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Antagonistic Interactions and Biofilm Forming Capabilities Among Bacterial Strains Isolated from the Egg Surfaces of Lake Sturgeon (*Acipenser fulvescens*)

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Abstract Characterization of interactions within a host-associated microbiome can help elucidate the mechanisms of microbial community formation on hosts and can be used to identify potential probiotics that protect hosts from pathogens. Microbes employ various modes of antagonism when interacting with other members of the community. The formation of biofilm by some strains can be a defense against antimicrobial compounds produced by other taxa. We characterized the magnitude of antagonistic interactions and biofilm formation of 25 phylogenetically diverse taxa that are representative of isolates obtained from egg surfaces of the threatened fish species lake sturgeon (*Acipenser fulvescens*) at two ecologically relevant temperature regimes. Eight isolates exhibited aggression to at least one other isolate. *Pseudomonas* sp. C22 was found to be the most aggressive strain, while *Flavobacterium* spp. were found to be one of the least aggressive and the most susceptible genera. Temperature affected the prevalence and intensity of antagonism. The aggressive strains identified also inhibited growth of known fish pathogens. Biofilm formations were observed for nine isolates and were dependent on temperature and growth medium. The most aggressive of the isolates disrupted biofilm formation of two well-characterized isolates but enhanced biofilm formation of a fish pathogen. Our results revealed the complex nature of interactions among members of an egg associated microbial community yet underscored the potential of specific microbial populations as host probiotics.

Keywords Microbiome · Antagonism · Antibiotic · Biofilm

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Introduction

Microbiologists have known for decades that intricate ecological linkages exist between a host and the hosted microbial community [e.g., 1, 2]. It has taken the era of genomics and next generation sequencing to fully appreciate that these linkages exist between all multicellular organisms and microbes. Now, we have more fully developed concepts of the metacommunity [3, 4] and the metaorganism [5, 6] and recognize the importance of long-term associations between a host and its microbiome and to potentially detrimental effects of increasing anthropogenic disturbances on host-microbe interactions. These concepts apply not only to the mature host, but to all developmental stages of the host. Of interest in this regard are early life stages of obligate aquatic vertebrates including amphibians and fish, the eggs and larvae of which develop in an aqueous solution of microbes that includes Bacteria, Archaea, viruses, and unicellular eukaryotes. In oviparous teleosts, the essentially sterile ovulated egg becomes another surface and substrate that bacteria rapidly colonize and changes to the aquatic microbial community [7–9] can modulate egg mortality. Once the microbial community on the surface of eggs is established, interactions between populations within the community can range from highly mutualistic to highly antagonistic [10] and in a community of high diversity, interactions between taxa can impact biofilm

formation. Relevant to antagonistic interactions are observations that biofilm formation can be induced by the presence of antimicrobial substances [11], and cells within a biofilm are more resistant to antimicrobial compounds [12, 13]. Biofilm is also formed to avoid host immune systems, which allows microbes to persist in host environments [14]. Thus, it is important to simultaneously analyze antagonistic interactions and biofilm formation in order to fully understand how interactions among microbes affect microbial community assembly [15–19].

Antagonistic microbial interactions have been studied in various microbial communities including those associated with sponges [20–22], corals [23], fish [24, 25], and marine water [26–30]. These studies revealed phylogenetic trends in antagonistic interactions, whereby some taxa aggressively inhibited the growth of others [22, 26–28]. Antagonistic interactions are not static, but rather dynamic, being influenced by environmental variables such as temperature [23, 29], oxygen levels [31, 32], biofilm formation [31], and nutrient levels [32]. One specific type of antagonistic interaction that has been studied extensively involves antagonistic interactions among host-associated microbes. A primary focus of these studies has been to identify antagonistic interactions against strains known to be pathogenic to the host [23, 29, 33, 34]. Identifying such interactions has important implications for potential probiotic application in systems in which the protection of hosts from pathogens is a priority [10, 23, 29, 33–35].

The biochemical and physiological properties of bacteria isolated from hosts have been the focus of considerable research. However, less is known regarding how the biochemical and physiological properties of individual isolates translate into broader functional or behavioral activities within complex microbial communities that exist in nature. It is therefore important to characterize individual isolates for "community" properties such as antagonistic interactions that directly link to survival of individuals in the context of community [10, 35]. Such an approach would yield new information about population dynamics within a complex community.

In this report, we describe interactions between microbial populations that have been isolated from the surface of sturgeon eggs. The lake sturgeon population of Black Lake MI has been geographically isolated from the Great Lakes by dams since 1903 [36]. This oviparous species spawns in the Black River between late April and early June each year. An early spawning subpopulation in late April and early May is followed by a late spawning subpopulation in late May and early June. The river is impacted by upstream dams and by spatially and temporally variable land use patterns that alter physical and biological features of the watershed. The demersal eggs experience high rates of mortality that may be due in part to microbial activity [37]. The objective of this study was to characterize antagonistic interactions and biofilm-forming abilities among host associated microbes. We hypothesized that some members of egg-associated communities exert antimicrobial behavior and modest abilities to form biofilm that allows them to compete for space and resources in a complex community on the egg's surface. To test this hypothesis, we measured antagonistic interactions and biofilm forming capability of 25 representative isolates from the egg surface of lake sturgeon at two temperature regimes that are relevant to the temperature range experienced during the spawning season of the lake sturgeon [38]. Greater understanding of how these different taxa-specific interactions among microbial community members may affect egg mortality and natural recruitment in fishes or other aquatic eukaryotes or how attributes expressed within the egg-associated community may identify hatchery protocols or probiotics that minimize egg mortality would be extremely helpful. The study also addresses the potential mechanisms of microbial community formation on egg surfaces during embryogenesis.

Materials and Methods

Study Site and Sample Collection

Microbes were isolated from the surface of lake sturgeon eggs from the Black Lake population near Onaway, Michigan. Two types of fertilized eggs were included in the study: those collected directly from spawning stream and those fertilized and reared in a streamside hatchery. A total of five eggs were collected from stream at two different spawning runs; 3 eggs from early run on May 7th and 2 eggs from late run on May 17th in 2009 in the upper Black River (45°23,577N, 84°19,996W), which is the sole spawning stream for the Black Lake population. Eggs were collected from the bottom of the stream in a net together with bottom substrates and were subsequently separated from the substrates using sterile tweezers. Eggs were rinsed with phosphate buffered saline (PBS) and each egg was placed in a 2-mL eppendorf tube with sterile PBS. The eppendorf tubes were placed in a cooler box and were transported to the streamside hatchery (45°24, 634N, 84°20, 096W) on the upper Black River within 30 min of collection.

The hatchery-fertilized eggs were collected in May 2010. Hatchery water, pumped from the spawning stream, was filtered through sock filters (100 μm) to remove large particulates and was gravity fed to the hatchery system. A total of 10 hatchery eggs were collected from fertilized eggs at different developmental stages reared in two water types (UV radiation-treated and untreated water) and at two temperature regimes (12 and 19 °C) to capture a diverse group of bacterial isolates. These two temperatures corresponded approximately to the early and late spawning temperatures of the Black Lake population, respectively [38]. The collected eggs were rinsed with PBS and placed in 2-mL eppendorf tubes with PBS.

Scanning Electron Microscopy

Eggs were collected in the field or hatchery and immediately placed in sodium phosphate (100 mM) buffered 4% glutaraldehyde and stored at 4 °C until returned to the laboratory (within 48 h). The eggs were briefly rinsed in 100 mM phosphate buffer (1 min) and dehydrated in an ethanol series (25, 50, 75, and 95%) for 1 h at each concentration followed by three 1-h changes in 100% ethanol. Samples were critical point dried on a Balzers Model 010 (Balzers Union Ltd.), Balzers, Liechtenstein) using liquid carbon dioxide as the transitional fluid with five 8-min changes. Samples were mounted on aluminum stubs using high vacuum carbon tabs (SPI Supplies, West Chester, PA.). Eggs were coated with osmium (~10 nm thickness) in a NEOC-AT osmium coater (Meiwafosis Co. Ltd. Osaka, Japan). Samples were viewed in a JOEL, JSM-7500F (cold field emission electron emitter) scanning electron microscope (JOEL, Ltd. Tokyo, Japan). At least ten fields at 5000× magnification were viewed for each egg from which representative fields were selected for presentation.

