

LONG TERM CO-CULTURE IN *ESCHERICHIA COLI* AND
PSEUDOMONAS AERUGINOSA

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LONG TERM CO-CULTURE IN *ESCHERICHIA COLI* AND

PSEUDOMONAS AERUGINOSA

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ABSTRACT

LONG TERM CO-CULTURE IN *ESCHERICHIA COLI* AND

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Most bacteria live in heterogeneous mixture in nature and as a result, constantly interact with other species. As bacteria are haploid, genetically tractable, and in some cases have generation times of less than one day, they are used as model organisms for evolution. In this study we used *Escherichia coli* strain MG1655 and two strains of *Pseudomonas aeruginosa* PA01 and PA14. *E. coli* and *P. aeruginosa* strains were grown as monocultures or as mixed cultures over a two month period, with the cultures being transferred daily. Throughout the culture protocol, representative cultures were collected and frozen for later analysis. When grown as batch cultures, *E. coli* did not survive with *P. aeruginosa* beyond 6-11 days. In contrast, when transferred regularly, the two organisms co-existed in approximately equal numbers in both planktonic and biofilm populations for two months (estimated 500

generations for monocultures). In co-culture experiments, evolved *P. aeruginosa* PA14 strain but not PAO1 lost fitness when grown with the parent *E. coli* strain. The evolved *E. coli* MG1655 strain lost fitness when grown with the parent PAO1 but not the PA14 strain. Several phenotypes changed during long-term culture. Indole production in *E. coli* increased during co-culture with both *P. aeruginosa* strains although the increase was significant only during co-culture with PA14. Pyocyanin production, commonly associated with *P. aeruginosa* competition, was decreased during long-term co-culture but in strain PAO1, increased significantly during long-term monoculture. Other *P. aeruginosa* phenotypes associated with quorum signaling were also reduced during long-term co-culture. Overall, this study shows how bacterial competition can be investigated as a component of natural selection during experimental evolution.

INTRODUCTION

In nature most of the bacteria live in heterogeneous communities where individual species interact with each other. Aside from predation, microbial interactions range from synergy to competition for nutrients, space and other resources (23). In the environment, beneficial changes in competitive traits can enhance ecological fitness. At the genetic level, changes occur through mutation or lateral gene transfer. Natural selection, a key driving force of evolution, is just as relevant to microbial competition as it is with higher organisms. As the microorganisms struggle for resources from they compete within the species and also in between the species also (13). I now address several aspects of microbial competition.

Bacteria have several different competition strategies. A population can (i) produce antibacterial molecules (e.g. bacteriocin and antibiotic) (7); (ii) produce metabolites such as short chain fatty acids that affects other organisms (22); (iii) produce high efficiency nutrient scavenging compounds such as Fe-scavenging siderophores (45) (16); (iv) by secreting predatory membrane vesicles (26) (38); and (v) disrupting signals thereby altering gene expression of competing organisms (53).

At the beginning of 20th century A. Gratia found that chemicals produced by some bacteria can kill other closely related bacteria. They were termed as bacteriocin. In several instances bacteriocins are named for the organisms that produce them, one example is colicin produced by *Escherichia coli* (7). They have a great importance in medical science as introduced pathogens can be killed by the bacteriocins produced by the normal microorganisms in the body. In contrast to antibiotics, bacteriocins are often strain-specific

in their bacterial targets (7) and so are not used widely. But due to increasing antibiotic resistance there is certainly renewed interest in alternative microbial control strategies including bacteriocins, identification of new antimicrobial compounds from plants (1) and bacterial signal disruption (21).

In the human genetic disease, cystic fibrosis (CF), the lung becomes permanently colonized by a mixture of bacteria and the resulting chronic pneumonia, coupled with a reduction of lung function, ultimately is a leading cause of death (20). The microbial ecology of CF lung flora is complex and there are a number of microbial interactions that occur. In one example, some organisms produce a metabolic by-product, acetate. In some cases these by-products are shown to be harmful to the other species. However in many instances they are utilized as second carbon sources by the other species other than the producer (22). Although *Pseudomonas aeruginosa* is a dominant organism in CF sputum, it typically colonizes subsequent to *Staphylococcus aureus*. During investigations of *S. aureus* – *P. aeruginosa* interactions, *P. aeruginosa* was shown to be able to kill *S. aureus* and use this organism as an iron source (37) (43).

Membrane vesicles (MV) are proven as signal traffic system for prokaryotes. *P. aeruginosa* can use MV as package for PQS quinolone signal (cell-cell communication) and produce PQS regulated group activity and without the MV they shut down those group behaviors (38). In other studies *P. aeruginosa* membrane vesicles were proved to lyse gram positive *Bacillus stearothermophilus* and *Aneurinibacillus thermoaerophilus* (26) and *S. aureus* during growth in CF sputum (38). These studies are example for the importance of membrane vesicles in co-culture of bacteria.

Bacteria can produce iron chelating molecule to scavenge iron molecules from the environment and they are known as siderophores (52). There are numerous siderophore-

mediated interspecies competition strategies. Many bacteria can use heterologous siderophores which are produced by other species and also differential binding affinity with the siderophores can mediate interspecies competition (23). *P. aeruginosa* in mix culture with *Staphylococcus aureus* is able to lyse *S. aureus* cell liberating free iron. In pure cultures, both *P. aeruginosa* and *S. aureus* produce siderophores that compete with the human host for iron (37).

Bacteria as single celled organisms have traditionally been thought to act as individuals. But recently about three decades ago Nealson and Hastings found community behavior of the bacteria *Vibrio fischeri* on Hawaiian squid light organ (41). The bacteria cells reach high density (10^{10} to 10^{11} cells/ml) in the light organ of squid and in high cell density, induce expression of genes required for bioluminescence which the squid use to protect themselves from predation. Bacteria communicate each other through different chemicals in a community and the technique is known as quorum sensing. This process includes producing, releasing, detecting and receiving some molecules which are known as autoinducers (AIs) or quorum signals (QS). Individual bacteria produce quorum sensing *N*-acyl homoserine lactones (AHLs) and when they reach threshold concentration, they change gene expression, and therefore behavior and start acting as a group. Originally quorum signaling was associated with the regulation of single phenomena such as light production in *V. fischeri* with the gene responsible for AHL synthase (*luxI*) and the gene responsible for AHL-induced transcriptional activation (*luxR*). Other gram negative bacteria capable of AHL synthesis and regulation thus have *luxI* and *luxR* homologues although the specific gene names will vary (19) (14). Whiteley et al. (1999) performed random insertions of promoterless *lacZ* reporter genes in the *P. aeruginosa* genome and found thirty nine genes to be regulated by AHL signaling in this organism (54). Based on this and subsequent work by

other laboratories, quorum signaling is now recognized as a global regulatory system (53). Quorum sensing is important for a bacterial community as it helps biofilm formation, turning on some virulent genes, induce production of secondary metabolite pyocyanin, hydrogen cyanide; and also help production of various virulent factors like elastase, extracellular protease, and rhamnolipid etc. (53).

Bacteria quorum sensing process can be classified into two different categories according to signal receptor proteins. Gram negative bacteria represent LuxR-type where two proteins LuxI and LuxR. LuxI is AHL synthase and produce AHLs. LuxR is a cytoplasmic AHL receptor and DNA binding transcriptional activator. Following production of AHLs in low density population the AHLs are easily diffused but when the cell density is high and signal reaches threshold level AHLs bind LuxR and change gene expression. Quorum sensing is a lot more complex in *Vibrio harveyi* there are three systems (53), and in *P. aeruginosa* there are two AHL systems and the PQS system. The “threshold level” for quorum signaling is relatively high in planktonic lab culture (typically $>10^8$), yet is activated normally at much lower cell numbers within biofilms and other situations, notably infections. To address this discrepancy, Connell et al. (10) grew *P. aeruginosa* in microscopic, diffusion-restricted polymerized albumen chambers and showed quorum signaling to be activated in more physiologically relevant ($>10^3$ CFU) cell concentrations.

Gram positive bacteria have quorum sensing transduction systems where they have membrane bound sensor histidine kinases as receptor. Signals in gram positives, are typically small peptides which may be circular or linear depending upon the species and have been associated with several functions including genetic competence, virulence, and stress response (36) (46). The signal is mediated by a phosphorylation cascade that influence the activity of DNA binding transcriptional regulatory protein termed a response regulator.

So the quorum sensing plays a very crucial role on bacterial physiology in a community. Also it is very important at the interface of different bacterial population and also host-pathogen aspect. So in nature when many different bacterial populations compete for limited resources it is very important for a bacterial population to have the ability to disrupt quorum sensing of other bacterial communities to get an advantage. It is also implied in the case of host to interfere bacterial cell-cell communication to prevent bacterial colonization (53). Zhang and his colleagues found that the auto-inducer inactivation or *aiiA* gene of *Bacillus* sp. can inactivate AHLs. They found *aiiA*-encoded AHL lactonase can hydrolyze the ester bond of homoserine lactone ring of AHLs. It has been also found that halogenated furanones on the coat of Australian red macroalga *Delisea pulchra* can inhibit quorum sensing (15). The process of interfering with quorum sensing is known as quorum quenching.

Most of microbiology research investigations have been carried out with pure cultures of bacteria, a concept that dates from the work of Louis Pasteur and Robert Koch (28) (5). As bacteria live in mixed culture in nature, it is very important to explore the bacterial physiology in co-culture. In this study, I am using two Gram-negative bacteria, *E. coli* and *P. aeruginosa* as model organisms. AHL-based quorum signaling has been documented in *P. aeruginosa* (14). *E. coli* is unable to produce AHL but it can respond to exogenously-produced AHLs (14) via *sdiA*, a *luxR* homologue (14). Also *E. coli* is capable of producing extracellular indole as a result of tryptophan biosynthesis (12). Evidence of indole produced by *E. coli* having impact on other bacteria phenotype is shown before (31). Indole increases biofilm formation in *P. aeruginosa* (32), and also indole can alter expression of different QS regulated genes and decrease production of quorum-regulated pyocyanin, rhamnolipid, *Pseudomonas* quinolone signal, and pyoverdine (31). It is also found that indole is nontoxic and

do not have any effect in growth rate of *P. aeruginosa* at 1.0 mM concentration, but at the concentration of 2.0 mM it has some effect in *P. aeruginosa* growth. (31).

Microbiology experiments can be conducted on the basis of both short term and long term culture of bacteria. It is expected that bacteria will respond to environmental changes when they are mixed with other species. For short term culture they may have some alterations in phenotypes, gene expression etc. But for the long term culture they may get some changes which can be permanent and also may be very significant than the wild types. These changes can be heritable and may lead towards natural selection. Bacteria have very short generation time, are haploid, and also highly mutable. Having these characteristics they are used as a model of experimental evolution. Maughan and her group have shown that sporulation was lost in *Bacillus subtilis* after culturing in nutrient rich media after 6,000 generations (40). They showed that it happened due to small scale changes in their genome. Richard Lenski and his colleagues from Michigan State University have shown adaptation of the microorganism in their environment and increasing divergence between different species in long term cultures (33). In his study Lenski has used twelve *E. coli* populations growing in glucose limited medium for 20 years (approximately 50,000 generations). Representative findings from Lenski's studies with *E. coli* include observing an increased mean fitness on the growth media; larger cell size of evolved bacteria, and an acquired ability to utilize citrate (33) (4). There are many studies using bacteriophage in microbial evolution experiments going on, especially to mention Luria and Delbrück using bacteriophage (34). Some other studies showed different adaptive behaviors using bacteriophage and Horizontal Gene Transfer (HGT). Long term exposure with increased temperature increases the heat tolerance (11). HGT of an external gene can introduce a novel characteristic in a species when grown for long time after the gene transfer and forced to live on particular environment. Like *E. coli*

after introducing genes need to catabolize sucrose and grown on sucrose showed increased fitness after long term culture (29). Due to adaptive behavior, fast growing resistance to antibiotic, highly mutable nature and phenotypic changes it is very important to study experimental evolution with bacteria. Long term culture with both monoculture and mix culture showed interesting phenotypic changes and genome sequencing after long term culture showed many short scale or large scale changes in their genomes.

