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Note

Induction of resistance to *Staphylococcus aureus* in an environmental marine biofilm

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ABSTRACT

The study of environmental biofilms is complicated by the difficulty of working with them under lab conditions. Nonetheless, knowledge of cellular activity and interactions within environmental biofilms could lead to novel biomedical applications. As a first step in this direction we propose a novel technique for inducing resistance to *Staphylococcus aureus* (*S. aureus*) in an intact environmental biofilm. Agar plates were prepared with or without the addition of 20% *S. aureus* spent culture media and immersed in coastal seawater (Boston Harbor, Massachusetts, USA) for four days to grow up an environmental biofilm. Nucleopore filters inoculated with an overnight culture of *S. aureus* were then applied to the surface of the agar plates with the environmental biofilms, incubated 4 h at 37 °C, removed and subsequently stained and analyzed. Marine environmental biofilms grown on agar containing *S. aureus* spent culture media were significantly more inhibitory of *S. aureus* growth than were marine environmental biofilms grown on plain agar.

In many environments multi-species bacterial consortia live in biofilms in which chemical signaling and other forms of cellular cross-talk underlie a complex array of cooperative interactions. These interactions include, among others, enhanced gene transfer, coordination of metabolic activity, and improved survival in harsh environmental conditions (Costerton, 2007). Constituents of microbial consortia in biofilms may also cooperate to protect colonized surfaces. This phenomenon has been investigated in living surfaces, some of which have recruited, or co-evolved with, specific epibiotic bacterial consortia with the ability to inhibit invasive bacteria (Gil-Turnes and Fenical, 1992; Wahl, 2008; Maximilien et al., 1998; Boyd et al., 1999; Holmstrom and Kjelleberg, 1999; Egan et al., 2000; Kelly et al., 2003; Dobretsov et al., 2005; Wigglesworth-Cooksey and Cooksey, 2005; Dobretsov et al., 2006; Kanagasabhapathy et al., 2006; Wahl, 2008; Armstrong et al., 2001). The ability of microbial consortia to ward off invasion by foreign microbes is related to species richness (He et al., 2010) and has been shown to depend upon synergistic activity between members of the consortia (Burmolle et al., 2006). Stable biofilm/microbiome consortia are known to be important for host health in humans as well as simpler systems such as marine algae (Burmolle et al., 2006). The complexities of epibiotic biofilms are just beginning to be appreciated, and so far little has been learned about the mechanisms involved in warding off invasive microbes (He et al., 2010; Matz et al., 2008).

While exploiting microbes in monoculture for their antibiotic potential has a long and rich history, resistance to antibiotics is a growing problem. Mechanisms employed by naturally occurring biofilms for warding off invaders have the potential for being evolutionarily conserved (Harder, 2009), and so possibly less susceptible to the development of resistance. Further research in this area has the potential to reveal details of bacterial processes which could be useful for novel biomedical and industrial applications. However, such endeavors are complicated by the difficulty of reproducing and studying environmental biofilms in controlled laboratory conditions. While some limited success has been reported (Kaeberlein et al., 2002; Penesyan et al., 2010), difficulties remain due to incomplete knowledge both of the diversity of biofilm microbial constituents, and environmental/physico-chemical variables which may promote the growth of rare and or unculturable bacteria (Piel, 2011). As a first step in the direction of further investigations into these complex phenomena we propose a novel technique for inducing resistance to a terrestrial human pathogen, *Staphylococcus aureus* (*S. aureus*), in a marine biofilm grown in its natural habitat.

Multi-drug resistant strains of *S. aureus*, such as methicillin-resistant *S. aureus* (MRSA), represent a growing public health threat, for which new therapeutic modalities are urgently needed (Bush et al., 2011). For example, the majority of soft tissue infections presenting to U.S. emergency departments (EDs) are caused by MRSA. What's more, the rate of ED visits and hospital admissions for this problem has increased dramatically in recent years (Talan

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et al., 2011). Previous work has shown that individual bacterial strains isolated from epibiotic marine biofilms are capable of inhibiting the growth of *S. aureus* (Mearns-Spragg et al., 1998). Additionally, individual marine biofilm isolates may be induced, through being grown in the presence of spent culture media from *S. aureus*, or in co-culture with *S. aureus*, to express increased activity against *S. aureus* (Kanagasabhapathy and Nagata, 2008; Burgess et al., 1999). In a similar fashion induced *Bacillus subtillus* activity against *S. aureus* has been shown (Tabbene et al., 2011) as has activity against *Pseudomonas aeruginosa* by marine epibiotic isolates (Dusane et al., 2011). These studies all started with selected epibiotic isolates which were known antibiotic producers. We, by contrast, used unselected biofilms which were grown on agar plates in coastal waters. Since our approach can produce a biofilm-wide response, our proposed technique has the potential to catch induced interactions between members of a biofilm consortia rather than just activity induced in individual isolates. Since in nature most bacteria exist in complex multi-species biofilms (Costerton, 2007), developing techniques for studying them in their natural state is an important goal.

As an initial step in this direction we tested the hypothesis that an environmental biofilm grown in its natural habitat but supplemented with media containing spent culture media from *S. aureus* will express increased resistance to *S. aureus*. The current study was conducted with biofilms grown in the coastal waters of Savin Hill Cove near Boston, Massachusetts.

Plates were marine agar, plain agar, or plain agar with the addition of 20% *S. aureus* spent culture filtrate. Spent culture filtrate was made with an overnight culture of *S. aureus* that was centrifuged at 10,000 rcf for 30 min, and then filtered through a 0.25-μm pore-size Whatman filter (Whatman PLC, Kent, UK). The liquid medium was trypticase soy broth (TSB).

