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Original Article

In Vitro demonstration of *Pseudomonas* Growth and Phenotypic Examinations of the Cells Under Cold Shock

Anika Bushra Moumita[1], Nafisa Tabassum[1], Ifra Tun Nur[1*]

[1] *Department of Microbiology, Stamford University Bangladesh, 51 Siddeswari Road, Dhaka 1217, Bangladesh.*

The present study attempted to detect the effect of cold shock on *Pseudomonas aeruginosa*, *P. fluorescence*, and *P. putida*; to deduce the culturable cells, the possible dead cells and viable but non-culturable (VBNC) cells; i. e., living but not capable of forming the colony forming units (CFUs) at 0 ºC and at 4 ºC. Estimation of VBNC at low temperature was performed by deducting the number of culturable cells (formed as CFUs) at 0 °C and at 4 ºC from the culturable cells at 37 °C. Maximum culturable cells (around 10^6 CFU/ ml) appeared at 24-48 hours, and after that, a gradual decline in the numbers of the culturable cells was observed. A minor but significant growth reduction was noticed in Minimal Broth compared to that in Luria Broth. Significant reduction in the culturable cells of all three strains was noticed under cold shock. However, a fraction of the population of each strain was observed to survive under cold stress. Interestingly while the number of culturable cells decreased in course of time under cold shock, the number of VBNC apparently seemed to be constant or to be increased. A comparatively lower number of VBNC was noticed under cold shock in case of *P. putida* which in turn affected culturability at ambient temperature.

Key words: *Pseudomonas aeruginosa*; *P. fluorescence*; *P. putida*; cold shock; culturable cells, viable but non-culturable (VBNC) cells.

Introduction

Years after years biologists have drawn their interests on bacterial growth to especially detecting the growth kinetics, bacterial generation time, survival and culturability (along with the determination of viable but non-culturable (VBNC) cells under various conditions like in nutrient limiting situation or certain exogenous or endogenous stresses[1-,3]. Many bacterial species have been noticed to undergo the VBNC state, which is indeed a sort of survival strategy when exposed to heat shock or the oxidative stress[4-7].A wide range of bacteria are known to activate different transcriptional regulatory network (TRN) at the stationary phase, improving the potential of stress defensive response [8- 10] against various stress transmitting stimuli; i.e., heat shock, pressure, irradiation, pH, osmolite/salt concentration, elevated level of reactive oxygen species (ROS), imbalance in redox-state and toxic chemicals[5]. Under specified stressed conditions, a portion of the cells of *Escherichia coli*, the model bacterium for the study the of stress response, have often been found to lose their ability to grow on standard nutrient agar plates at the early or mid stationary phase[5, 6, 11,]. Such cells may be dead, or in the VBNC state [12- 14]. Such an observation further drew the interests of the authors to examine the bacterial growth curve under cold shock state in order to detect the surviving cells. As the cells of *E. coli* are well known to respond against cold-shock [15], the present study was applied on to *Pseudomonas* strains.

Our earlier studies unraveled that *Pseudomonas* spp. had been incapable of colony forming ability on the agar plates when exposed to oxidative stress which is often related to the raise of temperature [11, 16]. The impacts of heat shock on bacterial species have been extensively studied revealing unfolded/ denatured proteins; the studies on the effect of cold shock apparently seem to be in scarce [2, 6, 7, 11, 15, 17]. However, among the environmental stresses posed on bacterial population, the theme of cold shock is not that new too [15, 18]. A number of physiological changes especially in *E. coli* and *Bacillus subtilis* have been noted in response to a temperature downshift. The decrease in membrane fluidity, reduced efficiency of translation and transcription along with inefficient folding of some proteins and the defects in ribosomal function were demonstrated [15].

One of the well-studied cold-shock response mechanisms underlies the discovery of the cold-shock proteins A (CspA) family members which are induced and function at low temperature[19]. Other proteins may include CspB, CspC and CspE which are over expressed under cold-shock and impart the traits of motility and biofilm formation under stress [19-22]. The roles of RpoS and (p)ppGpp are also significant for the cold shock stress management within bacteria [15].