Although there are many research projects studying experimental evolution using single species, very few studies are conducted on co-evolution. A mixed culture approach is highly applicable in that organisms naturally co-exist with others and mutation and other events leading to evolution do not occur in isolation. Few studies on mixed species co-evolution like host-pathogen interaction, between competitors have done. Evolution of plant immune response with R gene (Resistance gene) from microbial attack is an example of host-pathogen coevolution study (8). A separate study showed *P. aeruginosa* grown in vivo in cystic fibrosis patient for 39,000 generations where expression of several genes was found (24). It is important to mention in this context that cystic fibrosis lung is heterogeneous culture of bacteria and in this study they found the organism has acquired beneficial adaptive characteristics over time in the chronic disease case (24). There is very little research has been done on two bacterial species co-evolution. In a co-culture study with *P. putida* and *Acinetobacter* sp. grown on benzyl alcohol as carbon source showed that *P. putida* has grown increased ability to attach with *Acinetobacter* sp. and which helps them form better biofilm (9)(18). In real life situation coevolution study showed in human oral cavity *Streptococcus mutans* which is major cause of caries (42) were inhibited by *S. oligofermentans* and causes reduced caries (51). *S. mutans* enhance activity in the presence of lactic acid by which it

prohibits growth of other microorganisms. But interestingly with *S. oligofermentans* use lactic acid as substrate to produce peroxide, thus reduce the virulence of *S. mutans* (51).

The objective of this study is to observe competition between two organisms in both planktonic and biofilm culture for long time. In this study we seek for competition and/or cooperation between two bacterial species. As both of the species can produce and secrete cell signaling molecules they might alter phenotype of each other. Also it will be interesting to see how they will differentiate and evolve in mixed culture as well as in pure culture. In our experiment *P. aeruginosa* strains PA01 and PA14, and *E. coli* MG1655 were used as model organisms for the experiment. *P. aeruginosa* and *E. coli* are commonly found together in nature in soil and water, as well as the gastrointestinal tract. They also can be found on inanimate objects and serve as causative agents for hospital-borne or nosocomial diseases in immune-compromised patients (55). Also *P. aeruginosa* is ubiquitous and can also be found in the mammal intestine and cause sepsis (39) where *E. coli* is present. *P. aeruginosa* is responsible for chronic respiratory disease like cystic fibrosis where the organism develop for very long time, which is one reason for studying coevolution of *P. aeruginosa* for long time. *P. aeruginosa* PA01 is a prototype of *P. aeruginosa* lab strain and derived from burn infection (35); and PA14 strain is a human isolate and have multi host range (44). *E. coli* MG1655 is a common lab strain. All three strains have fully sequenced genome (49) (30) (3). In the long term experiment it is very important to know relative fitness (W) and Selection Rate (r) of each species. Relative fitness is a dimensionless quantity, which can be calculated as ratio of growth rate of one species with other species during competition. In this study it an important way of measuring competition between *E. coli* and *P. aeruginosa*. Two species were added in 1:1 volumetric ratio in LB broth and allowed to grow in normal growth

condition for 24 hours. Initial and final cell concentration was measured by plating them on LB agar (33).

MATERIALS AND METHODS

Bacterial strains and Media:

The bacterial strains used for this experiment are listed in Table 1. Cultures are grown in Luria-Bertani (LB) broth which contains Bacto-tryptone, yeast extract, NaCl and pH 7.5. All strains are stored in -80°C freezer with 50% glycerol and LB broth. Ampicillin (100µg/ml) and Cefsulodin (20µg/ml) antibiotics are used to isolate *P. aeruginosa* and *E. coli* respectively from the mix cultures (27).

Table 1: Strains used for long term mixed culture experiment.

Strain	Characteristics	Source
<i>E. coli</i> DS291/MG1655	Wild type CF 1648=MG1655	D. A. Siegele, Texas A & M University
<i>P. aeruginosa</i> PA01	Wild type	V. Deretic, University of Texas Health Science Center, San Antonio
<i>P. aeruginosa</i> PA14	Wild type	M. Whiteley, University of Texas

Co-culture in Batch Culture system:

Both strains of *P. aeruginosa* were mixed with *E. coli* at equal cell density (OD₆₀₀ 0.010) in 50 ml LB broth with glass beads. Serial dilution of the culture and then plating on antibiotic

media (ampicillin and cefsulodin to isolate *P. aeruginosa* and *E. coli* respectively) to isolate each species were performed to count the CFU at regular intervals.

Long term co-culture of Bacteria in pure and mixed culture:

Long term culturing was performed on pure cultures of *E. coli* MG1655, and *P. aeruginosa* PAO1 and PA14, as well as MG1655-PAO1 and MG1655-PA14 co-cultures. All the strains both pure and mix cultures were performed in triplicates. 5ml of fresh LB broth was aliquot in 16mm tubes with 4-5 glass beads of 4mm diameter from Fisher Scientific (Cat No. 11-312B), which are used for biofilm growth. The LB broth was preincubated to check contamination. On the first day of the long term mix culture project each strains were grown up to OD₆₀₀ 0.010 and then added in the respective tubes. Each day old culture was transferred every day into new media and also every week's culture were stored frozen at -80°C using glycerol (12.5% v/v) as a cryoprotectant. So we can use the frozen cultures for any experiment and that helps us go back in any generation of bacteria and perform tests. In average *E. coli* was generating approximately 8 generations per day where *P. aeruginosa* PAO1 and PA14 was giving 7-8 and 8 generations respectively. Contamination was checked by streaking them on LB plate for pure culture and also various biochemical tests.

Generation count:

We used a Bio-Rad Spectrophotometer (model SmartSpec™ Plus Spectrophotometer, Serial number 273BR01423) as a tool for reading absorbency of the cultures at optical density at 600nm (OD₆₀₀). We used “doubling” method of bacteria to count the numbers of generations evolved. As bacteria reproduce asexually where one mother bacteria go through binary fission process to give rise to two daughter cells. So absorbency readings are taken just after transferring in new media and after 22-26h of incubation. As for example if initial

OD₆₀₀ of culture is 0.01 and after incubation when the OD₆₀₀ is 0.02 then culture has doubled once. We have grown the bacteria for 60 days of daily transfer which corresponds 500 generations of the bacteria in pure culture.

Growth rate in mixed culture experiment:

During the period of two months long culture, the *E. coli* and *P. aeruginosa* cell numbers were measured using dilution plating with antibiotic containing media (described above) in weekly manner.

Measurement of Relative Fitness in mix culture using Malthusian parameter:

As stated in Lenski (33) let $N_1(0)$ and $N_2(0)$ are initial densities and $N_1(1)$ and $N_2(1)$ are final densities of *E. coli* and *P. aeruginosa* respectively. Then the average increase of growth or Malthusian parameter m_i for either competitor is,

$$m_i = \ln[N_i(1)/N_i(0)] \text{ per Day}$$

Hence the selection rate which is constant, r is equal to the difference between the Malthusian parameter of two competing strains. Now to calculate relative (Darwinian) fitness ration of Malthusian parameter of the species was used (33). Then the ratio of Malthusian parameters of the strain grown in mixed culture and pure culture were compared to check relative fitness.

Pyocyanin assay:

10ml of overnight culture was extracted with half a volume of chloroform and then the chloroform layer was removed in a 15ml falcon tube. Then the chloroform solution was centrifuged at maximum speed for 10minutes. Then it was extracted with 0.1N HCl, usually

1 ml of 0.1 HCl for 5ml of Chloroform. Then the aqueous layer was removed and absorbency was measured at 520nm.

Elastase Assay

Elastase activity produced by different *P. aeruginosa* was measured by Elastin Congo Red (ECR) assay. Cells were grown in a flask containing LB broth for 20-24h at 37°C with shaking. Then the cultures were centrifuged at 10000 x g for 10 minutes and the supernatant was collected. Supernatant was filtered through 0.45µm syringe filter. Then 1ml of culture filtrate was added to 1 ml of ECR buffer (0.1M Tris HCl/1 mM CaCl₂, pH-7.2) containing 20mg of ECR (Sigma-Aldrich). Then the tubes were incubated for 3h at 250 rpm at 37°C. Elastolytic activity is resulted in cleavage of ECR and releases a red soluble pigment. After incubation 0.2ml M Na₂EDTA was added to stop the reaction. The insoluble ECR was separated by centrifugation at 3500x g for 10 minutes. Then the absorbency was measured at 495nm. One sample without any culture was used as background activity and this value was subtracted from all samples (6).

Thin layer chromatography to isolate AHL's:

The AHL profile of different *P. aeruginosa* strains was evaluated by a thin layer chromatography (TLC) assay (47). 10ml of culture was grown overnight with shaking then the supernatant was collected after centrifugation at 3000 x g for 10 minutes. Then the AHL was extracted with three volumes of acidified ethyl acetate (0.1ml Acetic acid in 1 liter ethyl acetate) three times. Then it was evaporated in the fume hood and finally the residue was dissolved in 100µl ethyl acetate. Then TLC was performed using methanol: water (60:40, v:v). Then the TLC plate was air dried in the fume hood. The TLC plate was overlaid with a

reporter strain of *Agrobacterium tumefaciens* A136 (pCF218) (pCF372) seeded with X-Gal in agar.

Rhamnolipid Assay:

For detection of Rhamnolipid mineral salt medium supplemented with 200 μ g/ml cetyltrimethylammonium bromide (CTAB, Sigma), 5 μ g/ml methylene blue, and 1.5% agar were used (25). Mineral salt solution contained per liter: 0.7 g KH_2PO_4 , 0.9 g Na_2HPO_4 , 2 g NaNO_3 , 0.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2 ml of trace elements [per liter, 2 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.6 g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$] (17).

Biolog Assay:

BiologTM is a commercial assay based on reduction of tetrazolium dye by the utilization of a number of carbon sources (48). This is a plate based assay containing 95 carbon source and we have used to check alteration of ability to utilize different carbon source after being mix cultured. Actually this experiment was designed to identify microorganism from the environment (50). But in this study this test was used to check the ability to break different carbon sources of each strain after mix culturing for two months.

RESULTS

Co-culture in Batch Culture system:

P. aeruginosa PA01 in constant media (batch culture) survived longer than *E. coli* in both planktonic (Figure 1) and biofilm culture (Figure 2). Differences in the two populations became apparent after 4-6d. On the basis of cultivability, *E. coli* planktonic population survived longer (13d) than did biofilm populations (11d). *P. aeruginosa* PA01 remained viable through day 17 in both planktonic and biofilm culture.

