Identification of Genes Involved in Swarming Behavior of *Pseudomonas aeruginosa* PA14 strain Using Non-redundant Transposon Insertion Mutant Library

Genetic Regulators of Swarming Behavior in *Pseudomonas aeruginosa* PA14

A PROJECT SUBMITTED TO THE

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH (IISER) PUNE

TOWARDS FULFILMENT OF FIVE YEARS INTEGRATED DEGREE PROGRAMME

BS-MS (MAY 2017)

SUBMITTED BY

SHUBHAM DHANANJAY JOGE

(20121028)

UNDER THE GUIDANCE OF

Dr.VARSHA SINGH

DEPARTMENT OF MOLECULAR REPRODUCTION DEVELOPMENT AND GENETICS (MRDG)

INDIAN INSTITUTE OF SCIENCE, BANGALORE

THESIS ADVISOR

Dr.AURNAB GHOSE

DEPARTMENT OF BIOLOGICAL SCIENCES

IISER PUNE

CERTIFICATE

This is to certify that this dissertation entitled "**Identification of Genes Involved in Swarming Behaviour of *Pseudomonas aeruginosa* PA14 strain Using Nonredundant Transposon Insertion Mutant Library**" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research Pune represents original research carried out by **Mr.Shubham Dhananjay Joge** at **Indian Institute of Science, Bangalore** under the supervision of **Dr. Varsha Singh**, Department of Molecular Reproduction Development and Genetics (**MRDG**) during the academic year **2016-2017**.

Signature of Supervisor

Dr. Varsha Singh

Date: 20-03-2017

Dr. VARSHA SINGH
Assistant Professor
Department of Molecular Reproduction
Development and Genetics
Indian Institute of Science, Bangalore - 560 012

Signature of the Student

Shubham D Joge

Date: 20-03-2017

DECLARATION

I hereby declare that the matter embodied in the report entitled "**Identification of Genes Involved in Swarming Behaviour of *Pseudomonas aeruginosa* PA14 strain Using Nonredundant Transposon Insertion Mutant Library**" are the results of the investigations carried out by me at the Department of Molecular Reproduction Development and Genetics **(MRDG), Indian Institute of Science, Bangalore** under the supervision of **Dr. Varsha Singh** and the same has not been submitted elsewhere for any other degree.

Signature of Supervisor

Dr. Varsha Singh

Date: 20-03-2017

Signature of the Student

Shubham D Joge

Date: 20-03-2017

Dr. VARSHA SINGH
Assistant Professor
Department of Molecular Reproduction
Development and Genetics
Indian Institute of Science, Bangalore - 560 012

Abstract:

The present work describes the genetic regulators of swarming behavior in *Pseudomonas aeruginosa* strain PA14. Swarming motility is a collective coordinated group behavior exhibited by several pathogenic bacteria. Swarming in PA14 is an emergent behavior as a result of local interactions of individual bacteria and globally forming a well-structured dendritic pattern. Several studies have shown that in different bacteria swarming arises due to interaction with another individual, its surface environment and via sensing nutrient availability. All these factors integrate together and lead to genotypic and phenotypic changes in bacterial morphology and behavior.

P. aeruginosa is a well-known opportunistic human pathogen; causes infection in cystic fibrosis, burn injured and diabetic foot ulcer patients. It can show swarming in 0.6 % agar concentration and under nutrient depletes condition. Several parameters including temperature, moisture, pH, drying period etc. influence swarming pattern in PA14. In some bacterial species, swarming motility has been linked to virulence and antibiotic resistance. But there are very few studies on swarming and it's linked to virulence in *P. aeruginosa.* We have used PA14 NR transposon insertion mutant library comprising 5987 mutants and screen for all of them in order to find non-swarming mutants can also call as 'swarming promoting factors'. This study has found around 281 transposon mutants exhibiting swarming negative phenotype; 129 among them are newly identified in the screening. These set of genes belong to all the functional classes with the exception in non-coding RNA class; it comprises not only motility and attachment genes but also includes diverse sets of genes such as biosynthesis of amino acids, nucleotides, and cofactors, transcriptional regulators, transporters and two component regulators; 42 of them are hypothetical and unknown.

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Acknowledgement

Foremost, I would like to thank my thesis supervisor, Dr. Varsha Singh, for her guidance, support, and motivation throughout the duration of my project. I am especially grateful to her for giving me a very interesting and equally complex problem for my thesis work.

I would like to thank my thesis advisor committee member Dr. Aurnab Ghose for his constant inputs and suggestion.

I would like to thank Ayushi Mishra and Mohammad Ameen for their equal contribution and help in screening the entire PA14 NR library. Present work is a team effort and wouldn't have finished on time without them. I had a great time working with both of them especially with Ayushi the fun time we share while washing hundreds of test tubes in one go! Also, I am thankful to Vyshnavi for her help in the nutrient based studies.

I am very thankful to Akash Suman and Tuhin Chakraborty, Dr. Manoj Verma's Lab at Centre of Nano Science and Engineering, Indian Institute of Science, Bangalore (CeNSE) for their help in time-lapse imaging and analyzing the results.

I would like to thank All the Worm Lab Member for their patience and support in the lab, particularly while the hood crisis.

And I would like to thank IISER Pune and DST- INSPIRE for giving me fellowship during my project work.

Finally, I thank my parents, family, and friends for their belief and constant support.

Introduction:

Bacteria are a unicellular entity but can exhibit multicellular and collective behavior under certain environmental and ecological conditions. Biofilm, swarming and fruiting body are some of their social behavioral traits (Shapiro 1998, Mitri 2013, Wu 2007, Boyle 2013, Xavier 2011, Harshey 2003).

Biofilm is a surface assisted aggregation of the mostly sessile bacterial population. It is covered in an extracellular matrix of exopolysaccharides (Verstraeten 2008). The fruiting body is an aggregation of cells forming a multicellular structure having spores in them (Shapiro 1998).

Swarming is collective and coordinated group movement shown by several bacterial species over surfaces under specific conditions (Henrichsen 1972, Harshey 1994, Kearns 2010, Rashid 2000). It allows the bacterial population to move rapidly over the surface with very high speed and cover large distances in a shorter period (See Appendix A).

Swarming behavior are seen everywhere in nature. Schools of fish, flocks of birds, and a swarm of ants are the most common examples of swarming that can be seen around us. Even human being can also show swarming behavior. In the crowd of several thousand human populations; people behave collectively and coordinate with each other; formed a lane in order to move towards their destination is also an example of swarming.

The positive feedback mechanism is common for this kind of group behavior from bacteria to vertebrates to humans and it displays various patterns (Camazine 2001). Many bacterial species have developed different cooperative behavior to deal with hostile and unfavorable environmental conditions (Shapiro 1995, Ben-Jacob 1995, Appendix A). The condition for swarming may vary from species to species and strain to strain. It depends on their specific habitat and ecological niche (Appendix A). Swarming can provide a fitness advantage to the species. Bacteria can get a competitive advantage through swarming while searching for the nutrient-rich environment. Swarming is a social behavior that provides benefit to the group rather than an individual. It helps the bacterial population for accessing and exploring better

food resources that are difficult to reach for an individual and/or might also evolve as a defense mechanism against predations (Shapiro 1998).

Many studies have characterized and shown swarming in various genera of bacteria few of them are mentioned here and Appendix A will summarize the swarming pattern, required condition, habitat type and characteristic of some of the swarming bacteria in brief detail.

Aeromonas, Azospirillum, Bacillus, Burkholderia, Clostridium, Chromobacterium, Escherichia, Photobacterium, Proteus, Pseudomonas, Rhodospirillum, Rhizobium Salmonella, Serratia, Yersinia, Vibrio (Henrichsen 1972, Harshey 1994, Harshey 2003, Verstraeten 2008, Kearns 2010, Copland 2009). It is observed that within the same genus, the different strain shows a swarming pattern which may be differing in size, shapes, form as well as colors. In laboratory conditions, swarming pattern depends on a lot of factors including agar concentration, nutrient composition, incubation temperature, humidity, pH, water availability etc. (Tremblay 2007, Tremblay 2008, Rashid 2000). In nature, bacteria can show different types of motility which depends on their cellular morphology and habitat. Following table 1 will summarize different types of motility along with their respective characteristic features.

Table 1: Summary of the different type of motilities in bacteria:

	Types of Motility	Characteristic features
1	Swarming	Surface translocation; required multi-flagella, pili, and production of surfactant; cell-cell interaction; group phenomenon.
2	Swimming	Motility in a liquid medium; required flagella and fluid; individual phenomenon.
3	Gliding	Surface translocation; involves focal adhesion complexes; dependent on intrinsic motive forces; cell-cell interaction.
4	Twitching	Surface translocation; required the extension of pili; dependent on intrinsic motive forces.
5	Sliding	Passive mode of surface translocation spreading occurs by growth; production of surfactant.

6	Darting	Surface translocation; required expansive forces developed within cells; dependent on growth.

(Henrichsen 1972, Kearn 2010, Harshey 2003, Verstraeten 2008, Kolter 2006, Murray 2008).

Different bacterial strains exhibit swarming in a different configuration (Ben-Jacob 1995, Appendix A). The colony pattern formation varies between strains. For example, *E.coli* and *B.subtilis* move towards Petri dish periphery in a radial fashion; *Paenibacillus dendritiformis*, *Pseudomonas aeruginosa* PA14 can form dendritic or fractals; *P.mirabilis* moves in a concentric pattern (Shapiro 1995, Kearn 2010, Appendix A). Whatever the pattern may be, the ultimate aim here is common; to look and explore the territory for nutrient rich sources (Harshey 2003). Bacteria can adapt to various conditions depending on their requirement, available machinery, and resources.

Most bacteria show swarming motility on agar surface having a concentration in the range of 0.4 – 0.7 % (Hashey 2003, Appendix A). As the laboratory condition changes within a Petri dish swarming pattern also changes accordingly. Also in presence of some physical and chemical obstacle, it is seen that swarming gets disturbed and it changes its pattern (Shapiro 1995, Caiazza 2005). Due to the chemical diffusion of various molecules and formation of diffusion gradient of nutrients and pH over the agar plate surface are one of the reasons for such changes in patterns (Ben- Jacob 1995).

Initiation of swarming:

Initiation of swarming occurs via several extrinsic and intrinsic factors. Nutrient depletion, lack of specific micronutrient, surface contact, quorum sensing, rhamnolipid biosurfactant production these are some of the factors that can trigger swarming. They are explained in the following subsection. After inoculation of bacteria at the center of the agar plates and as the cell divides; there is an increase in population density. The nutrient gets exhausted and due to starvation bacterial population moves away in order to thrive and look for energy rich sources. This is when swarming is initiated (Shrout 2006). But how do bacterial cells know when and where to move out?

