

# **Analysis of inter-species interactions and metabolite excretion in bioluminescent marine bacteria isolates**

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## **Abstract**

**Bioluminescent microbes are an important component of marine eco-systems and a useful model system for the study of intercellular signaling. Natural isolates of bioluminescent bacteria show varying levels of luminous intensity. In this study, purified isolates of luminescent bacteria clustered in two major groups, *Allivibrio fischeri* isolates displaying dim luminescence and isolates of *Vibrio harveyi* displaying bright luminescence. Interestingly, bright luminescence of an *A. fischeri* isolate was observed when grown in co-culture with a natural isolate of *Pseudoaltermonas piscicida* that was obtained from the enrichment for bright luminescent organisms. HPLC analysis of culture extracts indicates that *A. fischeri* strains produce a variety of organic acids, typically at higher levels than the analyzed *V. harveyi* strains. Analysis of co-cultures of *A. fischeri* and *P. piscicida* resulted in acid reduction and brighter luminescence, presumably via uptake by *P. piscicida*. The implications of acid excretion on quorum sensing and symbiosis with eukaryotes are discussed.**

## Introduction

Bioluminescence is an important signaling mechanism in marine organisms (Herring, 1985). Unicellular marine dinoflagellates use a flash of bioluminescence to escape predation. It is utilized by some fish species, such as the angler fish *Melanocetus johnsonii*, as a lure to attract prey. The Black Dragonfish uses a combination of blue light and red light emission, with the red light thought to provide a stealth advantage since so few deep sea organisms see that wavelength of light (Douglas *et al.*, 2000). Bioluminescence in marine macrofauna can be self-generated or generated by a symbiotic relationship with an associated bacterium. The Hawaiian bobtail squid, forms a stable symbiosis with the gamma-proteobacterium, *Allivibrio fischeri*.

Bioluminescence generated by bacteria cultivated in the light organ of juvenile squid provides the squid with protection from predators by providing counter illumination (Visick and Ruby, 2006). This symbiosis is maintained throughout the life of the squid, and is highly specific. Other marine bacteria, even luminescent organisms such as *Vibrio harveyi*, are pumped through the body of the squid as it swims, but yet not cultivated by the squid. *A. fischeri* benefits from the symbiosis through nutrients provided by the squid.

Many bacterial species utilize quorum sensing to monitor the local population densities of self and non-self and regulate gene expression accordingly (Waters and Bassler, 2005). These quorum sensing pathways are important for the regulation of processes such as biofilm formation and pathogenesis. There are three well-described quorum sensing chemical families; the homoserine lactones produced by Proteobacteria, the oligopeptides produced by members of the Firmicutes, and the furanosyl borate diester (commonly called autoinducer 2, or AI-2) which is dependent on activity of the LuxS protein and present in many domains of bacteria. However, analysis of 818 currently available complete bacterial genomes indicates that only 322 (39%) of these genomes contain an identifiable LuxS homolog (MIST database). LuxS is not present in any of the 62 available Archaeal genomes. Thus it is possible, if not certain, that other inter-species signaling molecules exist that have not yet been identified. In addition to producing cell-cell signaling molecules, microbes also release a variety of metabolic by-products (Ruby and Neelson, 1977). Indeed, cell-cell signaling molecules may have evolved from the release of metabolic by-products. Thus, in addition to detecting and responding to molecules designed for cell-cell signaling, cells must also deal with responding to any other molecules in their environment. It is expected that toxic metabolic growth by-products will be a common stress that cells must have evolved to deal with. In this study we examine the metabolic products of *A. fischeri* and the impact of these molecules on cell-density dependent bioluminescence.

## **Methods**

### *Strains and media.*

All strains used in this study were isolated from sites near Woods Hole, MA by students of the 2009 MBL Microbial Diversity course (See Table 1). Strains were enriched and cultivated routinely using Sea Water Complete (SWC) media except where noted. All growth incubations were performed at 30 °C.

### *Species identification and phylogenetic analysis.*

Genus and species names were determined through colony PCR amplification of the 16S rDNA. PCR products were treated with ExoSap reagent. Direct sequencing of the PCR products was performed and the nucleotide sequences trimmed for quality using FinchTV software. Edited sequences were uploaded to the Ribosomal Database Project (RDP) website. Sequence alignments and tree building were performed using RDP.