Based on such background knowledge on cold shock response of *E. coli* and *B. subtilis*, present study further concentrated to observe the cold-shock response of *P. aeruginosa*, *P. fluorescence*, and *P. putida*. A preliminary growth pattern for each of the strain

Corresponding author:
Ifra Tun Nur, Lecturer, Department of Microbiology, Stamford University Bangladesh, 51 Siddeswari Road, Dhaka-1217. Tel: +880 2 8355626, ext. 472, Cell: +8801677268650, E-mail: tun.ifra@yahoo.com

was checked first under optimal condition, and then the respective growth rates were compared when the strains were subjected to cold stress. The culturable cells, and also the possible VBNC cells of *Pseudomonas* spp. were estimated.

Method and materials:

Bacterial stain, medium and culture conditions for generating *in vitro* growth curve

Preparation of pre-cultures: Laboratory stock cultures of *Pseudomonas aeruginosa*, *P. fluorescence*, and *P. putida* were used in this study. Minimal agar (MA) and Luria agar (LA) were used for the assay of culturability; i.e., the formation of viable and culturable cells[3]. Loopful bacterial culture from each of the three different bacterial strains was introduced into respective tubes consisting of 5 ml Luria broth (LB) and incubated at 37 °C with shaking at 100 rpm (rotation per minute) for 4-6 hours (preparation of pre-cultures of *P. aeruginosa*, *P. fluorescence*, and *P. putida*).

Preparation of main cultures: In each case, 30 µl of the pre-culture was introduced into 30 ml fresh LB and MB resulting in the initial OD_{600} (optical density or absorbance at 600 nm) of the main culture 0.1, In order to maintain the initial OD_{600} of the main culture around 0.1, from 10^{-7} dilutions of the pre-culture, 30 µl samples were transferred into 30 ml LB followed by incubation at 37 °C under 100 rpm; and from 10^{-3} dilutions 30µl samples were transferred into 30 ml MB followed by incubation at 37 °C under 100 rpm. The main cultures were let to be incubated at 37 °C with shaking at 100 rpm up to 3-5 days. *Assay of viable and culturable cells*: At specific time intervals (12 , 24, and 36 hours, and so on) an aliquot of 1 ml (from each of the cultures) of cell suspension was introduced into 9 ml of LB, and the dilutions were made up to 10^{-6} (for spreading onto LA and up to 10^{-3} (for spreading onto MA). For assaying the viable and culturable cells, 100 ml from each of the above- mentioned dilutions was spread; i.e., 10^{-6} dilutions for the three strains were considered for spreading onto LA plates; and 10^{-3} dilutions for the three strains were considered for spreading onto MA plates. Finally the plates were incubated at 37 °C for 12-24 hours for counting the respective colony forming units (CFUs).

Experiments on cold-shock

As described above, for the preparation of pre-cultures of *P. aeruginosa*, *P. fluorescence*, and *P. putida*, loopful pure colony of each of the bacterial culture from their respective plates was introduced into three respective tubes consisting of 5 ml LB; and was incubated at 37 °C with shaking at 100 rpm for 2-3 hours till the OD_{600} reached to indicating the exponential phase of the cells[7]. Then 150 µl from each of the cell suspension was added to the respective 150 ml LB and to the 150 ml MB (three sets of main culture) which were further incubated at 37°C with shaking at 100 rpm for 10 hours (generating the average OD_{600} of 0.6 - 0.8 (indicating the exponential phase of the cells).