Swarming and Surface contact:

Surface sensing involves multiple mechanisms. The surface interaction of the cells from the swarm center is one of the early stimuli for initiation of swarming (Kearn 2010).Surface sensing involved two-component sensors machinery which are crucial for sensing the initial changes on the surface environment (Rodrigue 2000). Also, bacteria can use their flagella not only for motility but for as a surface sensor by sensing viscosity and moisture over the agar plate (Wang 2005).Flagella can modulate the surfactant synthesis according to its requirement (Kearns 2013). Surface contact can induce many intracellular changes within the bacteria; making them the so-called swarmer proficient cell (Armbruster 2012). All these factors integrate together and change the overall morphology of the bacteria.

Morphology associated with swarming bacteria:

Several studies have shown that the swarmer cells are morphologically very distinct comparing with their planktonic state. They are generally longer, hyper-flagellated and multinucleated (Harshey 1994, Armbruster 2012).

Cell elongation and swarming:

Elongated cells have numerous advantages for swarming; it allows bacteria to move in a well-directed manner and provides a large surface area in order to contact with the other cells via cell-cell interaction (Fraser 1999).

The role of quorum sensing in swarming:

Quorum sensing in bacteria is a measure of their population density; allowing them to make a decision; communicate between individuals (Daniel 2004). Secretion of different biosurfactant depends on the quorum sensing. In PA14 the regulation of “public good” rhamnolipid; its production and secretion is quorum dependent (Caiazza 2005, Xavier 2011, Boyle 2013). Rhamnolipid play crucial role in swarming motility the primary function is to reduce the surface tension of the agar medium and allow smoother movement of bacteria over the surface.

Micronutrient and swarming:

Swarming is a highly regulated phenomenon over surfaces (Shapiro 1998). The conditions and triggers for swarming are quite distinct. Some bacteria swarm under nutrient-rich medium, for example, *E. coli and S. Typhimurium* (Harshey 1994), whereas other show swarming under the nutrient scarce condition including PA14 in this study and others (Rashid 2000). Some swarm in order to get particular micronutrient due to its limitation in the medium, for example, glucose, iron, glutamine (Allison 1993, Harshey 2003, Xavier 2011).

Therefore swarming is dependent on multiple stimuli such as bacterial cell density, surface sensing, a cell-cell interaction that induces physiological and morphological changes and production and secretion of biosurfactant, flagella biogenesis and type IV pili. Altogether this signal is integrated via two-component sensor, response regulators, quorum system, flagella and type IV pili and initiates swarming.

Swarming and Virulence:

Is swarming linked to virulence? Swarmer cells have the ability to promote infection in the host species. It is not just a surface translocation; it changes certain virulence factors and their gene expression. Adding to this, swarming bacteria show higher antibiotic resistance due to their ability to sense and move away further distances with very high speed (Overhage 2008, Butler 2010).

The linked of swarming with virulence is known among few pathogenic and swarming bacteria such as *Proteus mirabilis* (Allison 1992), where it is shown that swarmer cells invade human urinary tract. In swarming, both flagella and type IV apparatus are important (Appendix B). Inside the host, they are being used for movement and attachment respectively and facilitates in virulence along with other secreted factors.

Why study *Pseudomonas aeruginosa* swarming behavior?

Pseudomonas aeruginosa is pathogenic; rod-shaped; single polar flagellated; Gram-negative bacteria which belong to the class of *Gammaproteobacteria*. Due to its large genome size of around 6.3-6.9 Mega base pairs and over 66 % GC- content, which consisting 5500-6400 number of protein-coding open reading frames (ORF) makes it one of the complex prokaryotes (Klockgether 2011).This complexity shows

its physiological adaptability in diverse condition. Therefore it is found ubiquitously in nature. It can rapidly adapt and thrive to different ecological niches and environmental conditions including water, soil, plants and animals.

PA14 has 160 two component sensor and 430 transcriptional regulators making it one of the most adaptable organisms among prokaryotes (Rodrigue 2000, Campa 1993, http://pa14.mgh.harvard.edu).

P. aeruginosa is known as an “opportunistic” pathogen infecting diverse sets of the host as animals, plants, insects, as well as humans. It can colonize and invade any organ system, tissue type, and blood. The list of diseases cause by *P.aeruginosa* is quite long some of them are mentioned below:

Bacteremia, endocarditis, respiratory infections includes cystic fibrosis (Winstanley 2016), urinary tract infection, bone, and joint infection includes osteochondritis, wound infections includes burn infection and diabetic foot ulcers, skin infections, ocular infections includes keratitis of the eyes, endophthalmitis, otolaryngologic infections, dialysis infections, gastrointestinal infections such as typhlitis and perirectal and perianal abscess (Campa 1993).

P. aeruginosa can cause more serious diseases in immunocompromised individuals. Swarming motility can contribute for inhabiting different environmental niches within the host system (Kohler 2000, Overhang 2008). The secretion of various toxins coupled with swarming makes it more fatal.

Swarming in *Pseudomonas aeruginosa* PA14:

Pseudomonas aeruginosa strain PA14 shows swarming behaviour on surfaces having 0.6% agar under nutrient deplete condition. Cells at the moving tendrils or swarm tip are elongated and have two polar flagella with respect to swarm center and broth culture (Toutain 2005, Patridge 2013). These morphological changes can enable PA14 to move over the semisolid surface. Swarming in PA14 is dependent on the surface secreted surfactant called rhamnolipid (Caiazza 2005).

The role of rhamnolipid in swarming:

The role of rhamnolipid is not only to reduce the surface tension but also in dendritic pattern formation (Tremblay 2007).It is dependent on amount and diffusion of rhamnolipid through the surface. Its synthesis is dependent on the availability of carbon and nitrogen and their ratio (Xavier 2011). PA14 integrates various environmental and surface stimuli, carbon, nitrogen availability and coupling it with quorum sensing machinery to trigger rhamnolipid production.

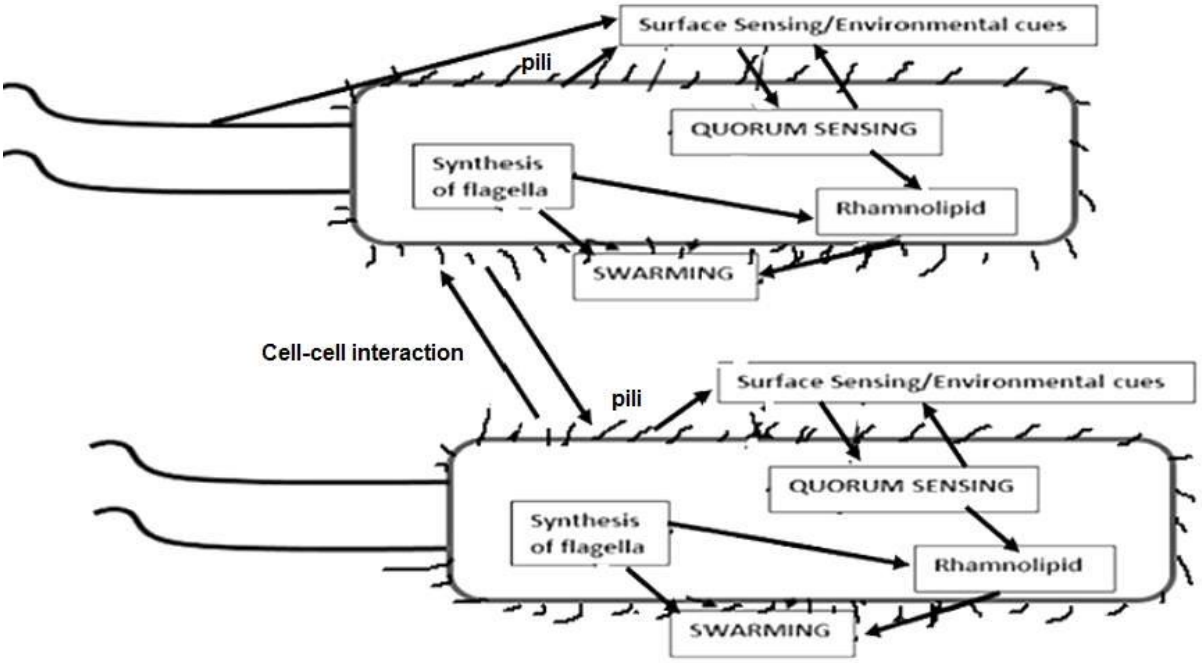


Figure 1: The integration of few of the factors that lead to the decision of swarming in PA14.

Swarming in PA14 is a collective migration with cooperation among individuals. But swarming has some cost and benefit. Cost being in synthesis and secretion of rhamnolipid biosurfactant which depends on the availability of carbon and nitrogen source. The benefit being a migration over surfaces for more nutrients (Xavier 2011).

Swarming in PA14 is an emergent behavior as a result of local interactions of individual bacteria and globally forming a well-structured dendritic pattern.

Dendritic pattern while swarming behavior of PA14:

The significance of dendritic pattern formation:

The dendritic pattern is one of the universal pattern seen in biological, chemical and physical systems. For example, dendritic growth is found in nerve cells, lungs bronchoalveolar and vascular system, rivers basin and deltas, crystals formation in some minerals, trees branching, coral reefs, and also found in bacterial colonies under low nutrient and environmental conditions (Shapiro 1995, Camazine 2001).

The dendritic pattern can form in swarming bacteria at specific agar and nutrient composition (Methods and materials). The dendritic pattern is efficient in covering more surface area and in the uptake of nutrients.