### *Photography.*

All pictures of petri dishes were captured using a Panasonic Lumix camera. Auto-focus and auto-exposure settings were used for visible light images. For images of bioluminescence, Manual settings were used as follows: Fstop = 2.8, exposure time = 15 sec, manual focus, and 2 sec delay. Images were cropped and brightness/contrast adjusted using Irfanview software.

### *Metabolic product analysis.*

3 ml of liquid SWC media were aliquoted into glass culture tubes and inoculated with the desired strain of bacteria. Cultures were grown overnight with shaking at 30 °C. Cultures were harvested and filtered through 0.2 µm syringe filters. The resulting cell free-extracts were either added directly to HPLC tubes in 550 µl aliquots or were stored at 4 °C until needed. 20 µl of sample were injected into a Shiamadzu HPLC equipped with a Biorad Aminex HPX-87H Ion Exclusion column for separation of acidic metabolic products. 550 µl of sulfuric acid in 2 L of Millipore pure water was used to run samples. UV spectra were captured and compared to known organic acid standards for peak identification. pH was determined using pH paper applied directly to the agar surface of plates for 10 sec and reading the color indicator.

## Results

### *Observation of differential luminescence*

Enrichment for luminescent marine bacteria from a decaying algal clump off the coast of stony beach resulted in the detection of a single, bright bioluminescent colony on a petri dish with ~400 total colonies. On the first attempt at purifying a clonal bioluminescent strain, bright bioluminescence was maintained, but there were also two distinct colony phenotypes. Luminescence correlated with yellowish-white colonies (strain 1H09), whereas pale white colonies were dark (strain 1A10). Both colony types were restreaked, and pure cultures obtained. After purification, strain 1H09 showed a markedly decreased bioluminescence; strain 1A10 remained dark. To determine if intense luminescence in strain 1H09 was dependent on the presence of strain 1A10, the strains were restreaked again in co-culture on the same petri dish (see Fig. 1). When co-cultured, bright luminescence of strain 1H09 was observed and the duration of luminescence lasted for several days. In contrast, mono-cultures of 1H09 show only a brief period of dim luminescence. Examination of other bioluminescent isolates indicated that dim luminescence is a common trait (See table 1). However, several isolated strains maintained bright luminescence in mono-culture. The causation for this difference in phenotypes will be examined further later.

PCR amplification of 16S rDNA indicated that strain 1H09 is closely related to characterized isolates of the *Allivibrio fischeri* species. Strain 1A10 is most closely related to *Pseudoaltermonas piscicida*. Both of these species are common to marine environments. *A. fischeri* is a well studied symbiont of eukaryotic animals such as the Hawaiian bobtail squid. *P. piscicida* is less well-studied, but has been implicated in a few cases of fish pathogenesis. This implies that these species may occupy similar niches, beyond the seawater sample used in the current enrichment.

### *Examination of secreted molecules in co-cultures*

Mono-cultures of *A. fischeri* 1H09, *P. piscicida* 1A10 and an *A. fischeri*-*P. piscicida* co-culture were grown overnight in 3 ml of liquid SWC media. *A. fischeri* displays bioluminescence transiently from ~12 – 18 h and then remains dark thereafter. *P. piscicida* does not luminesce, but forms a thick biofilm ring at the liquid-air interface of the culture medium. The co-culture shows physiological traits of both organisms, with a thick biofilm and luminescence. One interesting difference is that luminescence is more stable in the co-culture and observable over several days.

Cell-free extracts of all cultures were prepared using 0.2  $\mu\text{m}$  syringe filters. 550  $\mu\text{l}$  of cell free extracts were analyzed for organic acids with HPLC (see fig. 2). SWC is a complex medium and has a complex profile that can be used as the baseline for comparing culture extracts. *A. fischeri* produces a number of organic acids, the major peak at 9.5 min elution was not specifically identified, smaller peaks corresponding to lactate (13 min) and acetate (14.5 min) were also observed. *P. piscicida* culture shows little change in the acid profile compared to the SWC baseline. The co-culture shows no presence of the large unidentified peak at 9.5 min and lower lactate and acetate peaks. This indicates that organic acid production by *A. fischeri* is either inhibited by the presence of *P. piscicida* or alternatively, that the organic acid substrates released by *A. fischeri* are utilized by *P. piscicida* during growth. Determination of colony forming units after co-culturing indicates that both cultures are growing, with a slight growth (~5-fold higher density) of the *A. fischeri* cells.