S. aureus strain RN6390 was used with chloramphenicol resistance and a constitutively expressed GFP [kindly provided by Professor Ambrose Cheung of Dartmouth Medical School, Hanover, New Hampshire, USA] (Kahl et al., 2000). This strain is a derivative of NCTC 8325, a standard *S. aureus* lab strain which was originally isolated in Britain in 1960. NCTC 8325 and its derivatives are among the most highly studied *S. aureus* strains in use (Iandolo et al., 2002). An overnight culture of *S. aureus* in TSB with 10 μg ml^{-1} chloramphenicol was diluted 1:8000 in sterile water (0.125 μl ml^{-1}), then sonicated for 15 s to break up adherent clumps of cells. 4 ml aliquots of *S. aureus* were filtered through a 0.25-μm pore-size Whatman filter (Whatman PLC, Kent, UK) (Hobbie et al., 1977), prior to incubation, so that there were typically no more than two to three *S. aureus* cells singly, or in pairs, visible under the high-power oil immersion field (magnification at 1000×). Nucleopore filters were then placed, *S. aureus* face up, directly on the surface of agar plates with live or UV-killed biofilms, or onto the surface of agar plates without biofilms. *S. aureus*-inoculated nucleopore filters on the surface of experimental agar plates were then incubated at 37 °C for 4 h.

Biofilms were grown on agar plates with either plain agar (Difco, Becton, Dickinson Co., Sparks, MD, USA) or plain agar with 20% *S. aureus* spent culture filtrate (*S. aureus* agar). Plates were immersed at a depth of 2 m (low tide) in coastal seawater for 4 days prior to use in the study (University of Massachusetts at Boston, Savin Hill Cove, 100 Morrissey Boulevard, Dorchester, MA, 02125, USA). When removed from coastal waters the agar plates with overlying biofilms appeared as very slightly tinted brown with small specks of sediment scattered around the plate. This appearance was uniform between experiments. Inactivated biofilm plates were sterilized under high intensity UV in a standard class II biological safety cabinet for 1 h prior to use. Effective sterilization was demonstrated by lack of growth on marine agar of swabs from biofilm after UV treatment. Experiments were done in triplicate; plates grown with environmental biofilms not inactivated with UV light were stored at room temperature for 1 h after removal from coastal waters and prior to use.

After incubation *S. aureus* cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) (0.5 μg ml^{-1}) prior to microscopic examination.

Epifluorescence microscopy with a Zeiss Axioscope A1 epifluorescent microscope (Carl Zeiss Inc., Oberkochen, Germany) was used to evaluate the growth of *S. aureus* on the surface of the nuclepore filters. Digital images were obtained, and microcolonies were counted for 30 randomly selected fields in the vicinity of the center of each nucleopore filter. Counting of cells per microcolony (a colony of bacteria visible with low power microscopy) was done for filters that were incubated with *S. aureus* over living biofilms. In the case of UV-treated biofilms, growth of *S. aureus* was more robust, and counting of individual cells within microcolonies was not feasible. Growth of *S. aureus* under all experimental conditions was also estimated through the use of ImageJ (http://rsb.info.nih.gov/ij/) image analysis software (Papadopulos et al., 2007).

Two-tailed *t*-test with unequal variance assumed was used for all statistical tests (SPSS version 15). Analysis of variance (ANOVA) was performed for three experimental factors—control plus two treatment groups: *S. aureus* agar, and plain agar. Experimental protocol was performed on three separate occasions; results are based upon analysis of the pooled data.

After incubation on a biofilm grown on *S. aureus* agar, microcolonies with less than four cells comprised 91% of the total, versus 68% for the plain agar biofilm ($P = <0.001$) (Table 1; Fig. 1a). After incubation over UV-killed environmental biofilms, *S. aureus* microcolonies with fewer than 4 cells comprised 5% of colonies on *S. aureus* agar plates, and 53% on plain agar plates (Table 1 and Fig. 1b).

Image analysis software was used to estimate the area covered by *S. aureus* microcolonies. Growth of *S. aureus* over biofilms grown on *S. aureus* agar, 0.07%, was 46% less than *S. aureus* growth on biofilm grown on plain agar 0.13% ($P<0.001$).

S. aureus belongs to a group of human pathogens that are increasingly virulent and antibiotic resistant. Staphylococci are known to form biofilms rapidly on implanted medical devices, particularly catheters of various kinds (Costerton et al., 1999; Darouiche, 2004; Camargo et al., 2005; Henriques et al., 2005; Costerton, 2007), resulting in significant morbidity and mortality (von Eiff et al., 2005). Bacteria in biofilms are up to 1000 times more resistant to antibiotic killing than their planktonic counterparts (Brooun et al., 2000). For this reason, and because of advancing antibiotic resistance, new means for countering this disease-causing organism are greatly needed. We have proposed a novel technique for the induction of resistance to *S. aureus* in an intact environmental biofilm. Rather than a clinical *S. aureus* isolate we chose to use RN 6390, a highly studied *S. aureus* lab strain (Iandolo et al., 2002). The decision to use RN 6390 in the current study was made because findings in this

Table 1
Percentage of *S. aureus* microcolonies with less than 4 cells at baseline and after incubation for 4 h at 37 °C by treatment type.

	Percent<4 cells per microcolony	Standard deviation	Statistical comparison	P value
Baseline	78	±31	*S. aureus* agar with biofilm, no UV exposure	*<0.001*
S. aureus agar with biofilm no UV exposure	91	±24	Plain agar with biofilm, no UV exposure	0.02
Plain agar with biofilm no UV exposure	68	±37	Baseline	0.10[a]
S. aureus agar with biofilm, pos. UV exposure	5	±14	Plain agar with biofilm, pos. UV exposure	<0.001
Plain agar with biofilm, pos. UV exposure	53	±29		

[a] P value not significant at 0.05 alpha level.