Unravelling the Microbiome of Eggs of the Endangered Sea Turtle *Eretmochelys imbricata* Identifies Bacteria with Activity against the Emerging Pathogen *Fusarium falciforme*

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Abstract

Habitat bioaugmentation and introduction of protective microbiota have been proposed as potential conservation strategies to rescue endangered mammals and amphibians from emerging diseases. For both strategies, insight into the microbiomes of the endangered species and their habitats is essential. Here, we sampled nests of the endangered sea turtle species *Eretmochelys imbricata* that were infected with the fungal pathogen *Fusarium falciforme*. Metagenomic analysis of the bacterial communities associated with the shells of the sea turtle eggs revealed approximately 16,664 operational taxonomic units, with Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes as the most dominant phyla. Subsequent isolation of Actinobacteria from the eggshells led to the identification of several genera (*Streptomyces, Amycolaptosis, Micromomospora Plantactinospora* and *Solwaraspora*) that inhibit hyphal growth of the pathogen *F. falciforme*. These bacterial genera constitute a first set of microbial indicators to evaluate the potential role of microbiota in conservation of endangered sea turtle species.

Introduction

Sea turtles are one of the most endangered groups of animals worldwide with only seven species left [1]. Incidental by-catch, disturbance of nesting beaches, pollution and diseases are major causes of drastic population declines [2]. Among the emerging diseases, the fungal pathogens *Fusarium falciforme* and *F. keratoplasticum* are an increasing threat to sea turtle nests, especially to those experiencing environmental stress [3].

Several conservation strategies have been proposed to mitigate the impact of pathogens on endangered species. For example, establishment of *ex situ* colonies and ‘habitat bioaugmentation and biotherapy’ have been proposed to prevent dispersal of the fungal pathogen *Batrachochytrium dendrobatidis* in amphibian populations [4]. The latter two strategies encompass the use of protective microbiota, either indigenous or introduced, to limit pathogen infection and spread. These two approaches are adopted in agriculture to control plant diseases [5–8]. Also in mammals, the role of gut microbiota in health and disease is now widely studied [9–13]. In nature conservation programs, however, these approaches are not common yet. This is due, in part, to a lack of knowledge of the overall diversity of microbiota associated with endangered species and their role, if any, in protecting their hosts against pathogen infection [14].

The structure of microbial communities of different hosts, their genetic diversity, and ecological roles have been studied combining culture-based analysis with polymerase chain reaction (PCR) techniques [15–17]. For example, the high-density 16S ribosomal DNA (rDNA) oligonucleotide microarray, referred to as the PhyloChip [18,19] combined with bacterial isolations has helped identifying key bacterial and archaeal community members in the rhizosphere of plants grown in disease-suppressive soils [20]. In sea turtles, a limited number of culture-based and biochemical studies have allowed describing taxa of bacteria associated with egg failure in several species [21–27]. These studies have listed and reported on potentially pathogenic bacteria from unhatched sea turtle eggs. However, full characterization of the microbial community and its effect on hatching of sea turtle eggs has, to our knowledge, never been conducted.

In this study, we investigated the microbial community associated with *Fusarium*-infected eggs of the critically endangered sea turtle species *Eretmochelys imbricata*. To that end, we collected eggs from the nesting beach La Playita at Machalilla National Park, Ecuador, in order to survey for bacteria with antifungal activity. For this purpose, PhyloChip analysis was used to identify...
the bacterial community associated with the turtle eggs. Based on these analyses, targeted isolations of specific bacterial genera were conducted using culture-based techniques followed by in vitro assays to determine the potential antagonistic activity of the selected indigenous microbiota against *F. falciforme*, the fungal pathogen of sea turtle eggs [3].

Results

Fungal isolation and molecular characterization

A total of 10 fungal isolates were obtained from the eggshells (Figure 1, S1) and initially identified as *F. solani* based on NCBI BLAST analysis of the ITS nrDNA sequences (Table 1). Phylogenetic analysis of the ITS nrDNA showed that the 10 fungal isolates clustered within the previously described species *F. falciforme* (Table 1 and Figure S2).