Light Microscopy

Whole sturgeon eggs were washed serially through four 1-ml volumes of 80% ethanol and transferred to sterile water in preparation for microscopy and histological preparation. Images of whole eggs and thin sections were captured with a Canon 12D Digital camera. Histological preparations were performed at the MSU Histology Laboratory. All samples were formaldehyde fixed and paraffin embedded prior to sectioning. Sections were Gram stained or stained for lysozyme (human anti-lysozyme) using standard procedures and viewed with bright field or phase contrast on the Nikon Eclipse E600. Measurements on the light microscope were performed with an ocular micrometer calibrated with a stage micrometer (American Optical).

Isolation of Bacteria from Egg Surfaces

Five stream-collected and ten hatchery-collected eggs were processed to obtain microbial isolates using the following procedures. Eggs in PBS were vortexed for 3 min and the supernatant was diluted with PBS at different dilution factors. One hundred microliters of the diluted supernatant was plated on R2A medium (0.5 g proteose peptone, 0.5 g casamino acids, 0.5 g yeast extract, 0.5 g dextrose, 0.5 g soluble starch, 0.3 g dipotassium phosphate, 0.3 g sodium pyruvate, 0.05 g magnesium sulfate, and 15 mg agar in 1 L MiliQ Water). R2A medium was selected for its multiple carbon sources and low nutrient concentrations under the assumption that it would provide nutrients for a diverse collection of oligotrophic populations. The R2A plates were then incubated at two different temperatures: one at 5 °C in a refrigerator and the other at ambient temperature in the hatchery (10 to 18 °C). The plates with bacterial isolates were transported to a laboratory in Michigan State University for further processing. Each of the isolated colonies on R2A was re-streaked on a fresh R2A plate, and an isolated colony on the plate was grown overnight in R2B at room temperature in the laboratory (20–22 °C) and subsequently stored at −80 °C with a glycerol concentration of 15%.

16S rRNA Gene Sequencing and Phylogenetic Analysis

Isolated strains were grown in 10 mL of R2B overnight and harvested by centrifugation at 10,000 rpm in an SS34 rotor. Genomic DNA was extracted with a MoBio™ Power Soil DNA extraction kit (MO BIO Laboratories, Inc. CA). The 16S rRNA gene was PCR amplified using bacterial universal primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1389R (5′-ACG GGC GGT GTG TAC AAG-3′), and the resulting amplicons were purified with Qiagen™ PCR purification kit (QIAGEN Science, MD). Purified PCR products were sequenced at the Michigan State University Research Technology Support Facility using an ABI 3730 capillary electrophoresis system with a 27F primer. The phylogenetic relationships among the 92 egg isolates were inferred using SeaView V4 [39] after previously being aligned using Ribosomal Database Project (RDP) [40]. In SeaView, trees were constructed using neighbor joining [41] with the HKY substitution model and 481 shared characters. At each node, bootstrap values were calculated based on the frequency that the isolates appeared in the same cluster from 1000 replications. The sequences have been deposited at GenBank and have the accession numbers of KY075675-KY075766.

Selection of Isolates for Antagonistic Interactions and Biofilm Assays

We examined antagonistic interactions and biofilm-forming capabilities among 25 sturgeon egg isolates (Table 1). These isolates were chosen from the total of 92 isolate collection to best represent the phylogenetic diversity of the egg surface microbial community, which were determined using pyrosequencing data in our previous study [19]. The 25 isolates were revived in 10 mL of R2B overnight at room temperature (20–22 °C) for 2 to 3 days. Isolates that could not be grown in the broth were grown in R2A plate media at room temperature (20–22 °C) for 48 h.

Soft Agar Overlay Technique for Screening Antagonists

Antagonistic interactions among 25 isolates were screened by stamping 23 of the isolates on a soft agar lawn previously inoculated with one of the 25. The screening assays for

Table 1 List of isolates used for antagonistic interactions and biofilm assay

Assignment	Phylum/class	Genus/species	Strain ID	Source	Date	Accession
1	Bacteroidetes	Flavobacterium sp.	A20	Stream	5/7/2009	KY075719
2	Bacteroidetes	Flavobacterium sp.	D11	Stream	5/17/2009	KY075701
3	Bacteroidetes	Flavobacterium sp.	E17	Stream	5/17/2009	KY075734
4	Bacteroidetes	Flavobacterium sp.	B30	Stream	5/7/2009	KY075693
5	Bacteroidetes	Flavobacterium sp.	C6	Stream	5/7/2009	KY075723
6	Bacteroidetes	Flavobacterium sp.	B10	Stream	5/7/2009	KY075716
7	Firmicutes	Bacillus sp.	C20	Stream	5/7/2009	KY075739
8	Gammaproteobacteria	Aeromonassalmonicida	A12	Stream	5/7/2009	KY075690
9	Gammaproteobacteria	Aeromonassobria	A25	Stream	5/7/2009	KY075724
10	Gammaproteobacteria	Aeromonasenchereia	B25	Stream	5/7/2009	KY075741
11	Gammaproteobacteria	Pseudomonas sp.	D2	Stream	5/17/2009	KY075682
12	Gammaproteobacteria	Pseudomonas sp.	C22	Stream	5/7/2009	KY075722
13	Gammaproteobacteria	Serratia sp.	D14	Stream	5/17/2009	KY075703
14	Gammaproteobacteria	Rheinheimera sp.	F1	Hatchery	5/2/2010	KY075749
15	Betaproteobacteria	Massilia sp.	B13	Stream	5/7/2009	KY075696
16	Betaproteobacteria	Janthinobacterium sp.	F8	Hatchery	5/2/2010	KY075755
17	Betaproteobacteria	Janthinobacterium sp.	F13	Hatchery	5/12/2010	KY075765
18	Betaproteobacteria	Iodobacter sp.	D4	Stream	5/17/2009	KY075747
19	Betaproteobacteria	Deefgea sp.	D10	Stream	5/17/2009	KY075711
20	Betaproteobacteria	Vogesella sp.	F3	Hatchery	5/2/2010	KY075764
21	Betaproteobacteria	Undibacterium sp.	F9	Hatchery	5/4/2010	KY075752
22	Betaproteobacteria	Hydrogenophaga sp.	F14	Hatchery	5/4/2010	KY075763
23	Betaproteobacteria	Acidovorax sp.	F19	Hatchery	5/12/2010	KY075754
24	Alphaproteobacteria	Caulobacter sp.	F16	Hatchery	5/4/2010	KY075761
25	Deinococcus-Thermus	Deinococcus sp.	F4	Hatchery	5/2/2010	KY075762

antagonistic interaction were performed at both 14 °C and room temperature (20–22 °C). These two temperature ranges were used to create conditions in the lab that mimic stream temperatures that microbes experience during early (12–13 °C) and late (17–19 °C) spawning seasons. Isolates #1 and #5 (strains of *Flavobacterium* sp.) were removed from the stamp since they exhibited gliding on the soft agar. The soft agar overlay technique was previously described by Mendoza et al. 1997 [42]. The soft agar overlay technique consists of soft agar that is inoculated when molten with a single strain (denoted as "lawn" in this study) then poured on top of hard agar. The detailed procedure is as follows. The preparation of lawn was accomplished by pouring 6 mL of soft R2A (one half of the agar concentration relative to the regular R2A media) inoculated at 55 °C with 100 μL of each broth culture in the equivalent phase of growth for each trial (2–3 days of growth) or with 300 μL of plate culture suspension (3 loopfulls of plate culture suspended in 1 mL of R2B) on top of a 20-mL pre-solidified R2A plate. After soft agars were solidified, the 23 non-gliding strains were taken from broth culture or cell suspension in a 96-well plate and stamped in duplicate on the lawn using a 48-pin replicator (Fig. 4b). For each trial, three control plates with un-inoculated soft agar (no growth of organisms in the soft agar) were included at three different time points (in the beginning, the middle, and the end) during the stamp procedure to ensure that the stamp broth cultures were viable and stamped correctly. The stamped plates (including the three controls) were then incubated at 14 or 20–22 °C for 2 days. Antagonistic interactions were determined by the presence of zones of inhibition (zone of clearance in soft agar lawn) observed 2 days after stamping. The screening assay was performed three times with duplicates on each assay for a total of 6 replicates.

