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In Vitro Simulation of Growth and Survival of *Pseudomonas aeruginosa*, *P. fluorescence* and *P. putida* under Cold Shock

Syeda Muntaka Maniha, Tasmin Tabassum, Tahsin Tabassum, Rashed Noor*
School of Life Sciences (SLS), Independent University, Bangladesh
E-mail: rashednoor@iub.edu.bd

Abstract: The experimental demonstration of bacterial growth is of large interest within the scientific community to chalk out the specific mechanisms underlying the survival strategies under adverse conditions in nature. An array of research has been conducted on heat shock as well as the related oxidative stresses using a range of bacterial species including *Escherichia coli*, *Bacillus* spp., *Salmonella* spp. and *Pseudomonas* spp. Present study further focused on the cold shock response within the species of the ubiquitous bacterium *Pseudomonas*. *P. aeruginosa*, *P. fluorescence*, and *P. putida* were subjected to cold shock by placing the corresponding cultures at low temperatures (0°C and 8°C), and then their growth was assessed along with the necessary phenotypic observations. Interestingly, the pigmentation (green or bluish green pigments) was noticed almost in species at 37 °C compared to those growing at lower temperatures. While at 37°C, *P. fluorescence* showed the highest growth compared to the other two strains, at 8°C and even at 0°C, *P. putida* showed the maximal growth followed by the growth of *P. aeruginosa* and then *P. fluorescence*.
***Keywords*:** cold shock response, *Pseudomonas aeruginosa*, *P. fluorescence*, *P. putida*

1. Introduction

Lots of researches have so far shown that the bacterial growth and survival largely depends on (1) the limiting nutrients, (2) changes in temperatures, pH, aeration condition, etc., (3) toxic chemicals like ethanol and hydrogen peroxide (H_2O_2), accumulation of reactive oxygen species (ROS) especially in the early stationary phase of bacterial growth, (4) perturbations in other biotic and abiotic factors like the UV-irradiation, etc.[1-8]. Of them, temperature has been an important area of interest for many researchers to study the bacterial behavior under its variation; more specifically the stress response of bacterial cells especially under the high temperatures with the corresponding assessment of the culturable cells, the viable but non-culturable (VBNC) cells, expression of the stress responding relevant genes and proteins, and even by measuring the ROS levels at such stressed conditions [4,6,7,9-16]. The principal objective of these works probably underlies the investigation of the bacterial stress response since an extensive range of bacteria have been found to activate different transcriptional regulatory network (TRN) along with the up-regulation of an array of stress responsive genes under certain stress conditions [2,8,17]. As has been seen in our previous research, a large fraction of *Escherichia coli* cells lost culturability under heat stress and oxidative stress [4,6,7,15]. Our earlier studies also demonstrated the deterioration in the colony forming unit (CFU) of *Pseudomonas* spp. under oxidative stress. Compared to the study of heat shock, the effect of cold shock has not been investigated to a significant extent although the cold-shock responses involving the cold-shock proteins A (CspA), CspB, CspC, CspE, CapB as well as the induction of genes encoding the universal stress factors RpoS and (p)ppGppare well known [18-22].

Based on the background research on the bacterial cold shock response, present study further attempted to focus on the impact of such stress specifically on *Pseudomonas aeruginosa*, *P. fluorescence*, and *P. putida*. Examination for each of the strain's ability to survive at 0°C and 8°C was conducted and the corresponding culturability was assessed through their colony-forming ability.

DOI: https://doi.org/10.37256/amtt.112020285

2. Materials and methods

Laboratory stock cultures of *Pseudomonas aeruginosa*, *P. fluorescence*, and *P. putida*were were used. Growth was examined according to two aspects: (1) capacity of forming colony forming units (CFUs) on solid Luria-Bertani (LB) media under 37°C, 8°C, and 0°C; (2) observation of morphology and the phenotypes. Pre-cultures were prepared in 3mL LB broth media incubated at 37°C for 4 hours under shaking conditions; the optical density (at 600nm) was adjusted at 0.1; and finally 30µL of the pre-cultures were introduced into 30mL LB media following incubation at 37°C, 8°C, and 0°C [3, 15]. Culture growth was evaluated at different time intervals; 12, 24, 26, 48, 60, and 72h concerning the viability as colony-forming units (CFUs) on the agar plates using spot assay [3]. In addition, the morphological and phenotype characters of growing cultures were investigated.

