

Surface Charge Properties of and Cu(II) Adsorption by Spores of the Marine *Bacillus* sp. Strain SG-1

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Received 12 February 1997/Accepted 12 December 1997

Spores of marine *Bacillus* sp. strain SG-1 are capable of oxidizing Mn(II) and Co(II), which results in the precipitation of Mn(III, IV) and Co(III) oxides and hydroxides on the spore surface. The spores also bind other heavy metals; however, little is known about the mechanism and capacity of this metal binding. In this study the characteristics of the spore surface and Cu(II) adsorption to this surface were investigated. The specific surface area of wet SG-1 spores was 74.7 m² per g of dry weight as measured by the methylene blue adsorption method. This surface area is 11-fold greater than the surface area of dried spores, as determined with an N₂ adsorption surface area analyzer or as calculated from the spore dimensions, suggesting that the spore surface is porous. The surface exchange capacity as measured by the proton exchange method was found to be 30.6 μmol m⁻², which is equal to a surface site density of 18.3 sites nm⁻². The SG-1 spore surface charge characteristics were obtained from acid-base titration data. The surface charge density varied with pH, and the zero point of charge was pH 4.5. The titration curves suggest that the spore surface is dominated by negatively charged sites that are largely carboxylate groups but also phosphate groups. Copper adsorption by SG-1 spores was rapid and complete within minutes. The spores exhibited a high affinity for Cu(II). The amounts of copper adsorbed increased from negligible at pH 3 to maximum levels at pH >6. Their great surface area, site density, and affinity give SG-1 spores a high capability for binding metals on their surfaces, as demonstrated by our experiments with Cu(II).

Divalent manganese [Mn(II)] oxidation and subsequent formation of Mn (hydr)oxides are major controls on the speciation of Mn in sediments, soils, and natural waters. Mn (hydr)oxides are very reactive components of natural environments and affect the fate, transport, and bioavailability of a variety of heavy metals and organic compounds (6). Abiotic Mn(II) oxidation is a thermodynamically favorable but kinetically slow process under most natural conditions. In the environment, however, Mn(II) oxidation is believed to be largely due to the activities of microorganisms that catalyze the reaction (22) and increase the rate of Mn(II) oxidation by up to 5 orders of magnitude compared to abiotic Mn(II) oxidation (22, 37). For example, the Mn(II) oxidation rate near the sediment surface in a eutrophic lake calculated from a 4-year record of sediment trap data showed a distinct seasonal pattern, with maxima of up to 2.8 mmol m⁻² day⁻¹ during the summer (46). The average half-life of Mn(II) during stagnation in the summer was 1.4 days. This oxidation rate cannot be explained with the available knowledge concerning abiotic surface catalysis but is within the range typical of microbiological oxidation rates.

A wide variety of bacteria have been reported to oxidize Mn(II) in fresh and marine waters, soils, and sediments (10). Marine *Bacillus* sp. strain SG-1, which was isolated from a near-shore sediment enrichment culture, is one bacterium that has been extensively studied in terms of Mn(II) and Co(II) oxidation (40). In this bacterium, it is the spores that catalyze Mn(II) and Co(II) oxidation (19, 29) under environmentally relevant pH, temperature, and metal concentration conditions. The spores also bind a variety of other heavy metals, such as

Cd and Zn (38). Mn(II)-oxidizing activity is located on the spore coats and apparently is catalyzed by a protein (7, 42, 45). Recent evidence suggests that a spore surface protein related to the multicopper oxidase family of proteins is involved in Mn(II) oxidation (45). Consistent with this idea, low amounts of Cu(II) stimulate Mn(II) oxidation (45).

Although Mn(II) oxidation appears to be protein catalyzed, the mechanism of oxidation and precipitation of Mn(II) and Co(II) is not understood well. The process is envisaged as having two steps, binding and oxidation-precipitation, based on the observation that the precipitated Mn is generally found outside the cell (39). Soluble Mn(II) ions first bind to the negative sites on the spore surface; the bound ions are then enzymatically oxidized to Mn(III, IV), which precipitates as Mn (hydr)oxide solid phases. This two-step process is believed to be closely related to the surface charge properties of the spores.

In order to better understand heavy metal binding and the possible role of copper in Mn(II) oxidation by SG-1 spores, information about the characteristics of the surface charge and metal adsorption behavior of SG-1 spores is needed. In this study, we initiated investigations to examine the interactions of Cu(II) with SG-1 spores. The surface of SG-1 spores was first characterized in terms of surface area, surface site density, and surface charge. Cu(II) adsorption by SG-1 spores was investigated to quantify adsorption kinetics, adsorption capacity, and the effect of pH.