Confirmation of Antagonistic Interactions and Analysis

Stamp strains which exhibited antagonistic interactions with lawn strains in at least one of the 6 replicates were further examined at the two temperature regimes with 12 replicated spotting on a single plate to confirm the antagonistic interaction and to determine the extent of inhibition (Fig. 4c). Antagonistic interactions were confirmed to be positive when over 9 out of 12 replicates showed a zone of clearance. The extent of antagonism was calculated as the zone of inhibition (in mm) by subtracting the radius of the colonies from the radial zone of clearance. Stamp aggressiveness was defined

as cumulative number of inhibitions by each stamp strain, while lawn sensitivity was defined as number of stamp strains that each lawn strain was sensitive to. The effect of temperature on antagonistic interactions was examined using Fisher's exact test comparing lawn sensitivity of the two temperature regimes. Fisher's exact test was performed using the "fisher.test" function in R version 2.10.0 [43].

Assessment of Reciprocal Antagonistic Interaction

To investigate the reciprocal relationship of antagonistic interactions (the effect of a lawn strain on the growth of stamp strains), the radius of the colony of all 23 stamp strains was measured (in mm) for each lawn strain at both 14 and 20–22 °C after 4 days of incubation (the timing of measurements were synchronized for both 14 and 20–22 °C). The size of colonies on each lawn was compared to that on controls (without lawn organisms) to measure the effect of lawn strain on the stamp colony sizes. A total of six replicates were included for this assay. A general linear model was employed with the aggressiveness (defined in the previous section) as the independent variable and the average growth inhibition (reduction in colony radius relative to control) as the dependent variable to assess the effect of aggressiveness on the growth inhibition of colonies. The general linear model was performed using "lm" function in R version 2.10.0 [43].

Antagonistic Interactions Against Fish Pathogens

The soft agar overlay procedure was repeated to investigate the possible use of antagonistic mechanisms in controlling known fish pathogens. The top 4 most aggressive strains identified in this study were tested against six well-known or emerging fish pathogenic bacterial strains. Each aggressive strain was stamped with a 12 replicated spotting on soft agar in which one of six known fish pathogens was inoculated. These fish pathogens included motile *Aeromonas* sp. strain 060628-1-21, which was recovered from the kidney of a systemically infected lake whitefish (*Coregonus clupeaformis*) inhabiting Lake Michigan [44]; *Aeromonas salmonicida* subsp. *salmonicida* strains 051004-1-28 and 051006-58A, which were recovered from the internal organs of feral spawning Chinook salmon (*Oncorhynchus tshawytscha*) suffering from furunculosis in the Little Manistee River weir (Lake Michigan watershed) [45]; *Flavobacterium* sp. strain C05, which was recovered from the brains of hatchery-propagated coho salmon (*Oncorhynchus kisutch*) involved in a mortality event in Michigan (Lake Michigan watershed) [46]; *Flavobacterium columnare* strain 090702-1-3, which was recovered from the kidneys of a fingerling hatchery-reared Great Lakes muskellunge involved in a massive *F. columnare* disease outbreak (Lake Michigan watershed) (Loch & Faisal, in preparation); and the type strain of *Yersinia ruckeri* (ATCC 29473), etiological agent of enteric red-mouth disease [47].

Biofilm Assays

Biofilm forming capability of 23 isolates (excluding isolates #3 and #4 that were not culturable in broth) was investigated through an adaptation of a 96-well plate assay with crystal violet [48]. For each trial, 200 μL of culture broth of each of the 23 isolates was placed in duplicate into one half of a 96-well plate. In addition to the 23 isolates, *Pseudomonas aeruginosa* PA01 was used as a positive control and a single blank well was used as a negative control. A 48-pin inoculation stamp was used to transfer the broth cultures of isolates and controls into wells filled with 175 μL of sterile media. The biofilm assay was performed three times, yielding a total of 6 replicates per isolate. Three nutrient media were tested in the biofim assay: R2B (same as R2A above but without agar): M9 + Glucose (12.8 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 2 mL of 1 M $MgSO_4$, and 4 g glucose per liter**);** and M9 + Casamino Acids (M9+ 4 g Casamino Acids per liter). R2B was included because it was the media of isolation thus ensuring that all strains would grow. The minimal media was included because it represents oligotrophic stream nutrient conditions, and also because previous work indicated that biofilm was induced under limited nutrients [49]. The inoculated plates were incubated at 14 or 20–22 °C shaking at 100 rpm for 2 days. Post-incubation optical density at 600 nm was measured to quantify growth within the broth. Broth culture was decanted from the 96-well plate and the plate was gently rinsed with sterile water. Cells adhering to the surfaces of the plate were stained with 200 μL of 0.1% crystal violet dye for 15 min, rinsed in water baths, and then inverted to dry completely. The crystal violet stain was extracted from each well with the addition of 200 μL of 30% acetic acid for 15 min. The entire dye solution within each well was transferred into new 96-well plates, and the absorbance of the dye was measured at 600 nm using a microtitier plate reader. The statistical significance of biofilm formation relative to control was determined using multiple comparison tests within ANOVA. *P* values were controlled for multiple comparisons with Tukey's honest significant difference (HSD). The multiple comparisons with Tukey's HSD were run using "multicomp" package in R version 2.10.0 [43].

Inhibitor Effect of Strain C22 on Biofilm Formation

To determine the effect of strain C22 on established biofilms of *Caulobacter*, *Hydrogenophaga*, *Acidovorax*, and *Massilia*, a 2-day-old biofilm of each strain was challenged with C22. Biofilms of the four strains were established by the addition of 50 μL of an overnight culture grown in R2B supplemented with 100 μL of fresh media (R2B) in microtiter plate wells

(4 replicates), followed by 48 h of incubation at 25 °C and gentle agitation (110 rpm) on an orbital shaker. Established biofilm was washed with sterile media and the wells were loaded with overnight culture (50 μL) of C22 and supplemented with fresh media (100 μL). Plates were resealed and incubated an additional 48 h. The same protocol was used to test the effects of strain C22 on the biofilm of each fish pathogen. *Pseudomonas auruginosa* PA01 and uninoculated wells were used as positive and negative controls, respectively. The OD at 600 nm was measured to confirm growth and the established biofilms were challenged with strain C22. In both cases, biofilm was measured with the crystal violet assay as described above. The data presented are based on at least 4 replicates.

Results

Microscopy

To better understand interactions between bacteria on the surface of the egg, we provide results from microscopic examination of the surface and cross section of the sturgeon egg (Fig. 1). The ethanol preserved eggs seen in panel a are approximately 2 mm in diameter and have visible pigmentation. The pigmentation remained associated with the egg case after dissection and ethanol wash. Thin sections of the egg revealed the classic pattern of a thick chorion (also called zona radiata; Fig. 1b) with channels that appear to connect the inner egg to the outer microfibrous exterior. The inner layer of the chorion is approximately 15 μm thick while the outer layer approaches 20 μm. The outer microfibrous layer varied from 15 to 24 μm in thickness. These estimates are based on ethanol fixed and stained preparations. At a magnification of 1000×, morphologies consistent with bacteria can be detected on the fibrous surface (arrows in Fig. 1b). When stained with anti-human lysozyme, regions at the surface of the fibrous layer and at the basal layer of the chorion stained positive (dark brown precipitates, Fig. 1d). The microbial community viewed on an intact egg with SEM revealed remarkable morphological diversity with long filamentous rods, cocci, short rods with and without prostheca and bacteria loosely bound and some woven into the surface matrix in what appears to be a biofilm (Fig. 1c, e). Eggs that were aseptically harvested from gravid females were devoid of detectable attached bacteria (Fig. 1f).

