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Revised Model of Calcium and Magnesium Binding to the Bacterial Cell Wall

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Abstract

Metals bind to the bacterial cell wall yet the binding mechanisms and affinity constants are not fully understood. The cell wall of gram positive bacteria is characterized by a thick layer of peptidoglycan and anionic teichoic acids anchored in the cytoplasmic membrane (lipoteichoic acid) or covalently bound to the cell wall (wall teichoic acid). The polyphosphate groups of teichoic acid provide one-half of the metal binding sites for calcium and magnesium, contradicting previous reports that calcium binding is 100% dependent on teichoic acid. The remaining binding sites are formed with the carboxyl units of peptidoglycan. In this work we report equilibrium association constants and total metal binding capacities for the interaction of calcium and magnesium ions with the bacterial cell wall. Metal binding is much stronger and previously reported. Curvature of Scatchard plots from the binding data and the resulting two regions of binding affinity suggest the presence of negative cooperative binding, meaning that the binding affinity decreases as more ions become bound to the sample. For Ca^{2+} , Region I has a $K_A = (1.0 \pm 0.2) \times 10^6 \text{ M}^{-1}$ and Region II has a $K_A = (0.075 \pm 0.058) \times 10^6 \text{ M}^{-1}$. For Mg^{2+} , $K_{A1} = (1.5 \pm 0.1) \times 10^6$ and $K_{A2} = (0.17 \pm 0.10) \times 10^6$. A binding capacity (η) is reported for both regions. However, since binding is still occurring in Region II, the total binding capacity is denoted by η_2 , which are $0.70 \pm 0.04 \text{ } \mu\text{mol/mg}$ and $0.67 \pm 0.03 \text{ } \mu\text{mol/mg}$ for Ca^{2+} and Mg^{2+} respectively. These data contradict the current paradigm of there being a single metal affinity value that is constant over a range of concentrations. We also find that measurement of equilibrium binding constants is highly sample dependent, suggesting a role for diffusion of metals through heterogeneous cell wall fragments. As a result, we are able to reconcile many contradictory theories that describe binding affinity and the binding mode of divalent metal cations.

Keywords

Peptidoglycan; Teichoic Acid; Magnesium; Calcium; Metals

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INTRODUCTION

The importance of calcium and magnesium ions with regards to cell viability has been well documented (Webb 1949; Shooter and Wyatt 1955). Calcium ions participate in synergistic interactions with enzymes responsible for anchoring surface proteins to the cell wall, thereby affecting the bacterium's adhesion ability (Ilangoan, Ton-That et al. 2001; Naik, Suree et al. 2006). Some pathogens rely on Ca^{2+} for toxin activity (Van Nhieu, Clair et al. 2004). Magnesium ions play a role in peptidoglycan synthesis, cell wall strength, and the prevention of cell lysis (Rayman and MacLeod 1975). The surfaces of Gram-positive bacteria contain carboxyl, phosphoryl, hydroxyl, and amino functional groups (Beveridge and Murray 1980; Fein, Daughney et al. 1997). At physiological pH values, these groups are deprotonated and contribute to metal binding (Beveridge and Murray 1980; Fein, Daughney et al. 1997).

For Gram-positive bacteria, wall teichoic acid (WTA), lipoteichoic acid (LTA), and peptidoglycan are major bacterial components for sequestering metal ions from the environment. Peptidoglycan is a glycan (polysaccharide) backbone consisting of N-acetyl muramic acid and N-acetylglucosamine with peptide side chains (amino acids and diaminopimelic acid) that are cross-linked through peptide bonds to form a three dimensional structure. Teichoic acids are polysaccharides of either polyglycerol phosphate or polyribitol phosphate (depending on the bacterial strain) and are either anchored in the cytoplasmic membrane (LTA) or covalently bound to the cell wall (WTA) (Bhavsar, Erdman et al. 2004). The carboxyl groups on peptidoglycan are the anionic sites for metal binding, while phosphodiester groups are the primary metal binding sites for teichoic acids.

Previous studies on the metal binding behavior of *B. subtilis* have focused on the metal binding capacity and affinity. It was suggested through Hill plot analysis that negative cooperativity might exist (Doyle, Matthews et al. 1980). However, Scatchard plots do not exhibit the characteristic shape and curvature associated with negative cooperative binding (Doyle, Matthews et al. 1980). Nevertheless, K_A values of $4 \times 10^4 \pm 0.8 \times 10^4 \text{ M}^{-1}$ and $5.4 \times 10^4 \pm 3.3 \times 10^4 \text{ M}^{-1}$ for calcium and magnesium ions indicate a weak interaction (Doyle, Matthews et al. 1980). Binding capacities ($0.75 \pm 0.15 \text{ } \mu\text{mol/mg}$ and $0.91 \pm 0.54 \text{ } \mu\text{mol/mg}$) previously reported for calcium and magnesium have substantial error (Doyle, Matthews et al. 1980). These studies relied on either radioactive assays or atomic absorption spectroscopy. In contrast, an electrostatic model for metal sorption on the cell wall calculated a binding constants 3 orders of magnitude stronger ($6.31 \times 10^7 \text{ M}^{-1}$ for Ca^{2+} ions in low ionic strength solution with 0.001 M K^+ ions) (Yee, Fowle et al. 2004). These experiments utilized a modeling approach to extrapolate a binding constant rather than a typical linear regression Scatchard plot analysis.

