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RESPIRATION ACTIVITY IN FLEXIBACTER CHINENSIS UNDER STARVATION STRESS*

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Abstract – Electron transport system (ETS) activity was found to be related to the initial inoculum size, culturability and viability of *E. coli*. In this experiment, ETS activity of *Flexibacter chinensis* was measured by INT-method under a variety of conditions such as starvation media with different amendments and different temperatures. The relation between ETS activity, cell culturability, and viability was investigated. We found that the addition of glucose increased ETS activity without affecting cell culturability. Urea addition led to an increase in survival, but did not increase ETS activity.

Keywords – Electron transport system (ETS) activity, starvation, *Flexibacter chinensis*

1. INTRODUCTION

Bacteria, in their natural environment, are usually exposed to alterations in several environmental factors which may affect the respiration activity and ultimately the maintenance of culturability and viability of bacteria in the environment. The factors which may affect respiration activity include starvation [1], substrate concentration, temperature, dissolved oxygen [2] and some inhibitors [3].

During starvation, a large number of cells lose their ability to respire actively and in oligotrophic aquatic environments, the cells retain their ATP reserves for active transport mechanisms, which are required for the uptake of nutrients essential for the production of energy [1]. Bacteria, which have developed the ability to survive under starvation conditions, have the ability to reduce their endogenous respiration rate [4]. A dramatic decline in electron transport system (ETS) activity in *E. coli*, coupled with a decline in respiration rate and cell size reduction under starvation conditions have suggested that there is a link between the activity of the metabolic enzymes of the ETS and culturability [5,6]. Some nutrients also have a significant effect on respiration activity when supplied to cultures under starvation conditions. The addition of a variety of sugars increased the respiration activity of bacteria associated with the breakdown of n-alkanes markedly, but the bacterial viable counts showed no important variations whichever substrates were metabolized by the bacteria. In the natural environment it is probable that dissolved neutral monosaccharides, such as glucose and mannose, support a large fraction of bacterial respiration as well as biomass production [7].

Delgiorgio *et al.* [8] have reported that respiration activity in stressed cells is increased by the addition of organic carbon sources or inorganic phosphate. Pomeroy *et al*. [9] reported that respiration rate and bacterial secondary production increased when an inorganic phosphate source was added to water samples. Some amino acids, such as aspartic acid, also increase the rate of respiration while some others,

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such as leucine and lysine, decrease it [10]. However, there are some factors and chemicals which have an inhibitory effect on the respiration activity. Bacterial respiration activity decreases when the bacteria are incubated in the light [11]. Another factor which has a significant effect on respiration activity is temperature. Changes in temperature affect the potential QO_2 (specific oxygen uptake rate) and will therefore change the bacterial growth rate. The respiration rate of bacteria is a function of the rate of substrate uptake which is itself related to the substrate concentration and temperature [2]. Bacterial growth rate will also affect respiration rate because of the amount of nutrients diverted to macro-molecule synthesis rather than being reserved for respiration.

In this study, the respiration activity of *Flexibacter chinensis* under starvation conditions at a variety of temperatures and with the starvation medium amended with different sources has been investigated.

2. MATERIALS AND METHODS

a) Bacterial Strains

The bacterial strain used in this study was *Flexibacter chinensis.*

b) Bacterial Growth Media

All bacterial strains were routinely grown in Luria Broth (LB) (10g/l Bacto tryptone, 5g/l yeast extract, 5g/l NaCl, pH 7.2) or on Luria Agar (LBA) (10g/l bacto tryptone, 5g/l yeast extract, 5g/l NaCl, 15g/l Agar).

c) Nutrient Source Amendments

Carbon and KH_2PO_4 (as a phosphorus source) were used with 0.5 g/l and 100 mg/ml as final concentration respectively. In addition, urea, amino acids and ammonium sulphate were used at a maximum final concentration of 100 mg/l as nitrogen sources.

d) Starvation protocol

Flexibacter chinensis was grown in Luria broth at 30˚C for 24 hr. Culture (10 ml) was harvested by centrifugation at 5000 g for 10 min at 4˚C. The cells were washed twice using sterile distilled water (10 ml) and the pellet resuspended in the final volume of 10 ml of sterile distilled water. Resuspended culture (0.1 ml) was inoculated into 100 ml of sterile water to give an initial viable count of around 10^7 colony forming units/ml (cfu/ml). The flask was incubated at the required temperature without shaking in the dark at different times. Viable counts were determined on LBA plates after 48 hr incubation at 30˚C.