Isolation and Phylogenetic Affiliation of Egg-Associated Bacterial Populations

We purified 92 isolates from lake sturgeon egg surfaces and sequenced approximately 500 bases at the 5′ terminus (V1-V3 region) of 16S rRNA (Fig. 2). Out of the 92 isolates, 77 were isolated from the stream eggs with 45 isolates coming from

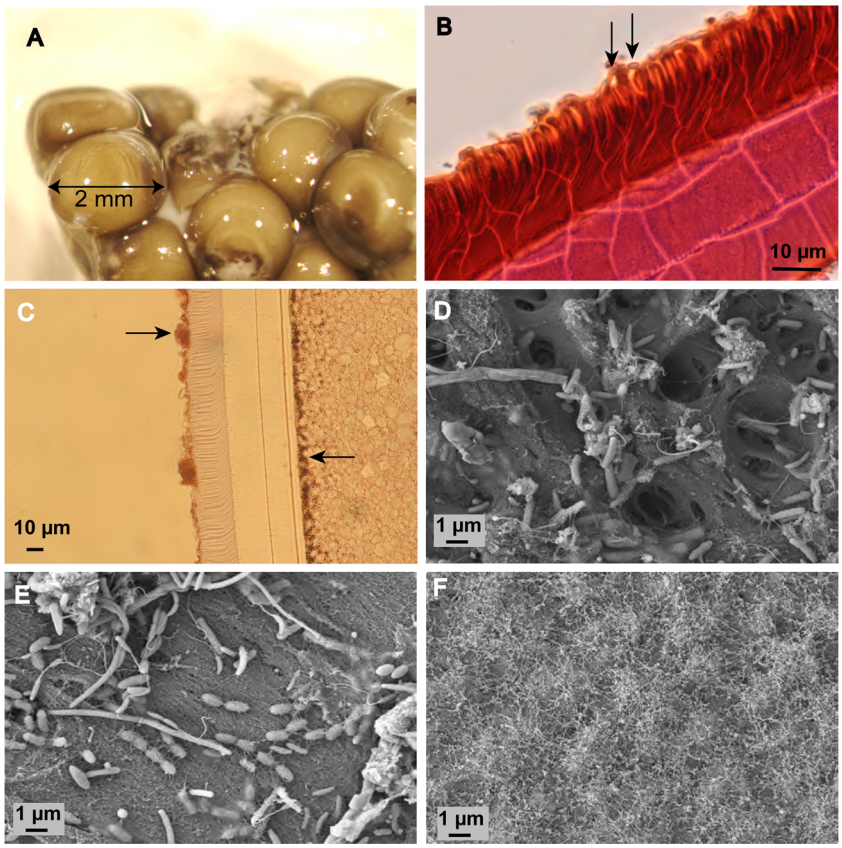

Fig. 1 Interactions of microbes with Sturgeon eggs. **a** Sturgeon eggs collected aseptically from female and preserved in 80% ethanol. **b** Egg thin sections Gram stained and viewed at 1000× magnification. **c** Thin section of sturgeon egg collected aseptically from female, preserved in 80% ethanol and stained with antibody against human lysozyme. **d**, **e** SEM of glutaraldehyde fixed eggs at 3 days viewed at 5000× magnification. **f** SEM of aseptically harvested egg fixed with glutaraldehyde and viewed at 5000× magnification

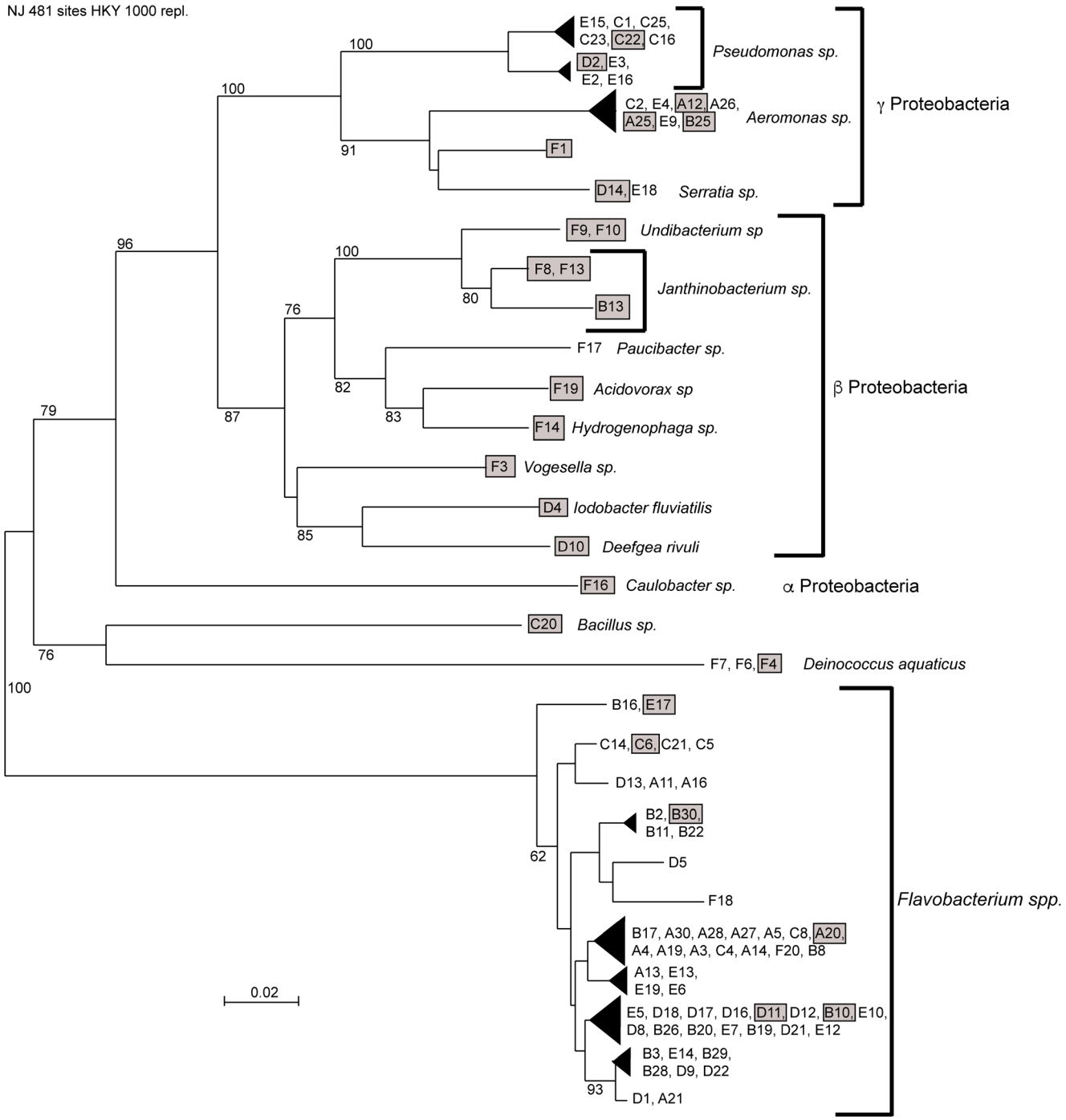

Fig. 2 Phylogenetic relationships among 92 sturgeon egg isolates inferred using the Neighbor-Joining method. A total of 481 characters within the 16S rRNA gene were used to construct the Neighbor-Joining tree in SeaView. The frequency that the isolates appeared in the same cluster after performing a 1000 bootstrap test is shown at each node (only above 60% are shown). The 25 isolates that were used in this study are *shaded*. Sequences deposited in GenBank are labeled SEI (Sturgeon Egg Isolate) followed by a microtiter plate coordinate

the early run (strain ID starts with A, B, C) and 32 isolates from the late run (strain ID starts with D and E). The remaining 15 isolates were from the hatchery reared eggs (strain ID starts with F). Taxa include representatives from phylum Bacteroidetes (all *Flavobacterium* spp.) and a broad spectrum of class ß-proteobacteria. However, only a single isolate, *Caulobacter* sp. F16, represented class α-proteobacteria. Twenty-five isolates that represent the egg surface microbial community diversity were selected out of the 92 isolates based on the previous pyrosequencing data and the phylogenetic tree topology of the isolates and were tested for antagonistic interactions and biofilm forming capabilities.

Antagonistic Interactions Between Isolates

The soft agar overlay technique proved to be a sensitive method for detecting antagonistic interactions (Fig. 4b, c). Among the 23 strains tested, 8 isolates (35%) were confirmed to be antagonistic to at least one of the other strains at one or both of the temperatures evaluated (Fig. 3). The number and the severity of antagonistic interactions were dependent on temperature. At 21 °C, isolates that exhibited aggressiveness to at least one other isolate (i.e., aggressor) increased their taxonomic range of interaction and intensity of antagonism relative to results from 14 °C (Fig. 3). The total number of antagonistic interactions also increased from 32 to 66 as temperature increased (Fig. 3). Fisher's exact test performed for lawn sensitivity columns between the two temperature regimes suggests that the effect of temperature on antagonistic interactions was nearly statistically significant ($P = 0.07$). The number of aggressors increased just by one from the low to high temperature regime, as *Bacillus* sp. showed aggressiveness only at 20–22 °C. The number of susceptible isolates