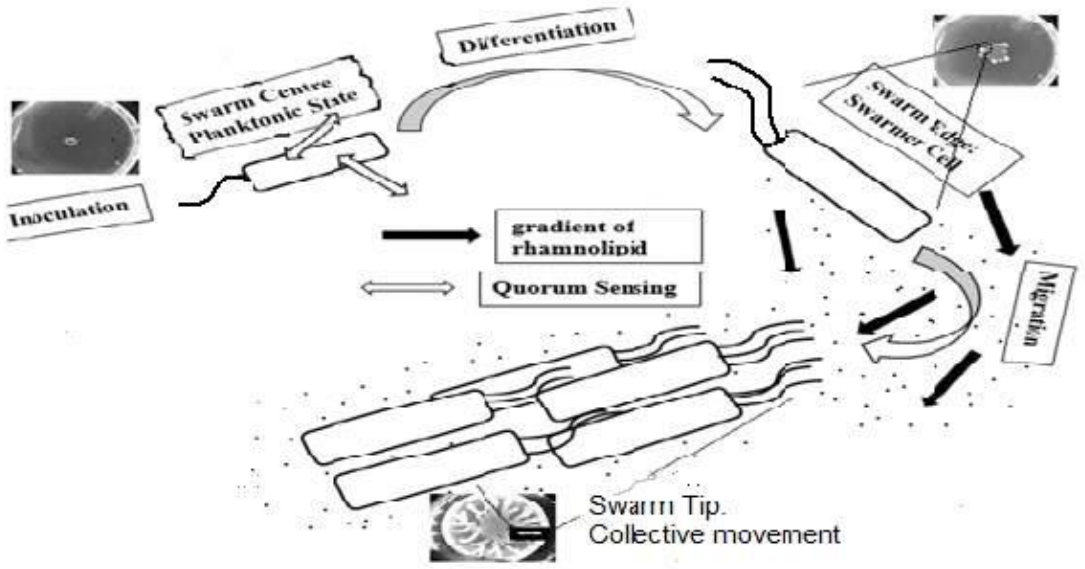


Figure 2: Probable course of events in PA14 Swarming behavior

Different physiological, morphological and genotypic changes can be seen in both at the swarm center and at the swarm tip of moving tendril. Swarm tip move due to forces coming from the swarm center and as the cell population number is increasing exponentially they started depleting the available resources.

The main aim of this study is to understand the regulatory network of swarming in PA14. What are the swarming promoting factors of PA14 which are crucial for such surface behavior?

MATERIALS AND METHODS:

Materials:

Screening of PA14 transposon insertion mutant library for identification of swarming negative phenotype:

In the present study, we have used *Pseudomonas aeruginosa* strain PA14 has a model system for studying its swarming behavior (Kohler 2000, Rashid 2000, Tremblay 2008, Xavier 2011, Badala 2008).

Bacterial Strains:

For our screening work, we have used *Pseudomonas aeruginosa* strain PA14 ordered, non-redundant transposon insertion mutant library gifted from Dr. Frederic Ausubel's Lab. The PA14 NR transposon mutant library was created by using *MAR2xT7* transposon which is a derivative of the mariner family transposes *Himar1.* The library has 5987 transposon mutants comprising 75 % of the total encoding genes from PA14 genome. Most of the transposon mutants have a single insertion in them. Mutants with *MAR2xT7* transposon have gentamicin antibiotic resistance (Liberati 2005).

Methods:

Swarming motility assay:

For swarming assay, we used NGM-SK medium (Nematode growth medium- slow killing) plates which are being used for feeding bacteria to nematode host *Caenorhabditis elegans* (Marsh 2012).

Preparation of NGM-SK plates:

Composition of NGM-SK 0.6% agar plates are 6 grams of bacteriological agar (Bacto agar), 3.2 grams of peptone, and 3 grams of sodium chloride (NaCl) added in 1 liter of distilled water. Autoclaved the medium at 121^{o}C for 30 minutes. After autoclaving the media, 1 mL of 1M $CaCl_2$ (Calcium chloride), 1 mL of 1 M $MgSO_4$ (Magnesium sulphate), 25 ml of 1M KPO4 and 1 mL of 5 mg/mL cholesterol were added into the medium and mixed properly.

25 mL NGM-SK were poured in each 90mm Petri plates and allowed them to solidify at RT (room temperature) for a half an hour under the laminar hood flow with the lid opened. And all the plates were kept at room temperature for 16-18 hours for further drying.

Screening of PA14 mutant for a defect in swarming:

Pseudomonas aeruginosa strain PA14 was freshly grown for 12-14 hours in 3 ml LB (Luria-Bertani) broth. And all the mutants were freshly grown individually for 12-14 hours in 3 ml LB (Luria-Bertani) broth with gentamicin antibiotic.

Screening is done with 2µl of 3.0 OD culture of PA14 and all transposon mutants spotted on NGM-SK plates.

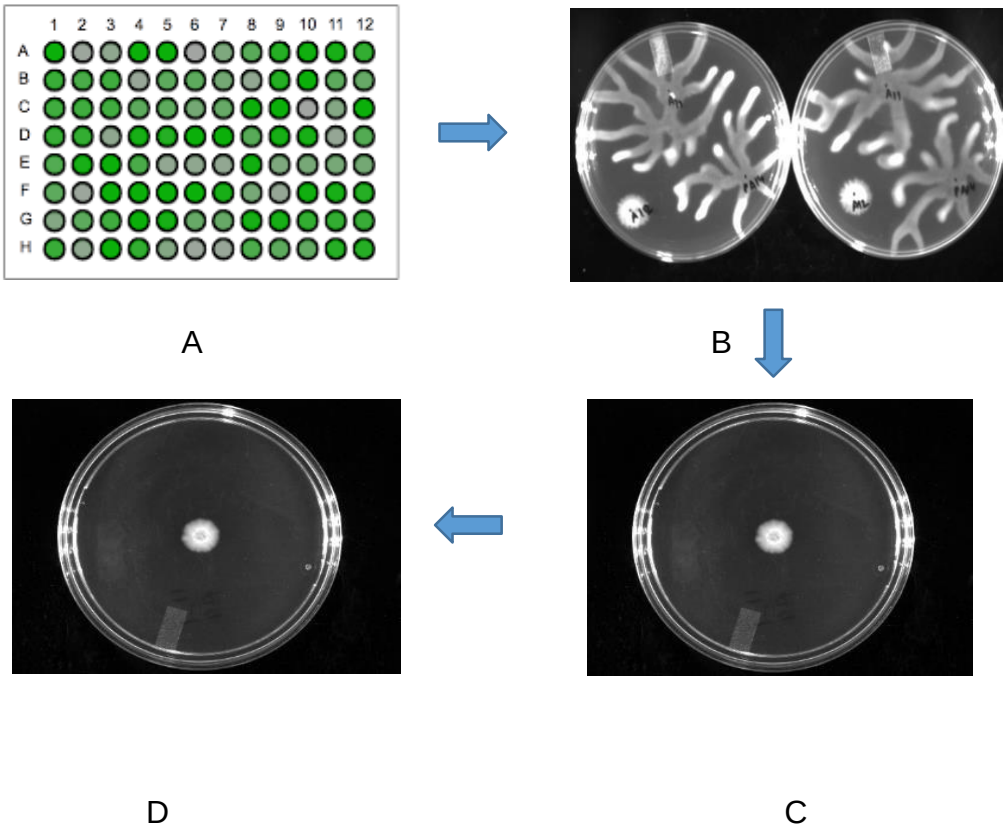


Figure 3. Overview of screening procedure: A: Transposon mutant library consisting of 5987 mutants, B: Primary screening is done with two mutants and one wild-type spotted on single plate in duplicates, C: Secondary screening is done for mutants showing less swarming or non-swarming phenotype based on primary screening were spotted at the center of the agar plates, D: Tertiary screening is done for validating secondary screening results.

Swimming Motility Assay: NGM-SK plates having 0.3% agar were used for performing swimming assay in wild-type PA14. By using a toothpick, the PA14 liquid culture of 3.0 OD was gently touched on the NGM SK plate. All the plates were incubated at 37^{o}C for 24 hours.

Twitching Motility Assay: Luria-Bertani (LB) broth having 1.5% agar were used for twitching assay. The 2µl PA14 liquid culture of 3.0 OD was stabbed to the bottom of LB agar plates. All the plates were incubated at 37^{o}C for 48 hours

Imaging:

The documentation of all the images is done by using Gel doc Alpha Imager from the common facility at MRDG, IISc, Bangalore.

Time-lapse imaging:

The time-lapse imaging is done at the Centre for Nano Sciences and Engineering, Indian Institute of Science, Banglore. The NGM SK plates were dried overnight and spotted with 2µl of 3.0 OD culture of PA14 at the center. Dried for half an hour inside laminar hood before keeping into the imaging chamber. Plates were sealed with parafilm tape. The chamber had controlled humidity and temperature for optimal growth. Allowed recording for 24-36 hours.

Library database:

We have classified our non-swarming mutant from the public database available for the PA14 transposon insertion mutant library (http:ausubellab.mgh.harvard.educgi-binpa14home.cgi).

Results:

Standardization of swarming assays in PA14:

In order to increase consistency and reproducibility of swarming pattern, we tried different substrate including different agar concentration and different medium composition. We have standardized different medium composition and found PA14 swarm better on (Nematode growth medium- Slow Killing) NGM – SK with 0.6 % Bacto agar concentration.

Table 2: Swarming motility of *Pseudomonas aeruginosa* strain PA14 under different medium composition

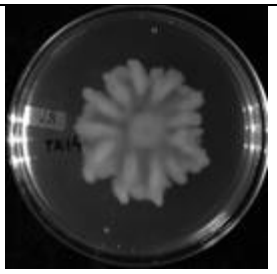
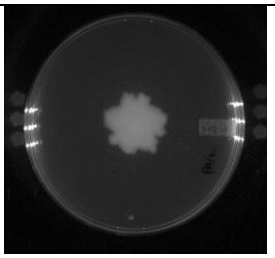
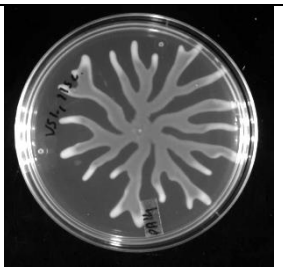
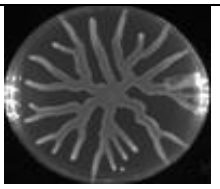
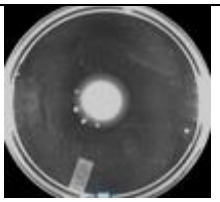
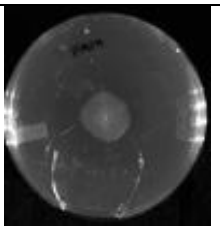
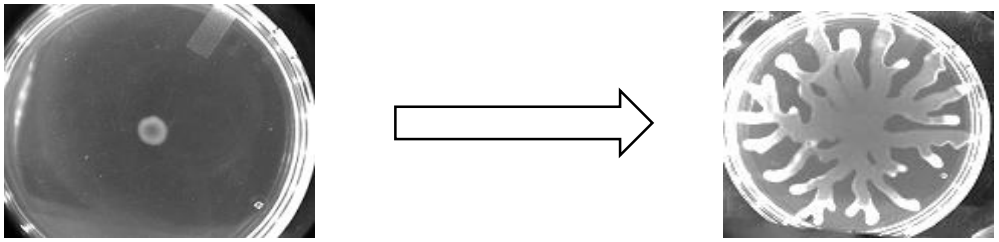
Medium	Conditions	Swarming Pattern
(Luria-Bertani) broth (LB) with 0.6 % Bacto agar	2 µl culture of PA14 were spotted at the center. Incubated at 37ºC for 24 hours.	
Brain Heart Infusion (BHI) with 0.6% Bacto agar	2 µl culture of PA14 were spotted at the center. Incubated at 37ºC for 24 hours.	
Nematode growth medium – Slow Killing (NGM SK) With 0.6% Bacto agar	2 µl culture of PA14 were spotted at the center. Incubated at 37ºC for 24 hours.	