Most Gram-positive cell walls have similar functional groups that contribute to metal binding (Beveridge and Murray 1980; Doyle, Matthews et al. 1980; Fein, Daughney et al. 1997; Yee, Fowle et al. 2004). Recent research with solid state NMR shows that Mg^{2+} preferentially binds to the phosphate groups, pushing the D-alanine away from the phosphate (Garimella, Halye et al. 2009). Solid state NMR experiments have also been performed to estimate the binding constant of wall teichoic acid using the ^{31}P chemical shift

based on the magnesium concentration used in the experiments (Kern, Giffard et al. 2010). Kern et al. (Kern, Giffard et al. 2010) published a dissociation constant of $600 \pm 300 \mu\text{M}$ ($K_A = 1.67 \times 10^4 \text{ M}^{-1}$) for WTA and Mg^{2+} by using purified cell wall that contained peptidoglycan and covalently attached WTA through the use of 1D NMR. Nonetheless, the great disparity in K_A values suggests a complicated system that warrants further study.

Calcium and magnesium ions are both important biologically active metal ions that are some of the most abundant divalent cations in nature. We find that electrostatic effects are responsible for a strong binding between metal ions. This strength of binding decreases as the negatively charged functional groups of the cell become neutralized by divalent metal ions. Purified cell wall fragments of *B. subtilis* 1A578 (containing peptidoglycan and WTA) give two binding regions. For Ca^{2+} , Region I has a $K_A = (1.0 \pm 0.2) \times 10^6 \text{ M}^{-1}$ and Region II has a $K_A = (0.075 \pm 0.058) \times 10^6 \text{ M}^{-1}$. Mg^{2+} ions give similar results in metal binding behavior. For Mg^{2+} , fragments of 1A578 yield a $K_{A1} = (1.5 \pm 0.1) \times 10^6$ and $K_{A2} = (0.17 \pm 0.10) \times 10^6$. A binding capacity (η) is reported for both regions. However, since binding is still occurring in Region II, the total binding capacity is denoted by η_2 . Fragments of 1A578 gave binding capacities of $0.70 \pm 0.04 \mu\text{mol/mg}$ and $0.67 \pm 0.03 \mu\text{mol/mg}$ for Ca^{2+} and Mg^{2+} respectively. As described below, these values will change when pH changes and also WTA is chemically removed from the cell wall. Thus, we envision a cell wall binding model of peptidoglycan providing sites for metal binding required for structural integrity whereas WTA provides sites for metals destined for bacterial biochemistry in the cytoplasm.

Materials and Methods

Cell Growth Protocol

A chloramphenicol resistant strain of *B. subtilis* 1A578 (Anderson, Henkin et al. 1984), was obtained from Bacillus Genetic Stock Center (BGSC, Department of Chemistry at the Ohio State University). An overnight culture of *B. Subtilis* 1A578 was inoculated with frozen stock and grown in 20mL of LB with 10 $\mu\text{g/mL}$ of chloramphenicol added. The following day a 1L culture was inoculated from the overnight culture by transferring a volume of the overnight culture equal to 1% of the new culture and adding an amount of antibiotic stock solution (10 mg/mL) to a final concentration of 10 $\mu\text{g/mL}$. The culture was allowed to grow (200 rpm, 37°C) to an OD_{600} of 1.0 and then centrifuged at 15000g for 25 minutes. After centrifugation, the supernatant was decanted and the pellet was re-suspended in 10 mL of Milli-Q H_2O in preparation for cell purification.

Cell Wall Purification

A modified procedure by Umeda et al. (Umeda, Yokoyama et al. 1992) was used to purify peptidoglycan with covalently bound WTA. Only peptidoglycan was purified from EB1451 in this method due to the absence of WTA in the sample. The cells were disrupted using a French press and the resulting fragments were re-suspended in 10 mL distilled deionized water and added drop wise with stirring to 100 mL of boiling 6% (w/v) sodium dodecyl sulfate. This step inactivates autolysins (Schleifer 1985; Majcherczyk, Langen et al. 1999) and removes the cytoplasmic membrane. In addition, the sample was kept cold (4°C) during the initial disruption procedure in order to prevent the degradation of peptidoglycan by

autolytic enzymes. The sample was rinsed 3 times with 200 mL of distilled deionized water and centrifugation at 15000g for 20 minutes at least three times. In order to remove any remaining cell wall associated proteins, RNA or DNA, the fragments were then re-suspended in TRIS buffer (pH 8.2) and treated with trypsin (200 µg/mL) and RNase/DNase (100 µg/mL) at 37°C in an incubator/shaker for 16–18 hours. The cell wall fragments were then spun down again, washed with fresh distilled deionized water, and then resuspended in ammonium acetate buffer (pH 4.7) and treated with pepsin (100 µg/mL) at 37°C for 2 hours. Finally, the cell wall fragments were centrifuged and washed with 50 mL of 50 mM EDTA overnight (4 °C) to remove any residual metal ion contamination, and then washed 3 times with 200 mL of ddH₂O. The cell wall fragments were then suspended in a approximately 5 mL of Milli-Q H₂O and then lyophilized, forming a white, charged, fluffy solid.