e) Viable count

The viable count was determined using a surface spread plate technique. Samples (1 ml) were taken from the flasks and serial dilutions prepared as a 10-fold serial dilution in Quarter-strength Ringers solution (2.25g/l NaCl, 0.12 g/l $CaCl_2$, 0.05 g/l $NaHPO_4$ and 0.105 g/l KCl in 1 liter Distilled water). 100 µl of the diluted samples was spread on duplicate LBA plates. The plates were incubated at 30˚C for at least 48 hr. Plates were counted manually by using an illuminated colony counter

f) Total Counts

Two different methods were used to determine the total count of bacteria in a sample. In early experiments, the total count was determined using a Coulter counter ZM (Coulter Euro Diagnostics GMBH) with a 30 µm orifice probe. The data were analyzed using Coulter channelyzer software to

estimate the size distribution. In later experiments, a CellFacts particle counter was used. This was connected to CellFacts analysis software. The sample in both methods were prepared for analysis in an identical fashion. The samples were diluted in an isotonic buffer containing 0.4% (v/v) glutaraldehyde to fix the cells. The samples were stored for not more than 10 days in the above solution at 4˚C. Total count is expressed as total particles/ml. The software also determines mean cell size and volume.

g) Electron Transport System Assay (INT method)

The activity of the Election Transport System (ETS) was measured using the method of Prin *et al.* [12]. Samples (10 ml) were added to 20 ml sterile plastic universals wrapped in foil. 0.1 ml of 0.2% (w/v) INT (2,9-p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride) was added and the universal was incubated at 25°C for 24 hr without shaking. INT activity was stopped by the addition of 0.1 ml of 37% (v/v) formaldehyde. The cells were pelleted by centrifugation at 13800 g for 10 min at 4°C. The supernatant was poured off and 1 ml methanol was added for 2 hr to extract the INT-formazan from the cells. The cell debris was removed by centrifugation, and the absorbance was read at 490 nm using methanol as a blank.

3. RESULTS

a) The activity of the electron transport system of Flexibacter chinensis in starvation medium

Preliminary experiments were carried out to determine the optimum conditions of inoculum size and incubation time required to be able to use the INT method to determine the accumulation of formazan deposits in *Flexibacter chinensis* cells under starvation conditions. The amount of INT reduction (recorded as change in absorbance) was increased after the initial inoculum size from 10^6 cfu/ml to 10^7 cfu/ml and increased again to 10^8 cfu/ml. Using the highest inoculum size led to sample coloration because the higher amount of INT chloride reduced to INT formazan during the reaction and subsequent experiments were carried out using an inoculum size of 10^7 cfu/ml.

The effect of incubation time on respiration activity was examined by analyzing samples after 10 min, 60 min and 24 hr incubation in the presence of INT and with an initial inoculum of 10^7 cfu/ml. INT reduction and formazan deposition increased over the incubation period, but the reaction rate was not linear. All future determinations were carried out after 24 hr incubation and this gave the highest reading and was felt to be more applicable to starved cultures where activity would be lower

b) The effect of temperature on ETS activity of Flexibacter chinensis under starvation stress

The cells were subjected to starvation for a period of up to 60 days at 4°C (Fig. 1a), 15°C (Fig. 1b), 25°C (Fig. 1c) and 30°C (Fig. 1d).

The loss of ETS activity at 4°C was less than that at the other temperatures and the greatest rate of loss of ETS activity was observed at the highest temperature used. The loss of cell viability was the same as the loss of ETS activity and both reached undetectable levels at about the same time. The exception to this is at 4°C where the viable count is still 10^4 cfu/ml and the ETS activity has virtually become undetectable. Cell viability was also lost faster at the higher temperatures than at 4°C where viable cells were still detectable after 54 days incubation in starvation media. ETS activity in these samples had virtually reached undetectable levels at this time. These results suggest that bacterial metabolism, especially ETS activity, was reduced considerably at higher temperatures under starvation conditions. Bacterial metabolism was slower at the lower temperatures and the loss of ETS activity was also slower. Only at 30°C was there still measurable ETS activity even though the viable count had become undetectable. In all cases the total count remained constant indicating that little or no cell lysis was taking

place under these starvation conditions. This suggests that the effect of the higher temperatures was to cause loss of cell culturability but these non-culturable cells retained the ability to respire and still possessed a functional ETS. The loss in cell size was the same as the loss of ETS activity more closely than the loss in culturability. It is possible that the decline in ETS activity is in some way connected to the loss in cell size. If this was an energy requiring process then the ETS activity would be essential to the cells as they undergo the miniaturization process and ETS activity would have to be retained until the process of miniaturization was complete.