Generation of cold shock and assay of viable and culturable cells: While one set of main cultures (total 6 cultures; i.e., 10 hour cultures of *P. aeruginosa*, *P. fluorescence*, and *P. putida* in LB and 10 hour cultures of *P. aeruginosa*, *P. fluorescence*, and *P. putida* in MB) was continued incubating at 37°C with shaking at 100 rpm for 3-5 days; another two sets were subjected to cold shock at 0ºC and at 4ºC. Indeed, to generate such cold shock on the *Pseudomonas* cultures, aliquots of 9 ml each from the respective 10 hour cultures (6 cultures; i.e., 10 hour cultures of *P. aeruginosa*, *P. fluorescence*, and *P. putida* in LB and 10 hour cultures of *P. aeruginosa*, *P. fluorescence*, and *P. putida* in MB) were poured onto the respective sterile cubes of ice trays. Considering two sets of low temperatures (0ºC and 4 ºC), for each of the six cultures, a maximum of 8 cubes were prepared, generating a total of 96 cubes; i.e., 8 cubes (considering the viable ad culturable cell assay time intervals: 12, 24, 36, 48, 60, 72, 96 and 120 hours) for each of the 3 strains in LB and in MB for keeping at 0 ºC and 8 cubes for each of the 3 strains in LB and in MB for keeping at 4ºC. For measuring the viable and culturable cells, bacterial cultures from the respective ice cubes of the marked three strains were dropped on to the LA plates (with an average amount of around 100 ml) followed by spreading. Plates were incubated at 37°C for 12-24 hours for enumerating .

Estimation the viable but non-culturable (VBNC) cells at cold shock: Estimation of VBNC at 0°C was performed by deducting the number of culturable cells (formed as CFUs) at 0°C from the culturable cells (formed as CFUs) at 37°C. Similarly the estimation of VBNC at 4°C was performed by deducting the number of culturable cells at 4°C from those at 37°C.

Result and Discussion

All 3 *Pseudomonas* strains showed standard growth in both Luria Broth (LB) and minimal broth (MB). Among the 3 species, *P. aeruginosa* showed an apparent ideal growth curve in both minimal and Luria broths. Maximum culturable cells; i.e., around 10^6 CFU/ ml in each case appeared at 24-48 hours, and after that a steady decline in the numbers of the culturable cells Was observed (Figure 1A-C). A reduction by an average of 1-log was noticed in case of the culturable cells from the MB compared to those from LB for all *Pseudomonas* species (1A-C).

Aliquots of 30 µl of each of the pre-cultures of the 3 bacterial strains were introduced into 30 ml fresh Luria Broth (LB, black bars) and 30 ml Minimal Broth (MB, grey bars), followed by incubation at 37°C under shaking condition (at 100 rpm). At specific time intervals, 100 ml from each of the respective dilutions of the main bacterial cultures were spread onto LA plates and onto the MA plates. After incubating the plates at 37°C for 12-24 hours, colony forming units (CFUs) were enumerated to measure the viable and culturable cells. Maximum culturable cells (around 10^6 CFU/ ml) appeared at 24-48 hours, and after that a gradual decline in the numbers of the culturable cells were observed (1A-C). A reduction by an average of 1-log was noticed in case of the culturable cells from the MM compared to those from LB in all cases (A-C).