WTA Hydrolysis

Portions of the freeze dried cell wall also underwent a 10% TCA (trichloroacetic acid) treatment at 4°C for 48 hours in order to remove the WTA (Bernal, Zloh et al. 2009). After treatment with TCA, the suspension was centrifuged at 15000 g and the insoluble portion containing peptidoglycan was washed 3 times with 200 mL of Milli-Q H₂O and then lyophilized for 2 days or until the sample was dry. Approximately 46% of the original mass remained after this procedure. Removal of WTA was affirmed by visual colorimetric phosphorus determination with addition molybdenum blue (Holman 1943).

Membrane Dialysis Procedure

A known mass of 10–60 mg of the purified cell wall material was suspended in 1–2 ml of Milli-Q H₂O, transferred to a 6.5 cm length, 1.8 cm width, 1000 MWCO Spectra/Por® 7 dialysis membrane and placed in a closed jar with a final total volume of 50 mL of Milli-Q H₂O. The jars with dialysis membranes were placed in a cold room and shaken on a New Brunswick G24 incubator/shaker at 4°C. Every 12 hours a 5 mL aliquot was removed from the jar and 5 mL of a known metal ion concentration in the range of 5–20 ppm were added in order to obtain a concentration gradient. This range was chosen by the method of trial and error. The concentration gradient had to be small enough to observe changes in the Scatchard analysis, but not so small as to have data points crowded into one region. Utilizing a Varian SpectraAA 55b Flame Atomic Absorption Spectrometer, the concentration of the aliquots was determined, and that data was used to calculate the amount of Ca²⁺ or Mg²⁺ bound to the cell wall inside the dialysis membrane. Binding constants from this data were determined with a Scatchard plot. The 12 hour time period between aliquot removals was determined by measuring the time required for the system to reach equilibrium. Additional equilibrium dialysis experiments with Ca²⁺ ions placed inside the dialysis membrane with extractions taken at specified intervals revealed equilibration was achieved was determined to be at 10 hours. Calibration curves of the SpectraAA response were used to determine metal concentration of each aliquot, measured in triplicate.

Additional experiments were performed with a low concentration of HEPES buffer, 0.001 M, at an adjusted pH of 7.25 in order to examine effects of pH on the binding constant and binding capacity of Mg²⁺. Mg²⁺ binding should increase at pH 7.25 as a result of the diaminoipimelic acid carboxyl groups becoming deprotonated and offering additional charge

density for binding metal ions. Barkleit *et al.* (Barkleit, Moll et al. 2009) determine three regions of pK_a values (4.55 ± 0.002 , 6.31 ± 0.01 , and 9.56 ± 0.03) when performing potentiometric titrations of purified peptidoglycan from *Bacillus subtilis*. These values were attributed to the carboxyl group on the glutamic acid, the carboxyl group on the diaminopimelic acid, and the amine/hydroxyl groups on the peptidoglycan sample, respectively. Site densities reported were 0.65 ± 0.17 , 0.76 ± 0.02 , and 1.45 ± 0.23 mmol/g of sample for the three sites.

RESULTS

Divalent metal cations are attracted to the negative charge of the cell wall. Initially, metal ions are strongly bound and only a small portion of metal ions remain in solution. This is demonstrated in the binding curve (Figure 1). Each standard addition increases the number of bound metal ions but also increases the concentration of excess ions in solution. Not only do additional cations occupy more binding sites, but the negative charge of the cell wall is partially neutralized. Attraction of free ions to the cell wall is diminished and the metal ion concentration gradient weakens. As the standard addition process continues, the unbound metal ion concentration increases and causes a decrease in the bound/unbound ratio. This behavior is commonly referred to as negative cooperative binding. Negative cooperativity is associated with a characteristic binding curve (Koshland and Hamadani 2002) and concave shape to the Scatchard plot (Alvarado, Klein et al. 2010). Negative cooperativity for cell-wall based metal binding has been suggested based on Hill plot analysis, but the expected curvature in the Scatchard plots was not observed in a previous report (Doyle, Matthews et al. 1980). Our data, obtained at lower metal ion concentrations, clearly demonstrate a concave curvature of the Scatchard plot. In this representation, the ordinate is a ratio of bound metal (micromoles per mg) over unbound metal concentration (micromoles per liter). Therefore the equilibrium concentrations determine the range of ordinate values required to find the association constant (K_A) and binding capacity (η). As described below, Scatchard plot analysis shows that metal chelation by the cell wall can be described with two distinct regions of equilibrium binding behavior. The designation of Region I and Region II are made from a Scatchard plot (Figure 2) which shows linear behavior and distinct slopes for each region. The first five data points of Figure 1 are not included in the Scatchard plot because the concentration of free ions in solution is negligible and thus the flame AA response is below the limit of quantification. Values for K_A and η are determined from the slope and x-intercept respectively. The correlation coefficient is improved if we exclude the data point in the transition between Regions I and II as shown in Figure 1 and Figure 2.