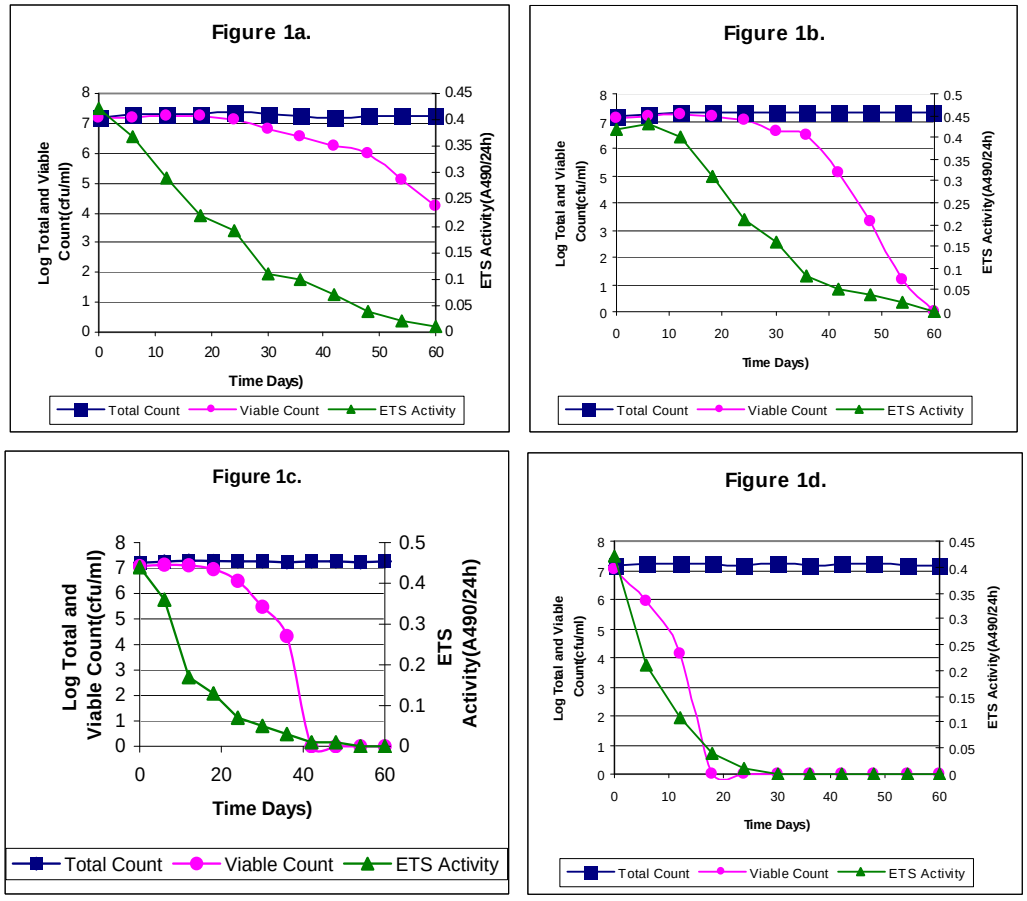

Fig. 1. A comparison of ETS activity, viable count, total count under starvation stress at different temperatures. Starvation microcosms were set and the flasks were incubated at 4°C (Fig. 1a), 15°C (Fig. 1b), 25°C (Fig. 1c) and 30°C (Fig. 1d) for up to 60 days

The total cell counts under starvation conditions remained constant at all the temperatures used, but the viable count decreased under starvation stress until there was no colony formation on agar plates. The rate of decline in viability was dependent upon the incubation temperature. The viability was retained the longest at 4°C but it took longer for visible colonies to be produced on agar plates than from samples starved at the higher temperatures. This suggests that it takes longer for the cells to recover from the effects of stress or that the response to starvation is more complete at this temperature.