#### *Examination of pH changes during acid excretion*

SWC is an unbuffered media and excretion of organic acids are likely to reduce the net pH of the system, which could impact energy intensive processes such as bioluminescence, growth and even survivability. To determine if dim bioluminescence is a consequence of the build up of organic acids excreted in the culture medium, *A. fischeri* 1H09 was restreaked onto SWC, SWC with 10 mM MOPS pH 7.2 and SWC with 10 mM Tris, pH 8.0 (see fig. 3). The presence of buffer significantly increases the overall brightness of luminescence after 24 h incubation. The most dramatic decrease in luminescence at this time point is observed in the highest density portion of the streak. Lower density regions containing isolated colonies have luminescence that is near to or as intense as the colonies on buffered media.

Over time, luminescence of *A. fischeri* decreases on all 3 of these media (see fig. 4). Probing of pH levels with pH sensitive strips indicates that mono-cultures of *A. fischeri* on unbuffered SWC can reach levels near pH 5 (orange strip). Even on buffered media, the pH was observed to decrease such that the pH was below 7 (yellow strips) after 96 h incubation. Co-culturing of *A. fischeri* with *P. piscicida* results in stable bioluminescence and stable maintenance of the pH level at or above 7 (green strips), such that bright luminescence is still observed after 96 h of incubation. These results indicate that regulation of bioluminescence is highly dependent on the local environment, and that *A. fischeri* can simultaneously release molecules that promote quorum-sensing dependent processes such as bioluminescence as well as bioluminescence inhibiting molecules such as acidic metabolic by-products. Thus, maximum bioluminescence in

this scenario is dependent on the presence of a neighboring species that can reduce the presence of extracellular acids.

#### *Phylogenetic analysis of luminescent isolates*

A total of 21 bioluminescent strains were isolated and the 16S rDNA sequenced. The sequences were aligned and grouped into a phylogenetic tree using RDP (see Fig. 5). All of the sequences fall into two major groups of *A. fischeri* and *V. harveyi* with the exception of strain 1A09 and 1F03. 1A09 resolved as a separate branch within the *A. fischeri*/*V. harveyi* group but shows a phenotype similar to *V. harveyi* isolates. Strain 1F03 grouped closely with *V. gazogenes*, but there are no known instances of bioluminescence from this species. Further sequencing would likely provide better resolution to the tree. Interestingly, the phylogenetic groupings corresponded with both the physiology and biogeography of the isolates (see table 1). *A. fischeri* isolates display dim luminescence (in mono-culture) and were isolated from seawater samples taken from Stony Beach, near Woods Hole, MA. In contrast, *V. harveyi* isolates show bright luminescence, faster colonial growth and were isolated from Eel pond in Woods Hole, MA. This indicates that there may be different selective advantages to growth in the two relatively similar environments.

#### *Analysis of metabolic diversity in luminescent isolates*

To determine if the acid production by *A. fischeri* strain 1H09 is typical of the *A. fischeri* species, cell free extracts of 6 strains of *A. fischeri* were prepared from liquid cultures as before. 4 strains of *V. harveyi* were also analyzed to determine if there are distinct metabolic signatures of the brightly luminescent *V. harveyi* and the dimly luminescent *A. fischeri*. *A. fischeri* strains were observed to excrete a number of organic acids, although an overlay of the traces indicates that not all strains produce the exact same acids or acid ratios (see Fig. 6). The large unidentified peak produced by strain 1H09 is unique to this strain. In general, malic acid (10 min) and the unidentified peak at 19.5 min are commonly excreted in *A. fischeri* strains, but not *V. harveyi* isolates. One strain of *V. harveyi* was observed to produce formic acid. Further analysis of acid production in response to various growth substrates should reveal if there is a significant difference in acid production and subsequent changes in pH and bioluminescence in the two species.

## **Discussion**

Every molecule excreted by a cell is potentially a signal. Some molecules are produced in direct proportion to cell density and when detected may function as quorum sensing molecules.