Table 1 is a compilation of K_A and η for Ca^{2+} and Mg^{2+} binding data. Equilibrium dialysis experiments were performed to evaluate the binding of Ca^{2+} to cell wall fragments of *B. subtilis* 1A578 containing both peptidoglycan and WTA. The K_A and η values were obtained from 6 trials, as presented in Table 2. The average binding capacity is 0.58 ± 0.07 $\mu\text{mol/mg}$ and 0.070 ± 0.04 $\mu\text{mol/mg}$ for Regions I and II, respectively. The average association constant is $1.0 \times 10^6 \pm 0.2 \times 10^6 \text{ M}^{-1}$ and $0.075 \times 10^6 \pm 0.058 \times 10^6 \text{ M}^{-1}$ for Regions I and II, respectively. The results of each trial show that K_A and η are sample independent in Region I, whereas values for the second stage of binding (Region II) are directly correlated with the bound / unbound range. Region I is characterized by higher

ranges of the bound/unbound ratio and binding in Region I is stronger than binding observed in Region II. Experiments using cell wall purified from strain 1A578 were repeated to evaluate Mg^{2+} binding. The binding affinity was $1.5 \times 10^6 \pm 0.2 \times 10^6 \text{ M}^{-1}$ for Region I and $0.17 \times 10^6 \pm 0.10 \times 10^6 \text{ M}^{-1}$ for Region II while a total binding capacity (represented by η_2) of $0.67 \pm 0.03 \text{ } \mu\text{mol Mg}^{2+}$ per mg of sample was measured.

A two tailed t-test was used to evaluate the statistical significance between the mean binding capacity and binding affinity of each metal ion. At a 95% confidence interval, the values of binding capacity (η) for both metals produced a t-value of 1.36 which was smaller than the critical t-value of 2.31. The smaller t-value suggests that a statistical difference in the binding capacities (η_2) cannot be accepted at a 95% confidence interval. However, for the binding affinities, there is a statistical difference between $K_A(\text{Ca}^{2+})$ and $K_A(\text{Mg}^{2+})$ for Region I only. We attribute the apparent higher affinity of Mg^{2+} to the higher sensitivity of atomic absorption measurements towards magnesium. Magnesium produces two usable wavelengths; one at 285.2 nm, for low concentrations, with a sensitivity of 0.007 ppm and one at 202.6 nm, for higher concentration samples, with a sensitivity down to 0.2 ppm. A wavelength of 422.7 nm is generally used for calcium. This wavelength produces a sensitivity in the 0.04 ppm range which is higher than that of the 285.2 nm wavelength for magnesium. As a result, we are able to observe lower Mg^{2+} concentrations. These lower concentrations correlate to higher bound/unbound values and an increase in the apparent binding affinity is observed. At an infinitesimal concentration, K_A would be significantly larger but unmeasurable.

Using TCA hydrolysis, we can separate WTA from peptidoglycan. At a buffered pH of 7.25, the metal binding capacity of the peptidoglycan increased and the metal binding capacity of the peptidoglycan with covalently bound WTA increased as well (Table 1). The affinity of metal to cell wall interactions in Region I remained similar at this higher pH. The increase in binding capacity is most likely a result of the diaminopimelic acid carboxyl functional groups becoming deprotonated above their pK_a value of 6.31 (Barkleit, Moll et al. 2009).

DISCUSSION

The observance of two distinct binding regions has not been demonstrated in previous studies of metal binding to the cell wall. Metal binding affinities have always been represented by a single value (Doyle, Matthews et al. 1980; Fein, Daughney et al. 1997) utilizing an experimental protocol where different samples were mixed with a single concentration of metal ions. Compared to literature reports (Doyle, Matthews et al. 1980), the binding affinity for Ca^{2+} in Region I ($1.0 \times 10^6 \pm 0.2 \times 10^6 \text{ M}^{-1}$) is 25 times larger than previously reported ($0.040 \times 10^6 \pm 0.008 \times 10^6 \text{ M}^{-1}$) for cell wall fragments containing both peptidoglycan and WTA. Additionally, Region II binding is 1.8 times larger than the previous report. The observation of Region I is important, as a misconception of weaker binding might arise if binding data is limited to only Region II. A similar trend is observed for Mg^{2+} binding to cell wall fragments. A binding affinity for Mg^{2+} in Region I is 28 times larger than previously reported ($0.054 \times 10^6 \pm 0.033 \times 10^6 \text{ M}^{-1}$) (Doyle, Matthews et al. 1980) for fragments of cell wall samples containing both peptidoglycan and WTA, whereas Region II binding is 3.1 times larger than the previous report.