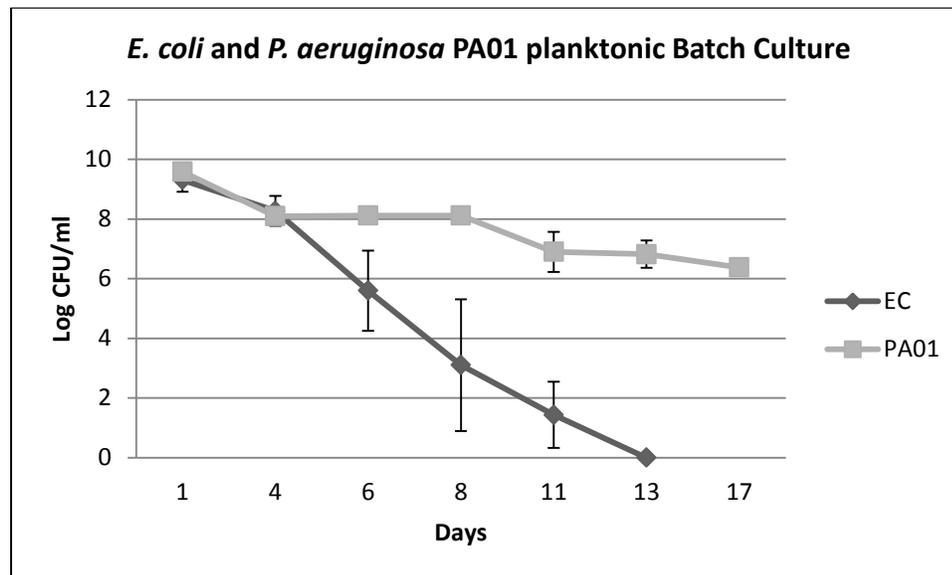


Figure 1: Planktonic mixed batch culture between *E. coli* and *P. aeruginosa* PA01 up to 17 days. Error bars represents standard error.

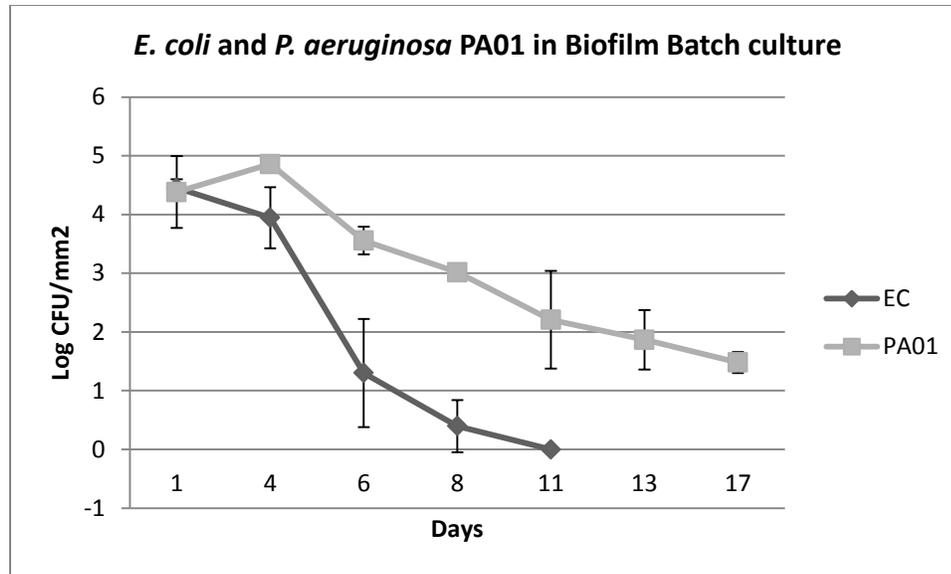


Figure 2: Mixed biofilm batch culture between *E. coli* and *P. aeruginosa* PA01 up to 17 days started with same cell density. Error bars represents standard error.

The PA14 strain of *P. aeruginosa* also showed greater survival ability in mixed culture in batch culture than *E. coli*. The *E. coli* cells were completely dead by the 6th day of mixed culture in both planktonic (Figure 3) and biofilm (Figure 4) cultures. Growth characteristics of PA14 in mixed culture differed slightly from PA01 (Figure 1 and 2) in that PA14 demonstrated slightly enhanced initial growth, but was not viable past day 11.

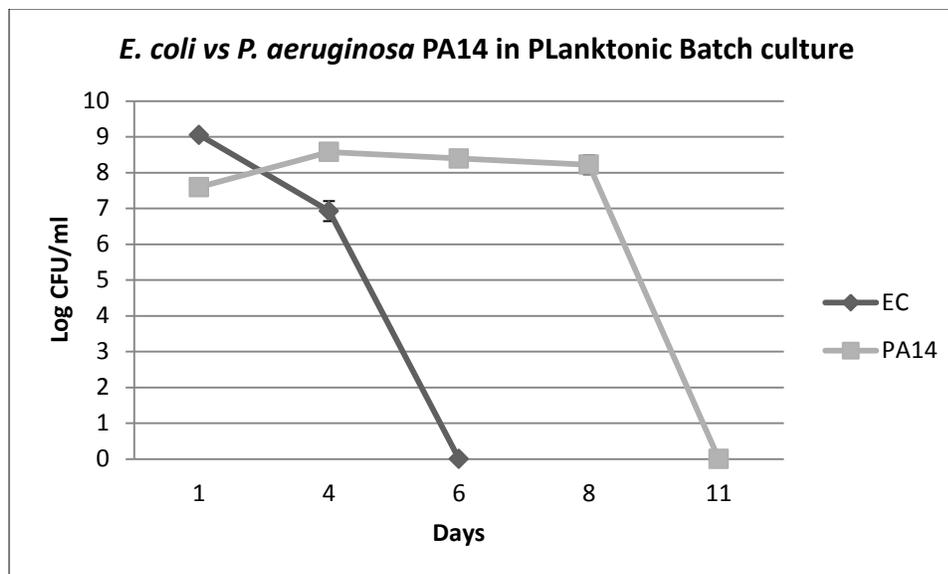


Figure 3: Planktonic mixed batch culture between *E. coli* and *P. aeruginosa* PA14 up to 11 days started with same cell density. Error bars represents standard error.

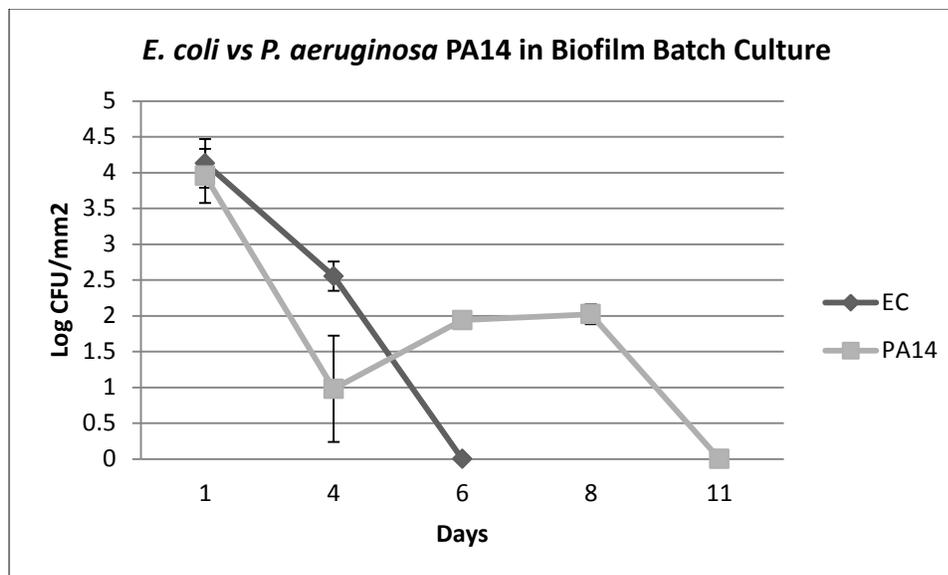


Figure 4: Mixed biofilm batch culture between *E. coli* and *P. aeruginosa* PA14 up to 11 days started with same cell density. Error bars represents standard error.

Long term co-culture for two months:

In contrast to the batch culture results (Figure 1-4), both *E. coli* and *P. aeruginosa* PA01 strains coexisted with daily transfers in equivalent numbers in mixed planktonic (Figure 5) and biofilm (Figure 6) populations for two months. Similar growth trends were seen in long term co-cultures of *E. coli* and *P. aeruginosa* PA14 planktonic (Figure 7) and biofilm (Figure 8) populations. In two instances (Figure 6 and 7), one organism was slightly more dominant ($P < 0.05$) in the first 24h, but the relative cell numbers stabilized thereafter.

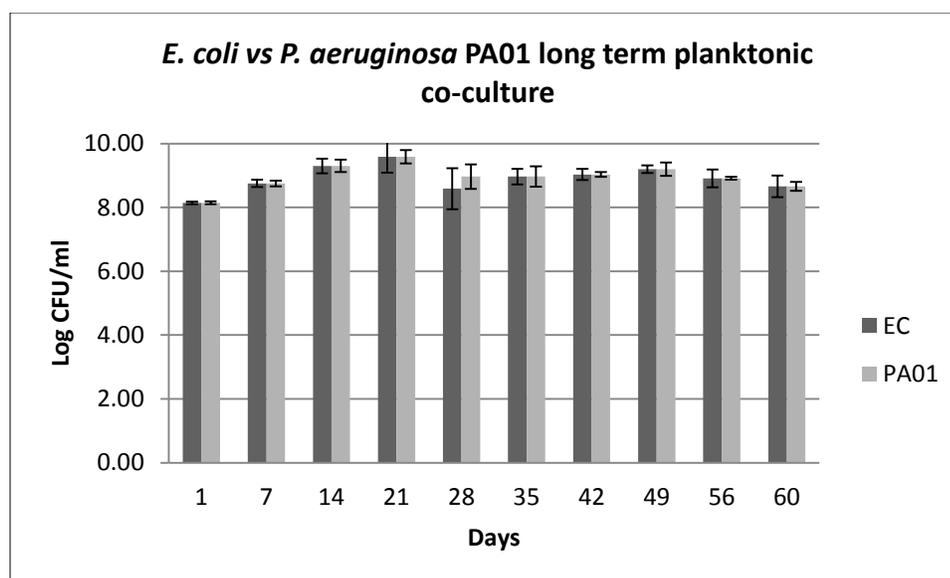


Figure 5: Two months co-culture with *E. coli* and *P. aeruginosa* PA01 in planktonic culture started with same cell density. Each week samples were collected and then after serial dilution they were plated in selective media. Error bars represent standard error.