Table 3: Different types of motility (Swarming, Swimming, and Twitching) seen in Pseudomonas aeruginosa strain PA14.

Types of motility	Agar concentration %	Incubation temperature	Motility pattern
Swarming	0.6 NGM-SK	37^{o}C 24 hours	
Swimming	0.3 NGM-SK	37^{o}C 24 hours	
Twitching	1.5 LB Agar	37^{o}C 48 hours	

PA14 swarming pattern on NGM-SK 0.6 % agar:

PA14 culture is inoculated at the center of the agar plate. (Methods and Materials). After around 24 hours post inoculation it can form dendritic branching pattern.



After 5 hours post inoculation After 22 hours post inoculation

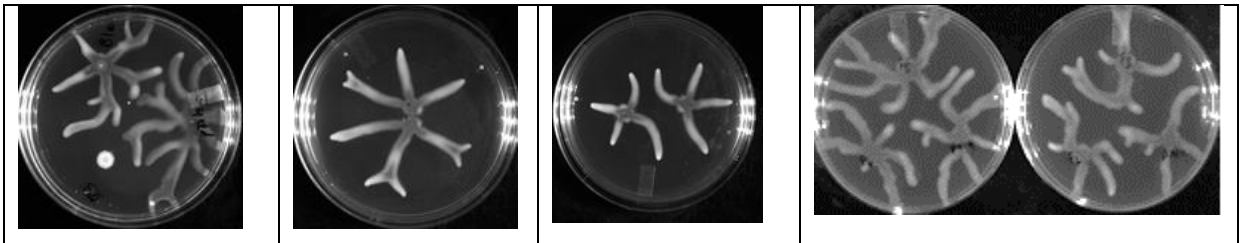
Figure 4: Time-lapse image: Course of events in PA14 swarming

Table 4: Characteristic features of PA14 swarming:

Characteristics associated with Swarming in PA14 on (90 mm plate)	Number estimated from 10 NGM-SK plates (range: min-max); Average values
Length of swarm tendrils	10 - 25 mm; 15 mm
Swarm center radius	5 – 12 mm; 8 mm
Swarming lag	8 -12 hours
Area covered	27 – 42 % ; 34 %
Number of primary branches	4 – 8; 5
Number of secondary branches	6 – 12; 8

The interspecies and intraspecies interaction between PA14 leads to differential branching pattern:

While screening we have seen some distinct or peculiar pattern by some of the mutants. They were having very thinner branching pattern and wider swarm center. Also, we have found some of the swarming mutants which touch the branch of other mutant or wild type. Suggesting us that these particular mutant are not able to sense the presence of another mutant. Might be these mutants are acting like a sensor which detects the presence of some unknown secreted factor. Apart from these cases the two moving tendril never touched each other due to the presence of some inhibitor as seen in figure 5. Figure 5 depicts how distinct swarming pattern arises when the culture of PA14 spotted at a different location on a Petri plate.



5a.	5b.	5c.	5d.

Figure 5. The interspecies and intraspecies interaction between PA14:

5a. swarming mutant, PA14 wild-type, and non-swarming mutant interaction.

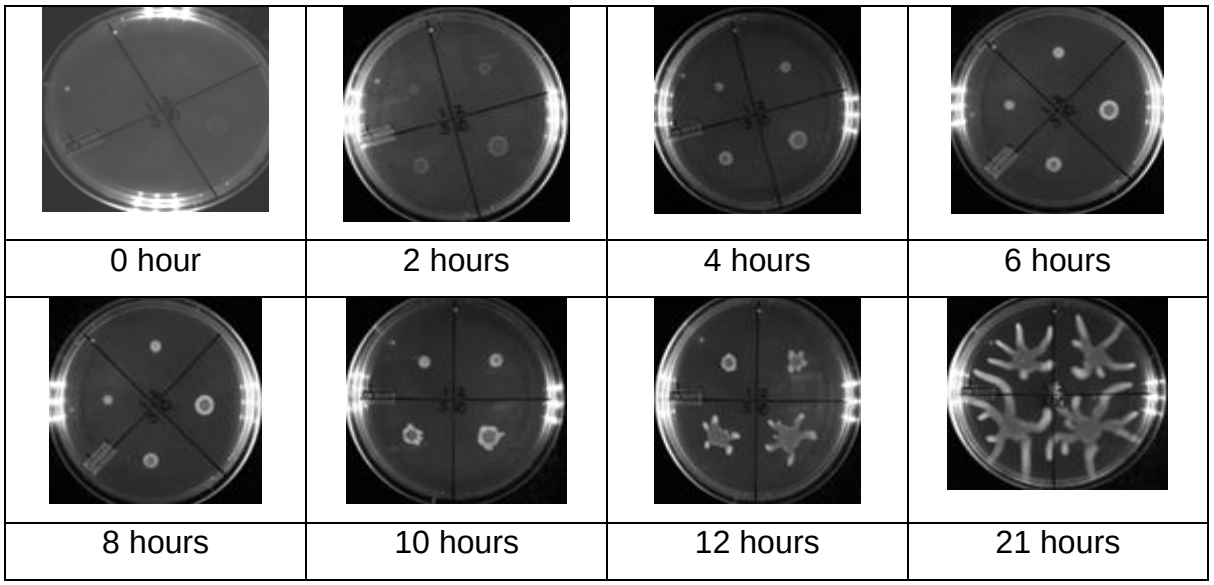
5b. Two PA14 wild-type interaction when spotted 0.5 cm apart.

5c. Two PA14 wild-type interaction when spotted close to 2.0 cm apart.

5d. Two swarming mutant and a PA14 wild-type interaction spotted at the edge of an imaginary triangle.

Swarming-associated phenotypes:

Swarm Lag: The swarming lag is seen in our swarm assay; when bacterial culture is transferred from liquid broth to the semi-solid surface; there is a lag of 8-12 hours before the formation of the tendril. We suspect that it occur due to the fact that cell needs to be adapted to the surface; in order to swarm. Here we have used different inoculation density varying from **1 µl, 2 µl,10 µl and 5 µl marked respectively in the four quadrants in a clockwise manner**. We can see 1 µl and 2 µl inoculation has longer lag time compares to 5 µl and 10 µl.



0 hour	2 hours	4 hours	6 hours
8 hours	10 hours	12 hours	21 hours

Figure 6: Different inoculation density and swarm lag:

Flagella, pili, and rhamnolipid are important for swarming:

In the following experiment, it can be seen that if two swarm negative mutant mixed together in equal proportion and spotted 2µl mixed culture at the swarm center and it can able to swarm. Here we used *rhlR* rhamnolipid synthesis mutant defective in surfactant production, *flgM* flagellar apparatus mutant defective in motility and *pilW* pili apparatus mutant which is impaired in twitching or type IV pili.

Panel A showing all the mutant are non swarmer.

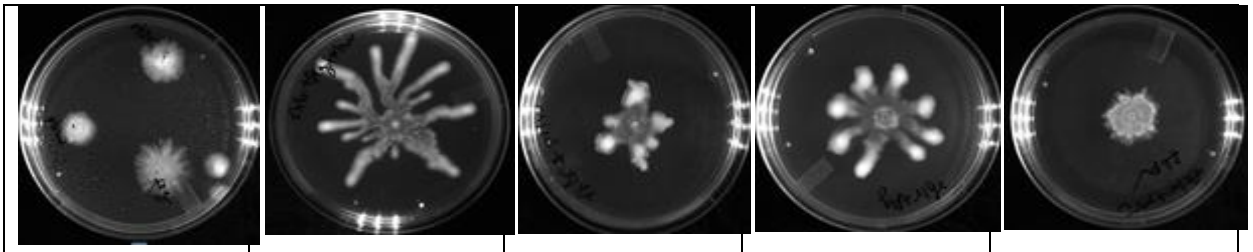
Panel B *rhlR+flgM+pilW* covering more area explaining more availability of surfactant leads to better swarming.

Panel C showing *rhlR+pilW* can swarm in some extent.

Panel D *rhlR+flgM* show swarming due to the exchange of surfactant.

Panel E is *flgM+pilW* no swarming at all since both are non-motile.

One explanation for this is that production of extracellular factors from one is being utilized by other.



Panel A	Panel B	Panel C	Panel D	Panel E
rhlR-top most *pilW*-left most *flgM*-rightmost	*rhlR*+ *flgM+pilW*	*rhlR+pilW*	*rhlR+flgM*	*flgM+pilW*

Figure7: Role of flagella, pili and rhamnolipid in swarming. The amount of rhamnolipid production, motility apparatus, as well as type IV pili, are needed for swarming in PA14. From above panels, there might be some positive feedback mechanism between flagella and type IV pili for rhamnolipid production.

Identification of genetic regulators of swarming motility: We have performed a genetic screening for identification of the genes underlying swarming behavior in *Pseudomonas aeruginosa* PA14**.** Swarming assays were conducted using PA14 transposon insertion mutants in three stages: primary, secondary and tertiary. In the primary and secondary screening, the non-swarming mutants are confirmed by screening each mutant in duplicate along with wild-type PA14. After the primary and secondary screening, we found around 700 mutants that were showing non-swarming, less swarming and non-distinguishable phenotype. These mutants were further analyzed for their swarming phenotype by performing the tertiary screening. After the tertiary screen, we have found total 281 transposon insertion mutants (218 non-swarming, 63 less swarming) from the PA14 non-redundant set (set of 5987 transposons mutant library) which were impaired in swarming related phenotype over NGM-SK 0.6 % agar plates. Around 129 transposon mutants were newly identified from this screening; their functional role is not previously known with respect to swarming in PA14. Appendix- B has listed all the mutant genes classified

according to their respective functional class after the tertiary screening. Following bar, plots show the total number of genes belonging to each functional classes from 1 to 28 (Legend shown at bottom).