As the cell wall gets close to saturation with metal ions, there is a gradual decrease in the slope of the Scatchard plot and thus a decrease in the apparent binding affinity. This decrease in K_A becomes more apparent as the equilibrium concentration increases (Figure 1). An increase in the equilibrium concentration causes a corresponding decrease in the bound/unbound ratio. Data taken from lower bound/unbound ratios will produce a line and slope that shows a weaker binding affinity. This can also be interpreted as a decrease in the electrostatic potential of cell resulting in a decreased affinity for metal ions. Data points were chosen for Region I based on limits of quantification and the point at which the curvature starts. Data points for Region II were chosen furthest away from the transition point at which the curvature begins in order to give the highest correlation coefficient.

The data in Table 2 show a variation in the bound/unbound ratio range for both Regions I and II for each trial. During the standard addition process, metal ions are added and become bound to the cell wall. After the initial group of ions is added, the unbound metal ion concentration is very low and undetectable. However, the equilibrium concentration required to produce a response depends on cell wall mass and varies between each trial. The difference between the bound/unbound ratios after each standard addition is observed in the two highest values in Figure 2. For Region II, the variation in the range of bound/unbound is less dramatic and a result of variation in the equilibrium concentration at the end of each experiment. A higher proportion of unbound metal ions in solution will cause the bound/unbound range to decrease when the cell wall sample reaches the saturation point for metal binding. Ideally, Scatchard plot analysis will provide a precise view of this equilibrium behavior independent of the bound/unbound range. The numerical values that describe the relationship between the bound and unbound metal ions should be very accurate. However, we cannot define a traditional equilibrium constant since it depends on the particulate size and electrostatic properties of each particulate. Measurements with *B. subtilis* sacculus (whole cell walls) produced a similar binding capacity of 0.62 $\mu\text{mol/mg}$ but a larger binding affinity is observed. In this sample a binding affinity of $4.5 \times 10^6 \text{ M}^{-1}$ was obtained for Region I and a binding affinity of $1.04 \times 10^6 \text{ M}^{-1}$ was obtained for Region II. The bound/unbound range was much higher than with the fragment samples. Nonetheless, equilibrium remains frustrated by hindered diffusion.

The large standard deviations in binding affinity can be attributed to a combination of diffusion effects and the dependence of the affinity constant, K_A , on concentration. The process of disrupting cells with a French Press creates a distribution of particle sizes and thus each trial is expected to have a different distribution of particle sizes. Peptidoglycan forms a three dimensional mesh where diffusion of metal ions through large fragments may be significantly different than small fragments. Within the distribution of fragments, each particulate will have a unique metal ion equilibrium between the interior and exterior of the cell wall fragment. Also affecting K_A is negative cooperativity of metal binding to the cell wall creates a situation where calculated values of K_A depend on the equilibrium concentration range. This is illustrated by the values for K_{A2} in Each trial (Table 2). The higher the bound/unbound region examined, a higher association constant is measured for Region II. The values of K_A for Region I are independent of the bound/unbound ratio.

