Variation in bacterial ATP concentration during rapid changes in extracellular pH and implications for the activity of attached bacteria

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ABSTRACT

In this study we investigated the relationship between a rapid change in extracellular pH and the alteration of bacterial ATP concentration. This relationship is a key component of a hypothesis indicating that bacterial bioenergetics – the creation of ATP from ADP via a proton gradient across the cytoplasmic membrane – can be altered by the physiochemical charge-regulation effect, which results in a pH shift at the bacteria's surface upon adhesion to another surface. The bacterial ATP concentration was measured during a rapid change in extracellular pH from a baseline pH of 7.2 to pH values between 3.5 and 10.5. Experiments were conducted with four neutrophilic bacterial strains, including the Gram-Negative *Escherichia coli* and *Pseudomonas putida* and the Gram-Positive *Bacillus subtilis* and *Staphylococcus epidermidis*. A change in bulk pH produced an immediate response in bacterial ATP, demonstrating a direct link between changes in extracellular pH and cellular bioenergetics. In general, the shifts in ATP were similar across the four bacterial strains, with results following an exponential relationship between the extracellular pH and cellular ATP concentration. The one exception occurred with *S. epidermidis*, where there was no variation in cellular ATP at acidic pH values, and this finding is consistent with this species' ability to thrive under acidic conditions. These results provide insight into obtaining a desired bioenergetic response in bacteria through (i) the application of chemical treatments to vary the local pH and (ii) the selection and design of surfaces resulting in local pH modification of attached bacteria via the charge-regulation effect.

Keywords: bacteria; ATP; activity; biofilm; bacterial attachment

23 INTRODUCTION

24 In recent work, we demonstrated that bacterial attachment to a surface can impact cellular
25 bioenergetics, with the effect related to the types and surface densities of acid/base
26 functional groups on the bacterial and solid surfaces [1-5]. Specifically, we have
27 demonstrated that surfaces with acidic functional groups can enhance bacterial activity,
28 measured as an increase in cellular adenosine triphosphate (ATP), whereas surfaces with
29 basic functional groups can decrease cellular ATP.

30 The current theory describing this effect is based on the charge-regulated nature of the
31 two surfaces, which results in both charge and electrostatic potential variation as a
32 function of separation distance [1-10]. When a surface with ionizable groups approaches
33 another surface, electroneutrality requires the counterion concentration to increase in the
34 solution between the surfaces to offset the decrease in inter-spatial volume. As H^+ and
35 OH^- are counterions for negatively- and positively-charged surfaces, respectively, their
36 concentrations next to the surface will vary, altering the local pH and electrostatic
37 potential (Figure 1) [7-9].

38 Through charge-regulation modeling, we have shown that the local pH at the adhesion
39 interface can drop below pH 5 or rise above pH 9, depending on the acid/base
40 characteristics of the bacterial and solid surfaces [1, 2, 4]. This is illustrated in Figure 2
41 for an *Escherichi coli* bacterium as it approaches both a glass (negatively-charged, acidic)
42 surface and an amine (positively-charged, basic) surface. The hypothesis states that this
43 variation in pH between the bacteria and solid surface can affect cellular bioenergetics,
44 which links catabolic and anabolic reactions. During bacterial respiration, catabolic
45 reactions establish an electrochemical proton gradient across the cytoplasmic membrane

by pumping protons to the outside of the cytoplasmic membrane (process A in Figure 1) [11-13]. This is analogous to charging a capacitor, with the energy stored in a proton gradient rather than an electron gradient. The energy stored in this electrochemical gradient is termed the proton motive force (Δp , in units of mV) and is composed of both a charge gradient ($\Delta\psi$) and a pH gradient (ΔpH) across the cytoplasmic membrane and can be expressed using the Nernst equation [14, 15]:

$$\Delta p = \Delta\psi - \frac{2.3RT}{F} \Delta\text{pH} \quad (1)$$

where R is the ideal gas constant, T is temperature and F is the Faraday constant. The energy stored in Δp is used to create ATP, which is used to drive many anabolic reactions. In this process, protons are allowed to cross back into the cytoplasm through the ATP Synthase enzyme complex and the energy stored in the proton gradient is used to convert adenosine diphosphate (ADP) to ATP (process B in Figure 1). Thus, an increase in ΔpH should increase Δp and be observed as an increase in cellular ATP concentration. Conversely, a decrease in ΔpH should decrease Δp and cellular ATP.