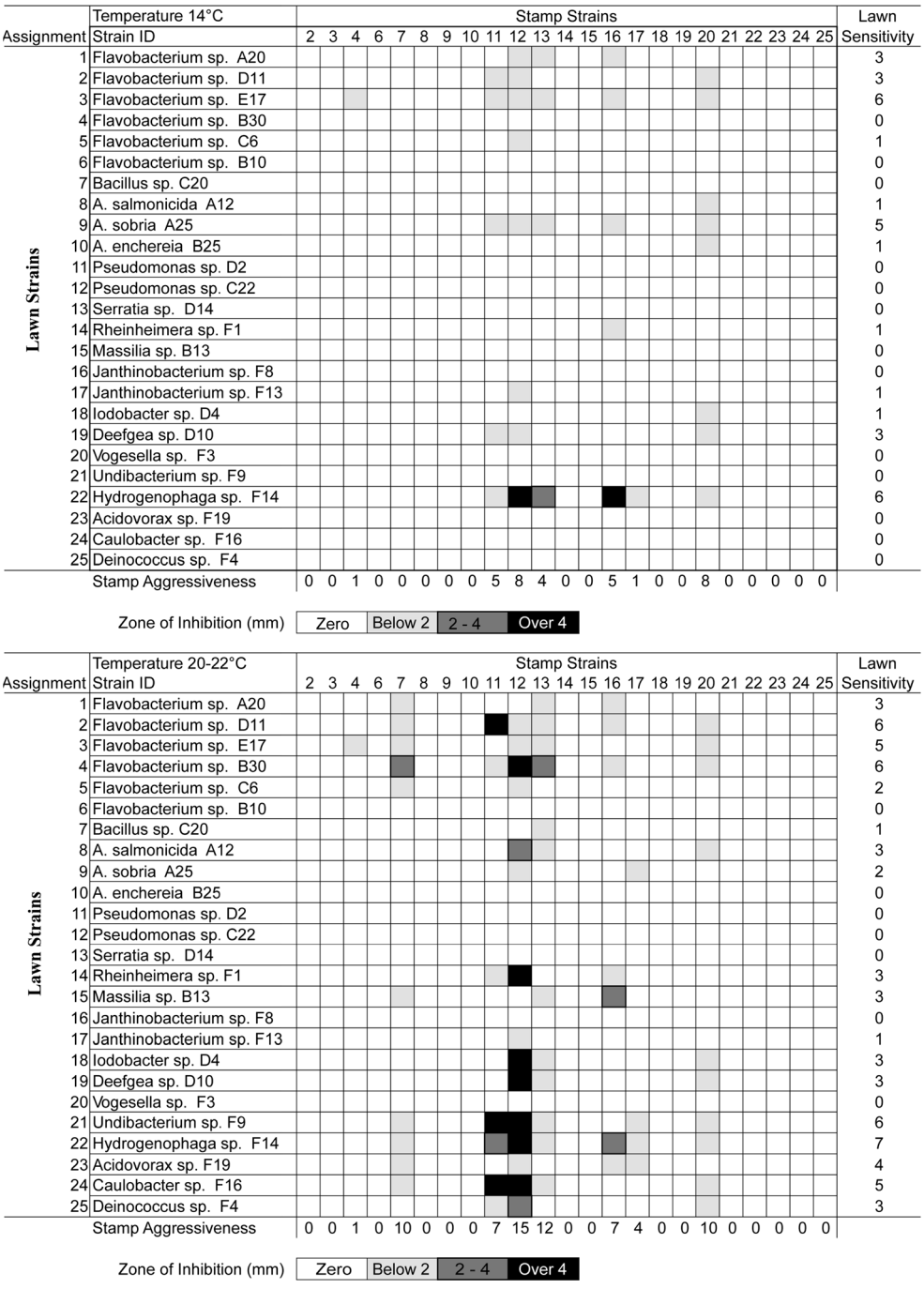

Fig. 3 Antagonistic interactions among 25 egg isolates at 14 and 20–22 °C. *Shading* indicates the size (mm) of zone of inhibition. The *bottom row* in the table shows cumulative number of inhibitions by each stamp strain (a measure of aggressiveness). The rightmost column summarizes the sensitivity of each lawn strain to stamp strains (sensitivity). *Flavobacterium* sp. A20 and C6 were removed from the stamp due to their gliding phenotype that obscured the results

increased from 12 to 18 (out of 25 lawn strains) as the incubation temperature increased (Fig. 3). Six isolates including *Flavobacterium* sp. B10 and *Caulobacter* sp. F16 exhibited susceptibility only at 20–22 °C. Overall, 48 to 72% (dependent on temperature) of the isolates we tested were susceptible to at least one of the isolates.

The degree of antagonism among our isolates is shown in Fig. 3. *Flavobacterium* isolates were found to be the least aggressive and one of the most susceptible taxa evaluated. The majority of β-proteobacteria was also susceptible to antagonistic interactions. *Bacillus* sp. and γ-proteobacteria including the genus *Pseudomonas* inhibited growth of *Flavobacterium* isolates and isolates of β-proteobacteria. Seven out of nine β-proteobacteria were susceptible to one of the γ-proteobacteria isolates at 21 °C and 5 out of 6 *Flavobacterium* isolates were susceptible to both *Bacillus* sp. and one of the γ-proteobacteria isolates. Genus *Pseudomonas* and *Bacillus* were found to be strongly aggressive. At a strain level, the most aggressive isolate was *Pseudomonas* sp. C22, which inhibited the growth of 15 out of 25 isolates. The most susceptible strain was *Hydrogenophaga* sp. F14 that was susceptible to 7 isolates, some with a large zone of inhibition (Fig. 3). Some isolates deviated from the general phylogenetic trend. Although about 80% of β-proteobacteria isolates were susceptible to at least one of the other isolates, strains *Vogesella* sp. F3 and *Janthinobacterium* sp. F8 showed aggressiveness to other isolates and were not susceptible to any aggressors. The strain *Flavobacterium* sp. B10 was also resistant to all aggressors tested.

Reciprocal Antagonistic Interaction

The antagonistic interactions remained evident in the reciprocal condition when the position of aggressive strains was switched from stamp to lawn. Evidence of antagonisms was detected when an aggressive strain (identified as a stamp strain) was placed in the soft agar as a lawn strain. The colony size of the sensitive stamped-isolates was severely diminished by presence of the aggressive strain in the lawn (Fig. 4a). A statistically significant negative linear relationship was observed between the degree of aggressiveness of the lawn strain and the average colony size of stamped strains on soft agar (Fig. 4a, $F_{1,21} = 16.8$, $p = 0.0005$). For example, *Pseudomonas* sp. C22, the strongest aggressor of all 25 isolates (inhibiting

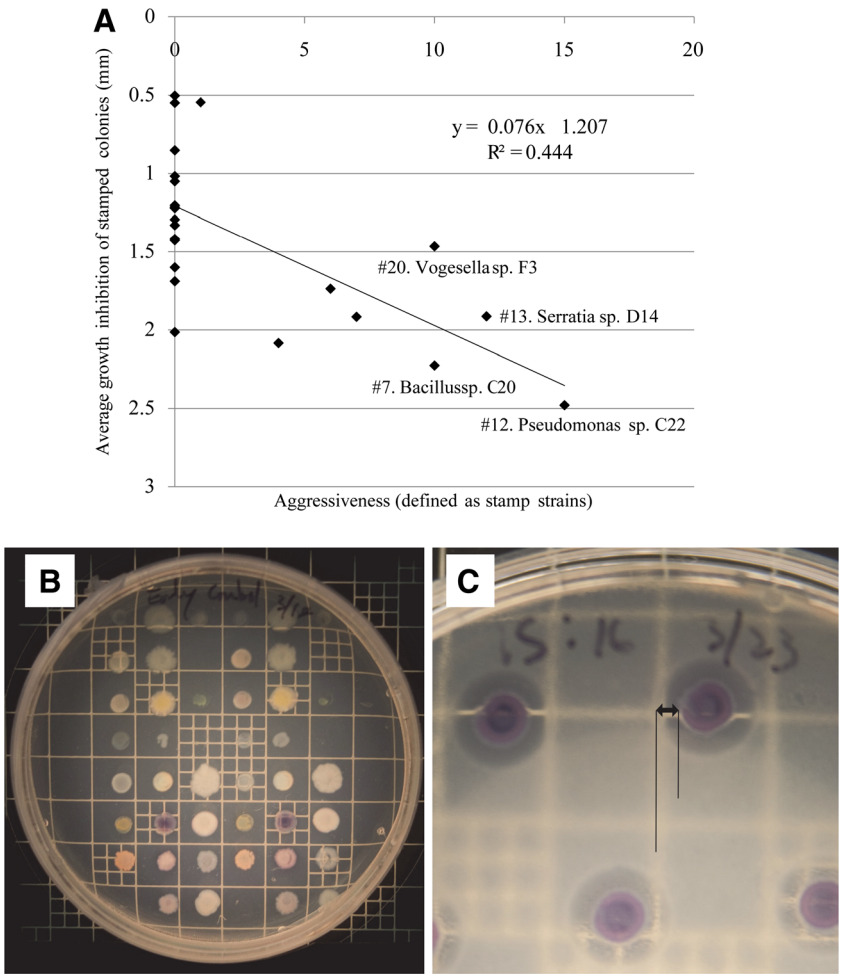

Fig. 4 **a** Reciprocal antagonistic interactions among 25 egg surface isolates when positions of aggressors were switched from stamp to lawn. Strong aggressors inhibited the growth of stamp colonies when used as lawn strains. The figure depicts the statistically significant negative correlation between the aggressiveness (defined in Fig. 3) and the average growth inhibition (the reduction in colony size relative to control) at 20–22 °C ($F_{1,21} = 16.8$, $p = 0.0005$). **b** Control stamped strains and **c** plate showing zone of inhibition