But sensing and responding to extracellular signals in the appropriate fashion will depend in large part on the other molecules in the environment, whether they be other quorum signals, metabolic substrates or toxins. It is paradoxical that in the laboratory mono-cultures of *A. fischeri* strains produce organic acids that build up to toxic levels at a similar time that quorum-sensing molecules build up to a level when they can effectively elicit a biological response. Co-production of these distinct molecules results in a brief bioluminescent output that is soon quenched and eventually leads to death of the culture. Interestingly, bioluminescence in *A. fischeri* can be prolonged by the presence of *P. piscicida*. It is not known if this relationship is ecologically relevant, and it may be that many organisms can fill the role of *P. piscicida* in removing acidic carbon sources from the co-culture. Since *V. harveyi* strains do not show this pattern, it is possible to speculate that *A. fischeri* strains are adapted to growth in symbiotic relationships where the organic acids they excrete are metabolized by symbiotic neighbors. It is unclear if *A. fischeri* ever engages in a symbiotic relationship with other bacteria such as *P. piscicida*, but the symbiosis between *A. fischeri* and eukaryotic hosts is well established. It is worth examining the metabolic by-products in future work to determine how these often over-looked molecules impact signaling status and the ability of organisms to appropriately respond to cell-cell signals.

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## Figure and Table Legends

**Table 1.** List of strain characteristics. Plate and well numbers refer to the position on sequencing plates and are used as unique identifiers for each strain in this study. N.d. = not determined.

**Figure 1.** Culture restreaks of *A. fischeri* strain 1H09 either in mono-culture (A, C) or in co-culture with *P. piscicida* strain 1A10 (B, D). Pictures were taken after 24 h incubation in the presence of visible light (A, B) and in darkness (C, D) to observe bioluminescence.

**Figure 2.** Organic acid profile from HPLC analysis of cell-free extracts from (A) 2 independent samples of SWC media and organic acid standards (B) *A. fischeri* strain 1H09 in mono-culture, (C) *P. piscicida* strain 1A10 in mono-culture, and (D) a co-culture of the two strains. Standard peaks represent (from left to right) oxalate, citrate, malate, pyruvate, succinate, formate, lactate, acetate, propionate, and butyrate.

**Figure 3.** Culture restreaks of *A. fischeri* strain 1H09 in mono-culture on (A) SWC agar, (B) SWC buffered with 10 mM, pH 7.2, or (C) SWC buffered with 10 mM, pH 8.0. Pictures were captured in darkness to observe bioluminescence after 24 h of incubation at 30 °C. Bioluminescence is reduced in the high density portion of the streak on unbuffered media.

**Figure 4.** Culture restreaks of *A. fischeri* strain 1H09 either in mono-culture (A, C, E) or in co-culture with *P. piscicida* strain 1A10 (B, D, F) after 96 h incubation at 30 °C. (A, B) SWC agar, (C, D) SWC buffered with 10 mM, pH 7.2, or (E, F) SWC buffered with 10 mM, pH 8.0. Pictures on the left depict colony appearance in visible light with pieces of pH paper added to the agar surface prior to photographing. The pH detected ranges from ~5 (orange) to 8 (green). Images on the right depict bioluminescence emitted.

**Figure 5.** Phylogenetic analysis of luminescent marine bacteria. 16S rDNA from novel strains isolated for this study were arranged in the tree based on nucleotide similarity. Type strain sequences from *Allivibrio fischeri*, *Vibrio harveyi*, *Vibrio gazogenes*, *Photobacterium phosphoreum*, *Pseudoaltermonas spongiae*, and *Pseudoaltermonas piscicida*, were added to provide a template phylogeny with *Pseudomonas syringae* as the outgroup. Most of the isolated

strains group with either *Allivibrio fischeri* or *Vibrio harveyi*. Exceptions to this are 1A09, 1F03, and 1A10.

**Figure 6.** HPLC analysis of luminescent marine bacteria organic acid profiles from (A) *A. fischeri* strains and (B) *V. harveyi* strains. Significant differences between the two species groups are labeled as (1) unknown, (2) malate, (3) unknown, and (4) formate.

Figures and tables

Table 1.