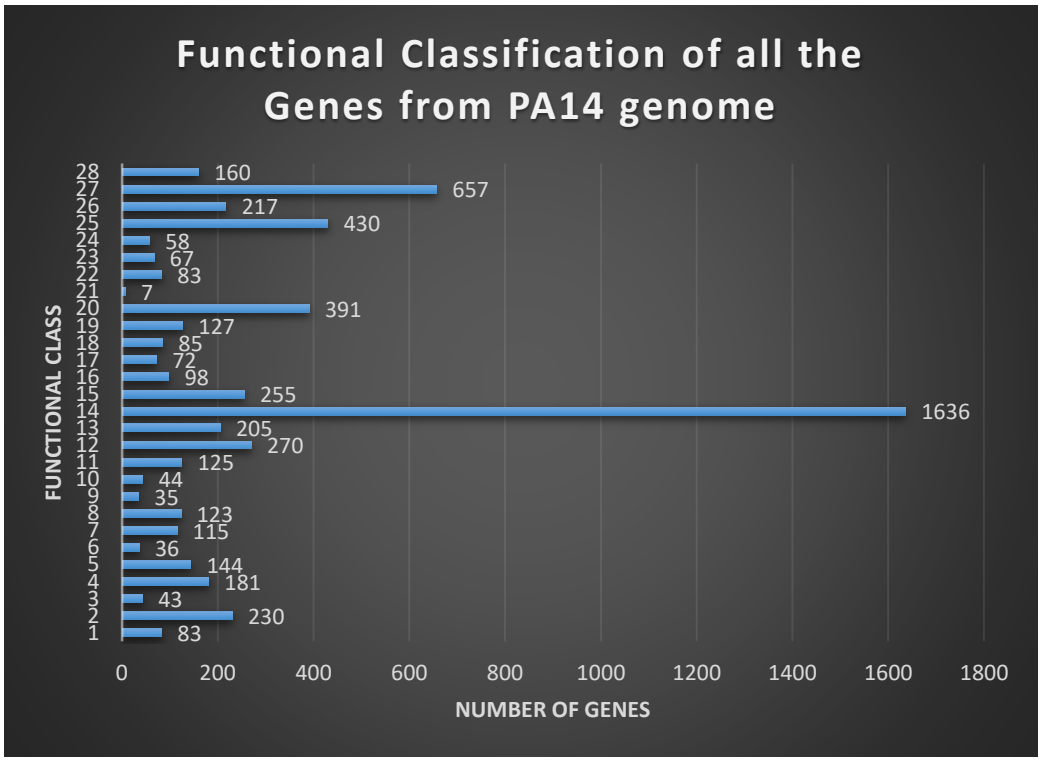


Figure 8A. Functional Classification of all the Genes from PA14 genome

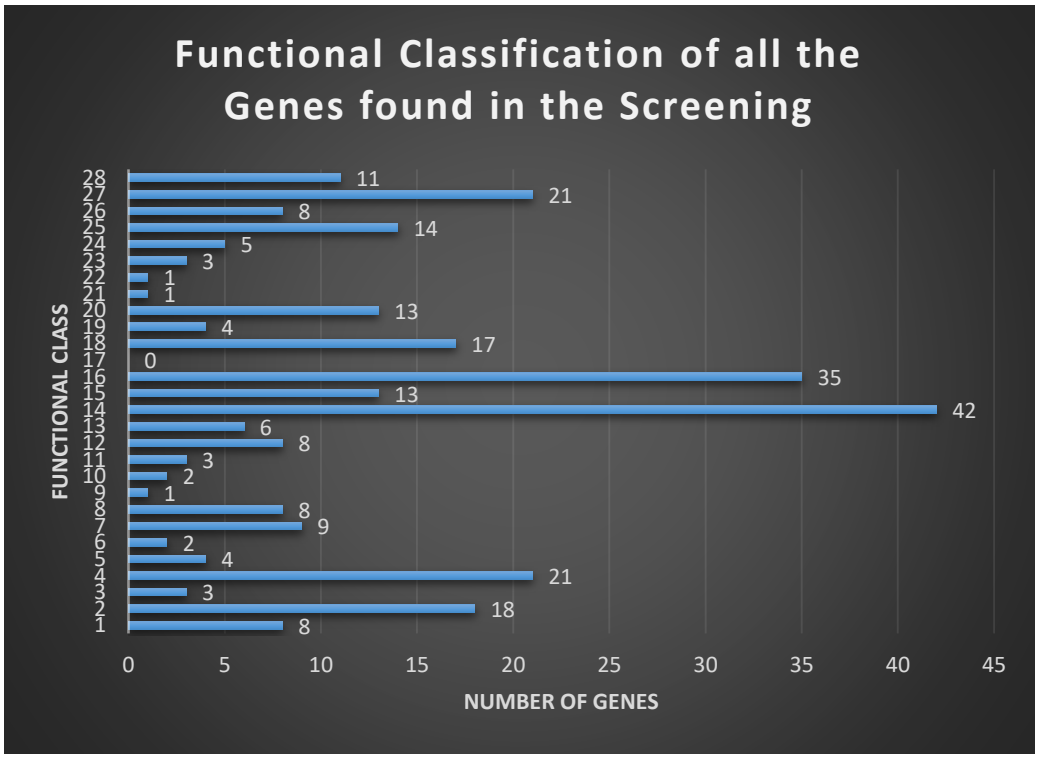


Figure 8B. Functional Classification of all the Genes found in the Screening

1	Adaptation, protection	15	Membrane proteins
2	Amino acid biosynthesis and metabolism	16	Motility & Attachment
3	Antibiotic resistance and susceptibility	17	Non-coding RNA gene
4	Biosynthesis of cofactors, prosthetic groups, and carriers	18	Nucleotide biosynthesis and metabolism
5	Carbon compound catabolism	19	Protein secretion/export apparatus
6	Cell division	20	Putative enzymes
7	Cell wall / LPS / capsule	21	Quinolone signal response
8	Central intermediary metabolism	22	Related to phage, transposon, or plasmid
9	Chaperones & heat shock proteins	23	Secreted Factors (toxins, enzymes, alginate)
10	Chemotaxis	24	Transcription, RNA processing, and degradation
11	DNA replication, recombination, modification and repair	25	Transcriptional regulators
12	Energy metabolism	26	Translation, post-translational modification, degradation
13	Fatty acid and phospholipid metabolism	27	Transport of small molecules
14	Hypothetical, unclassified, unknown	28	Two-component regulatory systems

Discussion:

Pseudomonas aeruginosa PA14 show distinct swarming behavior on different nutrient substrates:

We hypothesized that swarming in PA14 is coordinated, collective, group movement over the 2 D surface of the agar; in order to move towards higher nutrient gradient and to maximize the surface area.

For this, we have done standardization assay for swarming in PA14 on a different medium including Luria-Bertani broth (LB), Brain Heart Infusion broth (BHI) and NGM-SK (Rao 2009). We observed that swarming occurs only in NGM-SK which is relatively nutrient scarce than other two. This suggests us that swarming in PA14 is dependent on nutrient condition requires minimal nutrient composition.

PA14 swarm in a dendritic pattern on NGM SK plates with consistency in number and length of branching along with time lag of 8-12 hours.

Cells in the swarm lag might be changing their morphology from planktonic swimmer state to swarmer state. To become swarmer cell might be it takes several generations of bacteria explains the lag timing. Another explanation for this is while swarming PA14 has extra polar flagella thus the synthesis of entire new flagellar apparatus might be an energy consuming process involved a lot of time (Kohler 2000, Doyle 2004, Toutain 2005). We observed that swarm lag is dependent on the starting inoculum density, it is shorter for high-density inoculation and longer for low density. Suggesting role of quorum sensing in the swarming. Since at high-density cells can secret more rhamnolipid and initiates swarming early.

Swarming assays are always prone to several factors in laboratory conditions (Tremblay 2008). In order to have consistency and reproducibility, one should strictly follow the protocol that suits the question of interest. We have seen several factors that affect swarming assays throughout the screening process.

The agar concentration plays an important role in the swarming pattern. Since it changes the diffusion of molecules through it. Plate thickness also matters because the plate having higher medium can retain more moisture and can delay drying inside incubation chamber. A drying period of the plates inside laminar flow also

contributes to swarming pattern; too much drying timing inhibits swarming and too little can form undefined branches. So in order to get consistency in the results, we dried plates for half an hour only. The pH of the medium can also influence swarming by interfering with diffusion gradients created by secreted molecules of PA14. Incubation temperature is the most crucial we kept all the swarming plates at 37 °C for 24 hours before imaging. The morphology of the swarming cell differs with a change in temperature. Plate location within the stalks as well as within the incubator affects swarming as well.

Motility apparatus is essential for swarming of PA14 on NGM-SK agar plates:

Swarming motility is a complex behavior that involves swimming motility, twitching motility, and production of a surfactant called rhamnolipid. *Pseudomonas aeruginosa* PA14 displays complex dendritic swarming patterns that require a network of genes encoding for flagella for swimming, type IV pili for twitching, production of rhamnolipid in order to reduce surface tension, two-component sensors for sensing its environment, transport machinery for importing and exporting of nutrients. The main aim of this study is to understand the underlying genetic regulators and their complex network involved in swarming behavior of *Pseudomonas aeruginosa* PA14?

From this screening results as well as similar work done by Yeung *et.al* 2009 have found large sets of regulatory genes in swarming of *Pseudomonas aeruginosa* PA14. The main difference in both the studies is the medium used for swarming assay (Methods and Materials). They have used BM-2, 0.5 % agar swarming medium with CAA, glucose, some salts and ferrous sulphate supplemented in it (Yeung 2009).
The screening methodology is completely different for both the studies. They have grown 96 mutants in a 96 well plate and spotted for all of them at the same time on a single bigger plate. Yeung *et.al* were looking for a radial growth of the mutant not unlike us for a dendritic pattern which is the characteristic feature of PA14 for primary screening. Since they were scoring for mutant which having minimal or lesser radial growth. Also, it is possible that due to limited space available in the plate the mutant showed a non-swarming phenotype in their screening and turn out to be swarmer in this study.

The total number of non-swarming, less swarming and hyperswarming mutants found in Yeung *et.al* study is around 230. And around 150 transposon mutants are common in both the study.
All the mutants were grown well in the LB broth culture which were characterized by their color and confirm by their OD (optical density) measurement. It ruled out the possibility of whether the mutants were having defects in their growth. Also we have done swimming and twitching assays for some of them and they were showing phenotype again confirming they were not defective in their growth. In future we are planning to make clean knockouts for few of them and to do swimming and twitching for all of them in order to further validating our results.

Some of the previous studies have shown the role of flagella for motility, type IV pili and biosurfactant rhamnolipid are crucial factors for swarming in PA14 (Anyan 2014, Doyle 2004, Caiazza 2005, Kearns 2013, Kohler 2000).