Bacterial isolation and DNA extraction from sea turtle egg shells

The number of culturable aerobic bacteria, enumerated on 1/10th strength Tryptic Soy Agar (TSA) medium, ranged from $10^4$ to $10^7$ Colony Forming Units per area of eggshell (CFU/cm²) from hatched and unhatched turtle eggs respectively. The population density of culturable Actinobacteria, enumerated on semi-selective medium glycerol-arginine agar (GA), ranged from $10^6$ to $10^9$ CFU/cm² from hatched and unhatched eggs, respectively (Table S1). The Actinobacteria comprised on average, 0.2% of the total aerobic bacteria enumerated on 1/10th TSA (Table S1).

PhyloChip analysis

PhyloChip-based metagenomic analysis of the bacterial communities associated with the eggshells revealed the presence of 16,664 operational taxonomic units (OTUs). On average, Proteobacteria (52%), Actinobacteria (17%), Firmicutes (15%) and Bacteroidetes (8%) were detected as the most dominant phyla (Figure 2A, B). No significant differences were detected in overall bacterial phyla composition between hatched and unhatched eggs or between the two nests (Figure 2A). At family level, however, significant (Welsh test, $p<0.01$; $r=0.75$, Anosim) differences in abundance between the two nests were found for the Pseudomonadaceae, which comprised 24% of the Gammaproteobacteria (Figure S3A; Table S2). Furthermore, the Flavobacteriaceae, which comprised 51% of the Bacteroidetes detected, were significantly (Welsh test, $p<0.01$) more abundant on shells of hatched eggs than those of unhatched eggs (Figure S3B). Within the Flavobacteriaceae, *Chryseobacterium* was the second most abundant genus (10%) and *C. indologenes* and *C. gleum* were the most represented species (Welch test, $p<0.01$) in our PhyloChip analysis (Table S3). These two species represented 7 and 5% of the genus *Chryseobacterium*, respectively.

| Table 1. *Fusarium falciforme* isolates from eggshells of the sea turtle species *Eretmochelys imbricata.* |
|-----------------------------------------------|-------------------------------------------------|-----------------|-----------------|
| **Strain** | **Source** | *GenBank Accession* | **Maximum identity** |
| 326FUS | Hatched egg | KF179246 | 100% |
| 327FUS | Hatched egg | KF179247 | 99% |
| 328FUS | Hatched egg | KF179248 | 100% |
| 329FUS | Hatched egg | KF179249 | 100% |
| 330FUS | Hatched egg | KF179250 | 100% |
| 331FUS | Hatched egg | KF179251 | 99% |
| 332FUS | Unhatched egg | KF179252 | 100% |
| 333FUS | Unhatched egg | KF179253 | 100% |
| 334FUS | Unhatched egg | KF179254 | 100% |
| 335FUS | Unhatched egg | KF179255 | 100% |

*GenBank accession number of the *Fusarium* isolates.

In vitro activity assay and BOX-PCR based identification of Actinobacteria

The Actinobacteria was the second most abundant bacterial phylum detected on the sea turtle eggshells. Given their well-documented ability to produce an array of antibacterial and antifungal compounds [33-35], we isolated Actinobacteria from the eggshells and determined their activity against *F. falciforme*. Out of a total of 98 randomly selected Actinobacteria isolates from hatched (n = 69) and unhatched eggs (n = 29), thirty-one inhibited hyphal growth of *F. falciforme* (isolate 331FUS). Among these 31 isolates with antifungal properties, 23 different haplotypes were identified by BOX-PCR fingerprinting. Subsequent 16S rDNA sequencing and phylogenetic analysis indicated that these isolates belong to the genera *Streptomyces* (16), *Amycolaptosis* (3), *Micromonospora* (1), *Plantaectinospora* (4) and *Solivasaspora* (5) (Table 2). A total of 25 out of 31 of the antagonistic Actinobacteria isolates were

Figure 1. *Sea turtle nesting area sampled for this study.* A) Nests of the sea turtle *Eretmochelys imbricata* in La Playita beach at Machalilla National Park, Ecuador. B) Nest containing hatched and unhatched *Fusarium*-infected eggs. C) *Fusarium*-infected hatched eggs. D) *Fusarium*-infected unhatched eggs. doi:10.1371/journal.pone.0095206.g001
obtained from hatched eggs. Out of the 6 isolates obtained from unhatched eggs, 1 corresponded to the genus *Planctactinospora* and the other five to *Streptomyces*.