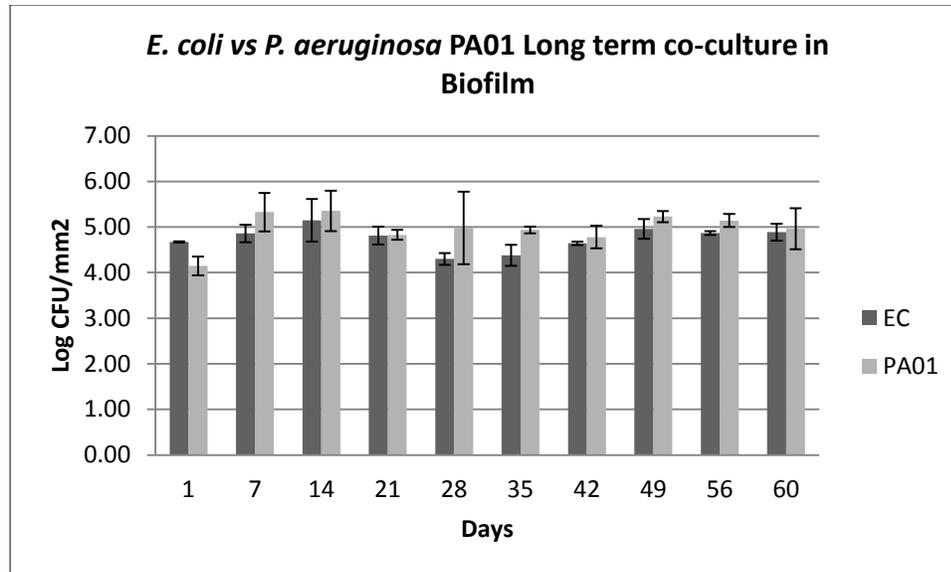


Figure 6: Two months co-culture with *E. coli* and *P. aeruginosa* PA01 in biofilm culture started with same cell density. Each week glass beads were collected and they were sonicated and vortexed. Then after serial dilution they were plated in selective media. Differences at day 1 were significant ($P=0.023$). Error bars represent standard error.

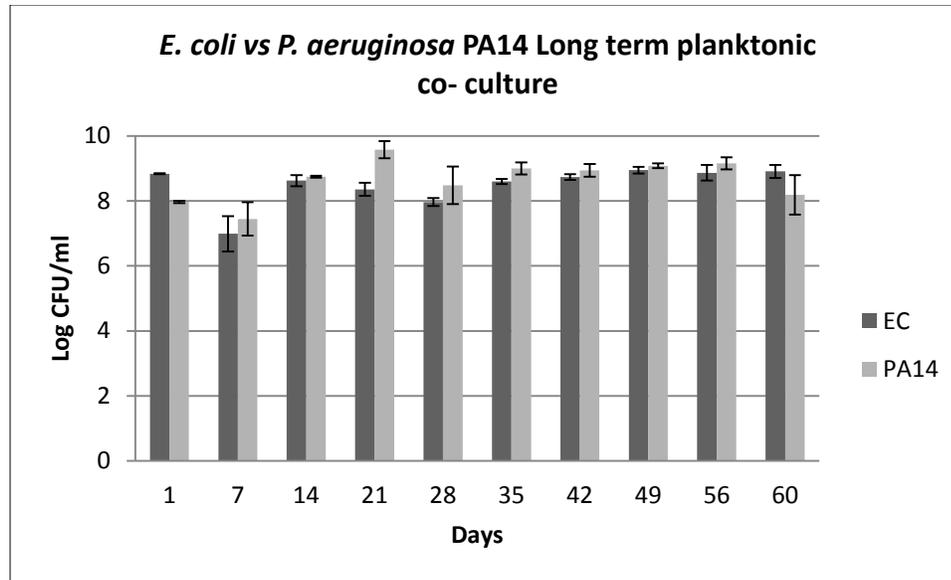


Figure 7: Two months co-culture with *E. coli* and *P. aeruginosa* PA14 in planktonic culture started with same cell density. Each week samples were collected and then after serial dilution they were plated in selective media. Differences at day one were significant ($P < 0.001$). Error bars represent standard error.

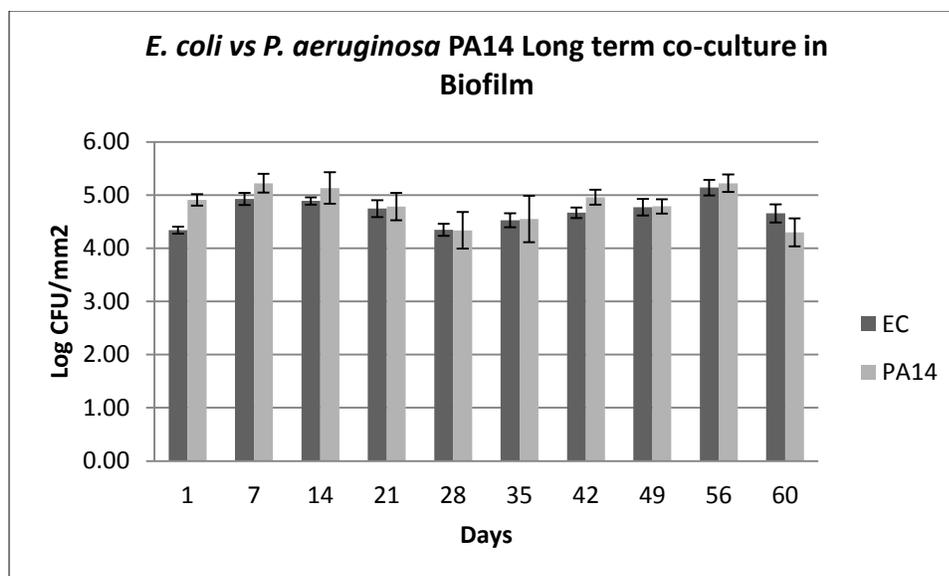


Figure 8: Two months co-culture with *E. coli* and *P. aeruginosa* PA14 in biofilm culture started with same cell density. Differences at day one were significant ($P=0.003$). Each week glass beads were collected and they were sonicated and vortexed. Then after serial dilution they were plated in selective media. Error bars represent standard error.

Co-culture with different strains:

In order to investigate whether long-term co-culture affected the competitive abilities of either *E. coli* or *P. aeruginosa*, I conducted co-culture experiments using different strains from the whole evolution experiment timeline. In the co-culture between *E. coli* starting culture and *P. aeruginosa* PA01 evolved strain in mix culture (Figure 9), both species grew equally in planktonic culture, but the evolved PA01 grew better than *E. coli* (Figure 9). In case of *E. coli* starting culture when mixed with *P. aeruginosa* PA14 evolved in mix culture *E. coli* grew significantly higher than PA14 in both planktonic and biofilm culture (Figure 10).

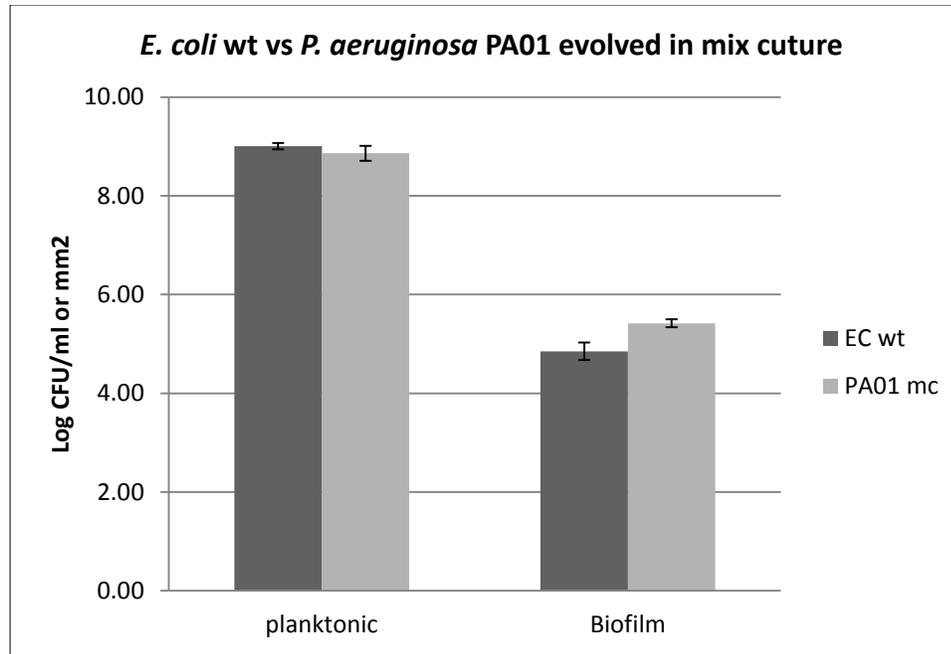


Figure 9: *E. coli* starting culture was co-cultured with *P. aeruginosa* PA01 which is evolved in mixed culture with *E. coli* after two months. The biofilm values were significantly different ($P=0.015$). Error bars represent standard error.

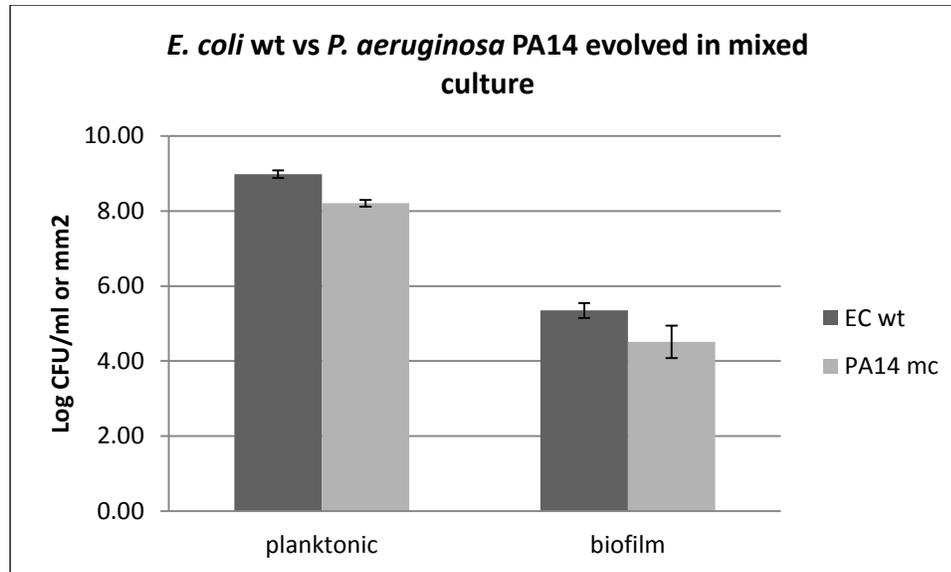


Figure 10: *E. coli* starting culture was co-cultured with *P. aeruginosa* PA14 strain which was evolved in mixed culture with *E. coli* after two months. Differences in planktonic ($P=0.001$) but not biofilm values ($P=0.067$) were significant. Error bars represent standard error.

In co-culture with *E. coli* strain evolved in two months mix culture, *P. aeruginosa* PA01 starting culture showed greater growth in both planktonic and biofilm culture (Figure 11). On the other hand PA14 starting culture grew equally when mixed with *E. coli* strain evolved in mix culture (Figure 12).

