng μl^{-1} (Waar *et al*. 2005) and slides were mounted with ProLong gold antifade. Slides were imaged through a 100x objective on a Nikon eclipse E400 microscope with a Nikon DS-fi1c using a G2-A and UV filter set and images were captured with software NIS-Elements (Nikon) and ImageJ (open source).

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Juvenile sponge structure prior to experimentation. The image shows the sponge channel system and the gemmule from which it hatched.

Figure S2. Experimental protocol to test if sponges facilitate conjugal transfer of antibiotic resistance between *Enterococcus faecalis* where MW05 = *Ent. faecalis* (rifampicin resistance), MF36 = *Ent. faecalis* (vancomycin resistance), van = vancomycin, rif = rifampicin, C = control, S = sponge.