The present study also has found a number of motility and attachment related mutants validating our results. We have around 35 flagellar biosynthesis related mutants (*flhABF, flgBCEFIJKLH, fliIJQ*) as well as pili mutant (*pilY1, pilX, pilF, pilW, fimU, pilT)* that were showing swarm negative phenotype. Suggesting crucial role of flagella and type IV pili in swarming of *Pseudomonas aeruginosa* strain PA14.
Also, we have found *rhlR* which is a transcriptional regulator for rhamnolipid biosurfactant proving our results to be correct.

The total number of genes that found from our screening comprises approximately 5 % of the total annotated genes from PA14 genome. Suggesting how complex regulatory mechanism is governing this surface behavior. We have found all the genes from every functional class except gene belonging to non-coding RNA functional class.

We have found enrichment in genes belonging to following functional classes; some of them are mentioned in the parenthesis:

Amino acid biosynthesis and metabolism (*argG,metH,argH,thrC*), Two component sensors (*fleR,fleS,Hpt*), Transport of small molecules (*dppD,cysQ, lon,miaA,rpsD*), Adaptation and protection (*pvdDENH*), Energy metabolism (*norB,ccoN,cycH*), Membrane protein (*opmD, oprF*), Central intermediary metabolism (*metF,tpiA, galU,ppsA*), Biosyntheis of cofactors, prosthetic groups and carriers (*panBC,*

bioAD,nadD, thiG), Nucleotide biosynthesis and metabolism *(pyrBDF, apaH,purHM, carA)* and few other functional classes. Around 42 of genes were identified in our screening belong to a hypothetical, unknown and unclassified category.

Conclusion:

Overall this study suggests that swarming is a complex form of surface associated behaviour which involves a large set of genes belongs to diverse functional classes.

The present study has found around 281 swarming associated genes through screening of each mutant from PA14 NR Set. Importantly, we have found 129 new transposon insertion mutants to control PA14 swarming. These genes are likely to throw light on pathways which enable bacteria to execute a motility pattern that requires extensive communication and coordination. These genes can be organized into a network based on known interaction of some of the genes and also by studying their ability to regulate the motility apparatus, surfactant production or inter- bacterial communication.

This study of swarming behavior will give us a better understanding of social traits shown by microbes as well as higher order organisms. Biofilm formation and virulence are social traits that involve quorum sensing and collective behavior. Swarming is another such trait which may also be linked to PA14 pathogenesis. We will be studying a number of genetic regulators for virulence phenotype, using *Caenorhabditis elegans* as a model host for PA14.The present work raises many interesting questions related to the evolution of social behavior in bacteria in general and PA14 in particular. Some of these of immediate interest are:

a. Does swarming behaviour provide a fitness benefit?
b. Why there is a long swarm lag?
c. Do bacteria sense nutrient gradient? How?
d. Does PA14 sense the space available in its vicinity?
e. How does surface interaction of PA14 is translated into the swarming phenotype?
f. How does PA14 form dendritic pattern while moving on the agar surface?
g. Is a dendritic swarming pattern is seen in the wild?

h. Does PA14 swarm in lungs filled with thickened mucus, such as in patients with cystic fibrosis.

In conclusion, swarming is a complex adaptive social behavior shown by *Pseudomonas aeruginosa PA14* which depends on several physical and chemical stimuli and requires irreversible morphological changes within.

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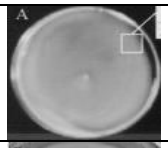
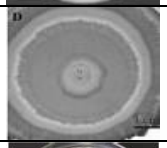
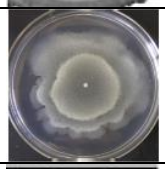
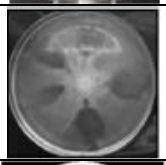
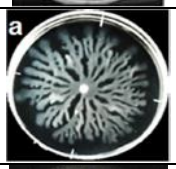
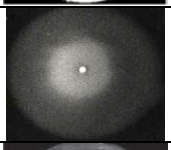
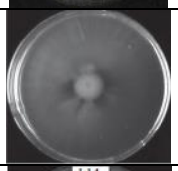
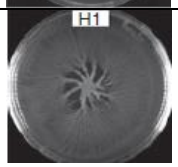
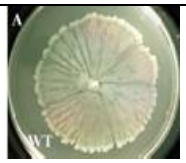
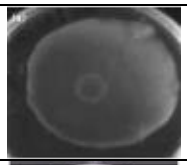
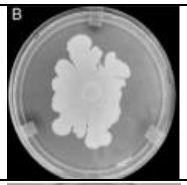
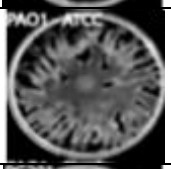
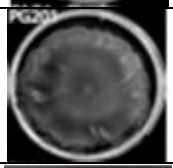
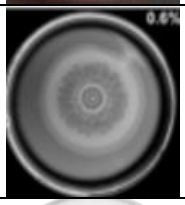
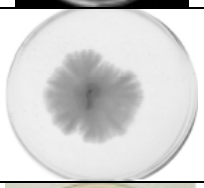
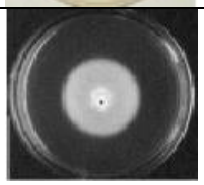
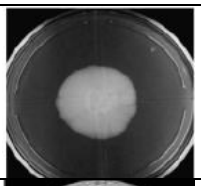
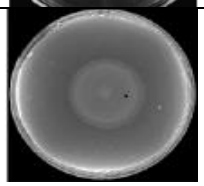
APPENDIX-A

Bacterial species	Swarming pattern	Conditions	Habitat/ Features/ Characteristic	Source
Aeromonas hydrophila		Eiken agar 0.5% (wt/vol) in Difco Broth Incubated at 30°C	Fresh water, GI tract.	Kirov (2002)
Bacillus subtilis strain 3610		LB medium 0.7 % agar, Incubated at 37°C for 24 hours	Soil, Air (spores).	Julkowska (2016)
Bacillus cereus ATCC 14579		TrA-NaCl (1.0% tryptone, 0.5% NaCl, 0.7% agar) incubated at 37°C for 24 hours	Soil, Air (spores).	Senesi (2010)
Burkholderia pseudomallei sp.K96243		0.5 % agar Incubated at 37 °C for 18 h.	Soil; Fresh Water Gram negative pathogen.	Adler (2016)
Burkholderia glumae BGR1		M9 medium 0.5 % agar Incubated at 34°C for 18 hours	Soil; Fresh Water Phytopathogen of rice.	Nickzad (2015)
Campylobacter jejuni 11168		BBA medium 0.4% agar Incubated at 37 °C	Intestinal tract, Oral cavity	Adler (2014)
Dickeya didantii 3937		MG plates containing 0.4% agar	Gram negative Plant pathogens	Yi (2010)
Edwardsiella tarda H1		Tryptic Soy Broth plates (0.5% agar, 0.5% glucose) Incubated for 48 hours	Entero-bacteriaceae, Gram-negative bacterium Pathogenic to fishes	Xu (2014)
Escherichia coli MG1655		Difco agar 0.5% plates dried for 5–6 hours at RT Incubated at 37°C for 24 hours	Gastrointestinal Tract	Manfredi (2007)

Mesorhizobium tianshanense		M1 medium 0.6 % agar Incubated at 28 °C in the dark for 4 days	Nodules forming Rhizobium on the roots of plants	Zheng (2015)
Paenibacillus lautus NE3B01		0.5 % agar	Marine Bacterium	Mangwani (2014)
Paenibacillus alvei CCM 2051T		1% agar Incubated at 37°C for 48 hrs.	Gram positive bacterium	Janesch (2013)
Photorhabdus temperata		2% m/v PP3, 0.5% yeast extract, 0.5% NaCl, 0.65% agar, Incubated at 28°C for 24 hours	Insect pathogen	Michaels (2011)
Proteus mirabilis		2.0 % agar Incubated for 48 hours	Soil, Water, Plant Surface, Urinary tract, GI tract	Czirok (2000)
Pseudo alteromonas sp.SM9913		0.5 % agar Incubated at 15°C for 5 days	Marine bacterium	Liu (2016)
Pseudomonas aeruginosa PAK		0.5 % agar 1 hour drying period Incubated at 30°C for 24 hours	Water, Soil , Plant Surface	Tremblay (2008)
Pseudomonas aeruginosa PAO1		0.5 % agar 1 hour drying period Incubated at 30°C for 24 hours	Water, Soil , Plant Surface	Tremblay (2008)
Pseudomonas aeruginosa PG201		0.5 % agar 1 hour drying period Incubated at 30°C for 24 hours	Water, Soil , Plant Surface	Tremblay (2008)
Pseudomonas syringae (PssB728a)		KB plates 0.4% agar Incubated for 24 hours	Plant pathogen	Chatna parat (2015)

Pseudomonas aeruginosa M18		Medium: 0.5 % glucose, 0.5% Agar. Incubated at 28 °C for 24 hours	Water, Soil , Plant Surface	Wei (2013)
Rhodospirillum centenum ATCC 51521		CENMED (0.25% agar) Incubated aerobically in the dark at 42°C for 4 days.	Water, Marine bacterium	Bird (2011)
Salmonella enterica strain 14028		0.6 % agar with 0.5 % arabinose Incubated at 37 °C	Gastrointestinal Tract, Feces	Partridge (2013)
Stenotrophomonas maltophilia E77		BM2 0.5 % agar Incubated at 30°C for five days	Gram negative nosocomial pathogen	Yero (2015)
Serratia marcescens strain PIC3611		LB medium with 0.6% agar	Soil, Water, Plant Surface	Stella (2015)
Treponema denticola 35405		TYGVS medium 0.8% agarose Incubated for 7 days.	Causative factor for patho-genesis of periodontal diseases	Ikegami (2004)
Vibrio shilonii ATCC BAA-91 (AK-1)		0.5% agar Incubated at 30°C for 36 h.	Salt water	Venegas (2010)
Yersinia enterocolitica		0.6% agar Incubated at 26°C For 24 h.	Mammalian Entero-pathogen ; Rodents; Flea; Gastrointestinal Tract	Atkinson (2006)