Figure 2 summarizes the ETS activity data and reveals more clearly how starvation leads to a slow down in the metabolic activity and the respiration rate of the bacteria. ETS activity at 4°C and 15°C was detectable for a longer period of time than at 25°C and 30°C as was bacterial viability (as determined by culturability). The rate at which ETS activity was lost was slower at 4°C than at 15°C, while the most rapid decline was seen at 30°C. ETS activity at 4°C and 15°C was not detectable when the cells were not

culturable, but at 30°C ETS activity was detectable even when the viable count had declined significantly. This suggests that, under long term starvation conditions the cells lose ETS activity and viability as a direct result of starvation, but in short term starvation at the higher temperatures (30°C). Although the cells rapidly become non-culturable because of the effect of the higher temperatures, they lose the ability to respire more slowly (Fig. 2). At 15°C, closer to the optimum growth temperature of the bacteria, ETS activity appears to be slightly more stable than at the other temperatures including 4°C. ETS Activity could be lost faster at 4°C than at 15°C because overall metabolic activity decreased at the lower temperature following normal thermodynamic considerations, and hence it is suggested that proteins might have been degraded or not synthesized in these starving bacteria.

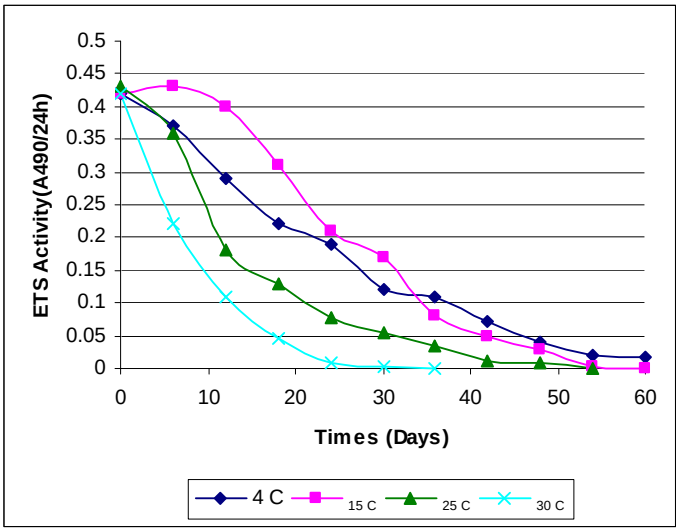

Fig. 2. The effect of different temperatures on the ETS activity in a starvation medium. Starvation were set up and the flasks were incubated at 4°C, 15°C, 25°C and 30°C for up to 60 days

c) The effect of nutrient amendments on the ETS activity of Flexibacter chinensis at 15°C in a starvation medium

Some nutrient sources, such as urea and glucose, increased the viable count of *Flexibacter chinensis* and led to an increase in the period of time for which cell viability was retained under starvation conditions. The addition of the inorganic phosphate source had no effect on cell viability, cell culturability or ETS activity.

The total cell count in these amended samples did not decline over the starvation period suggesting that cells become non-culturable over the starvation period rather than lysing. Here, the ETS activity was examined over a 60 day starvation period after the addition of different nutrient sources to the starvation medium to investigate the relationship between the increased survival seen under these conditions and the ETS activity of the bacterial cells. The results are presented in Fig. 3 for changes in ETS activity and in Fig. 4 for changes in viable count. There was a large increase in ETS activity from a typical starting rate of 0.7 units to 1.99 units when the starved cells were amended with D-glucose at a final concentration of 5 mg mg-c l^{-1}. However, there was no significant increase in the viable count (Fig. 4) compared with the viable count of the control. Also, there was no increase in total count. The decline in ETS activity in all cases matched the loss of culturability of the cells. This reinforces the belief that cells lose their ability to grow on agar plates as they become more stressed. This stress is shown here by the gradual loss of ETS activity. In all cases, activity was virtually undetectable after 54 days regardless of the amendment made and the starting value. Both urea and D-glucose increased the length of time for which the cells remained

culturable but only D-glucose increased ETS activity. Therefore, it has to be concluded that the increased time of culturability seen with these amendments is not due to the increased ETS activity.