We have experimentally demonstrated that adhesion of bacteria under non-growth conditions results in significant shifts in cellular ATP, and numerical modeling of the charge-regulation effect indicates that these shifts were due to local pH variations up to two units away from the pH of the bulk solution [1-3]. Full development of this hypothesis, however, requires development of the relationship describing how cellular ATP concentration responds to external pH shifts under non-growth conditions (process C in Figure 1).

There has been research with actively growing cells that examined the effects of bulk pH on Δp . These studies examined acidophiles, neutrophiles and alkaliphiles, using a variety of electron donors and acceptors [16-28]. In these studies, the extracellular pH was varied within the optimal range for growth of the different species and the results demonstrated that Δp is highest under acidic pH (with acidophilic bacteria) and it decreases as the pH increases through neutral pH (with neutrophilic bacteria) to basic pH (with alkaliphilic bacteria).

We have identified three studies that demonstrated the effects of a large external pH shift on the ATP levels of non-growing bacteria. In these studies, the pH of a suspension of *E. coli* cells was lowered from ~8 to ~3 in a single, rapid step and this resulted in an increase in cellular ATP concentrations [29-31]. One study then increased the pH back to pH ~8 and the cellular ATP concentration returned to a lower level [30]. This data is in agreement with the working hypothesis (Figure 1), but given that these studies focused on a single data point, the relationship between changes in external pH and cellular ATP levels (process C in Figure 1) remained unresolved.

A goal of this study was to develop a general form of this relationship. We can approach this mathematically by considering the free energy of phosphorylation, ΔG_p (kJ/mol), which provides a mathematical relationship between Δp and ATP [32]:

$$\Delta p = -\frac{\Delta G_p}{nF} = -\frac{1}{nF} \left[\Delta G_p^\circ + RT \cdot \ln \left(\frac{[ATP]}{[ADP][P_i]} \right) \right] \quad (2)$$

Here, ΔG_p° is the standard free energy for ATP hydrolysis with a reported value of 30.1 kJ/mol [33]; $[P_i]$ is the intracellular inorganic phosphate concentration (mol/L); $[ATP]$

and [ADP] are the concentrations of ATP and ADP, respectively (mol/L), and n is the number of protons translocated by the ATP synthase to generate one ATP molecule. The value of n is typically reported in the range of 2 to 4 for bacteria [33-37] and there is strong evidence that n varies as an inverse function of Δp , i.e., n decreases as Δp increases [37]. It is important to note that the relationship described by equation 2 is a reversible thermodynamic process: if Δp increases, then the concentration of ATP will increase, and if Δp decreases, then ATP will decrease [38].

Combination of equations 1 and 2 then provides a relationship describing the cellular ATP concentration as a function of ΔpH :

$$[ATP] = [ADP][P_i] \exp \left\{ \frac{nF}{RT} \Delta \psi - 2.3n \Delta pH - \frac{\Delta G_p^\circ}{RT} \right\} \quad (3)$$

While this is a simplistic analysis, as [ADP] and $[P_i]$ will vary inversely with [ATP] [1, 3, 33, 39-41] (we have previously provided example calculations for *Escherichia coli* in a study on the activity of attached bacteria [1, 3]), it does demonstrate that the ATP concentration should be exponentially related to the pH gradient across the cytoplasmic membrane. Assuming changes in extracellular pH affect ΔpH , we would then anticipate an exponential relationship between external pH and the cellular ATP concentration.

The focus of this study was to experimentally elucidate this exponential relationship for neutrophilic bacteria by varying extracellular pH and rapidly measuring cellular ATP concentrations. These experiments were conducted under non-growth conditions, with local pH values varied both within and well outside of the cell's optimal range, thus allowing exploration of process C in Figure 1. Specifically, this was accomplished by artificially manipulating the external (bulk) pH between the values of 3.5 to 10.5 and

rapidly measuring changes in cellular ATP levels in four different neutrophilic bacterial strains (two Gram-Negative and two Gram-Positive). The results provide further evidence in support of the working hypothesis by elucidating process C in Figure 1, and demonstrate that an exponential relationship between pH and cellular ATP concentrations was observed in agreement with equation 3.