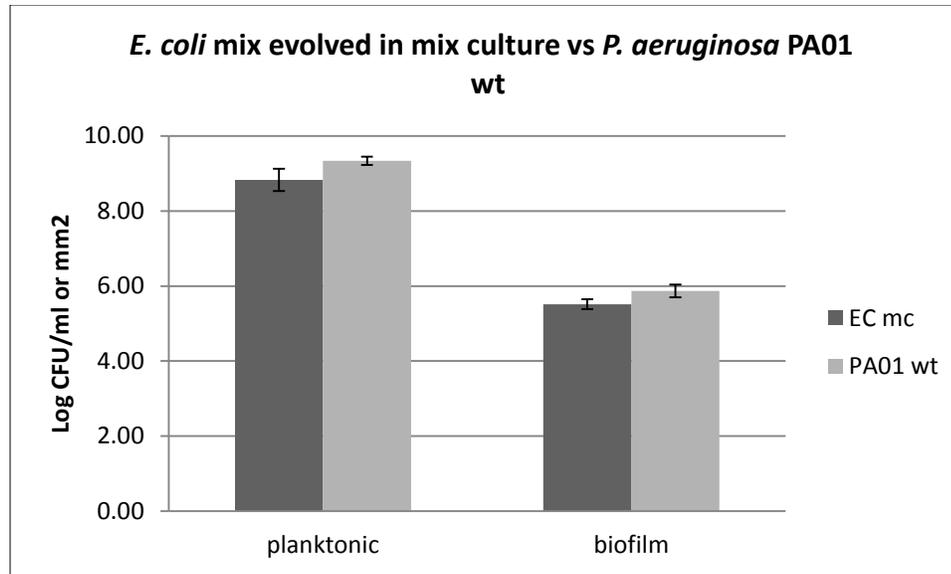


Figure 11: *E. coli* strain grown in mixed culture with *P. aeruginosa* PA01 was isolated and the co-cultured with *P. aeruginosa* PA01 starting culture. Differences in planktonic ($P=0.05$) and biofilm ($P=0.082$) were not significantly different. Error bars represent standard error.

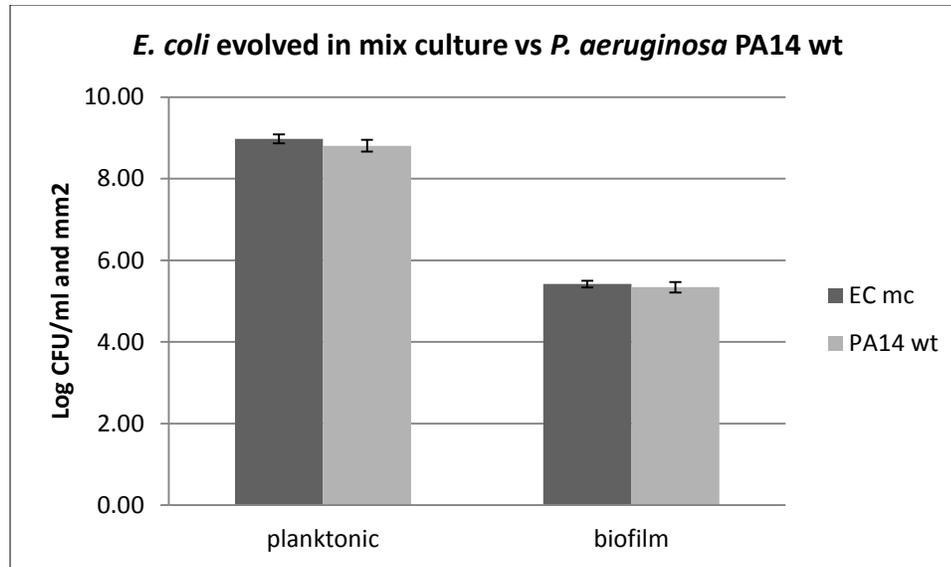


Figure 12: *E. coli* strain evolved in mixed culture with *P. aeruginosa* PA14 for two months and then isolated and co-cultured with *P. aeruginosa* PA14 starting culture. Differences were not statistically significant. Error bars represent standard error.

When the PA01 strain of *P. aeruginosa* evolved in two months pure culture was mixed with *E. coli* starting culture it showed greater growth than *E. coli* in planktonic culture but it grew equally in the biofilm (Figure 13).

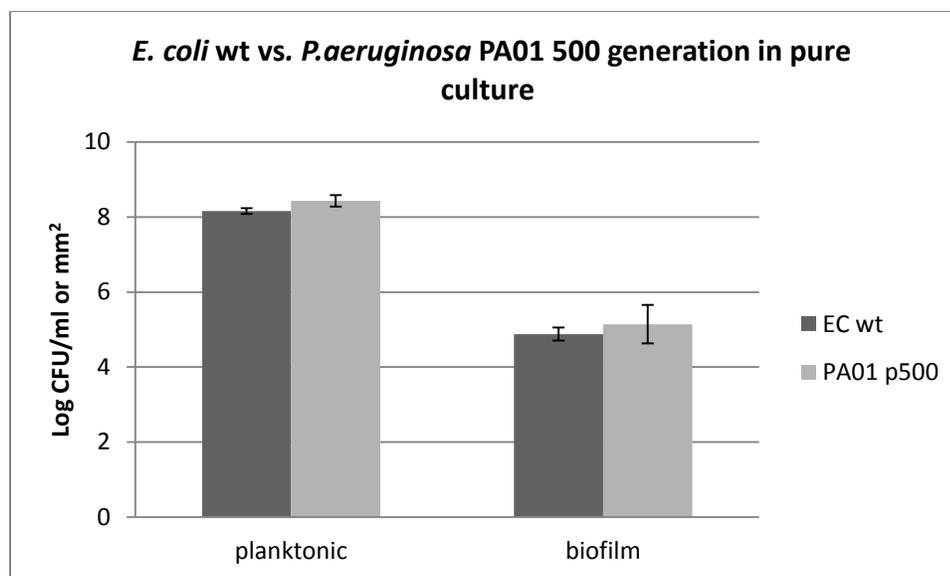


Figure 13: *P. aeruginosa* PA01 grown in pure culture for two months was co-cultured with *E. coli* starting culture. Differences were not significant. Error bars represent standard error.

Table 2: Calculation of relative fitness by using Malthusian parameter, in the co-culture experiments using different strains from different time point. (mc= evolved in two months mix culture).

Experiment	Formula	Result	Conclusion
<i>E. coli</i> with <i>P. aeruginosa</i> PA01	EC mc/EC wt	1.45	EC mc grows 45% faster
<i>E. coli</i> with <i>P. aeruginosa</i> PA14	EC mc/EC wt	1.06	EC mc grows 6% faster
<i>P. aeruginosa</i> PA01 with <i>E. coli</i>	PA01 mc/PA01 wt	1.45	PA01 mc grows 45% faster
<i>P. aeruginosa</i> PA14 with <i>E. coli</i>	PA14 mc/PA14 wt	1.17	PA14 mc grows 17% faster

Pyocyanin production:

P. aeruginosa PA01 strain showed an increase in pyocyanin production when it was grown in pure culture for two months or equivalent to 500 generations. In mixed culture, pyocyanin production decreased (Figures 14 and 15).



Figure 14: Pyocyanin production of *P. aeruginosa* PA01 (A) starting culture, (B) pure culture grown for two months and (C) Grown in mixed culture for two months.

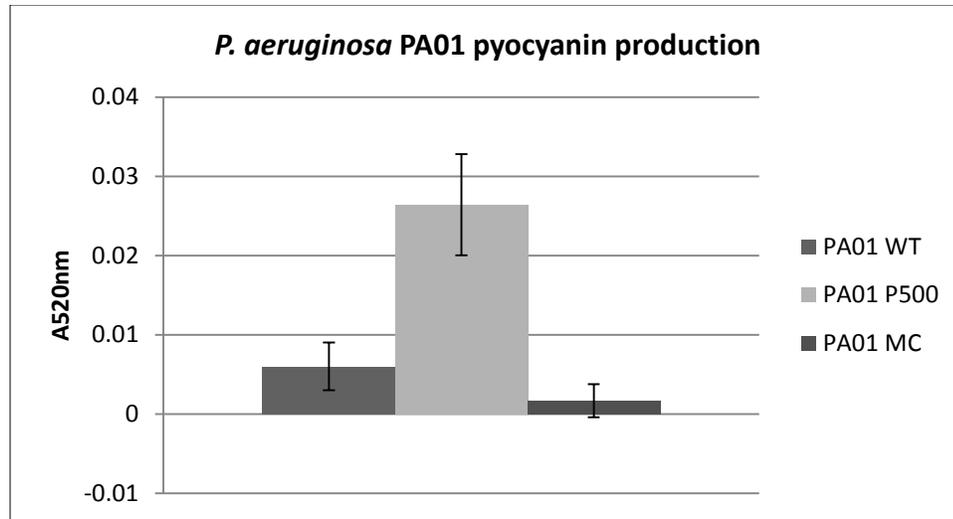


Figure 15: Pyocyanin production per 10^9 cells of *P. aeruginosa* PA01 starting culture (wt), evolved strain in two months pure culture (P500), and evolved strain in two months mixed culture (MC).

On the other hand pyocyanin production in *P. aeruginosa* PA14 decreased when grown in pure culture for two months, and no pyocyanin was observed in long-term mixed culture (Figures 16 and 17).

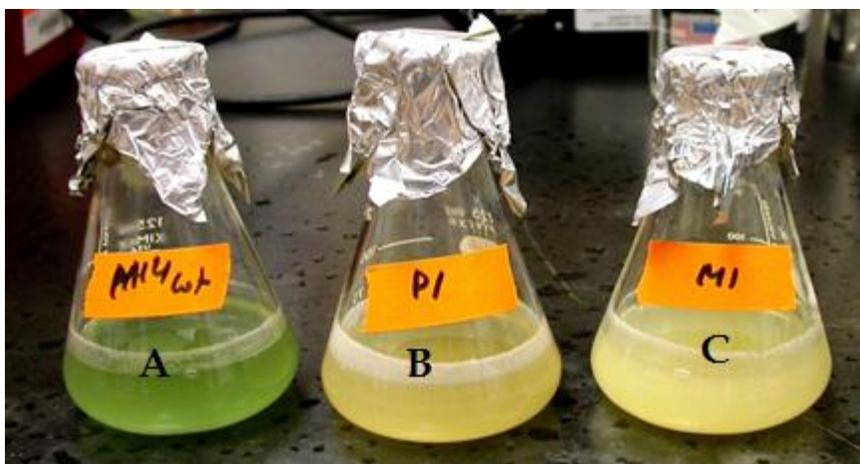


Figure 16: Pyocyanin production per 10^9 cells of *P. aeruginosa* PA14 (A) starting culture (B) Evolved strain in two months pure culture and (C) Evolved strain in two months mixed culture.

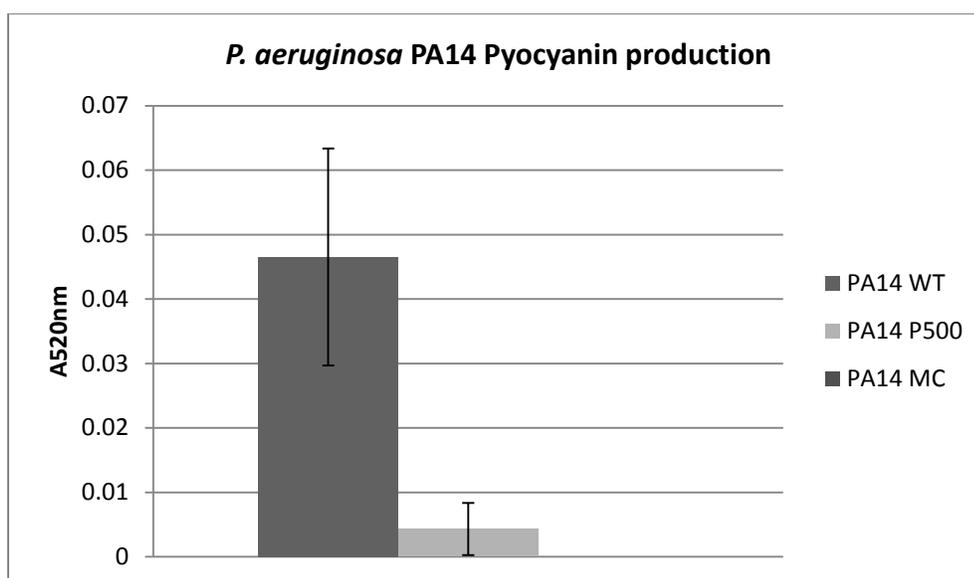


Figure 17: Pyocyanin production per 10^9 cells of *P. aeruginosa* PA14 starting culture (WT), evolved strain in two months pure culture (P500), and strain evolved in two months mixed culture (MC).