Based on phylogenetic analysis of the 16S sequences, the antagonistic *Streptomyces* isolates clustered in three different groups within the 364 *Streptomyces* OTUs detected by the PhyloChip (Figure S4). The antagonistic isolates classified as *S. mutabilis* and *S. albogriseolus* clustered with OTUs classified as the same species detected by the PhyloChip (BS = 74% and BS < 50% respectively). The antagonistic *S. variabilis* isolate clustered with OTUs detected by the PhyloChip classified as *S. variabilis* and *S. aureofaciens* (BS = 58%).

Similarly, phylogenetic analysis of the 16S sequences of the antagonistic isolates belonging to *Amycolaptosis* sp. and *Micro-

Discussion

In this study, we described the microbial community of *Fusarium*-infected sea turtle eggs from the critically endangered species *Eretmochelys imbricata*. Due to the extreme difficulties to obtain samples and export permits from authorities for studies on endangered and critically endangered species, only four eggs were allowed to be collected. Hence, the results presented here provide a first ‘glimpse’ into the microflora associated with sea turtle eggs.

The PhyloChip analyses showed that the bacterial community associated with the eggs is mainly represented by the phyla Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes. In studies on the microbiome of the rhizosphere, members of the Proteobacteria and Firmicutes were described as the most dynamic taxa associated with disease suppression [20]. The potential implication of these bacterial taxa in protection of turtle eggs against *Fusarium* disease is not yet known.

No significant differences were detected in overall bacterial phyla composition between hatched and unhatched eggs or between the two nests. However, differences in abundance of two representative families of the microbial community of the sea turtle eggs were found. The significant difference in Pseudomonadaceae abundance among nests may reflect the variation in environmental conditions in the nesting area. Honarvar et al [23] demonstrated that bacterial diversity and richness increased with nest density and is higher in the zones closer to vegetation. *Pseudomonas* species have been previously isolated from cloaca of sea turtle females and eggs [22,28]. They have been associated with diseases of captive
Table 2. 16S rDNA sequence identities of the Actinobacteria, isolated from sea turtle eggshells, that inhibited the hyphal growth of Fusarium falciforme.

| Isolate | Source | Identity of best BLAST hit | GenBank accession | Score | Identity |
|---------|--------|---------------------------|-------------------|-------|----------|
| 2       | Hatched egg | Solvaraspora sp. | KF179216 | 979 | 99% |
| 121     | Hatched egg | Micromonaspora sp. | KF179221 | 1246 | 99% |
| 13       | Hatched egg | Micromonaspora sp. | KF179222 | 1222 | 98% |
| 14       | Hatched egg | Micromonaspora sp. | KF179223 | 1222 | 98% |
| 20       | Hatched egg | Micromonaspora sp. | KF179224 | 1226 | 98% |
| 125      | Hatched egg | Solvaraspora sp. | KF179225 | 1232 | 99% |
| 1       | Hatched egg | Solvaraspora sp. | KF179226 | 1260 | 100% |
| 16       | Hatched egg | Solvaraspora sp. | KF179227 | 1260 | 100% |
| 19       | Hatched egg | Solvaraspora sp. | KF179228 | 1260 | 100% |
| 23       | Hatched egg | Solvaraspora sp. | KF179229 | 1245 | 100% |
| 108      | Hatched egg | Solvaraspora sp. | KF179230 | 1245 | 100% |
| 145      | Hatched egg | Amycolaptosis coloradensis | KF179218 | 1134 | 99% |
| 151      | Hatched egg | Amycolaptosis coloradensis | KF179219 | 1238 | 99% |
| 152      | Hatched egg | Amycolaptosis coloradensis | KF179220 | 1245 | 99% |
| 147      | Hatched egg | Streptomyces mutabilis | KF179231 | 1265 | 100% |
| 150      | Hatched egg | Streptomyces albogriseolus. | KF179232 | 1250 | 99% |
| 146      | Hatched egg | Streptomyces variabilis | KF179217 | 1065 | 100% |
| 148      | Hatched egg | Streptomyces variabilis. | KF179233 | 1215 | 100% |
| 149      | Hatched egg | Streptomyces variabilis | KF179234 | 1264 | 100% |
| 153      | Hatched egg | Streptomyces variabilis | KF179235 | 1273 | 100% |
| 154      | Hatched egg | Streptomyces variabilis | KF179236 | 1270 | 100% |
| 155      | Hatched egg | Streptomyces variabilis | KF179237 | 1273 | 100% |
| 156      | Hatched egg | Streptomyces variabilis | KF179238 | 1273 | 100% |
| 157      | Hatched egg | Streptomyces variabilis | KF179239 | 1273 | 100% |
| 162      | Hatched egg | Streptomyces variabilis | KF179240 | 1278 | 100% |
| 164      | Hatched egg | Streptomyces variabilis | KF179241 | 1272 | 100% |
| 166      | Hatched egg | Streptomyces variabilis | KF179242 | 1269 | 100% |
| 167      | Hatched egg | Streptomyces variabilis | KF179243 | 1281 | 100% |
| 169      | Hatched egg | Streptomyces variabilis | KF179244 | 1244 | 100% |
| 170      | Hatched egg | Streptomyces variabilis | KF179245 | 1277 | 100% |