<b>Plate</b>	<b>Well</b>	<b>Scientist</b>	<b>Genus</b>	<b>Species</b>	<b>Luminosity</b>	<b>Isolation site</b>
2	C8	James Boedicker	<i>Allivibrio</i>	<i>fischeri</i>	Dim	seawater, fish skin
2	C12	Carie Frantz	<i>Allivibrio</i>	<i>fischeri</i>	Dim	Seawater, algae clump
1	H1	Emily Balskus	<i>Allivibrio</i>	<i>fischeri</i>	n.d.	Stony beach seawater
2	D8	James Boedicker	<i>Allivibrio</i>	<i>fischeri</i>	Dim	seawater fish skin
1	D3	Julia Rezende	<i>Allivibrio</i>	<i>fischeri</i>	Dim	Seawater
2	D6	Jenna Morgan	<i>Allivibrio</i>	<i>fischeri</i>	Dim	seawater MBL beach
1	C4	Alex Petroff	<i>Allivibrio</i>	<i>fischeri</i>	Dim	sea
2	B8	James Boedicker	<i>Allivibrio</i>	<i>fischeri</i>	Dim	seawater fish skin
1	G7	Yuko Hasegawa	<i>Allivibrio</i>	<i>fischeri</i>	Dim	Stony beach seawater
1	H9	Jeb Berleman	<i>Allivibrio</i>	<i>fischeri</i>	Dim	Sea water, buzzard bay
1	G1	Emily Balskus	<i>Allivibrio</i>	<i>fischeri</i>	n.d.	Stony beach seawater
1	F8	Terry Legg	<i>Allivibrio</i>	<i>fischeri</i>	Dim	seawater at MBL beach
1	A9	Teresa Kirschling	<i>Vibrio</i>	<i>harveyi</i>	Bright	Eel Pond
1	D7	Roland Hatzenpichler	<i>Vibrio</i>	<i>harveyi</i>	Bright	Eel Pond
1	E7	Roland Hatzenpichler	<i>Vibrio</i>	<i>harveyi</i>	Bright	Eel Pond
1	D10	Bolaji Okulate	<i>Vibrio</i>	<i>harveyi</i>	n.d.	Eel Pond
1	G2	Jan Blees	<i>Vibrio</i>	<i>harveyi</i>	n.d.	Eel Pond
1	H2	Jan Blees	<i>Vibrio</i>	<i>harveyi</i>	n.d.	Eel Pond
1	D1	Ben Tully	<i>Vibrio</i>	<i>harveyi</i>	Bright	Eel Pond, dock
1	B2	Monisha Brown	<i>Vibrio</i>	<i>harveyi</i>	Bright	Eel Pond
1	F3	Jenna	<i>Vibrio</i>	<i>gazogenes?</i>	dim	seawater
1	A10	Jeb Berleman	<i>pseudoaltermonas</i>	<i>Piscicida</i>	dark	Sea water, buzzard bay