MATERIALS AND METHODS

Bacterial Cultivation

Four neutrophilic bacterial strains were used in this study, including the Gram-Negative strains *Escherichia coli* K-12 (ATCC29181) and *Pseudomonas putida* (ATCC12633) and the Gram-Positive strains *Bacillus subtilis* (ATCC23059) and *Staphylococcus epidermidis* (ATCC 35984). Bacterial cultures were grown to the exponential phase in 500 mL of Luria Bertini broth (LB broth, Fisher Scientific) and then stored with 15% glycerol at -86°C using the glass bead method [3, 4, 42]. For each experiment, bacteria from the frozen stock were cultivated in 500 mL of LB broth at 30°C. After 20 hours of growth the bacteria were harvested and washed by centrifuging the suspension at 3500xg for 15 minutes, followed by re-suspension of the bacterial pellet in phosphate buffer solution (PBS, 0.258g KH_2PO_4 and 0.470g K_2HPO_4 in 1 liter of deionized water with the pH adjusted to 7.2 using 1M NaOH). The bacterial suspension was sampled into 50 mL centrifuge tubes and placed on a rotatory shaker at room temperature. The test tubes were removed after either a 24 hour (1 day) starvation period or a 168 hour (1 week) starvation period and the bacteria were washed a second time. The use of two starvation periods was to demonstrate if cells respond differently after undergoing extended

depletion of their energy reserves. The resulting bacterial suspensions were diluted with PBS to a concentration of approximately 10^8 cells, determined via Acridine Orange direct counts [43]. These final bacterial suspensions were used as described below.

Experimental Methods

For each experimental condition, an 8 ml sample of the bacterial suspension was withdrawn and its pH was adjusted from its current value of 7.2 to a desired value in the range of 3.5 to 10.5 using 1 M HCl or 1 M NaOH. After a specified waiting period (discussed below), 1 mL of the sample was removed and treated with 1 mL of nucleotide releasing buffer solution (NRB) [3]. NRB consists of 0.05% alkyl-dimethyl-benzyl-ammonium-chloride (benzalkonium chloride) in Tris-Mg²⁺ buffer (20 mM Tris, 2 mM EDTA and 10 mM magnesium acetate adjusted to a pH of 7.75) and it is used to lyse the bacterial cells and inactivate ATPase so that the enzyme does not degrade the released ATP. The samples treated with NRB were shaken well for 15 seconds and then analyzed for ATP as described below.

Analysis of Bacterial ATP

The ATP assay was performed following Hong and Brown (2009) using a Sirius Luminometer (Titertek-Berthold) and freshly prepared Luciferin-Luciferase solution. Luciferase solution was prepared by adding 1 ml Tris buffer (20 mM Tris and 2 mM EDTA, adjusted to a pH of 7.75) to 1 mg of Luciferase (Sigma). 25 μ l aliquots of this Luciferase solution were stored at -20°C in amber colored bottles until required for use. Prior to each experiment, the Luciferin-Luciferase solution was prepared by adding 1 mg of Luciferin (Sigma) and 10 ml of Tris Albumin buffer (20 mM Tris, 2 mM EDTA, 150

mM magnesium acetate, 50 μ M dithiothreitol and 1 g bovine serum albumin adjusted to a pH of 7.75) to a Luciferase aliquot. The solution was gently mixed and allowed to sit at room temperature for 30 minutes before use.

For the ATP measurement, 100 μ l of Tris Mg^{2+} buffer was pipetted into a luminometer tube containing 200 μ l of the bacterial-NRB sample. The contents were mixed thoroughly for 15 seconds using a vortex mixer. The tube was then placed in the luminometer and 100 μ l of the Luciferin-Luciferase solution was injected into the sample. The light generated by the reaction of ATP and the Luciferin-Luciferase was measured by the luminometer and quantified as Relative Light Units (RLU), which is a direct measure of the ATP concentration in the vial. To allow comparison of the change in ATP as a function of solution pH between the four different bacterial strains, the RLU values obtained at different pH values for each strain were normalized by the RLU value obtained at a pH of 7.2 and the units are provided as normalized RLU (nRLU).