*ACT corresponds to the acronym of the Actinobacterial isolates.
BLAST hit corresponds to the Greengenes database (greengenes.lbl.gov/cgi-bin/nph-blast_interface.cgi).
The data represent the best BLAST hit with 16S rDNA sequences from the GreenGenes database (greengenes.lbl.gov/cgi-bin/nph-blast_interface.cgi).
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Insights into the Microbiome of Eggs of the Endangered Sea Turtles

sea turtles although their pathogenicity was not resolved [29]. In soil, the Pseudomonadaceae contribute to natural suppressiveness against several fungal pathogens including Fusarium [20,30,31]. For the Flavobacteriaceae, C. indologenes and C. gleum were the most represented OTUs (Welch test, p<0.01) in our PhyloChip analysis (Table S2). Chryseobacterium indologenes has been previously isolated from unhatched eggs of the loggerhead sea turtle Caretta caretta [22] and associated with shell disease of captive freshwater turtles [32]. Conversely, Chryseobacterium sp. strains are also known to exhibit antifungal activity [33]. Hence, the role of Flavobacteriaceae and/or the Pseudomonadaceae in mitigation of Fusarium infections of sea turtle eggs remains unclear. With the combined sample size of 4 eggshells, a first representative analysis of the microbial families that are associated with turtle eggs was performed (Figure 2). However, the differences observed between conditions (nests, hatched, unhatched) on family composition should be interpreted carefully due to the limited sample size per condition (n = 2).

The second most abundant bacterial phylum detected on the sea turtle eggshells was the Actinobacteria. Given their well-documented ability to produce an array of antibacterial and antifungal compounds [34–36], we isolated Actinobacteria from the eggshells and determined their activity against F. falciforme. The in vitro activity assays showed that isolated Actinobacteria of the genera Streptomyces, Amycolaptosis, Micromonaspora and Plantactinospora are able to inhibit hyphal growth of F. falciforme. Interestingly, most of the antagonistic isolates described in this study were obtained from hatched eggs (Table 2). The majority of the antagonistic isolates belonged to the genus Streptomyces and this genus was the most representative group of the Actinobacteria (Table 2). In plants, Streptomyces species have been implicated in the protection against bacterial [37] and fungal pathogens including Fusarium [38,39]. Species of the genus Streptomyces and other Actinobacteria with antifungal activity are also well known for their symbiotic associations with insects, protecting these from fungal pathogens [40]. The results of this study suggest that Streptomyces are a
component of the bacterial community that reduce infection or proliferation of Fusarium on sea turtle eggs. Whether the Streptomyces, and other antagonistic Actinobacteria species, identified in this study can be used as a bioindicator, or as a component of protective microbiota in the nesting areas, to minimize sea turtle infections by Fusarium or other fungal pathogens remains to be investigated.