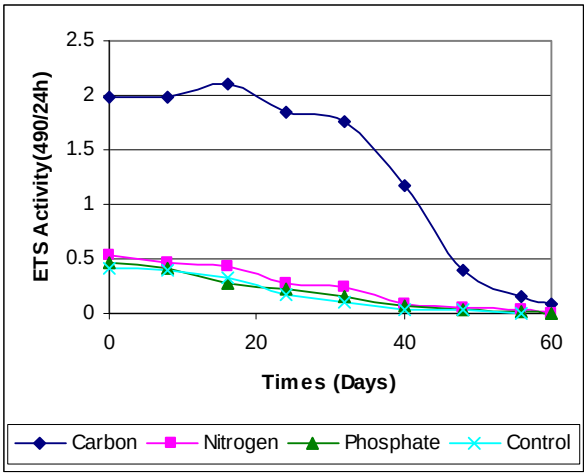

Fig. 3. The effects of nutrient amendments on ETS activity at 15°C in starvation media. D-glucose, urea and KH_2PO_4 were added at final concentrations mentioned in materials and methods

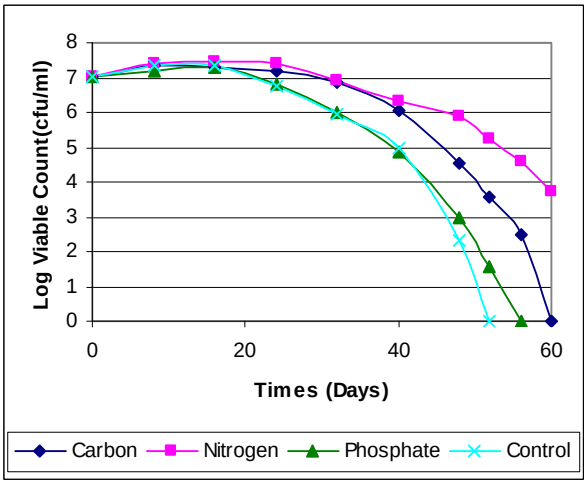

Fig. 4. The effect of nutrient amendments on the viable count at 15°C in starvation media. D-glucose, urea and KH_2PO_4 were added at final concentrations mentioned in materials and methods

4. DISCUSSION

In this experiment, the effect of the cell numbers on ETS activity at 25°C was examined using different inoculum sizes of *Flexibacter chinensis* to determine the best cell density to use in future experiments. An initial inoculum size of approximately 10^6 cells gave an ETS activity of 0.208, an initial inoculum size of 10^7, an ETS activity of 0.431, and finally, an initial inoculum size of 10^8 gave an ETS activity of 0.941. Each log increase in the inoculum size resulted, approximately, in a doubling of ETS activity. Although this log-linear relationship is assumed from only three points, further investigation would determine whether or not this could be used as a means of determining the number of cells which have ETS activity in culture. Ozkanca [13] reported a similar result for *E. coli* where ETS activity at 30°C increased as the inoculum size increased.

The effect of starvation on ETS activity was determined by incubating the cells at a variety of temperatures in sterile distilled water, measuring ETS activity, and then correlating this to total and viable counts and the reduction in cell size as the cells respond to starvation stress. In this experiment, the maximum ETS activity was obtained at 25°C after 24 hr incubation, however, Ozkanca and Flint [14] reported a maximum ETS activity for *E. coli* at 30°C occurred after only 80 min incubation time. ETS activity was reduced faster at 30°C and 25°C than at 4°C and 15°C. ETS activity was still detectable after 60 day starvation; in some cases even when the viable count had become undetectable. These results demonstrate a relationship between ETS activity and cell viability; Both decline fastest at 30°C and slowest at 4°C. There was no reduction in the viable counts at 4°C and 15°C over the first 20 days of the starvation period, but the ETS activity started to decline almost immediately. Although it is not possible to measure ETS activity in an initial inoculum size at less than 5 x 10^5 cfu/ml, here activity was still measurable in cultures where the viable count had declined to 10^4 cfu/ml or lower. Again this is evidence that the cells are still viable although they have lost their ability to grow on agar plates. Ozkanca and Flint [14] reported similar results with *E. coli* subjected to starvation conditions in sterile autoclaved lake water. A large amount of energy is needed for *E. coli* actively respiring at 30°C and the cells will reduce their ETS activity to preserve the small amount of available energy under starvation conditions at this temperature [2]. Similarly, *Flexibacter chinensis* reduced its ETS activity as a response to decreased amounts of nutrients in the starvation medium. As reported previously there was no decline in total cell count during the 60 day starvation period indicating that there was no cell lysis. Lim [15] reported the same results with *A. hydrophila*, and suggested that there was no occurrence of cell lysis even though the viable count decreased to an undetectable level over a starvation period of 90 days in sterile natural water samples.