RESULTS AND DISCUSSION

Temporal variation in cellular ATP during rapid extracellular pH change

In prior studies, it was demonstrated that the bacterial ATP concentration rose and quickly plateaued after a rapid drop in pH, and then began to decrease after a few minutes, presumably due to protein denaturation [29, 30]. The first experimental series in this study examined these temporal changes for the four different bacterial strains, with the goal of selecting a specific waiting period between the rapid change in solution pH and the ATP measurement. Variations in ATP as a function of time are shown in Figure 3 for *E. coli* K-12 at a solution pH of 7.2 and when the solution pH was rapidly changed

from 7.2 to both 4.5 and 9.2. As anticipated, the ATP concentration increased upon lowering the solution pH and it decreased when the pH was increased.

Temporal variations in ATP were observed upon changing the pH, with notable differences for the one-day and one-week starvation periods. With one-day starvation, the ATP concentrations rapidly reached a stable value within 30 seconds (the time of the first measurement) and remained stable for approximately 2-3 minutes, after which they started to decline. With one-week starvation, the ATP concentrations took 2-4 minutes to reach a stable value (which was very similar to the stable value for the one-day starvation), with a decline in ATP then starting soon afterwards. Similar temporal results were found with the other bacterial strains, and based on the results of these experiments the remaining analyses used ATP values obtained during the stable period (90 seconds for the one-day starvation period and up to four minutes for the one-week starvation period).

Response of ATP concentration to rapid changes in extracellular pH

The second experimental series measured the bacterial ATP concentration after a rapid change from a pH of 7.2 to values within the range of 3.5 to 10.5. It was anticipated that a rapid decrease in bulk pH would induce an increase in the proton gradient across the cell membrane, enhancing Δp and increasing the cellular ATP concentration, and as shown in Figure 4 this was directly observed. The nRLU values obtained for various pH levels are in direct proportion to the H^+ concentration in the bulk solution and, with the exception of *S. epidermidis*, which will be discussed below, the ATP concentrations showed a two- to four-fold increase as the pH of the sample was decreased to 3.5. Conversely, it was anticipated that a rapid increase in bulk pH would decrease the proton

gradient across the cell membrane, depleting Δp and decreasing cellular ATP. As shown in Figure 4, the ATP decreased as the bulk H^+ concentration decreased, with the ATP concentrations dropping by 20% to 70% as the pH was increased to 10.5. The results also demonstrate that the response to the rapid change in pH was identical under both the one-day and one-week starvation periods. Overall, these results are consistent with the hypothesis shown in Figure 1 and suggest a distinct relationship between bacterial ATP content and solution pH, with the ATP concentration inversely related to the pH.

Relationship between ATP and extracellular pH

To test for the exponential relationship between ATP and ΔpH suggested by equation 3, the data for each bacterial strain were aggregated and examined for their ability to be fit via a general exponential function. The results, shown in Figure 5, demonstrate that the exponential function accurately describes the experimentally-observed ATP/pH relationship.

To examine this relationship further, a comparative analysis of the four bacterial strains was performed and the results are presented in Figure 6. *E. coli*, *B. subtilis* and *P. putida* all demonstrated similar metabolic responses across the entire pH range. This finding indicates that the hypothesis outlined in Figure 2 is a common mechanism for influencing Δp via changes in pH outside of the cell and occurs with both Gram-Negative and Gram-Positive bacteria.

S. epidermidis, on the other hand, showed no response under acidic conditions and a steeper slope under basic conditions than the other three bacterial strains. *S. epidermidis* is a well-studied inhabitant of human skin, which has a pH range of 4.0 to 5.6 with an

average value around 4.7 [44-49]. This indicates that this species, although neutrophilic, has adapted to survive and grow under acidic conditions [50]. *S. epidermidis* has been shown to grow at both pH 7.0 and 5.5 with an identical growth rate and minimal lag period, whereas at a pH of 8.5 it had a an extensive lag period and a much lower growth rate [51]. The results found here, with no variation in *S. epidermidis* ATP levels under acidic conditions and an enhanced decrease in ATP under basic conditions, are consistent with these prior studies.

Statistical analysis of the data of the four bacterial species supports the similarity between the bacterial strains and the difference with *S. epidermidis*. Linearization of the exponential relationships in Figure 5 gives

$$\ln(nRLU) = m \cdot pH + b \quad (4)$$

where m is the slope and b is the intercept of the line fits. The 95% confidence intervals on m and b for each of the bacterial strains are presented in Figure 7. *E. coli*, *B. subtilis* and *P. putida* responded similarly to changes in pH and this is seen by the grouping of their confidence intervals, whereas *S. epidermis* shows distinct deviation from this grouping. When considered in context with our prior work on the variation in bacterial ATP upon adhesion [1-5], these results suggest that *S. epidermis* adhesion to acidic surfaces would have no effect on cellular bioenergetics, while its adhesion to basic surfaces will have an enhanced effect compared to other neutrophilic bacteria. This information may be useful in identifying and developing antibacterial materials, coatings and solutions to discourage *S. epidermidis* colonization of surfaces.