**Fig. 1.**

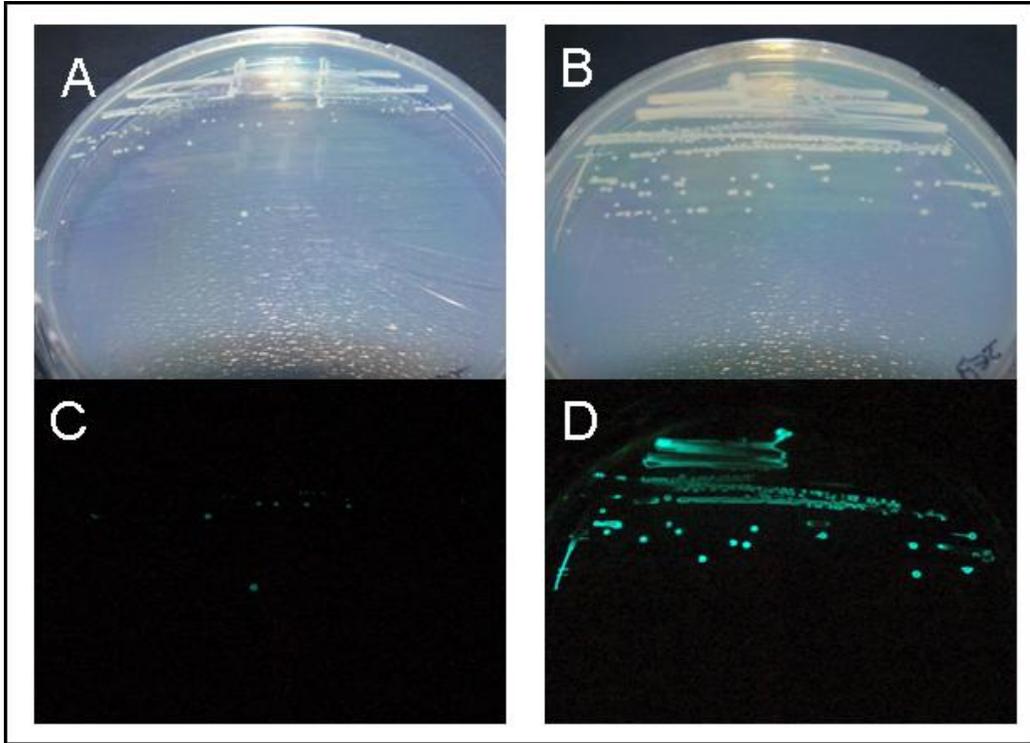
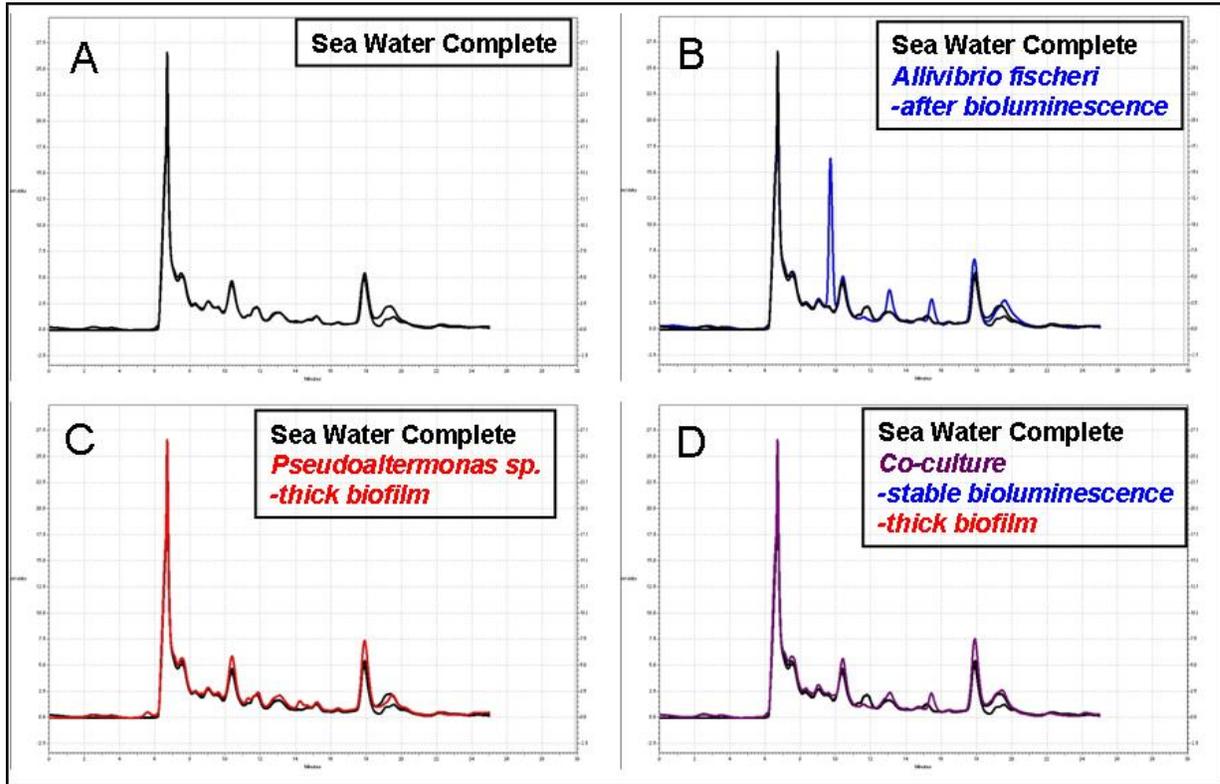


Fig. 2.