Elastase Production:

P. aeruginosa PA01 strain when grown in pure culture for two months or equivalent to 500 generations showed significant increased production of elastase than the starting culture. But in mixed culture after two months it showed no significant change in the production of elastase (Figure 18).

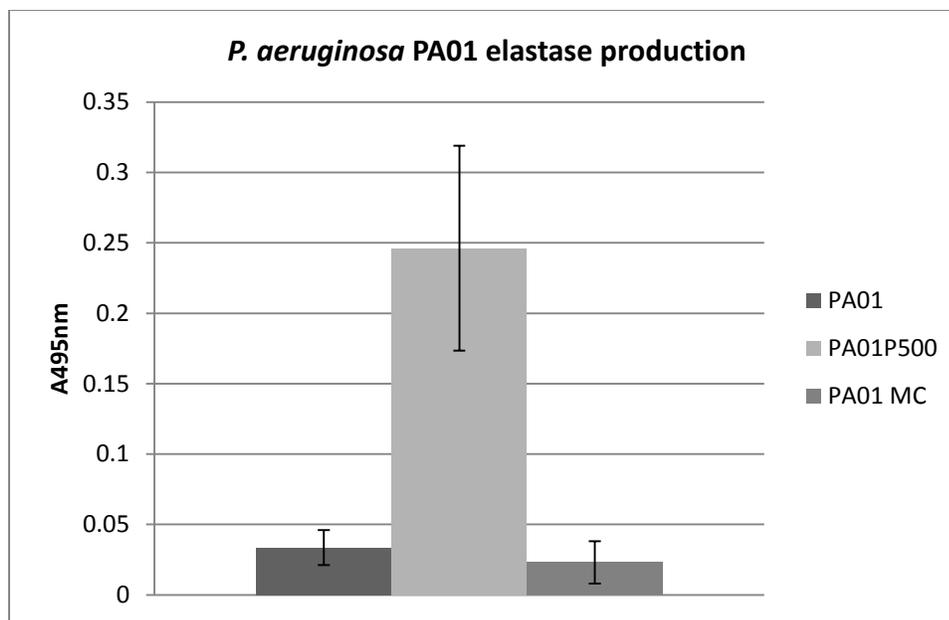


Figure 18: *P. aeruginosa* PA01 elastase production per 10^9 cells by starting culture (PA01), evolved in two months pure culture (P500), evolved in two months mixed culture (MC). Error bars represent standard error.

On the other hand PA14 strain of *P. aeruginosa* showed a significant decreased production of elastase when grown two months in both pure culture and mixed culture (Figure 19).

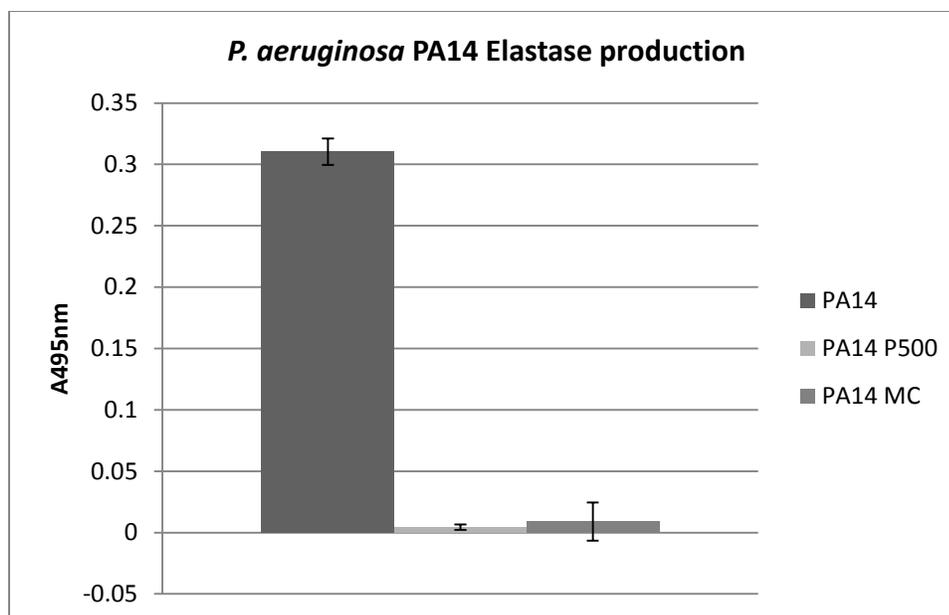


Figure 19: *P. aeruginosa* PA14 elastase production per 10^9 cells by starting culture (PA14), pure culture grown for two months (P500), strain evolved in mixed culture for two months (MC). Error bars represent standard error.

Rhamnolipid production:

P. aeruginosa PA01 showed rhamnolipid production in wild type or starting culture and also in two evolved strain in pure culture or 500 generation equivalent culture. But PA01 strain did not show any production of rhamnolipid in the two months old mixed culture (Figure 20).



Figure 20: Rhamnolipid production by *P. aeruginosa* PA01 (A) starting culture, (B) pure culture grown two months and (C) mixed culture two months. The cultures were grown in CTAB methylene blue agar and the halo around the holes is positive rhamnolipid reaction.

Indole Production:

Indole production as inter-species signaling molecule of *E. coli* is observed in mixed culture with both strains of *P. aeruginosa*. The *E. coli* strains mixed culture with *P. aeruginosa* PA01 have showed no change in indole production after two months of both pure and mix culturing (Figure 21). In the mixed culture with *P. aeruginosa* PA14, *E. coli* showed significant increase in indole production than pure culture (Figure 22).

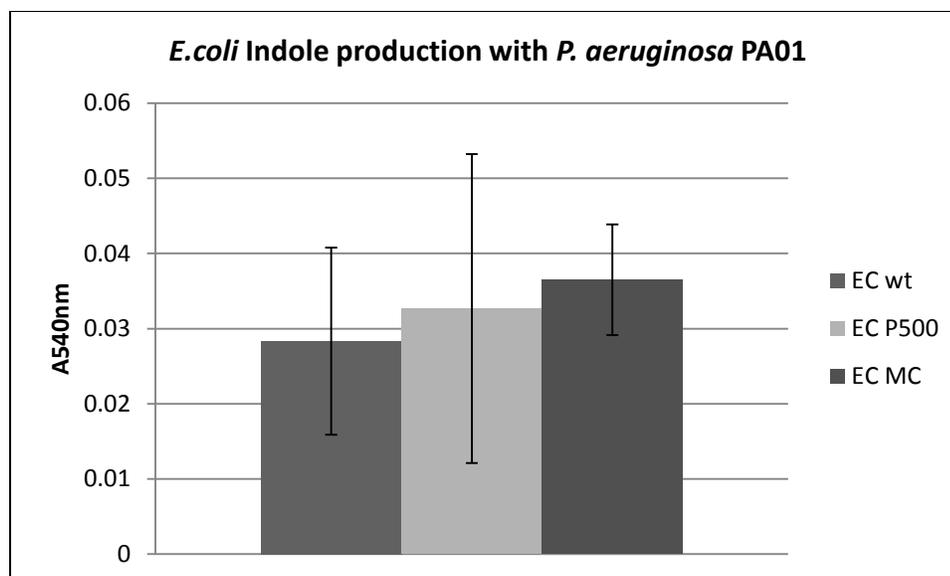


Figure 21: *E. coli* indole production per 10^9 cells of starting culture (EC wt), evolved in two months pure culture (EC P500), evolved in two months mixed culture with *P. aeruginosa* PA01 (EC MC). Error bars represent standard error.

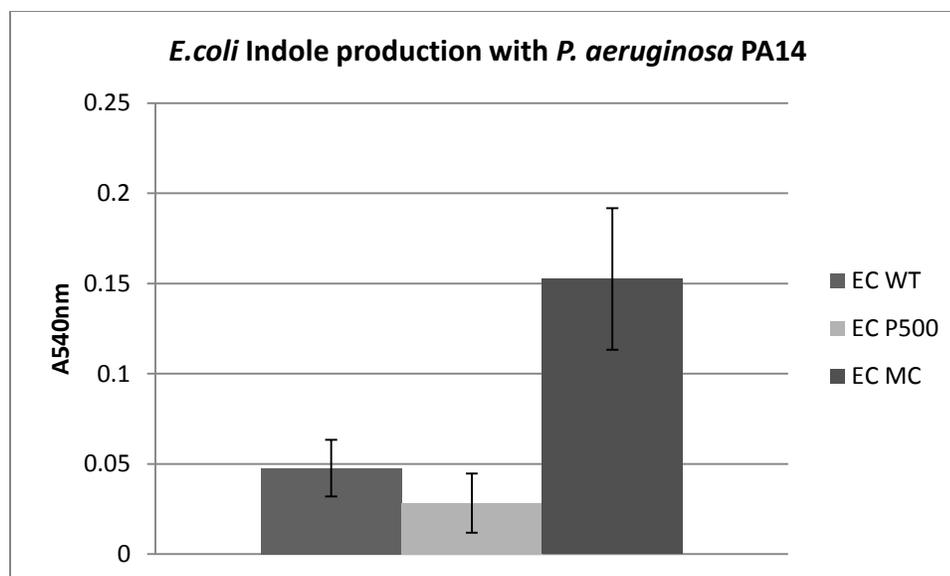


Figure 22: *E. coli* indole production per 10^9 cells of starting culture (EC WT), evolved in two months pure culture (EC P500), evolved in two months mixed culture with *P. aeruginosa* PA14 (EC MC). Error bars represent standard error.

Indole Degradation by *Pseudomonas*:

P. aeruginosa PA01 evolved strains both grown in pure and mix culture showed faster indole degrading ability than the starting culture (Figure 23). While in case of the PA14 strain the evolved strains both in pure and mix culture showed less indole degrading ability than the starting culture (Figure 24).