This study provides a first survey of the composition of the bacterial microflora on eggs of endangered sea turtles. Understanding not only the diversity and abundance of bacteria and other microorganisms associated with endangered species, but also the role of these microorganisms in disease suppression may have direct applications for nature conservation programs.

**Material and Methods**

**Ethics Statement**

Collection of sea turtle eggshells was done under permissions: 002 RM-DPM-MA and CITES 003/V.S. None of the experiments involved sacrificing animals and, therefore, we did not require a specific approval from any institutional animal research ethics committee.

**Sample collection**

Samples were collected from two selected nests of the sea turtle species Eretmochelys imbricata located in La Playita beach at Machalilla National Park (Ecuador) during the nesting season of 2012 (Figure S1). Four eggs (two hatched and two unhatched) were collected (Figure 1). Immediately after hatching of the eggs (approximately 45 days after the start of the incubation), one hatched and one unhatched egg (containing a nonviable embryo) were collected per nest, all with signs of Fusarium infection [41] (Figure 1). Samples were collected using sterile latex exam gloves and maintained at 4°C in individual bags during 2 days.

**Fungal isolation and molecular characterization**

To confirm that the turtle eggs were indeed infected by Fusarium species, fragments of the eggshells (1 cm²) were placed on Peptone Dextrose Agar (PDA) and on Malt Agar (Figure S1), both supplemented with rifampicin (100 µg/ml) to prevent bacterial growth, and incubated at 25°C. Pure cultures of the fungal outgrowths were obtained by transferring single hyphal tips to fresh agar media. Pure cultures of the isolates are kept in the culture collection of the Laboratory of Phytopathology at Wageningen University, The Netherlands and the Real Jardin Botánico-CSIC, Spain.

To characterize the fungal isolates, DNA was extracted from mycelium (10 mg) collected from pure cultures. The mycelium was collected in 1.5 ml sterile tubes and 90 µl of NaOH (0.5 M) and two glass beads were added to the suspensions. The suspensions were placed in the Mixer Mill MM400 for 3 min to a frequency of 25°C. Pure cultures of the fungal outgrowths were obtained by transferring single hyphal tips to fresh agar media. Pure cultures of the isolates are kept in the culture collection of the Laboratory of Phytopathology at Wageningen University, The Netherlands and the Real Jardin Botánico-CSIC, Spain.

The amplification products were sequenced in both forward and reverse direction (MACROGEN, Amsterdam, The Netherlands). Sequencing results were processed by Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, Michigan, USA) and initially compared with sequences in the National Centre of Biotechnology Information [NCBI] nucleotide databases using BLAST [42]. For precise identification of the Fusarium spp., a phylogenetic analyses was carried out. The generated ITS nrDNA sequences from isolated Fusarium (Table 1), 136 NCBI-GenBank sequences of Fusarium turtle egg isolates (Table S1), and 60 selected sequences of Fusarium spp. from other hosts and environments were included (Table S2). The program Se-Al 2.0a11 Carbon [43] was used for manual alignment of the sequences. Maximum parsimony analysis (MP) [44] was inferred using the heuristic search option in PAUP* v4.0b10. Nonparametric bootstrap support (BS) [45] for each clade was tested based on 10,000 replicates, using the last-step option. Newly obtained sequences were submitted to GenBank with accession numbers KF179246 through KF179255.