Dawes [4] suggested that the ability of bacteria to survive during starvation is related to their ability to reduce their endogenous respiration rate. ETS activity is related to the metabolic activity of bacteria and, therefore, an estimate of the ETS activity under different conditions could give an indication of the physiological status of a microbial population. The loss of ability to grow on agar plates could be the result of a change of the uptake system for organic nutrients [16]. Consequently the relationship between the amendment of the starvation medium with a single nutrient, ETS activity, viable and total counts was investigated. When D-glucose was used as an amendment there was an increase in respiration activity in cultures starved at 15°C from the usual starting value of 0.7 units to more than 1.99. This value then declined in line with the control and reached an undetectable value after 54 days of starvation. There was no real increase in the viable count in these amended flasks and the total count remained constant. The increase in ETS activity might have been due to a real increase in the ETS activity which is associated with each cell or better accessibility of INT electron acceptor active sites access. This accessibility is presumed to be actively taking up D-glucose from the medium. The increase in ETS activity did not lead to a significant improvement in culturability compared to the unamended control and the viable count declined at the same rate in both the test and control flasks. Smith and McFeters [17] reported that the numbers of cells capable of using INT as an electron acceptor was higher in cultures which had been nutrient amended than in the unamended control and that the numbers capable or respiration in aquatic samples using this method was always higher than the plate count.

Other flasks were amended with urea as a nitrogen source as Lim and Flint [18] had demonstrated that nitrogen sources had a dramatic effect on *E. coli* survival in natural aquatic environments. Amendment experiments for *Flexibacter chinensis* also showed that there was an increased survival in flasks amended with a nitrogen source. However there was no increase in ETS activity compared to the unamended controls when urea was added to the flask although the culturability of the cells was retained longer.

The addition of inorganic phosphate had very little effect on ETS activity or the viable count of *Flexibacter chinensis*. There was no significant difference between the ETS activity, viable count, total count or cell size changes between the amended and unamended flasks.

The results from the carbon and nitrogen amended flasks would suggest that there is no direct relationship between survival and ETS activity. On the other hand, there is no link between the processes necessary for the establishment of the survival state (miniaturization for instance) and ETS activity as the process occurred as quickly in cells even with a lower ETS activity. The viable but non-culturable state has been demonstrated for a variety of bacterial species. Although the induction of this state in water microcosms is temperature dependent [19], the temperature dependence must be related to the ability of the cells to synthesize the proteins necessary for the survival state rather than simply to the need of the cell to provide energy. In this experiment, the cell size reduction of *Flexibacter chinensis* at 30°C and 25°C was more than the cell size reduction at 4°C and 15°C. Similar results were reported for *A. hydrophila* cell size reduction by Lim [15] and on *E. coli* by Ozkanca [14]. As with ETS activity, the cell size started reducing from the beginning of the starvation period, but the viable count at 4°C and 15°C remained constant for up to 18 d. In *E. coli*, it was suggested that the decline in ETS activity indicated that the metabolic enzymes involved in the ETS reactions were becoming inactivated and that this was related to cell culturability and to cell size reduction under starvation stress [5]. In *Flexibacter chinensis* it seems that there is no relationship between loss of ETS activity and cell size reduction or loss of culturability.

Although Dawson *et al.* [20] reported that starvation stress resulted in cell size reduction and that this is a strategy for long term survival, during starvation stress there is no relationship to respiration activity. In *Vibrio fluvialis*, again, it has been suggested that there is a relationship between cell size reduction and low respiration rate [6]. The results presented here suggest that this relationship is not causal and that both reduction in ETS activity (respiration rate) and cell size reduction are responding to a third parameter determining the survival strategy of cells under starvation stress. These changes in bacterial physiology and morphology are more likely to be the result of a chain-like reaction responding to changes in temperature and nutrient conditions to give the bacterial cells the optimum strategy to protect themselves against starvation stress.

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