15 out of 25), decreased the average stamped colony size by 2.5 mm when inoculated in lawn. The negative effect of lawn strains on growth of stamped strains was also observed for non-antagonistic pairs of isolates presumably due to competition for resources.

Antagonistic Interaction Against Known Fish Pathogens

The four strong aggressive strains *Bacillus* sp. C20, *Pseudomonas* sp. C22, *Serratia* sp. D14, *Janthinobacterium* sp. F8 were tested against six known fish pathogens. All four aggressors inhibited at least one of the six fish pathogens, and all of the six fish pathogens were inhibited by at least one of the four aggressors (Fig. 5). *Pseudomonas* sp. C22, which displayed the highest aggressiveness among the 25 isolates, inhibited the growth of five out of the six fish pathogens. The most susceptible fish pathogen was *Flavobacterium* sp. C05, whose growth was inhibited by three out of the four aggressors we tested. In contrast to *Flavobacterium* sp. C05, *F. columnare* 090702-1 was susceptible to only one aggressor, *Janthinobacterium*. The most aggressive strain, C22, was ineffective against *F. columnare*.

Biofilm Forming Capability

The attachment of bacterial strains to the egg's surface is, phenotypically, the formation of biofilm. We therefore tested our strains for biofilm formation using the crystal violet assay [48]. Biofilm forming capabilities were both temperature and media dependent (Fig. 6). At 14 °C, five populations had biofilm staining greater than 0.5 A_{600} while eight populations achieved this measure at 22 °C. The pattern of biofilm formation and temperature/medium dependency was strain dependent. *Flavobacterium* sp. D11 formed biofilm at 20–22 °C when grown on minimum medium plus glucose. *Caulobacter* sp. F16 formed biofilm in R2B at both temperatures while two β-proteobacteria isolates (*Hydrogenophaga* sp. F14 and *Acidovorax* sp. F19) formed biofilm when grown on either R2B or minimum medium plus casamino acid at 20–22 °C. *Pseudomonas* sp. D2 and *Serratia* sp. D14 formed biofilm in five out of the six conditions (temperature/medium combinations) we tested.

Planktonic growth did not always directly correlate with biofilm forming capability. For instance, *Flavobacterium* sp. D11 grew well in R2B and minimum medium plus glucose at 20–22 °C, but biofilm formation was detected only in the minimum medium plus glucose. In addition, planktonic growth in broth medium was not a necessary condition for biofilm formation. For instance, *Pseudomonas* sp. D2 did not grow well planktonically at 14 °C in minimum medium plus glucose but displayed the maximum biofilm formation under these conditions.

The Effect of an Antagonist on Biofilm Formation

To determine if an antagonist detected via the soft agar technique was effective in inhibiting biofilm formation, we tested *Pseudomonas* sp. C22, our most aggressive strain, against *Caulobacter*, and *Acidovorax* (strong biofilm, >0.2) and *Hydrogenophaga* and *Massilia* (weak biofilm, <0.2) in a standard biofilm assay in which the four strains were allowed to establish a biofilm for 48 h followed by a 48 h challenge with *Pseudomonas C22* added to the wells. Strain C22 grew well in R2B but formed only small amounts of biofilm under these

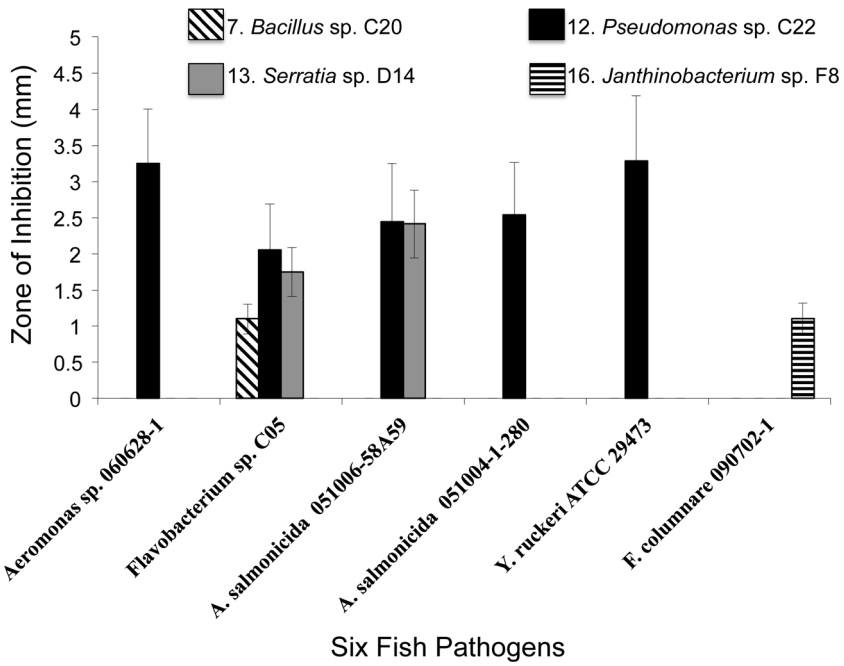

Fig. 5 Antagonistic interactions against six known fish pathogens by the four most aggressive isolates. Each fish pathogen was measured for growth inhibition using the soft agar overlay technique as described above. Mean and standard deviation are from 6 replicates