Implications to bioenergetic response of attached bacteria

We have previously demonstrated that bacterial adhesion to surfaces results in changes in cellular ATP, with the variation a function of the surface properties as described by the working hypothesis [1-3]. However, the direct elucidation of the link between surface pH changes and variation in cellular ATP had not been established (process C in Figure 1). The results provided herein demonstrated that a distinct response in cellular ATP to changes in pH at the cell surface does exist. This finding fills in a key knowledge gap in the hypothesis suggesting that charge-regulation-induced pH changes at the cell surface can induce a bioenergetic response in bacteria [1-5].

Specifically, and most importantly, this study elucidated a means to predict changes in cellular ATP as a function of changes in the local pH. Charge-regulation modeling can provide an estimate of the local pH upon a bacterium adhering to a surface [1, 2, 4, 7-9] and an example was provided in Figure 2. The exponential relationship elucidated in Figures 5 and 7 provides a means to estimate the relative variation in cellular ATP that may occur during this adhesion-induced pH change. In summary, we have demonstrated how cellular ATP levels are influenced by rapid changes in the extracellular pH, and this information may aid in the selection and design of surfaces that provide a desired bioenergetic response in bacteria (e.g., a passively antimicrobial surface) through local pH variations via the charge-regulation effect [1-4].

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Figure 1. The working hypothesis describing the effect of adhesion on bacterial metabolic activity, depicted here for a Gram-negative bacterium adhering to a negatively-charged surface, links cellular bioenergetics to the charge-regulation effect. (a) In cellular bioenergetics, protons are pumped across the inner (cytoplasmic) membrane (IM) during respiration, setting up pH and electrostatic gradients across the IM, which are quantified as the proton motive force (Δp). (b) The protons are then allowed back across the IM via the ATP-Synthase enzyme complex and the energy is captured to produce ATP from ADP and phosphate. When cells approach a charged surface, the charge-regulation effect alters the proton concentration at the cell surface. We hypothesize that the alteration in proton concentration at the cell surface (c) propagates through the outer membrane and affects the pH gradient across the IM. Similar results would be expected with Gram-Positive bacteria.

Figure 2. Example charge-regulation results showing the pH at the *E. coli* cell surface (solid lines) and glass and amine-coated surfaces (dashed lines) as the bacterium approaches each of the two surfaces. Following the hypothesis outlined in Figure 1, the decrease in pH upon adhesion to the glass surface should result in an increase in cellular ATP and the increase in pH upon adhesion to the amine surface should result in a decrease in cellular ATP.

Figure 3. Total ATP concentration of *E. coli* suspensions as a function of time at three solution pH values. Data presented as RLU normalized to the average RLU value at pH 7.2 (nRLU) for both one-day (solid symbols) and one-week (hollow symbols) starvation periods. For the pH 4.5 and 9.2 data, zero minutes represents the time when the pH was changed from 7.2 to the specified pH.

Figure 4. ATP concentration, presented as Relative Light Units normalized to the value at pH 7.2 (nRLU), as a function of the solution pH. Grey and black symbols are replicate experiments with bacteria starved for one day. White symbols are bacteria starved for one week.

Figure 5. Exponential curve fits of the consolidated experimental data. Hollow symbols for *S. epidermidis* not included in the curve fit.

Figure 6. Comparison of ATP levels between the four bacterial species as a function of solution pH. (a) Experimental data. (b) Exponential curve fits.

Figure 7. Comparative analysis of the slope (m) and intercept (b) from equation 4 for the four bacterial species. Error bars represent the 95% Confidence Intervals. Results demonstrate that *E. coli*, *B. Subtilis* and *P. putida* have similar responses to pH changes (delineated by circle) and *S. epidermis* deviates from this response. The data for *S. epidermidis* was obtained using the upper pH range indicated in Figure 5.

Figure(s)

Low extracellular pH,
Increased cellular ATP

High extracellular pH,
Depleted cellular ATP