**Bacterial isolation and DNA extraction from sea turtle eggshells**

For bacterial isolation and DNA extraction, the eggshells (4 cm²) were individually suspended in 10 ml of sterile tap water and vortexed for 2 min. The suspensions were sonicated using an ultrasonic bath (Transonic 460, Elma) for 2 min and vortexed for an additional 2 min at maximum speed. Each suspension was divided in 1.5 ml aliquots in eppendorf tubes and centrifuged at 10,000 rpm for 3 min. Pellets were resuspended in 100 µl of sterile tap water by vortexing and pipetting and then pooled in a sterile eppendorf tube to a final volume of approximately 700 µl. A 50 µl aliquot of each suspension was mixed with 50 µl of 80% glycerol and these samples were stored in the freezer at −20°C until processed for bacterial isolations. The remaining suspension (approximately 650 µl) was centrifuged at 13,000 rpm during 30 min, supernatants were discarded and pellets were stored at −80°C until processed for DNA extraction. For bacterial isolations, glycerol suspensions were diluted in 10-fold steps up to 10,000 times and, for each dilution, two replicates of 50 µl were plated on 1/10th TSA for total aerobic bacteria and on the semi-selective medium GA supplemented with Nalidixic acid (20 µg/ml) and Trimethoprim (20 µg/ml) for Actinobacteria (Figure S1). Both media were additionally supplemented with Delvocid (100 µg/ml) to prevent fungal growth. TSA plates were incubated at 25°C for 5 days and GA plates were incubated at 30°C for 21 days. Colonies were collected from GA medium. Based on the colony counts, the number of CFU/cm² was calculated.

**PhyloChip analysis**

To identify the bacterial and archaeal communities on the shells of Fusarium-infected sea turtle eggs, metagenomic DNA was isolated from the cell pellets extracted from the hatched and unhatched eggs (Figure S1). The PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc.) was used for DNA isolation according to the manufacturer’s instructions. The DNA concentration was determined by a Nanodrop 1000 Spectrophotometer (Thermo Scientific). The microbial profile for each sample was generated by G3-PhyloChip analysis (Second Genome, CA, USA). All PCR conditions and universal primers used for amplification of 16S rDNA genes of bacteria and archaea were previously described by [18]. Fragmentation of the 16S rDNA amplicons, labelling, hybridization, staining, and scanning of the PhyloChip, as well as data processing to determine absence/presence and HybScores of OTUs was performed according to methods
described by [18]. Phyla represented by over 10% of the detected OTUs were analysed in detail. These Phyla were also analysed at the family and genus level. Comparisons of composition between samples were performed using the Bray-Curtis distance with the average as the clustering method. Statistical analyses of the PhylodChip data were performed by Primer-E 6 software (PRIMER-E Ltd., UK).

In vitro activity assay and BOX-PCR based identifications of Actinobacteria

Because Actinobacteria have the ability to produce an array of antibiotic and antifungal compounds [34–36], all the bacterial isolates obtained from GA medium were purified and screened for in vitro antagonism against Fusarium isolate 331FUS, which was obtained from the sea turtle eggs in this study. For each bacterial isolate, one 5 mm diameter agar plug from 3-week-old culture plates was inoculated at the periphery of a quadrant of 1/5 strength PDA plates (four plugs per plate in total) and incubated for 4 days at 30°C. After this period, a 5 mm diameter agar plug from a 7-day-old Fusarium plate culture was transferred to the centre of the plate. After an additional 7 days of incubation at 30°C, inhibition of hyphal growth by each of the four bacterial isolates was measured and expressed relative to radial hyphal growth of Fusarium on plates without bacteria.

The genotypic diversity of the bacterial isolates with antagonist activity against Fusarium was assessed by BOX-PCR using the 22-mer BOXA1R oligonucleotide [46,47]. DNA was extracted from pure cultures using 2 mg of the colonies by microwave treatment as described previously [48]. The suspensions were centrifuged at 13,000 rpm for 30 s and supernatants were used for amplifications. Amplification reactions were performed in 25 μl containing 1 μl of DNA sample, 5 μl of 5x Gitschier buffer [49], 1 μl the BOXA1R primer (10 μm), 1.25 μl of mix of dNTPs (100 mM), 0.4 μl of BSA (10 mg/ml), 2.5 μl of 100% DMSO, 0.4 μl of 5 μl of GoTaq DNA polymerase (Promega Co, Ma, US) and 13.45 μl of MiQiQ water. Amplification was performed following an initial denaturation at 95°C for 2 min; 30 cycles at 94°C for 3 s, 92°C for 30 s, 50°C for 1 min and 65°C for 8 min [50]. PCR amplification products were detected by electrophoresis in 1% (w/v) agarose gels (5h at 45W). DNA fingerprints were visually compared for similarity; variations in intensities of bands were not taken into account in the analysis. For each isolate, one 5 mm diameter agar plug from 3-week-old Fusarium plate culture was transferred to the centre of the plate. After an additional 7 days of incubation at 30°C, inhibition of hyphal growth by each of the four bacterial isolates was measured and expressed relative to radial hyphal growth of Fusarium on plates without bacteria.