**Fig. 3.**

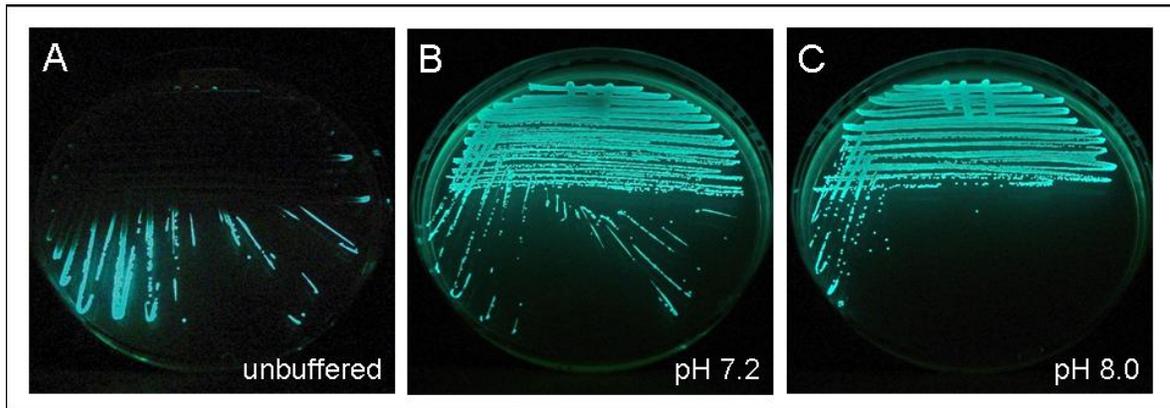


Fig. 4.