3. Results and discussion

3.1 Pigmentation during growth of *Pseudomonas* spp

While *P. aeruginosa* and *P. fluorescence* are well known to produce phenazines pigment, the *P. putida* strains have been reported to produce anthocyanin pigment [23-25]. The pigmentation capacity of the strains was noticed in the following order: *P. aeruginosa* > *P. fluorescence* > *P. putida*. *P. putida* was not noticed to produce any greenish pigment; even at prolonged incubation; nevertheless, a brownish was seen after 72 hours (Table 1, Figure 1). As expected, cultures of the bacterial strains under investigation are colorless at 0°C.

Table 1. Pigmentation pattern of *Pseudomonas* species at different temperatures

Pseudomonas species	0°C	8°C	37°C
P. aeruginosa			
24 hours incubation	No pigmentation	No pigmentation	Dark greenish (pyocyanin) Pigment
72 hours incubation		Appearance of bluish green pigment	Dark greenish (pyocyanin) Pigment with more fluorescence
P. fluorescence			
24 hours incubation	No pigmentation	No pigmentation	Blue-greenish- and brownish pigment
72 hours incubation		Appearance of pale greenish pigment	Dark greenish (pyocyanin) Pigment with more fluorescence
P. putida			
24 hours incubation	No pigmentation	No pigmentation	Dark brown pigment
72 hours incubation		Appearance of pale brownish pigment with a light bluish ring	Dark brown pigment with a little fluorescence

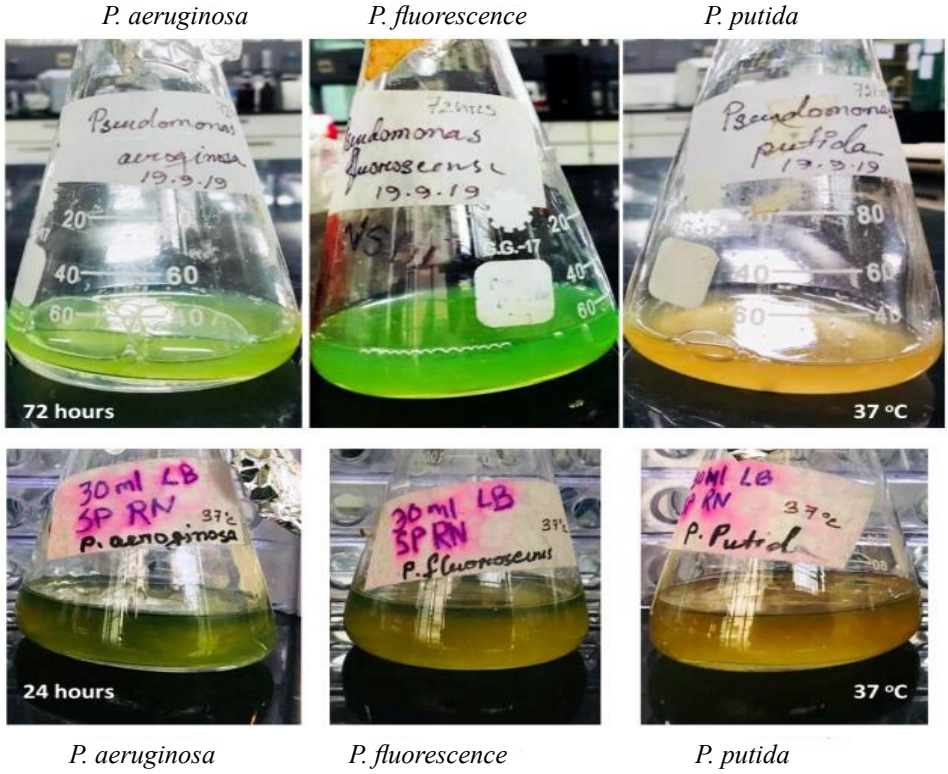

Figure 1. Pigmentation pattern of *Pseudomonas* species at different temperatures. The representative image of pigment production at 37 °C has been shown. For clarity of the observation, a tabular note has been inserted within the image. The pigmentation capacity of the *Pseudomonas* spp. strains was noticed to be in the order of *P. aeruginosa* > *P. fluorescence* > *P. putida*

The bluish green coloration of the *P. aeruginosa* and *P. fluorescence* is due to phenazines; it is a class that approximately contains fifty derivatives of heterocyclic secondary metabolites, which are usually produced during the stationary phase. *P*henazines have long been reported as the reductants for molecular oxygen and ferric ions; participating in cell-to-cell communication, as well as they are known as antimicrobial agents [23, 26]. Pyocyanin, the blue green phenazine pigment, is considered as a key virulence factor of *Pseudomonas* spp. Pyocyanin has been reportef to inactivate the host catalases and survive upon exposure to the reactive oxygen species (ROS) generated by the host tissues [23]. In addition, this pigment has also been reported to induce the destruction of neutrophils [27]. The results of the present study revealed the relatedness between the pigmentation rate of *Pseudomonas* spp. and the incubation temperature as well as the impact of cold shock on the pigmentation, which, so far our knowledge, has been noticed for the first time.