Revising models for metal binding require a comparison to the previous literature. The dependence of the Region II K_A on the bound/unbound range may lie with electrostatic interactions between the cell wall, metal cations, and counter ions. The cell wall is a polyelectrolyte in terms of the peptidoglycan and teichoic acid components, which both exhibit a strong negative charge based on deprotonated carboxyl and phosphoryl functional groups near pH 7. The metal binding behavior of other polyelectrolytes has been investigated (Leroy and Guéron 1977; van den Hoop, Porasso et al. 2002). However, no studies have been directed towards the cell wall. Studies with two polyelectrolytes, RNA and humic acid, have exhibited similar binding behavior when compared with our cell wall samples. Humic acid is a natural organic polyelectrolyte containing multiple carboxyl and phenolate groups. Using voltammetry, it has been shown that the stability constant of the metal ion complex decreases with increasing metal ions concentration and decreases with increasing ionic strength values (van den Hoop, Porasso et al. 2002). Increasing Zn^{2+} concentration from 10^{-7} M to 3×10^{-6} M caused the stability constant to decrease from $5.01 \times 10^5 \text{ M}^{-1}$ to $1.26 \times 10^5 \text{ M}^{-1}$ (van den Hoop, Porasso et al. 2002). A gradual increase in the ionic strength of the solution from 0.001 M KNO_3 to 0.1 M KNO_3 with a constant Zn^{2+} concentration of 10^{-6} M caused a decrease in the observed stability constant from $3.16 \times 10^5 \text{ M}^{-1}$ to $7.94 \times 10^4 \text{ M}^{-1}$ (van den Hoop, Porasso et al. 2002). Additionally, just as there was little difference in the metal binding characteristics of Mg^{2+} and Ca^{2+} in our cell wall metal binding data, there was little difference in the metal binding characteristics of Zn^{2+} and Cd^{2+} (van den Hoop, Porasso et al. 2002). RNA has a poly(ribose phosphate) backbone similar to the poly(glycerol phosphate) backbone of teichoic acid. Mg^{2+} ions binding to RNA were also found to exhibit curvature on the Scatchard plot (Misra and Draper 1998). This behavior was explained as either the result of changing electrostatics, which would lead to negative cooperativity, or two classes of binding sites (Misra and Draper 1998). Class 1 is a group of strongly bound ions whereas class 2 is a group of more weakly bound ions that are responsible for the trailing region of the Scatchard plot (Misra and Draper 1998). As a result, a traditional equilibrium constant cannot be defined because diffuse ion binding is based on long range electrostatic interactions and does not follow the laws of mass action (Wyman and Gill 1990). Other studies into RNA have demonstrated that the ionic strength plays a huge role in the determination of binding affinity with RNA and metal ions, thereby providing evidence against the classification of two classes of binding sites (Leroy and Guéron 1977). Application of this electrostatic model at low sodium concentrations has shown to be a good fit to experimental data (Leroy and Guéron 1977). Electrostatic effects have been shown to cause a decrease in the degree of curvature of Scatchard plots, and subsequently a decrease in affinity, as the concentration of competing monovalent cations increases (Leroy and Guéron 1977). As a result, reported stability constants for metal binding to polyelectrolytes is directly dependent on a number of variables including ionic strength, temperature, and divalent metal ion concentration (Leroy and Guéron 1977; van den Hoop, Porasso et al. 2002). The dependence on ionic strength appears to have a greater influence on Region I in the Scatchard plot (commonly referenced as class 1 binding sites in other publications) (Leroy and Guéron 1977). Individual experiments at varying ionic strengths were not performed, although it is expected that the affinity within Region I would decrease as ionic strength increases, based on observations with other polyelectrolytes (Leroy and Guéron 1977). Electrostatic effects are less prominent when the electrostatic

potential of the cell wall has already been neutralized through the binding of cationic metal ions.

It has been reported that peptidoglycan contributes half of the cell's metal binding capacity by comparing the binding of both cell wall fragments (containing peptidoglycan with covalently bound WTA) and peptidoglycan alone (Matthews, Doyle et al. 1979). These results show that calcium has a binding constant of $25 \times 10^3 \text{ M}^{-1}$ and a binding capacity of $0.78 \text{ } \mu\text{mol/mg}$ for cell wall containing both peptidoglycan and WTA (Matthews, Doyle et al. 1979). Peptidoglycan alone was determined to have a binding constant of $18 \times 10^3 \text{ M}^{-1}$ and a binding capacity of $0.45 \text{ } \mu\text{mol/mg}$ (Matthews, Doyle et al. 1979). Although our binding constants are much larger for region I, the binding capacity data are similar to the Matthews *et al.* (Matthews, Doyle et al. 1979) report. For *B. subtilis* 1A578, peptidoglycan accounts for 47% of Ca^{2+} binding whereas it is 34% of Mg^{2+} binding. The apparent difference in binding capacities with Ca^{2+} and Mg^{2+} with peptidoglycan is not statistically different at a 95% confidence interval. The observance of calcium binding to peptidoglycan with values similar to that of Mg^{2+} is in contrast to previous reports that teichoic acids were solely responsible for Ca^{2+} binding to the cell wall (Beveridge and Murray 1980). A strong dependence of metal binding to the electrostatic properties of the cell is not expected to show complete specificity for or against certain divalent cations.

Based on a statistical t-test analysis, we do not observe an obvious difference between the affinities or binding capacities of specific metal ions to the cell wall. In a subsequent study by Doyle *et al.* (Doyle, Matthews et al. 1980), Ca^{2+} , Mn^{2+} , Ni^{2+} , Sr^{2+} , Zn^{2+} , and Mg^{2+} were reported to have binding constants of 40 ± 8 , 52 ± 11 , 33 ± 11 , 36 ± 11 , 39 ± 7 , and 54 ± 33 respectively (which are $\times 10^3 \text{ M}^{-1}$). Likewise the binding capacities of all divalent metal ions appeared to be highly similar with values of 0.75 ± 0.15 , 0.74 ± 0.16 , 0.64 ± 0.20 , 0.86 ± 0.26 , 0.97 ± 0.16 , and 0.91 ± 0.54 (in units of $\mu\text{mol/mg}$) for Ca^{2+} , Mn^{2+} , Ni^{2+} , Sr^{2+} , Zn^{2+} , and Mg^{2+} respectively (Doyle, Matthews et al. 1980). Without knowing how many samples were taken to obtain the standard deviations reported, it is impossible to perform any statistical tests on the sets of data. Nonetheless, similarity of values for divalent metal ions, coupled with large standard deviations, show that there is little preference in binding affinity or capacity between divalent cations.