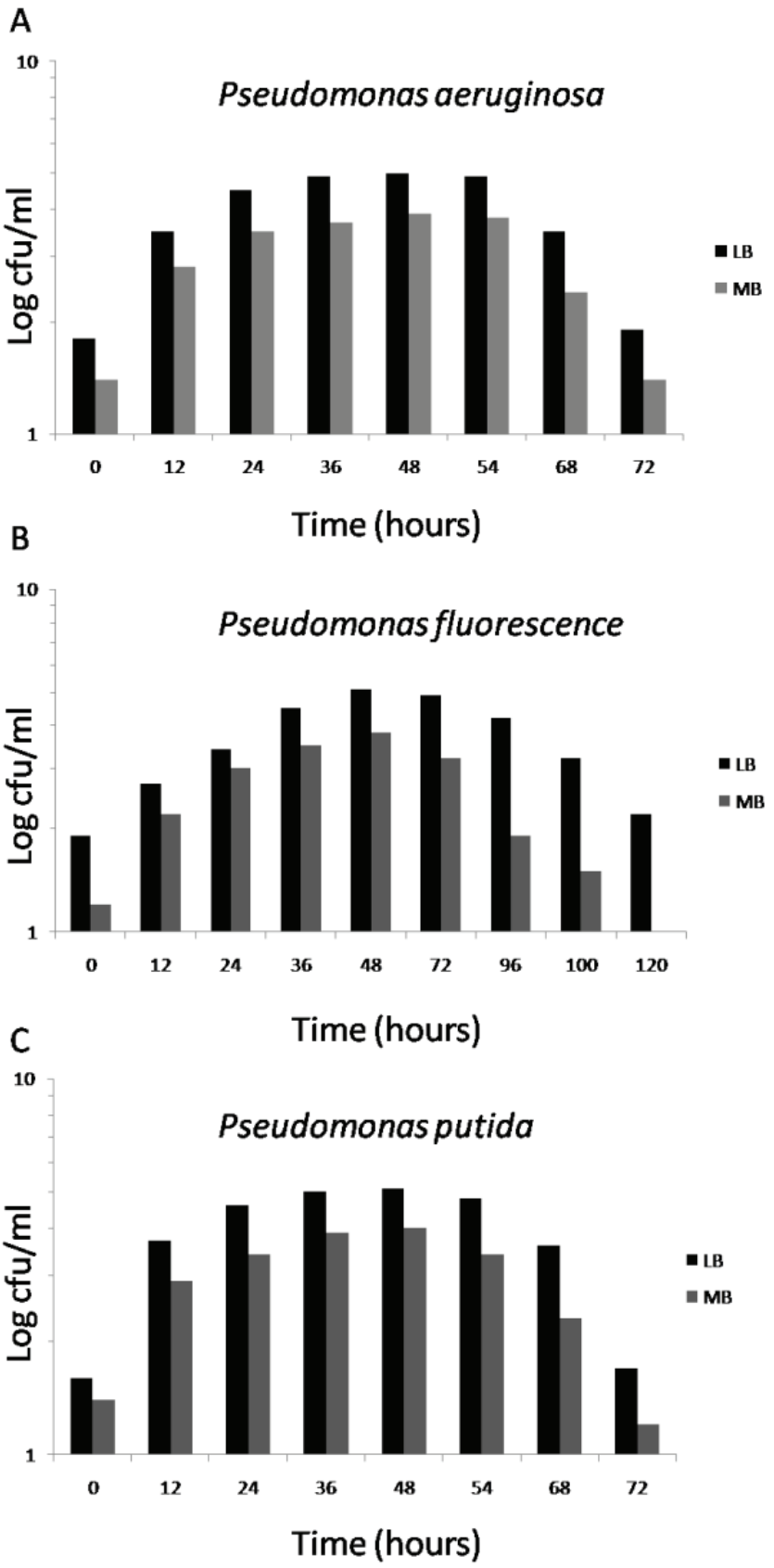

Figure 1. Assay of viable and culturable cells of A. *Pseudomonas aeruginosa*, B. *P. fluorescence*, and C. *P. putida* at optimal temperature.