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APPENDIX – B

Functional Class	"Active" Gene Locus	"Active" Gene ID	"Active" Gene Name	"Active" Gene Description
Adaptation, Protection	PA14_33720	GID1230	*pvdN*	putative aminotransferase
	PA14_68670	GID2327		putative carboxypeptidase
	PA14_12300	GID2843		Putative Mg2+ and Co2+ transporter CorC
	PA14_33500	GID959	*pvdH*	2-ketoglutarate 4-aminotransferase
	PA14_33690	GID648	*pvdE*	pyoverdine biosynthesis protein PvdE
	PA14_33650	GID2	*pvdD*	pyoverdine synthetase D
	PA14_14680	GID2898		inositol-1-monophosphatase
	PA14_45940	GID3828	*lasI*	autoinducer synthesis protein LasI
Amino acid biosynthesis and metabolism	PA14_53000	GID4861	*phhB*	pterin-4-alpha-carbinolamine dehydratase
	PA14_23920	GID751	*purF*	amidophosphoribosyltransferase
	PA14_62130	GID2003	*ilvC*	ketol-acid reductoisomerase
	PA14_35520	GID1810	*bkdA2*	2-oxoisovalerate dehydrogenase, beta subunit"
	PA14_18740	GID1386	*argG*	argininosuccinate synthase
	PA14_12010	GID1408	*proA*	probable gamma-glutamyl phosphate reductase
	PA14_67240	GID2881	*hutG*	N-formylglutamate amidohydrolase
	PA14_05220	GID1061		cystathionine beta-synthase
	PA14_05480	GID807		putative monoamine oxidase
	PA14_23280	GID1777	*pheA*	prephenate dehydratase
	PA14_40670	GID31	*metH*	methionine synthase
	PA14_69500	GID991	*argH*	argininosuccinate lyase
	PA14_18740	GID1386	*argG*	argininosuccinate synthase
	PA14_23920	GID751	*purF*	amidophosphoribosyltransferase
	PA14_16090	GID934	*thrC*	Threonine synthase
	PA14_14700	GID2969	*cysE*	serine O-acetyltransferase
	PA14_65110	GID1885	*alr*	biosynthetic alanine racemase
	PA14_66600	GID1769	*aroB*	3-dehydroquinate synthase
Antibiotic resistance and susceptibility	PA14_48280	GID1736		putative multidrug resistance efflux pump
	PA14_41280	GID464		putative beta-lactamase
	PA14_20110	GID1684		putative ABC-type multidrug transport system, permease component
	PA14_07170	GID1869	*epd*	D-erythrose 4-phosphate dehydrogenase
	PA14_39010	GID222	*pqqF*	pyrroloquinoline quinone biosynthesis protein F
	PA14_00700	GID3365		putative protein-disulfide isomerase
	PA14_26510	GID3221	*cobI*	precorrin-2 methyltransferase CobI
	PA14_19630	GID4047	*folE1*	GTP cyclohydrolase I precursor
	PA14_07740	GID2135	*pdxA*	pyridoxal phosphate biosynthetic protein PdxA

Biosynthesis of cofactors, prosthetic groups and carriers	PA14_46470	GID1669	*pdxB*	erythronate-4-phosphate dehydrogenase
	PA14_72450	GID3639	*dsbA*	thiol:disulfide interchange protein DsbA
	PA14_38820	GID2257	*pqqB*	pyrroloquinoline quinone biosynthesis protein B
	PA14_62580	GID3061	*panB*	3-methyl-2-oxobutanoate hydroxymethyltransferase
	PA14_12400	GID3878	*thiE*	possible thiamin-phosphate pyrophosphorylase
	PA14_06510	GID1530	*bioF*	8-amino-7-oxononanoate synthase
	PA14_62590	GID2804	*panC*	pantoate--beta-alanine ligase
	PA14_05460	GID862	*bioA*	adenosylmethionine-8-amino-7-oxononanoate aminotransferase
	PA14_06570	GID3613	*bioD*	dethiobiotin synthase
	PA14_54290	GID3309	*pdxJ*	pyridoxal phosphate biosynthetic protein PdxJ
	PA14_04980	GID3057	*thiG*	thiamine biosynthesis protein, thiazole moiety
	PA14_06540	GID2882		putative biotin synthesis protein BioC
	PA14_62590	GID2804	*panC*	pantoate--beta-alanine ligase
	PA14_62570	GID4374	*folK*	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase
	PA14_12020	GID3602	*nadD*	NadD nicotinic acid mononucleotide adenylyltransferase
Carbon compound catabolism	PA14_38530	GID1173	*fahA*	fumarylacetoacetase
	PA14_54000	GID788	*prpD*	propionate catabolic protein PrpD
	PA14_53950	GID1675	*prpC*	citrate synthase 2
	PA14_38860	GID350	*exaA*	PQQ-linked alcohol dehydrogenase
Cell division	PA14_22020	GID3011	*minD*	cell division inhibitor MinD
	PA14_30290	GID181	*ftsK*	cell division/stress response protein
Cell wall / LPS / capsule	PA14_12080	GID1950	*sltB1*	soluble lytic transglycosylase B
	PA14_23470	GID381	*wbpM*	
	PA14_72000	GID2437	*rmd*	oxidoreductase Rmd
	PA14_23460	GID3451	*wbpL*	putative glycosyltransferase L
	PA14_23400	GID6270	ORF_8	
	PA14_48790	GID4118		putative lipoprotein
	PA14_57320	GID2282	*ddlB*	D-alanine--D-alanine ligase
	PA14_23460	GID3451	*wbpL*	putative glycosyltransferase L
	PA14_66670	GID161	*ponA*	penicillin-binding protein 1A
Central intermediary metabolism	PA14_37360	GID3220		putative short-chain dehydrogenase
	PA14_05590	GID2625	*metF*	5,10-methylenetetrahydrofolate reductase
	PA14_38350	GID2749	*galU*	UTP-glucose-1-phosphate uridylyltransferase
	PA14_23080	GID3428	*pgl*	6-phosphogluconolactonase
	PA14_62830	GID3276	*tpiA*	triosephosphate isomerase
	PA14_05620	GID906	*sahH*	S-adenosyl-L-homocysteine hydrolase
	PA14_41670	GID193	*ppsA*	phosphoenolpyruvate synthase
	PA14_17960	GID709	*glpK*	glycerol kinase

Chaperones & heat shock proteins	PA14_41240	GID3668	*clpP*	ATP-dependent Clp protease proteolytic subunit
Chemotaxis	PA14_45540	GID2597	*motB*	chemotaxis protein
	PA14_31400	GID670		putative methyl-accepting chemotaxis transducer
DNA replication, recombination, modification and repair	PA14_00810	GID1853		putative DNA repair photolyase
	PA14_25110	GID125	*topA*	DNA topoisomerase I
	PA14_25230	GID42	*mfd*	transcription-repair coupling factor
Energy metabolism	PA14_68500	GID1718		putative alcohol dehydrogenase, iron-containing
	PA14_06830	GID921	*norB*	nitric-oxide reductase subunit B
	PA14_37090	GID5904		putative aldehyde dehydrogenase
	PA14_44370	GID808	*ccoN*	cytochrome oxidase subunit (cbb3-type)
	PA14_57540	GID2924		putative cytochrome c1 precursor
	PA14_32520	GID2106		putative flavin-dependent oxidoreductase
	PA14_45280	GID1531	*cycH*	cytochrome c-type biogenesis protein
	PA14_18950	GID4038		putative NADH:ubiquinone oxidoreductase, subunit RnfA
Fatty acid and phospholipid metabolism	PA14_21340	GID576	*fadD2*	long-chain-fatty-acid--CoA ligase
	PA14_43420	GID1674		putative acyl-CoA dehydrogenase
	PA14_26670	GID372		putative biotin carboxylase/biotin carboxyl carrier protein
	PA14_25690	GID1367	*fabF1*	beta-ketoacyl-acyl carrier protein synthase II
	PA14_24440	GID3830		putative lipoprotein
	PA14_68360	GID427		putative beta-ketoacyl synthase
	PA14_29710	GID5122		conserved hypothetical protein
	PA14_57910	GID3978		conserved hypothetical protein
	PA14_24360	GID6249		putative serine protease
	PA14_07710	GID4828		putative apaG protein
	PA14_42950	GID1569		conserved hypothetical protein
	PA14_45120	GID4902		hypothetical protein
	PA14_52730	GID5057		hypothetical protein
	PA14_61380	GID6726		conserved hypothetical protein
	PA14_28950	GID4687		conserved hypothetical protein
	PA14_54540	GID2157		conserved hypothetical protein
	PA14_24480	GID86	*pelA*	conserved hypothetical protein
	PA14_62030	GID5194		putative paraquat-inducible protein A
	PA14_56280	GID5453		conserved hypothetical protein
	PA14_07370	GID5487		conserved hypothetical protein
	PA14_49930	GID2795		conserved hypothetical protein

Hypothetical, unclassified, unknown	PA14_51690	GID2876		conserved hypothetical protein
	PA14_39070	GID4825		conserved hypothetical protein
	PA14_66140	GID843		conserved hypothetical protein
	PA14_63860	GID4065		conserved hypothetical protein
	PA14_01510	GID5208		putative plasmid stabilization system protein
	PA14_63680	GID4258		conserved hypothetical protein
	PA14_39730	GID4915		conserved hypothetical protein
	PA14_28520	GID6153		hypothetical protein
	PA14_15600	GID6437		
	PA14_49320	GID3512		hypothetical
	PA14_57070	GID1942		conserved hypothetical protein
	PA14_43310	GID3806		conserved hypothetical protein
	PA14_00730	GID4531		conserved hypothetical protein
	PA14_08330	GID5366		hypothetical protein
	PA14_26980	GID4450		conserved hypothetical protein
	PA14_04020	GID3700		conserved hypothetical protein
	PA14_68840	GID5080		conserved hypothetical protein
	PA14_14210	GID2143		hypothetical protein
	PA14_53890	GID4117		hypothetical protein
	PA14_57850	GID4514		conserved hypothetical protein
	PA14_59000	GID4176	RL108	Conserved hypothetical protein
	PA14_07430	GID93		conserved hypothetical protein
	PA14_41730	GID2087		conserved hypothetical protein
	PA14_57910	GID3978		conserved hypothetical protein
	PA14_24260	GID2885		hypothetical
	PA14_00300	GID1956	*plcB*	phospholipase C, PlcB
	PA14_45710	GID1361		conserved hypothetical protein
Membrane proteins	PA14_36170	GID786		putative integral membrane protein
	PA14_02410	GID191		putative TonB-dependent receptor
	PA14_41590	GID3116		putative cytoplasmic membrane protein
	PA14_37900	GID124		putative TonB-dependent receptor
	PA14_08500	GID4692		putative integral membrane protein
	PA14_14500	GID1798		putative permease
	PA14_60730	GID878		putative outer membrane protein
	PA14_05300	GID2318		putative tonB domain protein
	PA14_18720	GID2249		putative outer membrane protein precursor, OmpA family
	PA14_63970	GID1538		putative membrane protein
	PA14_09500	GID919	*opmD*	outer membrane protein
	PA14_41570	GID1901	*oprF*	major porin and structural outer membrane porin OprF precursor
	PA14_44460	GID3465		putative membrane protein
	PA14_50140	GID517	*fliF*	Flagella M-ring outer membrane protein precursor