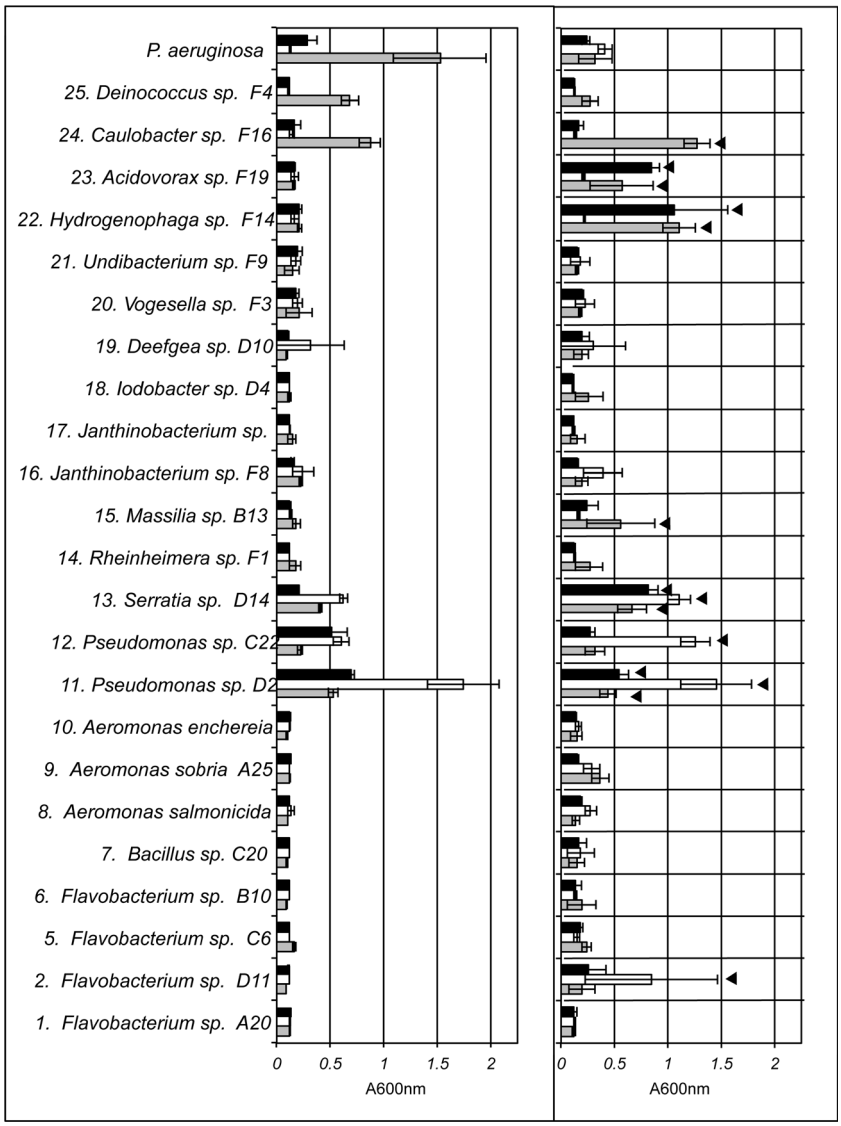

Fig. 6 Quantification of biofilm formation by 25 isolates in different media at two temperatures. Biofilm was measured with the crystal violet staining protocol. Statistical significance relative to control was declared using multiple comparisons with Tucky's honest significant difference (HSD) and was displayed above the respective bar (*white square* corresponds to $p < 0.001$). *Left panel*, 14 °C; *right panel*, 20–22 °C; *black*, R2B; *white*, M9-Glucose; gray, M9-Case Amino Acids

growth conditions. The results presented in Fig. 7a show that biofilm formed by *Caulobacter* and *Acidovorax* were substantially reduced in the presence of strain C22. *Massilia* and *Hydrogenophaga* formed only small amounts of biofilm and the observed reduction by C22 was not statistically confirmed. The positive control *P. aeruginosa* was not tested against C22. To extend this analysis to the fish pathogens described in Fig. 5, we set up the analogous biofilm assay, first establishing biofilm with the pathogens grown on R2B (Fig. 7b). Under these conditions, two *Aeromonas* isolates (060628-1 and 051006-58A59) showed reduced biofilm in the presence of C22 and three pathogens showed weakly positive responses to C22 but none of these responses were larger than the amount of biofilm that C22 forms alone. However, *F. columnare* was an outlier in this experiment in that there was a robust increase of biofilm when *F. columnare* biofilm was challenged with strain C22. This was repeatable in independent experiments.

Discussion

Our prior work indicated that the egg-associated microbial community of the sturgeon changes in predictable ways over the course of the maturation of the egg [19]. Changes in the community structure on the egg could be due to (i) changes in the aquatic microbial community from which the egg surface draws colonizing bacterial populations, (ii) egg surface chemistry and alterations in the quantity or quality of waste excreted from the egg that selects for bacterial metabolic guilds, (iii) antimicrobial activities of the fish immune system [50,51], eg. the presence of lysozyme (Fig. 1), (iv) competition between bacterial populations on the egg, and (v) predation of the bacterial community by phage, protists, or invertebrates. To obtain a more complete understanding of the assembly and maturation of the egg-associated community, we initiated a reductionist approach to study potential interactions between bacterial populations isolated from the sturgeon egg. The isolation

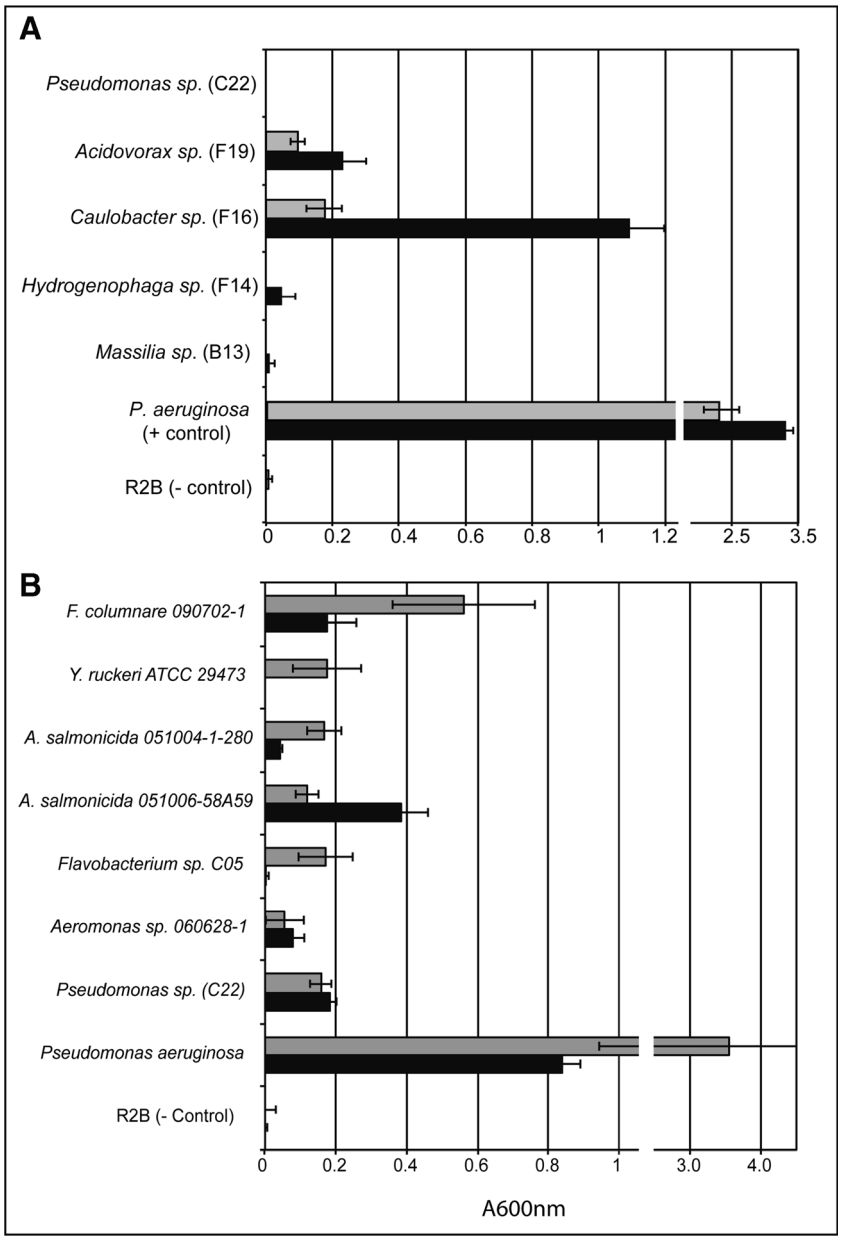

Fig. 7 Disruption of biofilm by strain C22. **a** The disruption of 48 h established biofilm of *Acidovorax*, *Caulobacter*, *Hydrogenophaga*, and *Masillia* by *Pseudomonas* strain C22 tested with the standard biofilm assay ("Material and Methods"). *Black*, 48 h unchallenged biofilm; *gray*, response of 48 h biofilm to strain C22 challenge after additional 48-h incubation; note discontinuity of the abscissa scale. **b** Disruption of biofilm of fish pathogens by *Pseudomonas* strain C22; *black*, 48 h isolate biofilm; *gray*, established biofilm (48 h) of isolates followed by a 48 h challenge with strain C22. Note discontinuity of the abscissa scale

effort obtained a diverse group of microbes from egg surfaces which covered much of the known egg surface microbial community diversity previously identified using pyrosequencing analysis [19]. The egg isolates were collected from both stream and hatchery under different environmental conditions and different developmental stages; hence the diversity of isolates was maximized and the obtained results were roughly comparable to those found using pyrosequencing. Similar to our previous results from pyrosequencing, the 92 isolates were dominated by phylum Bacteroidetes and class β-proteobacteria. However, the collection was underrepresented in the α-proteobacteria, which accounted for nearly 40% of third trimester egg associated microbial community in pyrosequencing reads [19]. The 25 isolates used in this study encompassed 16 genera including 5 isolates from genus *Flavobacterium*, the most dominant of all genera identified on the egg surfaces using pyrosequencing during the incubation periods in the natural stream. The 16 genera also covered the egg microbiome diversity observed throughout the successional changes associated with the embryo's developmental stages, regardless of flow rates. The 16 genera tested in this study represented 10 (*Flavobacterium*, *Undibacterium*, *Rheinheimera*, *Acidovorax*, *Massilia*, *Vogesella*, *Hydrogenophaga*, *Pseudomonas*, *Aeromonas*, *Caulobacter*) of the top 20 early dominant genera, 7 (*Flavobacterium*, *Undibacterium*, *Massilia*, *Iodobacter*, *Pseudomonas*, *Aeromonas*, *Hydrogenophaga*) of the top 20 middle dominant genera, and 5 (*Flavobacterium*, *Aeromonas*,

Hydrogenophaga, *Pseudomonas*, *Acidovorax*) of the top 20 late dominant genera previously identified using pyrosequencing. A diverse group of microbial strains that colonized the egg surface of lake sturgeon was tested for both antagonistic interactions and biofilm forming capabilities under different environmental conditions. We demonstrated that some isolates from the egg surfaces exhibited antagonistic interactions and biofilm forming capabilities that could help them to compete for resources and space on the egg when interacting with other members of the egg-associated microbial community.