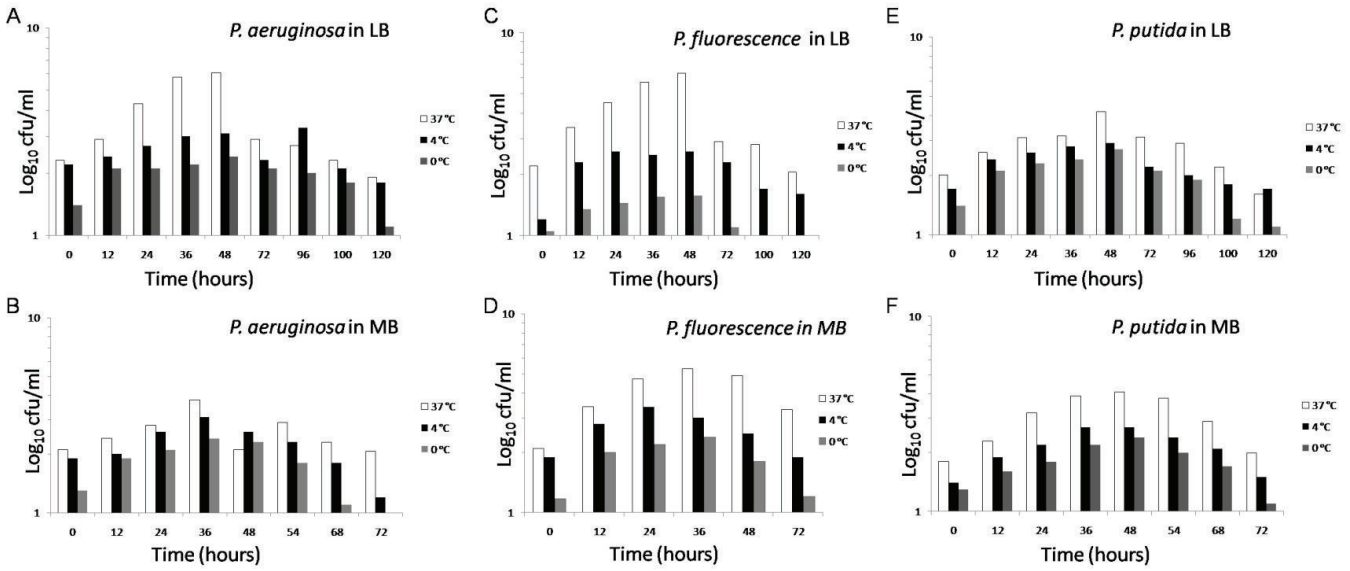

Figure 2. *Assay of viable and culturable cells of A, B. Pseudomonas aeruginosa, C, D. P. fluorescence, and D, E. P. putida under cold shock in LB (A, C, E) and in MB (B, D, F).*

Two sets of low temperatures (grey bars indicating 0ºC and black bars indicating 4ºC) were considered for cold shock in comparison to normal condition (white bars indicating 37ºC). For the assay of the viable and culturable cells under cold shock, ice cubes of all the bacterial strains were generated, which were later used for plating for the enumeration of the colony forming units (CFUs). The reduction of culturable cells of all 3 strains was noticed under cold shock at each specific time points of assay. Estimation of viable but non-culturable cells (VBNC) at these two low temperatures (0°C and 4°C) was performed by deducting the number of CFUs at 0 °C (grey bars) and 4°C (black bars) from the CFUs formed at 37°C (white bars). While the number of culturable cells decreased in course of time under cold shock, the number of VBNC apparently seemed to be constant.

Reduction of viable and culturable cells at cold shock

The reduction of culturable cells of all 3 strains was noticed under cold shock at each specific time points of the assay. In case of *P. aeruginosa*, at 0ºC the highest reduction (5-log reduction) in the amount of the culturable cells was observed at 36 hours of growth in LB media while in MB the reduction was scored around 2-log (Figure 2A, B). At 4ºC cold shock condition, 4-log reduction in the amount of the culturable cells was observed at 36 hours of growth in LB media while in MB the reduction was scored nearly 1-log. In case of *P. fluorescence*, at 0ºC the highest reduction (6-log reduction) of the culturable cells was observed at 36 hours in LB media while in MB the reduction was half. At 4ºC, 2-log reduction in the amount of the culturable cells was observed at 36 hours in both LB media and in MB (Figure 2C, D). In case of *P. putida*, at 0ºC the highest reduction (3-log) in the amount of the culturable cells was observed at 36 hours of growth in MB while in LB the reduction was 1-log. At 4ºC cold shock condition, 1-log reduction in the amount of the culturable cells was observed at 36 hours of growth in LB media while in MB the reduction was scored approximately 2-log (Figure 2E, F). Comparing to the steady state growth curve at 37ºC, the culturable cells at 0ºC and at 4ºC were found to gradually decrease. In case of *P. aeruginosa*, no culturable cells were detectable after 68 hours. A comparatively lower number of VBNC was noticed under cold shock in case of *P. putida*.

Constant numbers of VBNC were noticed in P. aeruginosa at cold shock

While grown at 0ºC, although the number of VBNC (culturable cells at 37 ºC and culturable cells at cold shock) in case of both *P. aeruginosa* was noticed to increase within 24-48 hours during growth both in LB and (MB) (Figure 2 A, B); however, that was minor and afterwards the number was decreased. While grown at 4ºC, the VBNC number seemed to be decreased than the number calculated from the growth at 0ºC. Indeed in MB, VBNC seemed to be in a lower number than those in LB.