Motility & Attachment	PA14_60310	GID409	*pilY1*	type 4 fimbrial biogenesis protein PilY1
	PA14_45810	GID4290		putative flagellar protein FliL
	PA14_50110	GID2984		probable flagellar assembly protein
	PA14_60300	GID4945	*pilX*	type IV pilus biogenesis protein
	PA14_14850	GID3242	*pilF*	type 4 fimbrial biogenesis protein PilF
	PA14_45830	GID1366		putative flagellar hook-length control protein FliK
	PA14_45720	GID1761	*flhB*	flagellar biosynthetic protein FlhB
	PA14_50470	GID4632	*flgC*	flagellar basal-body rod protein FlgC
	PA14_45660	GID1380	*flhF*	flagellar biosynthesis protein FlhF
	PA14_45760	GID5283	*fliQ*	flagellar biosynthetic protein FliQ
	PA14_45680	GID323	*flhA*	flagellar biosynthesis protein FlhA
	PA14_45770	GID3316	*fliP*	flagellar biosynthetic protein FliP
	PA14_50130	GID2191	*fliG*	flagellar motor switch protein FliG
	PA14_50270	GID1093	*fliD*	flagellar capping protein FliD
	PA14_50290	GID1029	*fliC*	flagellin type B
	PA14_50100	GID1146	*fliI*	flagellum-specific ATP synthase FliI
	PA14_45800	GID2241	*fliM*	flagellar motor switch protein FliM
	PA14_45680	GID323	*flhA*	flagellar biosynthesis protein FlhA
	PA14_60290	GID4752	*pilW*	type 4 fimbrial biogenesis protein PilW
	PA14_50480	GID4759	*flgB*	flagellar basal-body rod protein FlgB
	PA14_50380	GID1523	*flgJ*	flagellar protein FlgJ
	PA14_60280	GID5422	*fimU*	type 4 fimbrial biogenesis protein FimU
	PA14_45830	GID1366		putative flagellar hook-length control protein FliK
	PA14_50340	GID1270	*flgL*	flagellar hook-associated protein type 3 FlgL
	PA14_50420	GID3427	*flgH*	flagellar L-ring protein precursor FlgH
	PA14_50410	GID1846	*flgI*	flagellar P-ring protein precursor FlgI
	PA14_50450	GID987	*flgE*	flagellar hook protein FlgE
	PA14_50440	GID3270	*flgF*	flagellar basal-body rod protein FlgF
	PA14_11080	GID152	*cupB3*	usher CupB3
	PA14_50430	GID3086	*flgG*	flagellar basal-body rod protein FlgG
	PA14_50080	GID4588	*fliJ*	flagellar protein FliJ
	PA14_50360	GID352	*flgK*	flagellar hook-associated protein 1 FlgK
	PA14_50380	GID1523	*flgJ*	flagellar protein FlgJ
	PA14_05180	GID2040	*pilT*	twitching motility protein PilT
	PA14_71620	GID4383	*purE*	phosphoribosylaminoimidazole carboxylase, catalytic subunit
	PA14_70370	GID3731	*pyrE*	orotate phosphoribosyltransferase
	PA14_62910	GID52	*carB*	carbamoylphosphate synthetase large subunit
	PA14_15740	GID24	*purL*	phosphoribosylformylglycinamidine synthase
	PA14_52050	GID3563	*purN*	phosphoribosylaminoimidazole synthetase
	PA14_51240	GID3358	*purC*	phosphoribosylaminoimidazole-succinocarboxamide synthase

Nucleotide biosynthesis and metabolism	PA14_26890	GID3483	*pyrF*	orotidine-5'-phosphate decarboxylase
	PA14_07700	GID2604	*apaH*	bis(5'-nucleosyl)-tetraphosphatase
	PA14_18710	GID1907	*pyrC*	dihydroorotase, homodimeric type
	PA14_62930	GID1643	*carA*	carbamoyl-phosphate synthase small chain
	PA14_05250	GID1358		noncatalytic dihydroorotase-like protein noncataly
	PA14_64930	GID3447		putative ADP-ribose pyrophosphatase
	PA14_64200	GID674	*purH*	phosphoribosylaminoimidazolecarboxamide transferase
	PA14_52040	GID1887	*purM*	phosphoribosylaminoimidazole synthetase
	PA14_05260	GID2156	*pyrB*	aspartate carbamoyltransferase
	PA14_24640	GID2054	*pyrD*	dihydroorotate dehydrogenase
	PA14_05250	GID1358		noncatalytic dihydroorotase-like protein noncataly
Protein Secretion and Export apparatus	PA14_55920	GID1384		putative type II secretion system protein
	PA14_10330	GID1043		putative outer membrane protein precursor
	PA14_23970	GID417	*xcpQ*	general secretion pathway protein D
	PA14_66980	GID2973	*tatC*	sec-independent protein translocase TatC
Putative enzymes	PA14_50710	GID925		probable NADH dehydrogenase
	PA14_50820	GID1505		probable esterase
	PA14_68040	GID3305		putative short-chain alcohol dehydrogenase
	PA14_52610	GID2188		possible threonine aldolase
	PA14_07480	GID7177		putative reverse transcriptase
	PA14_11970	GID3223		methylpurine-DNA glycosylase family protein
	PA14_49360	GID133		probable glucosyl transferase
	PA14_30090	GID4634		putative acyltransferase
	PA14_34540	GID866		putative xenobiotic compound monooxygenase, DszA family
	PA14_27880	GID3932		putative phosphohydrolase
	PA14_58560	GID142		Sulfite reductase
	PA14_57570	GID3783		putative cytochrome c reductase, iron-sulfur subun
	PA14_53300	GID3849		probable alkyl hydroperoxide reductase
Quinolone signal response	PA14_51420	GID2747	*pqsB*	Homologous to beta-keto-acyl-acyl-carrier protein synthase

Related to phage, transposon, or plasmid	PA14_08210	GID4271		putative major tail protein V
Secreted Factors (toxins, enzymes, alginate)	PA14_54410	GID2295	*mucB*	negative regulator for alginate biosynthesis MucB
	PA14_19110	GID1159	*rhlB*	rhamnosyltransferase chain B
	PA14_54390	GID1011	*mucD*	serine protease MucD precursor
Transcription, RNA processing and degradation	PA14_69560	GID4114	*hcpA*	secreted protein Hcp
	PA14_64190	GID5047	*fis*	DNA-binding protein Fis
	PA14_05560	GID401		putative ATP-dependent RNA helicase, DEAD box family
	PA14_07620	GID1403	*cca*	tRNA nucleotidyl transferase
	PA14_69190	GID1379	*rho*	transcription termination factor Rho
Transcriptional regulators	PA14_40600	GID2105		putative transcriptional regulator
	PA14_13510	GID2313		putative transcriptional regulator, LysR family
	PA14_63170	GID4726		putative transcriptional regulator
	PA14_06260	GID2424		putative transcriptional regulator, LysR family
	PA14_46060	GID2574	*gbuR*	transcriptional regulator
	PA14_00460	GID2617	*trpI*	trpBA operon transcriptional activator
	PA14_48830	GID1038		putative transcriptional regulator
	PA14_19120	GID3229	*rhlR*	acylhomoserine lactone dependent transcriptional regulator
	PA14_50220	GID846	*fleQ*	transcriptional regulator FleQ
	PA14_62490	GID4517	*dksA*	suppressor protein DksA
	PA14_52570	GID5444	*rsmA*	RsmA, regulator of secondary metabolites
	PA14_20730	GID5107	*flgM*	putative negative regulator of flagellin synthesis, FlgM
	PA14_45630	GID3299	*fliA*	motility sigma factor FliA
	PA14_30650	GID3719	*gacA*	response regulator GacA
Translation, post-translational modification, degradation	PA14_25600	GID2146		putative peptidase
	PA14_58050	GID1160	*pmbA*	PmbA protein
	PA14_41220	GID199	*lon*	Lon protease
	PA14_64180	GID2131		putative tRNA-dihydrouridine synthase
	PA14_65320	GID2284	*miaA*	delta 2-isopentenylpyrophosphate transferase
	PA14_09100	GID3802	*rpsD*	30S ribosomal protein S4
	PA14_57950	GID5141		putative ribosomal subunit interface protein
	PA14_64180	GID2131		putative tRNA-dihydrouridine synthase
	PA14_14380	GID2793		putative transmembrane component of ABC transporte
	PA14_15920	GID1260		putative major facilitator family transporter
	PA14_57100	GID530		putative permease

Transport of small molecules	PA14_03670	GID2774	*cysW*	sulfate transport protein CysW
	PA14_40620	GID1683		putative MFS transporter
	PA14_56640	GID1705		putative MFS transporter
	PA14_71030	GID2267		putative glycine betaine/L-proline ABC transporter, periplasmic component
	PA14_63710	GID2199		putative glycosyl transferase
	PA14_10440	GID1261		putative porin
	PA14_73120	GID2363		putative periplasmic transport protein
	PA14_58470	GID2224	*dppD*	putative dipeptide ABC transporter
	PA14_15180	GID1425		putative ABC transport system, membrane protein
	PA14_64590	GID1133		putative MFS transporter
	PA14_00780	GID4103		putative carbonic anhydrases
	PA14_44440	GID175		putative cation-transporting P-type ATPase
	PA14_68800	GID3598		putative phosphate transport regulator
	PA14_61080	GID2164		putative C4-dicarboxylate-binding protein
	PA14_68370	GID2819	*cysQ*	3'(2'),5'-bisphosphate nucleotidase
	PA14_65850	GID969		putative amino acid ABC transporter, permease protein
	PA14_67050	GID2972		putative amino acid ABC transporter, periplasmic amino acid-binding protein
	PA14_56470	GID1216		putative MFS transporter
Two-component regulatory systems	PA14_13660	GID3618		putative protein phosphatase
	PA14_20800	GID4990		putative histidine-containing phosphotransfer (HPt) domain
	PA14_50180	GID993	*fleR*	two-component response regulator
	PA14_21700	GID41		putative sensory box histidine kinase/response regulator
	PA14_38900	GID3671		putative two-component response regulator
	PA14_50200	GID1559	*fleS*	two-component sensor
	PA14_17670	GID3703		putative DNA-binding response regulator, LuxR family
	PA14_27950	GID4438		putative anti-anti-sigma factor
	PA14_52260	GID113		sensor/response regulator hybrid
	PA14_45590	GID261		putative two-component sensor
	PA14_61020	GID4129		ankyrin-like protein

Note: Text highlighted in green were showing less swarming phenotype.