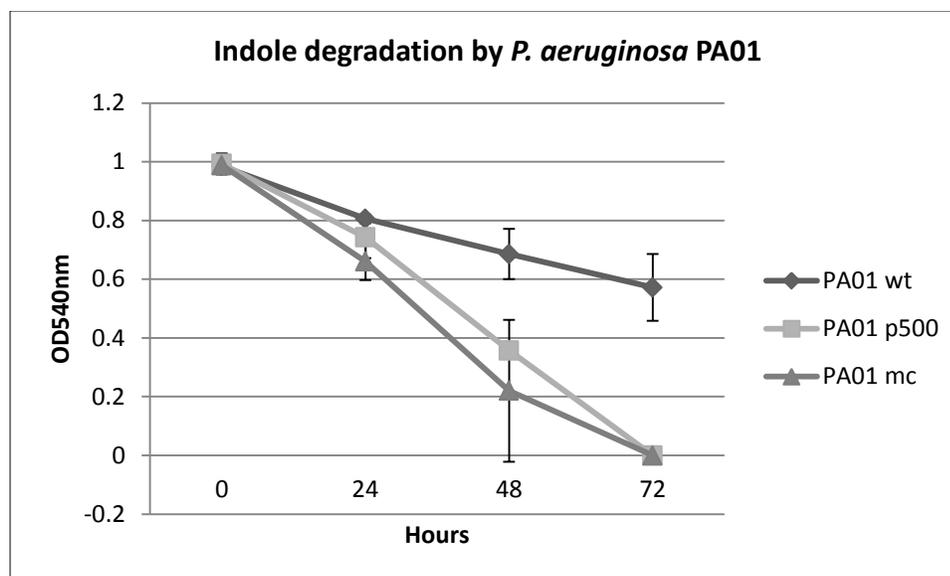


Figure 23: Indole degradation by *P. aeruginosa* PA01. *P. aeruginosa* can degrade 0.5mM indole in 50ml LB broth. Each strain started with same number of cells and indole concentration was measured every 24 hours. Error bars represent standard error.

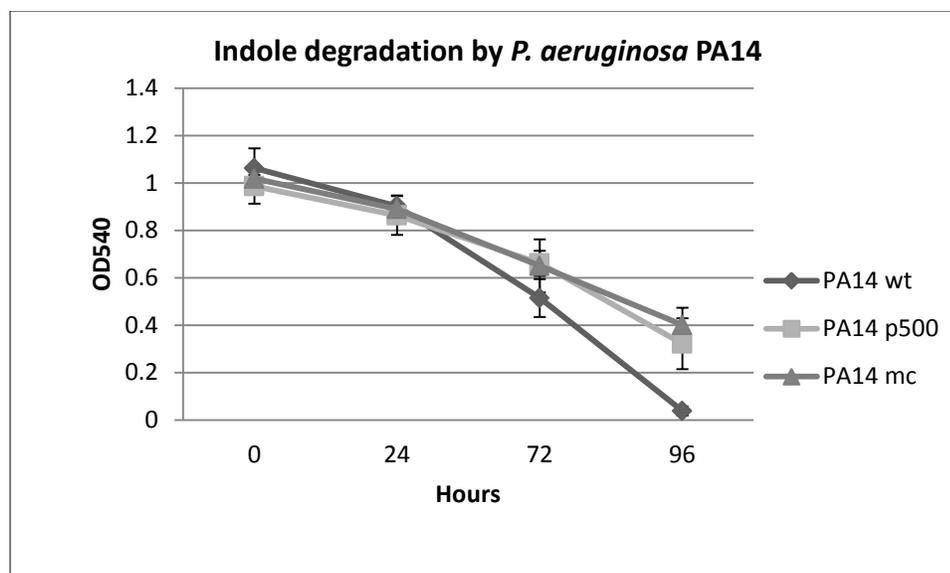


Figure 24: Indole degradation by *P. aeruginosa* PA14. *P. aeruginosa* can degrade 0.5mM indole in 50ml LB broth. Each strain started with same number of cells and indole concentration was measured every 24 hours. Error bars represent standard error.

TLC assay:

Thin layer chromatography with the AHL extracts showed varied results in both of the strains of *P. aeruginosa*. The PA01 strain showed no change from the starting culture or the wild type strains (Figure 25). But the PA14 strain showed deletion of AHL molecule in evolved strains both in pure and mix culture. In the evolved pure culture strain PA14 showed one spot in all three replicates but the evolved strain in mix culture showed complete loss of all AHLs except one replicate (Figure 26).

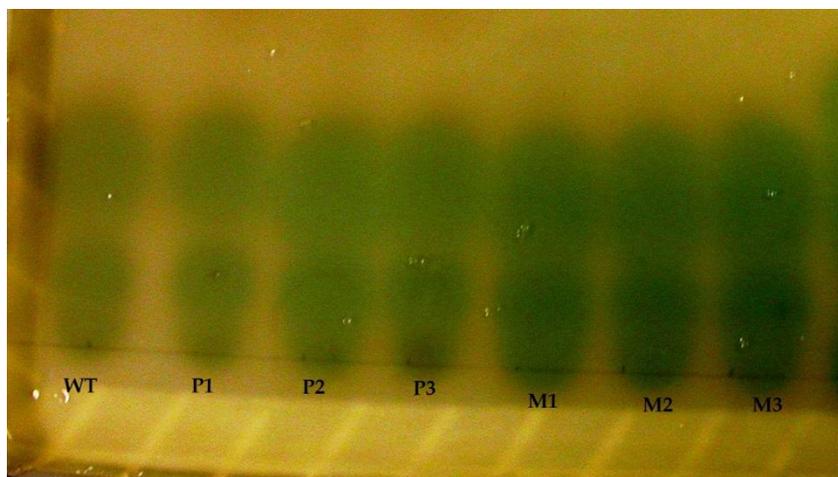


Figure 25: TLC plate showing AHL profile of *P. aeruginosa* PA01 strain. WT represents the starting culture or wild type, P1-P3 are the three replicates grown up to two months in mixed culture, and M1-M3 are the three replicates grown with *E. coli* for two months.

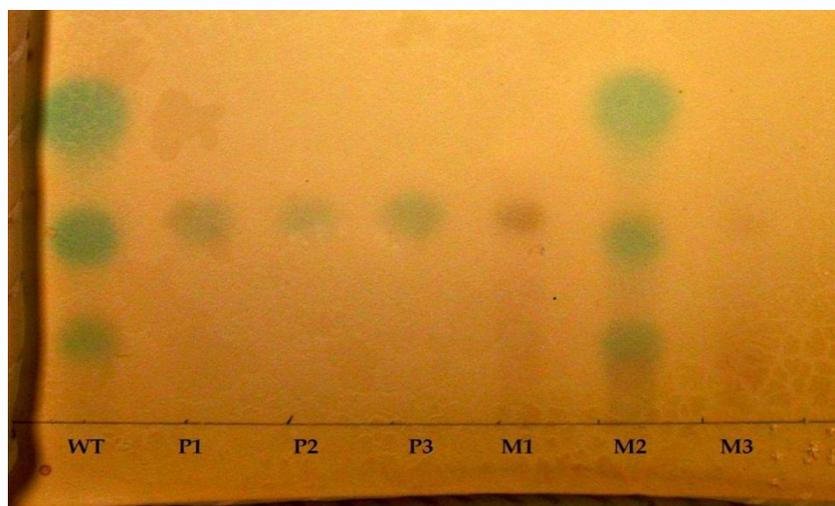


Figure 26: TLC plate showing AHL profile of *P. aeruginosa* PA14 strain. WT represents the starting culture or wild type, P1-P3 are the three replicates grown up to two months in mixed culture, and M1-M3 are the three replicates grown with *E. coli* for two months.

Biolog Assay:

Biolog assay showed altered ability of metabolizing different carbon sources of both species after two months mixed culture. *E. coli* has acquired and also lost ability to breakdown some C-sources. The *P. aeruginosa* PA01 strain only lost ability to breakdown some C-sources (Table 3).

Table 3: List of carbon sources to which *E. coli* and *P. aeruginosa* have acquired and/or lost ability to utilize after two months of mixed culture.

<i>E. coli</i>		<i>P. aeruginosa</i> PA01
Acquired ability to metabolize	Lost ability to metabolize	Lost ability to metabolize
α -Hydroxybutyric acid	N-Acetyl-D-Galactosamine	Succinic acid Mono-Methyl ester
α -Ketoglutaric acid	Lactulose	β -hydroxybutyric acid
	Pyruvic acid Methyl ester	Malonic acid
	Succinic acid Mono-Methyl Ester	Succinic acid
	D, L, α -Glycerol Phosphate	Succinamic acid
		L-Histidine
		L-Serine
		2-Aminoethanol
		Pyruvic acid Methyl Ester

Fitness assay by calculating Log (24h CFU/0h CFU) both in mono and co-culture:

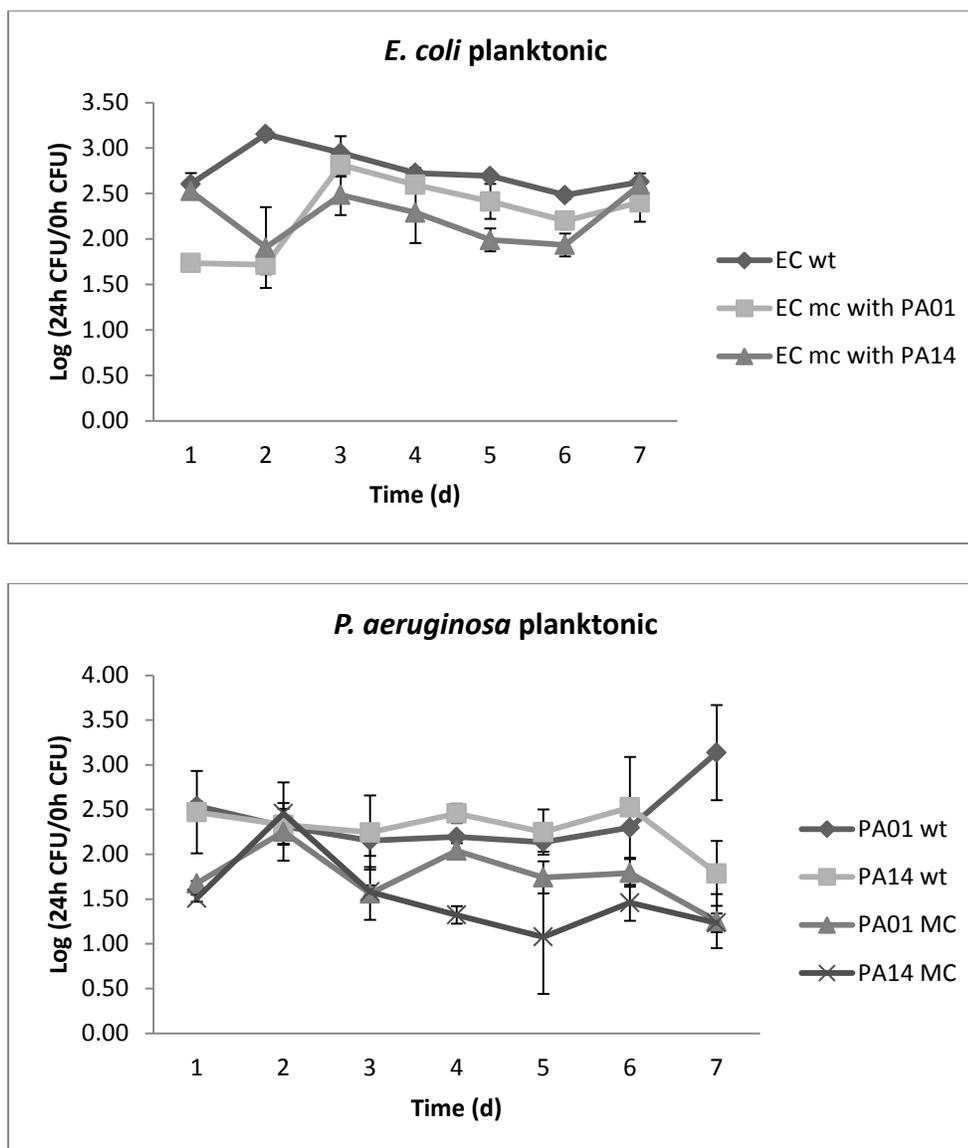


Figure 27: Fitness assay of *E. coli* and *P. aeruginosa* in both planktonic pure and co-culture.