Increased numbers of VBNC cells were noticed in P. fluorescence at cold shock: a possible reason for better survival

In contrast to *P. aeruginosa*, higher numbers of VBNC were noticed in *P. fluorescence* in both LB and MB with a higher ratio of VBNC cells in the LB medium (Figure 2 C, D). Interestingly the culturable cells in *P. fluorescence* were higher than the culturable cells of both *P. aeruginosa* and *P. putida* (Figure 2 C - F). The larger number of VBNC cells in *P. fluorescence* than the other strains might be an evidence of survival (reflected by the ability of this strain to form culturable cells) of *P. fluorescence* at cold shock. The largest number of culturable cells among all 3 *Pseudomonas* strains was noticed in case of *P. fluorescence* while grown at cold temperatures (Figure 2 A - F).

Relatively low numbers of viable and non-culturable (VBNC) cells were noticed in *P. putida at cold shock*

In contrast to *P. aeruginosa* and especially to *P. fluorescence*, relatively lower numbers of VBNC were noticed in *P. putida* in both LB and MB (Figure 2 E, F). In turn, the number of the culturable cells (formed as CFUs) in *P. putida* was found to be lower than those from *P. aeruginosa* and *P. putida* (Figure 2 A - D).

Discussion

Our previous studies on the VBNC formation was based on the generation of temperature stress and oxidative stress in mostly *Escherichia coli*, *Bacillus* spp., and to *Pseudomonas* strains to a little extent [6, 7, 11, 16]. Results of the current study met the objective where the formation of VBNC was expected at cold temperature stress. Besides, a number of studies have been performed using cold shock on *E. coli* and *Bacillus* spp. whereas reports on the cold shock effect on *Pseudomonas* spp. is not that abundant [15, 19, 23, 24, 25,]. Even though the current study lacks the examinations of the cold-shock genes of *Pseudomonas* spp.; however, the data found here clearly reveals that *P. fluorescence* cells could survive better than *P. aeruginosa* and than *P. putida* at cold temperatures as scored by their ability to form culturable cells.

E. coli cells are likely to undergo a decrease in viable cell number in the early stationary phase when grown in rich media [26]. In the current study a similar scenario has been noticed in case *Pseudomonas* strains since the growth was abundant in LB (nutrient rich medium) compared to that in MB (Figure 1A - C).

Initially, at the stationary phase, the s^E-dependent lysis of the dead cells and the damaged cells has been extensively observed[26]. Later some suggestive data clearly showed that VBNC cells may accumulate in the early clearly stationary phase in addition to those dead cells and the damaged cells due to nutrient depletion, heat shock or due to the oxidative stress [6, 7]. Accumulation of such lysis was clearly visible as the aggregate accumulated in the culture media [6, 7, 11, 17 , 27-30]. Interestingly, the accumulation of the aggregates has also been noticed during the current experiments when *Pseudomonas* spp. were grown at 37ºC (data not shown). Such cell lysis of the defective cells; i.e., a combination of dead cells and damaged cells or the VBNC was earlier hypothesized to be nutritionally required for the maintenance of the living cell population in the prolonged stationary phase [26]. Thus the survival strategy drawn by the VBNC of significant a cold shock as has been demonstrated in the present study (Figure 2). Moreover, since there are corresponding genes and homologues to the *rpoE* gene (encoding s^E, systems similar to the *E. coli* s^E-dependent dead cell lysis may exist in many microorganisms including in *Pseudomoans* spp. Such system may be crucial for cell turnover in stressed growth condition as indicated as the low temperature in the current study. Perhaps the lysis of the dead cells and of the VBNC cells provided nutrients for the remaining population seen at 4ºC an at 0ºC (Figure 2). Further studies on such aggregate accumulation and the lysis of *Pseudomoans* would be interesting to decipher the mechanism of *Pseudomonas* response against cold shock.

Conflict of interest: None

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