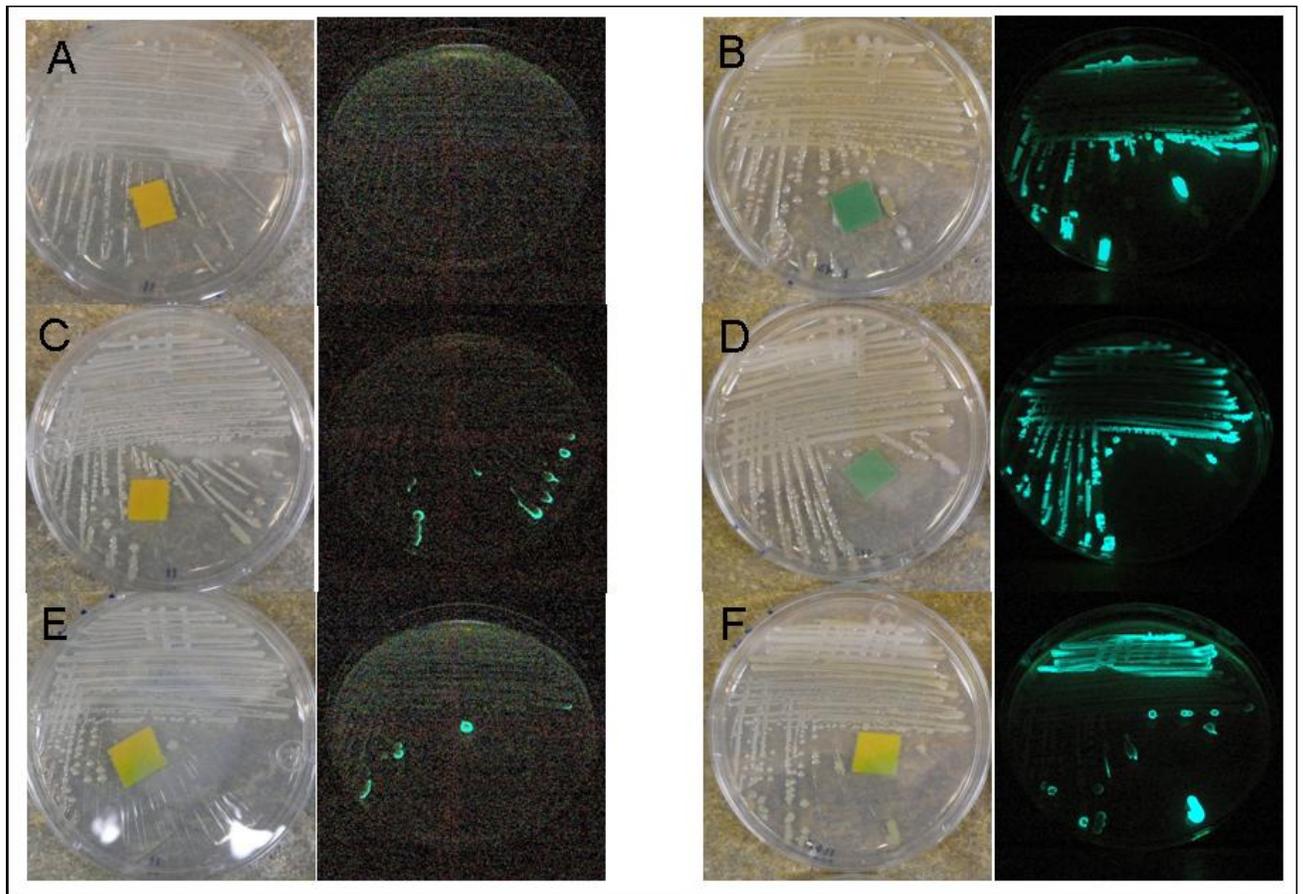


Fig. 5.

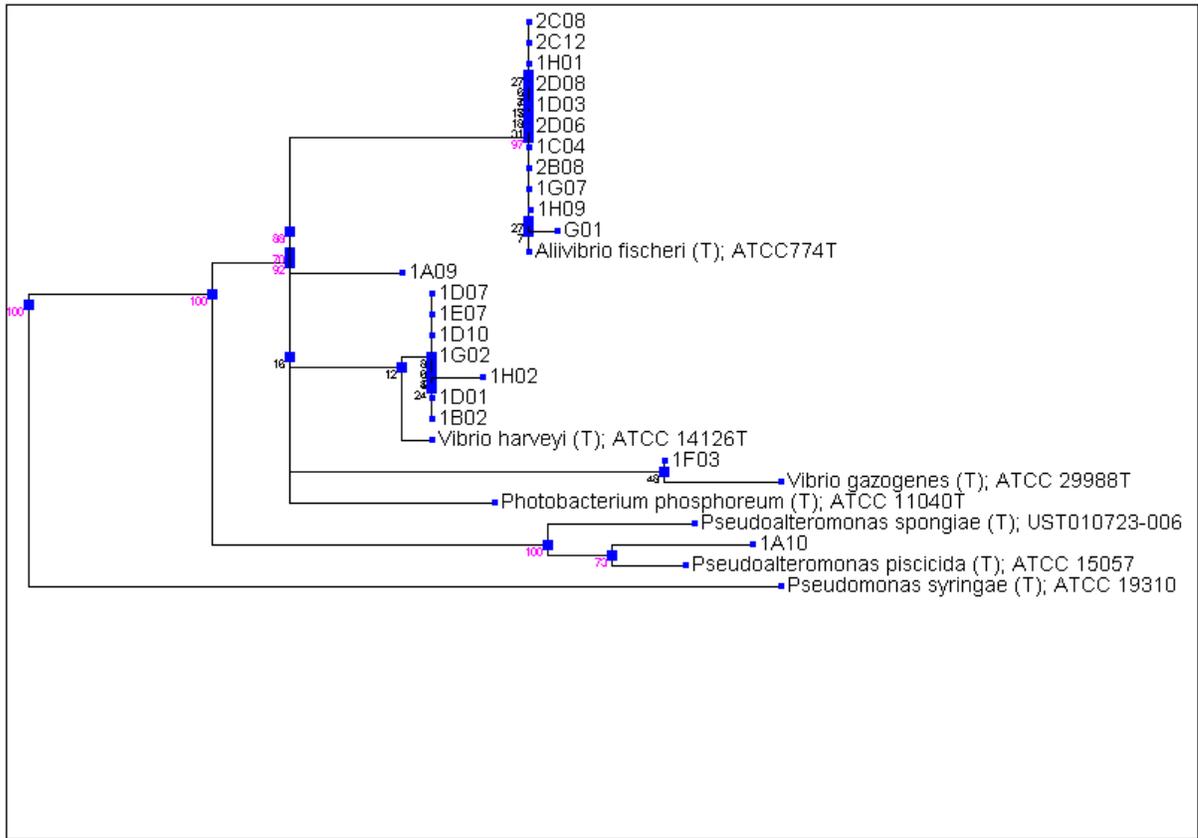


Fig. 6.