Supporting Information

Figure S1 Schematic presentation of the metagenomic and classical microbiological approaches and techniques. The scheme represent the approaches used to isolate, identify and characterize the fungal and bacterial community from eggs of the sea turtle species Eretmochelys imbricata nesting at La Plavita beach, Machalilla National Park, Ecuador. (TIF)

Figure S2 Out-group rooted cladogram of the ITS nrDNA region of isolates within the Fusarium solani species complex. One of the most parsimonious trees inferred from the ITS nrDNA sequence data of 136 sea turtle fungal isolates and 60 non-sea turtle fungal isolates. The numbers on the internodes indicate the bootstrap values (BS) of the parsimony analysis. Highlighted isolates correspond to those obtained in this work (n=10). The arrow indicates the F. falciforme isolate, i.e., 331FUS, used in the dual culture assays to determine the activity of the Actinobacteria. (TIF)

Figure S3 Cluster analysis (Bray-Curtis) of the microbiome of hatched and unhatched eggs infected by Fusarium falciforme. A) Dendrogram of family Pseudomonadaceae (n=949 OTUs). B) Dendrogram of family Flavobacteriaceae (n=710 OTUs). Abbreviations as in Figure 2. (TIF)

Figure S4 Out-group rooted phylogenetic tree inferred from the 16S rDNA sequence data from isolates of Streptomyces spp. Data includes isolates of Streptomyces spp. (n=16) with activity against Fusarium falciforme, and those detected by the PhyloChip analysis (n=364). The numbers at the internodes indicate the bootstrap values (BS) of the parsimony analysis. (TIF)

Figure S5 Out-group rooted phylogenetic trees inferred from sequence data from isolates of the Amycolaptosis sp. and Micromonosporaceae. Phylogenetic trees were inferred from the 16S rDNA data from isolates from both taxa, with activity against Fusarium falciforme, and those detected by the PhyloChip analysis. A) Phylogenetic tree from the isolates of the Amycolaptopsis sp. (n=3) with activity against F. falciforme, and those detected by the PhyloChip analysis (n=29). B) Phylogenetic tree from isolates of the Micromonosporaceae (n=11) with activity against F. falciforme, those detected by the PhyloChip analysis (n=33), and additional GenBank strains (n=5). The numbers at the internodes of the phylogenetic trees indicate the bootstrap values (BS) of the parsimony analysis. (TIF)

Table S1 Number of bacteria isolated from the shells of hatched and unhatched eggs of the sea turtle species Eretmochelys imbricata on 1/10th TSA agar medium (total aerobic bacteria) and on GA medium (semi-selective for Actinobacteria). Presented are the Colony Forming Units (CFU/cm²) for each of the two media and for each of the two hatch statuses. For each hatch status, a mean value of 2 eggs is given. SD refers to the standard deviation. (DOCX)

Table S2 Most abundant microbial communities from Fusarium-infected eggshells of the sea turtle species Eretmochelys imbricata. Data shown represent the most abundant phyla and families detected by the PhyloChip. The
families highlighted in grey are most represented (with >10%) per phylum.

**Table S3** *Chryseobacterium* species found significantly more abundant on eggshells of hatched than of unhatched eggs of the sea turtle species *Eretmochelys imbricata* (Welsh test, p<0.01; r=1, Anosim).

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**Author Contributions**
Conceived and designed the experiments: JMSR MvdV JMR JDU. Performed the experiments: JMSR. Analyzed the data: JMSR MvdV JMR. Contributed reagents/materials/analysis tools: JMR JDU. Wrote the paper: JMSR MvdV JMR JDU.

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