3.2 Growth of *Pseudomonas* spp. at different temperatures

The growth of ***Pseudomonas*** cells was assessed by the number of colony-forming units (CFUs). At 37°C, the number of viable and culturable cells was increased as follows; *P. fluorescence* > *P. aeruginosa* > *P. putid*a whereas the order is completely reversed at 8°C and 0°C. Regarding results of spot assay, three *Pseudomonas* species under investigation at 48h fail completely to grow on the agar plates at 0°C. At 12h, bacteria grew well at 0°C as observed in figures 2 and 3.

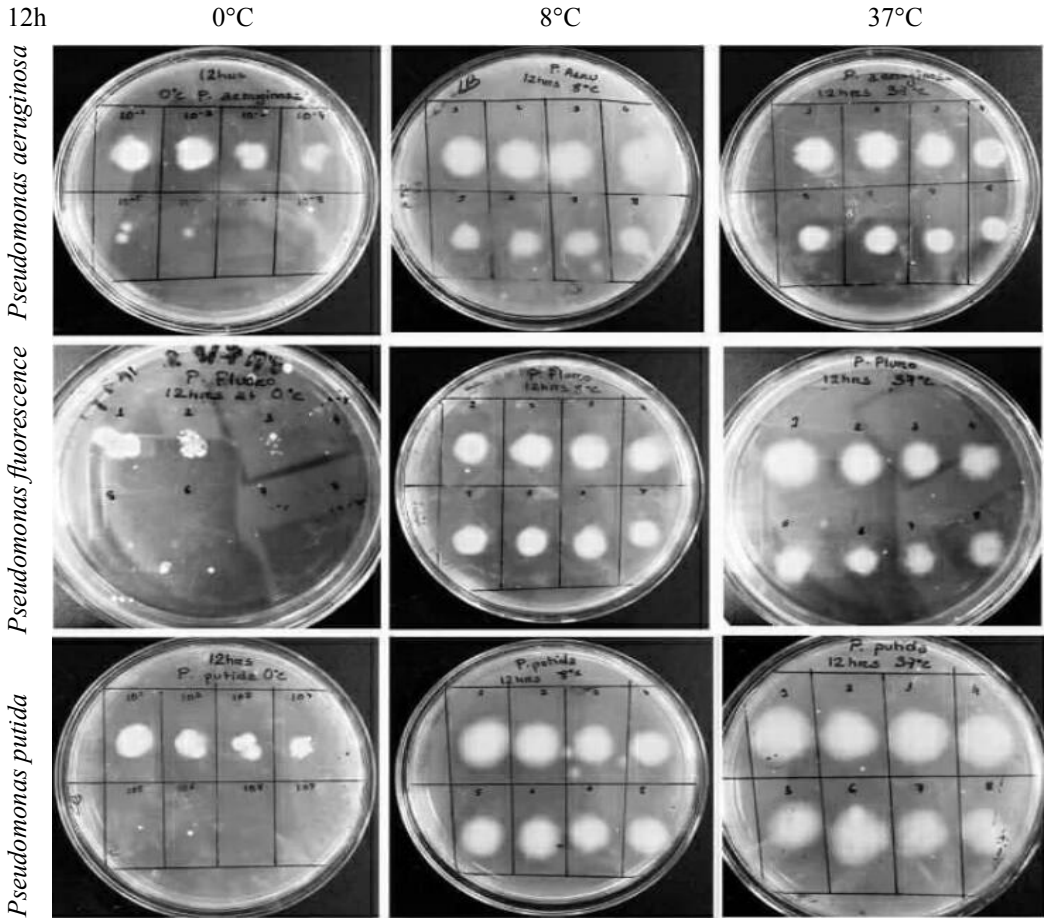

Figure 2. Stock cultures of *Pseudomonas aeruginosa*, *P. fluorescence*, and *P. putida* were subjected to be cultivated as discussed in the Materials and Methods. At 37°C, the growth frequency was noticed in the order of *P. fluorescence* > *P. aeruginosa* > *P. putida*. Interestingly such order was completely opposite while growing at 8°C and 0°C