We found that temperature influenced the detection of antimicrobial activity among isolates, suggesting that the nature of microbial interactions on the egg surface microbial community could vary across the prolonged spawning season for lake sturgeon, since ambient water temperature changes during the egg incubation period and fluctuates daily [19]. Temperature dependency of antimicrobial interactions has previously been reported in other systems [23, 29], but has particular relevance to the lake sturgeon host because adult sturgeon can spawn at different times during the season, which normally correspond to comparatively 'colder' and 'warmer' stream temperatures [17] as contrasted in our experimental design.

The phylogenetic trends we observed in antagonistic interactions were revealing in highlighting the evolutionary origins of microbial interactions. We found that *Pseudomonas* spp. and *Bacillus* sp. were most aggressive of all isolates tested in this study, and *Flavobacterium* spp. were the least aggressive and the most sensitive. Previous studies found similar antagonistic interactions in other microbial communities [22, 26–28]. Studies on microbial communities in marine water found that Bacteroidetes was the least aggressive phylum and the most susceptible to antagonistic interactions [28], and genus *Flavobacterium* spp. were the least aggressive genus in the community [26]. Strong inhibitory activities by *Bacillus* spp. [26] and γ-proteobacteria including *Pseudomonas* spp. [24, 52] were also reported. Our study also revealed variability in antagonism among strains within the same genus, suggesting that this phenotype is strain-specific [27].

The antagonism displayed by the aggressor isolates against known fish pathogens indicates that there is potential for microbes to protect fish eggs against colonization and proliferation of pathogenic bacteria. *Pseudomonas* sp. C22 could be considered for potential future use in probiotic treatment for eggs of lake sturgeon, since *Pseudomonas* sp. C22 was the most aggressive isolate against five emerging fish pathogens. Furthermore, *Pseudomonas* sp. C22 also inhibited the growth of several *Flavobacterium* spp. that are known to become dominant on the lake sturgeon egg surface microbiome specifically during the early embryonic developmental stage [19] (the presence of *Flavobacterium* spp. on hatchery reared eggs correlates with higher egg mortality [18]). *Pseudomonas* spp. have been used as probiotics to protect other fish species and found to be effective [24, 52–54].

Biofilm formation is relevant to microbial interactions since susceptible isolates may persist in a community by escaping the effects of antimicrobial substances through establishment of biofilm [12, 13]. The dependence of biofilm formation on both temperature and nutrient medium highlighted the complex nature of biofilm formation, corroborating observations in other systems [55, 56]. Our findings together with observations that egg metabolites can change during embryogenesis [57, 58] suggest that biofilm formation on the egg surfaces may be dynamic during embryogenesis. One should note here that the phylogenetic diversity of the microbial world is quite possibly recapitulated in the diversity of environmental conditions that elicit biofilm formation in diverse strains of bacteria. Predicting the community structure of the egg-associated community based on a handful of described phenotypic traits is impossible.

We documented a correlation between antagonistic interactions and biofilm formation in certain strains. For instance, *Hydrogenophaga* sp. F14 and *Caulobacter* sp. F16 were among the most susceptible strains and also were substantial biofilm formers. The fact that we could isolate these susceptible strains from the community where antagonists are present suggests that they may persist on the egg surface microbial community by evading the antagonistic interactions through biofilm formation, as previously reported by other studies [12, 13] or through non-uniform colonization of the surface. We also found that *Pseudomonas* strain C22 was capable of disrupting established biofilm of *Caulobacter* and *Acidovorax*. Similar observations that antagonists reduced the biofilm formation were also reported previously [59, 60]. Our observation that strain C22 inhibited the growth of five known fish pathogens highlights the fact that strain C22 may be a good candidate for probiotic treatment. However, we also note that the biofilm formed by *F. columnare* increased in response to the presence of *Pseudomonas* strain C22. The crystal violet biofilm assay does not inform us about which strain was present in the biofilm. However, in a separate experiment using culture-independent analyses (Illumina sequencing), we have identified both *F. columnare* and *Pseudomonas* strain C22 present in the biofilm at a ratio of 30:70, respectively (Angoshtari, R. & Marsh, T.L., in preparation). This finding was consistent with the apparent insensitivity of *F. columnare* to strain C22 in the soft agar over lay experiment. The complex interactions that we detect between strain C22 and pathogens and non-pathogens reveal the complexity of potential interactions among populations on the egg's surface. Concluding that a strain is a suitable probiotic because it carries antimicrobial activity against a few putative pathogens may be misleading as a shift in the community could be unfavorable to some pathogens while assisting

others. Nonetheless, some researchers have utilized applications of *Pseudomonas* strains to control plant diseases and find that pathogens have been successfully removed from the system [61].

The antagonistic interactions we observed could also help explain the process of community formation we observed in our earlier studies [19]. Pryosequencing analysis revealed that genus *Hydrogenophaga* increased its relative abundance toward the late egg developmental stage, and genus *Flavobacterium* decreased toward the late egg developmental stage. Our biofilm assays demonstrated that genus *Hydrogenophaga* was capable of forming a biofilm, which might allow for an increase in the relative abundance of this genus in the egg-associated microbial community toward the end of the egg developmental stage. The fact that *Flavobacterium* was one of the most susceptible genera to antagonistic interactions could contribute to the decline of this microbe in the community toward the later period of egg development. Although there are a number of other factors that could affect community assembly on the egg surface including dispersal from water columns [62, 63], pH [64], and local effects [65] such as egg metabolites [57, 58], and host immunity [66, 67], antagonistic interaction may be an important factor as previously described in some ecological systems [68]. Other recent studies also reported that antagonisms are important processes in the succession of microbial communities, specifically before long-term co-existence was established [21, 69, 70]. In our study, strains participating in egg-associated microbial communities were isolated from a relatively transient state, lasting for only the developmental period of the embryo (~10 days). Microbial succession on the egg and all interactions between strains is conducted within this short time frame [19].

We do not know how our findings translate into the natural setting where microbes interact on the egg surface in streams. Although our experimental regimes mimicked temperature ranges that eggs experienced during the spawning season, the nutrient content, concentrations, and physical structure of R2A agar and microtiter plates are different from the egg surface environment. Our antagonistic interactions represent those between a pure colony and a pure cell aggregate (lawn), which may differ from the situation on the egg surfaces where the density of microbes is considerably less. Furthermore, we tested the antagonistic interactions on a one-on-one basis, whereas in nature, the community is complex and local population demographics are unknown. We also incubated soft agar plates in the atmosphere, while microbes in the natural setting are submerged in a stream where dissolved oxygen is considerably lower. Previous studies demonstrated that the level of oxygen affects the induction of antimicrobial substances [31, 32]. Both nutrient uptake and delivery of antimicrobial substances could also be affected by stream flow. Although we tested biofilm-forming capability using pure cultures in broth media, the presence of antagonistic strains or antimicrobial substances can also induce biofilm formation [11]. The implications of our findings should to be viewed with constraints associated with our experimental design in mind.

In summary, our results revealed the complex nature of microbial interactions among the members of the egg surface microbial community. To our knowledge, this is the first study on any system to simultaneously analyze antagonistic interactions and biofilm formation, an approach that was informative in revealing the complex nature of microbial interactions. Although our findings may not fully represent microbial interactions that directly occur on the egg surfaces in the natural environment, our results can serve as baseline information to develop a model for microbial community formation on embryos throughout the incubation period. Our results also suggested a potential use of *Pseudomonas* sp. C22 and several other strains for probiotic application as a management tool to inhibit the proliferation of potential fish pathogens on the egg surfaces of this threatened fish species.

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