Log (24h CFU/0h CFU) was calculated and the fitness was compared in different cultures.

Error bars represent standard error.

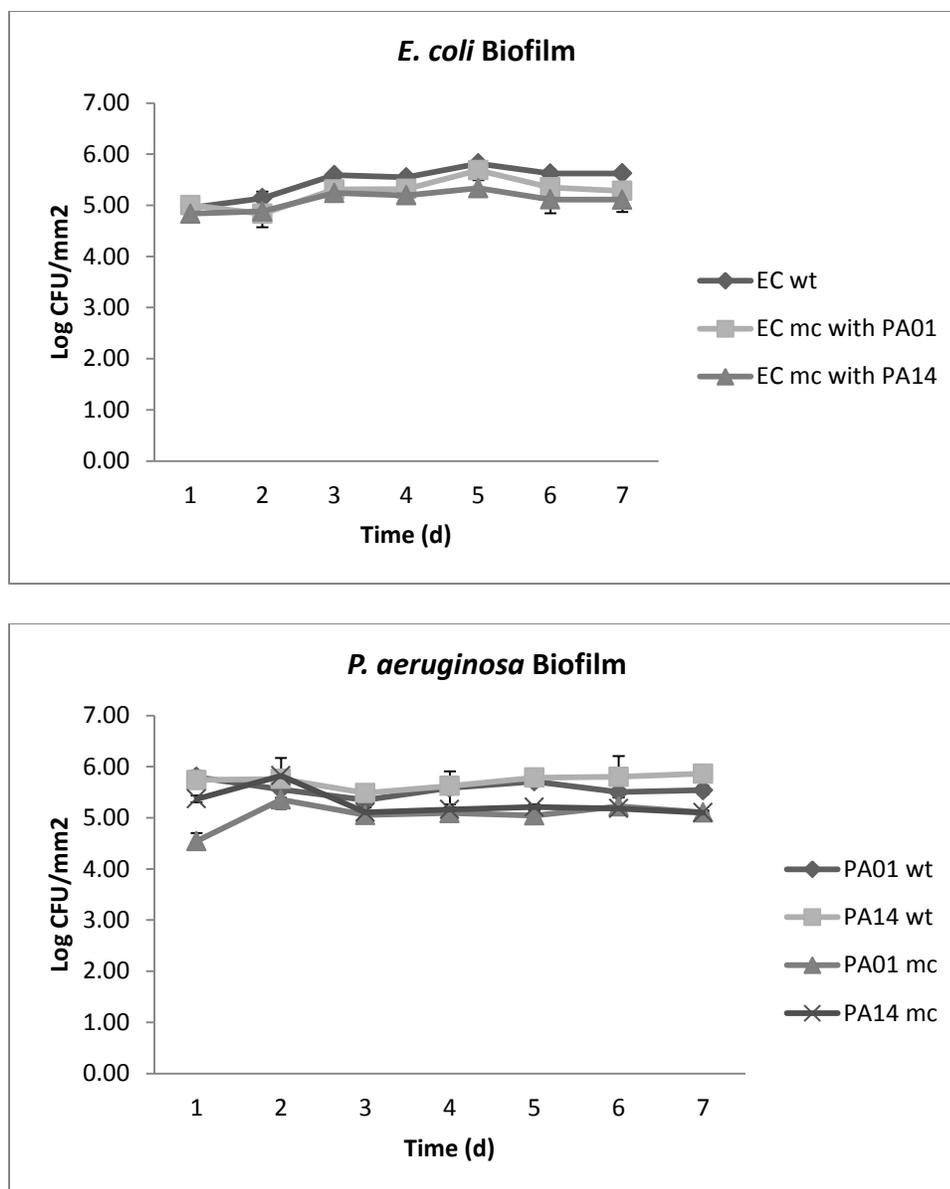


Figure 28: Biofilm formation of *E. coli* and *P. aeruginosa* in pure and mixed culture for one week. Error bars represent standard error.

This study showed that *E. coli* planktonic (Figure 27) and biofilm (Figure 28) both in mono culture and in co-culture was approaching an equilibrium after one week. Similarly, *P. aeruginosa* biofilms (Figure 28) were equilibrating although the PA14 was slightly less

competitive in mixed culture than in monoculture. In contrast, the planktonic *P. aeruginosa* cultures (Figure 27) were less competitive during the first week of co-culture.

DISCUSSION

In the long term batch culture both strains of *P. aeruginosa* PA01 and PA14 survive more than the *E. coli* strain in co-culture. However the PA01 strain exhibited better survival than the PA14 strain (Figures 1-4). In contrast to the batch culture results, the long term mix culture both *E. coli* and *P. aeruginosa* showed equal growth throughout the two months period of mix culture (Figures 5-8). Unlike in the batch culture where there is no change of media, none of them was dead or had significantly inhibited growth. Initially PA01 in biofilm and PA14 in planktonic culture were dominated by *E. coli* on the very first day of mixed culture. But after a week both strains have improved growth and grew equally with the *E. coli* in both planktonic and biofilm culture. This study proved that *E. coli* MG1655 can coexist for two months with either *P. aeruginosa* PA01 or PA14 strain provided there is a constant supply of media present.

Similar to the long term evolution experiment of Richard Lenski in this study mean fitness in growth was calculated over time (33). In this study both *E. coli* and *P. aeruginosa* strains showed to have increased fitness after two months of mixed culture (Table 2). One question that arose during this study was whether their fitness was altered by long-term co-culture. To address this question, mix culture experiments were performed using starting cultures (Figures 9-12) of one organism and the evolved strain of the other species. In this competitive fitness study with both of the strains of *P. aeruginosa* which were evolved in mixed culture were dominated when they were cultured with *E. coli* starting strain in the planktonic culture (Figures 9-12). When the starting cultures of *P. aeruginosa* were grown with

evolved *E. coli* strains, *P. aeruginosa* PA01 starting culture grew faster than *E. coli*. However, the PA14 starting culture grew more slowly than the evolved *E. coli* strain. As stated previously, the biofilm culture forms anew during each culture trial so it was not possible to calculate the relative fitness during biofilm growth. The fitness study showed similar trend for both *E. coli* mono culture and co-culture but in case of *P. aeruginosa* only PA01 strain grown in planktonic mono culture showed increased fitness after a week (Figure 27 and 28).

P. aeruginosa has many quorum-regulated virulence factors including: elastase, pyocyanin, rhamnolipid, and pyocyanin. In related work from the laboratory (57), *E. coli* indole production was shown to be the major factor facilitating competitiveness with *P. aeruginosa* by inhibiting quorum-regulated virulence. Previous study showed that indole can alter gene expression of different QS regulated virulence factor of *Pseudomonas* and affect production of them (31).

PA01 strain of *P. aeruginosa* decreased production of pyocyanin pigment after two months of mixed culture. But in pure culture when it was grown for two months or equivalent to 500 generations it showed a significant increase in pyocyanin production. During the period of two months it has gained some unknown change by which it acquired increase production of pyocyanin. On the other hand PA14 strain showed decreased production of pyocyanin in both evolved pure and mixed culture. PA01 strain also did not show much difference in other quorum sensing regulated virulence factor elastase after mixed cultured for 2 months. But it also showed increased production of elastase when it was grown in pure culture for two months. On the other hand PA14 showed decreased production of elastase in both long term mixed and pure cultures. Thin layer chromatography assay showed that after two months of culturing in both pure and mixed culture PA14 strain lost AHL molecules, with the effect being more pronounced in mixed

culture (Figure 26). This result suggests either a loss of AHL production in PA14 (supported by the pure culture results), or an increased susceptibility to *E. coli*-produced indole (supported by the mixed culture results) and would also explain the decreased quorum-regulated virulence factor production. On the other hand PA01 strain showed no loss of AHL molecule in the TLC assay, which indicates unlike PA14 it had intact quorum sensing profile and it retained those phenotypes intact. The genomes of strains PA01 and PA14 have been fully sequenced (30) (49), and while very similar are not identical. As a result, future studies could explore the strain differences between these two *P. aeruginosa* strains in the context of evolutionary success.

Recent studies showed that *E. coli* secreted inter species signaling molecule indole can regulate *Pseudomonas* quorum sensing gene expressions (31). We performed indole quantification from different strains of *E. coli* from different culture times. The result showed in mix culture with PA01 strain *E. coli* still producing indole without any significant change in the production quantity. But with PA14 strain *E. coli* showed significantly higher production of indole and which indicates that this higher indole concentration might be an important factor regulating *P. aeruginosa* PA14 phenotypes in mix culture. So in the long term mix culture both loss of AHL and increased production of indole could be the regulating factor to down-regulate quorum sensing controlled phenotypes.

Recent studies also showed that *P. aeruginosa* is able to degrade indole (31). In our study different strains were isolated and then they were cultured with 0.5mM indole and then their indole degradation ability was measured. In case of PA01 evolved strains both in pure and mix culture showed faster degradation of indole than the starting culture. While in the PA14 evolved strains showed less indole degrading ability than the starting culture. This result again suggests PA14 strain of *P. aeruginosa* lost virulence after long term culture in both

mixed and pure culture. Using a commercially-available (Biolog) metabolic profiling technique (48) (50), I performed additional tests on the bacteria to see whether long-term culture affected other metabolic capabilities. As shown in Table 3, *E. coli* acquired the ability to utilize two new carbon sources α -hydroxybutyric acid and α -ketoglutaric acid, but lost ability to metabolize five carbon sources during long-term co-culture. On the other hand *P. aeruginosa* PA01 has lost ability to utilize nine different carbon sources and did not acquire the ability to utilize new carbon sources. It was difficult to perform this test with *P. aeruginosa* PA14 evolved mixed culture strain as it was not suspending properly with the inoculating fluid of the BiologTM kit.

So from this whole study it can be inferred that if *E. coli* strain MG1655 and *P. aeruginosa* PA01 and PA14 strains grown in batch culture where there is no supply of continuous media *Pseudomonas* can grow longer than the *E. coli*. But in long term co-culture where the cultures are transferred into new media every day the both species can coexist. Also the PA01 strain of *P. aeruginosa* showed better fitness in mix culture than the PA14 strain. PA14 strain lost its quorum sensing regulated virulence factor after being propagated in long term pure and mixed culture as well. The PA01 strain of *P. aeruginosa* if grown in pure culture for two months in has increased quorum sensing virulence. The Lenski group has showed single nucleotide polymorphism has occurred at different time point of 20 years evolution study of monoculture *E. coli* (2). As future study expression of quorum sensing genes by reverse transcriptase, quantitative PCR (RT-qPCR) can be checked to find out if the changes are just because of altered gene expression or whether a mutation occurred. Also a DNA microarray can be performed to check expression of other genes after long term pure and mix cultures.

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