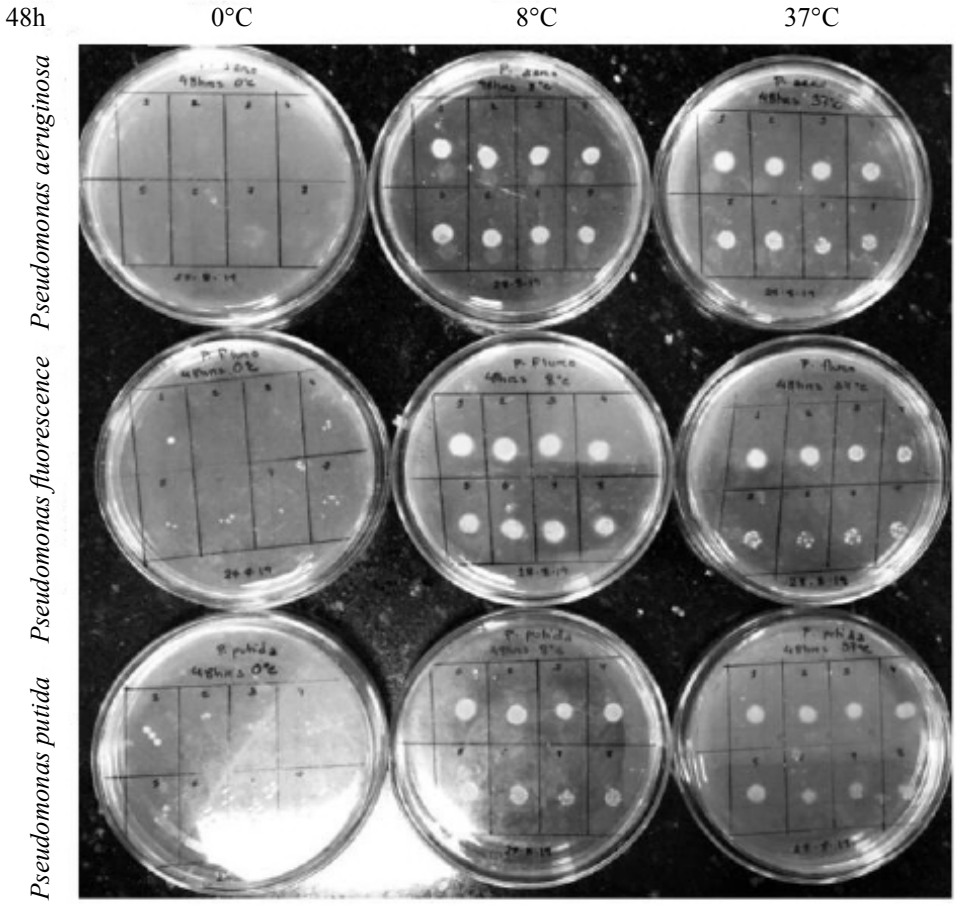

Figure 3. Spot tests were performed as stated in Figure 2. The only difference is that in this case the aliquots from the stationary phase (48h) were used. All species failed to grow on the agar plates at 0°C after 48h

With a similar mode of our findings, an array of *Pseudomonas* species have been found to grow glowing between 5°C and 10°C, and even at 0°C as reported earlier [28]. Due to the ubiquitous nature of *Pseudomonas* spp., knowledge on the cold shock resistance traits from the current research would be fruitful because of their particular clinical impacts (multidrug-resistant, MDR- and the carbapenem-resistant *P. putida and P. aeruginosa)* and their capacity to contaminate different food items like milk, meat, fish, vegetables, etc. [29, 30]. Additionally, the pigmentation of *Pseudomonas* species as evaluated in the current study may be further investigated for their possible role in disease progression.

Finally, it would be more interesting to ponder the molecular strategies for the growth and survival of *Pseudomonas* species against cold shock; particularly by studying the *cap*B, *csp*A genes (encoding the major cold shock proteins) [19, 30]. However, our study clearly demonstrated the cold shock tolerance traits of different *Pseudomonas* species which may further come in help to study not only the protective management system against environmental stresses but also would benefit the understanding of the propagation of food spoilage microorganisms and the clinically oriented pathogens.

Acknowledgement

Authors are thankful to the Microbiology Laboratory of the School of Life Sciences (SLS), Independent University, Bangladesh (IUB) for the logistic support. However, no research grant was received to conduct this research.

Funding

There was no fund for conducting this research.

Competing Interest

Authors declared that they have no conflict of interest.

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