Initially, experiments were performed at a measured pH of 5.65, which was the pH of the distilled deionized Milli-Q water due to dissolved carbon dioxide (Bradfield 1942). Additional experiments were performed with a low concentration of HEPES buffer, 0.001 M, at an adjusted pH of 7.25 in order to examine effects of pH on the binding constant and binding capacity of Mg^{2+} . The binding capacity increases because there are more binding sites. This makes sense for both Regions I and II because of binding to the diaminopimelic acid carboxyl group. Increasing pH with NaOH creates competition for the binding site between Na^+ and Mg^{2+} and lowers the observed K_A . However, Mg^{2+} will overwhelm Na^+ binding and preferentially replaces the Na^+ due to its higher charge density, leading to a higher binding capacity. There appears to be a change in the K_A value for Region I and II when the pH is increased from 5.65 to a buffered pH of 7.25. We attribute the decrease in affinity constant for Region I to the minute addition of NaOH to adjust the pH to 7.25. The slightly higher ionic strength is expected to cause a decrease in the electrostatic potential of

the cell wall (Barkleit, Moll et al. 2009). However, we observe an increase in K_A for Region II though this increase is not statistically different between pH 5.65 and 7.25.

The two regions of binding affinity in the Scatchard plot are sometimes described as two classes of binding sites. The purified cell wall does contain two types of binding sites (carboxyl and phosphoryl groups). However, two regions of binding affinity are also seen for peptidoglycan purified from 1A578 after TCA hydrolysis. A two site model predicts similar capacities for Region I between the two samples, which is not observed. Rather, the binding capacities of Region I and II are best interpreted by electrostatic effects but we cannot attribute these two regions to specific functional group.

Conclusion

In these experiments, we measured the binding constants and binding capacities of metal ions (Ca^{2+} and Mg^{2+}) with purified cell wall fragments of *B. subtilis* containing either peptidoglycan or peptidoglycan with covalently attached WTA. We found much higher metal ion stability constants than previously reported. We have shown metal binding capacities similar to those reported by Doyle *et al* (Doyle, Matthews et al. 1980) and no significant difference between the two ions tested (Ca^{2+} and Mg^{2+}). This is in contrast to results of binding capacity published by Beveridge. (Beveridge and Murray 1980) that showed binding capacities of 0.399 $\mu\text{mol/mg}$ for Ca^{2+} and 8.226 μmol for Mg^{2+} , while also showing that teichoic acids were solely responsible for Ca^{2+} binding to the cell wall. Metal binding affinity values were found to depend on the amount of metal bound to the sample based on electrostatic effects. For the conditions used in these experiments, a binding constant much higher than previously reported was obtained.

Our data enables an updated model of metal binding, which appears to be a largely electrostatic phenomenon at low ionic strengths. This model should be able to explain how bacteria can grow in a variety of conditions, such as the amount of divalent metal ions present. At low ionic strength, we show that the cell wall has a strong affinity for metal ions in solution. This property can be part of a survival mechanism of the cell to capture important bioactive divalent metal ions and sequester them in the cell wall. Metal ions are required for cell wall integrity and binding to peptidoglycan provides this stability, yet metals associated with the cell wall also provide a reservoir from which the cytoplasm can draw metals when extracellular metals are scarce. Initial metal binding events are very strong and eventually transition to weaker binding as metals become abundant in the cell wall. Weaker binding suggests that metals can return to the extracellular fluid, but these metals can also traverse the cell wall and enter the cytoplasm (Figure 3). When metals are abundant, these weakly interacting cations are harnessed for biochemical processes without the need to extricate metals bound to peptidoglycan.

The data in Region I reveal the maximum amount of metal ions that are bound to this particular region. However, we are reluctant to assign an exact chemical interaction because binding is still occurring at Region II. There is no evidence to suggest that these binding events occur independently, it is possible that the initial binding events cause a reorganization of the cell wall and teichoic acid architecture to expose additional binding

sites. It is also possible that heterogeneous cell fragments with an array of metal binding groups from the carboxyl groups associated with D-alanine, D-glutamate, diaminopimelic acid, and phosphate groups associated with teichoic acid provide distinct binding sites that compose the overall binding affinity. In the low ionic strengths of our experiments, these distinct changes would be difficult to distinguish due to the large electrostatic contribution of each group towards binding. It is also likely that the chemical nature of the binding site is described by a combination of carboxylic acid and phosphate groups acting in concert to form a binding pocket.

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FIGURE 1.

The cell wall binds most Ca^{2+} ions during an initial metal uptake phase, after which metal binding slows. These two regions of the binding curve were used to obtain binding constants. The equilibrium concentration was determined from the concentration of Ca^{2+} ions outside of the dialysis membrane. In this particular sample, the first four points on the binding curve correspond to the same measured concentration of $5.89 \times 10^{-7} \text{ M}^{-1}$, which also correlate to an absorbance value of 0.001. These points were taken at a value near the limit of quantification and were not used in the analysis. Due to an apparent curvature in the Scatchard plot, indicative of negative cooperative binding, two bind regions are examined as presented by the dotted and solid selections. The dotted selection indicates a higher affinity region. As more metal ions become bound to the cell wall, a lower binding stability constant can be extracted with the data points in the solid circle.

Scatchard Plot for the Binding of Ca^{2+} to 1A578 Cell Walls

FIGURE 2.

A linear transformation of the binding data provides the binding affinity (denoted by the negative value of the slope) and the binding capacity (denoted by the x-intercept). From this Scatchard plot analysis, a binding constant of $1135 \times 10^3 \text{ M}^{-1}$ is obtained from the points selected with a dotted line and a lower affinity constant of $43 \times 10^3 \text{ M}^{-1}$ is obtained from the points selected with solid line. The binding constant changes as more metal ions become bound to the sample, a property of negative cooperativity resulting from electrostatic effects in the cell wall samples. Hill plots of samples (not shown) have also pointed towards negative cooperative binding effects of the cell wall.

FIGURE 3.

A model for the binding of Mg^{2+} ions to the cell wall of *B. subtilis* bacteria. The binding sites may compose functional groups from peptidoglycan, teichoic acid, or both. We are able to determine that two different interactions occur, a strong binding that could be for cell wall strength and a weaker interaction that enables metals to easily reach the cell cytoplasm.

Table 1
Metal Binding Properties of *B. subtilis* purified cell wall components at T=4°C

Composite data table showing the metal binding properties for each sample

Type of Sample	Metal Ion	pH	Region I		Region II	
			η ($\mu\text{mol/mg}$)	$K_{\text{assoc.}}$ (M^{-1})	η ($\mu\text{mol/mg}$)	$K_{\text{assoc.}}$ (M^{-1})
IA578 (Peptidoglycan + WTA)	Ca^{2+}	5.65	0.58 ± 0.07	$(1.0 \pm 0.2) \times 10^6$	0.70 ± 0.04	$(0.075 \pm 0.058) \times 10^6$
IA578 (Purified Peptidoglycan)	Ca^{2+}	5.65	0.27 ± 0.05	$(1.0 \pm 0.3) \times 10^6$	0.33 ± 0.04	$(0.080 \pm 0.084) \times 10^6$
IA578 (Peptidoglycan + WTA)	Mg^{2+}	5.65	0.50 ± 0.10	$(1.5 \pm 0.1) \times 10^6$	0.67 ± 0.03	$(0.17 \pm 0.10) \times 10^6$
IA578 (Purified Peptidoglycan)	Mg^{2+}	5.65	0.19 ± 0.05	$(0.7 \pm 0.5) \times 10^6$	0.23 ± 0.04	$(0.098 \pm 0.024) \times 10^6$
IA578 (Peptidoglycan + WTA)	Mg^{2+}	7.25	0.63 ± 0.08	$(1.0 \pm 0.2) \times 10^6$	0.77 ± 0.07	$(0.26 \pm 0.12) \times 10^6$

Table 2
Metal Binding Properties of *B. subtilis* (1A578) with Ca^{2+} at pH 5.65 and $T=4^{\circ}\text{C}$

Individual trials of the metal binding properties of *B. subtilis* (1A578) with Ca^{2+}

Trial	Mass (mg)	η ($\mu\text{mol}/\text{mg}$) (Region II)	η ($\mu\text{mol}/\text{mg}$) (Region I)	Bound/Unbound range $\times 10^3$ (Region I) ^a	$K_{\text{assoc.}}$ (M^{-1}) (Region I)	Bound/Unbound range $\times 10^3$ (Region II) ^a	$K_{\text{assoc.}}$ (M^{-1}) (Region II)
#1	35.1	0.739	0.659	231–29.4	1.180×10^6	13.2–6.54	0.172×10^6
#2	34.7	0.666	0.628	146–25.9	0.660×10^6	4.41–2.67	0.031×10^6
#3	30.2	0.756	0.512	166–6.14	1.098×10^6	4.71–3.54	0.043×10^6
#4	32.4	0.681	0.633	224–14.8	0.955×10^6	7.02–3.75	0.121×10^6
#5	30.3	0.685	0.529	149–17.1	1.039×10^6	7.64–4.79	0.047×10^6
#6	11.7	0.645	0.502	84.9–5.61	1.160×10^6	5.61–2.96	0.035×10^6
Average:		0.70	0.58		1.0×10^6		0.075×10^6
Std. Dev. :		0.04	0.07		0.2×10^6		0.058×10^6

^aThe ratio of bound ions ($\mu\text{mol}/\text{mg}$) over the unbound ion concentration ($\mu\text{mol}/\text{L}$) decreases as the amount of free ions increases at later stages of the standard addition procedure. Size effects of cell wall fragment heterogeneity causes variation in these values. These are values on the Scatchard plot denoting regions of different equilibrium behavior.