MATERIALS AND METHODS

Unless indicated otherwise, all reagents used in this study were analytical grade or better. All vessels and storage containers were polyethylene or polycarbonate and were acid washed prior to use. Solutions were made with water purified by passage through a Milli-Q water system.

Preparation, purification, and pretreatment of SG-1 spores. *Bacillus* sp. strain SG-1 was grown and maintained in K medium. K medium contains (per liter of 75% seawater) 2 g of peptone and 0.5 g of yeast extract. After autoclaving,

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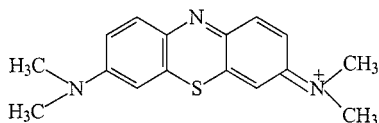


FIG. 1. Molecular structure of methylene blue.

20 ml of 1 M filter-sterilized *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES) buffer (pH 7.4 to 7.8) and 0.1 ml of 1 M filter-sterilized MnCl_2 were added per liter. For agar plates, 15 g of agar per liter was added before autoclaving.

SG-1 cultures were grown at room temperature in 1 liter of K medium in 2-liter flasks placed on a rotary shaker at 150 rpm. Usually more than 95% of the bacteria produced endospores within 5 days. Fully sporulated cultures consisted of spore clumps and few individual spores, as observed by phase-contrast microscopy. Spores were harvested by centrifugation at $16,000 \times g$ and 4°C for 10 min and were purified to remove precipitated Mn oxides and adsorbed trace metals from the spore surface as follows. Spore pellets were washed with water by using a Teflon tissue homogenizer and recollected by centrifugation. Spores were then homogenized and treated with 0.01 M ascorbic acid with shaking for 10 min, washed three times with 1 M NaNO_3 -0.01 M EDTA, and finally washed five times with Milli-Q water. Unless indicated otherwise, prior to the ascorbic acid treatment spores were treated with 2% glutaraldehyde for 2 h at 37°C (29, 30) to prevent spore germination that would have resulted in changes in resistance and could have caused trace metal release. The glutaraldehyde-treated spores still possessed the ability to oxidize Mn(II), as observed in previous experiments (29). After homogenization, spores were stored in Milli-Q water at 4°C ; spores in the suspensions usually formed aggregates. The number of spores in a suspension was determined by direct counting with a Petroff-Hausser counting chamber (Arthur H. Thomas Co., Philadelphia, Pa.) after passage through a French pressure cell at $8,000 \text{ lb/in}^2$ to disperse the clumps. A total of 44 small squares were counted. The average number of spores in each small square was 23.4, and the standard deviation was 6.8.

Surface area. (i) BET method. The purified spore suspension was freeze-dried, and the dried spores were stored in a desiccator until they were used. The specific spore surface area was measured with a model NOVA-1000 BET surface area analyzer. The dried spores were first degassed by evacuation for 2 h at 80°C , and the surface area was then determined by using five datum points.

(ii) Dimension measurement. Spore surface area was calculated from spore dimensions obtained by scanning electron microscopy. The lengths and widths of 30 individual spores were measured, and the surface area (S_a) was calculated with the following equation by assuming that each spore is a perfect prolate ellipsoid of revolution (5):

$$S_a = 2\pi b^2 + \frac{2\pi a^2 b}{\sqrt{a^2 - b^2}} \sin^{-1} \frac{\sqrt{a^2 - b^2}}{a} \quad (1)$$

where a and b are the semimajor and semiminor axes, respectively. The volume of a spore (V) was obtained by using the following equation:

$$V = 4\pi ab^2/3 \quad (2)$$

(iii) Methylene blue adsorption method. The methylene blue adsorption technique has been used to successfully measure the surface areas of kaolinite, illite, and montmorillonite (25). Methylene blue has the molecular structure shown in Fig. 1. This molecule can be regarded as an approximately rectangular box with dimensions of 1.7 by 0.76 by 0.325 nm (25), and the projected areas of the molecule surfaces are 1.3, 0.55, and 0.25 nm^2 . The methylene blue used in this study (Aldrich Chemical Co., Inc., Milwaukee, Wis.) has a molecular weight of 373.9, which corresponds to the molecular weight of the methylene blue hydrochloride with three H_2O molecules. The methylene blue adsorption experiments were conducted by using concentrations of $<7 \mu\text{M}$ since methylene blue molecules form dimers when the methylene blue concentration exceeds $7 \mu\text{M}$ (4).

A spore suspension containing 10^7 spores per ml was prepared and adjusted to pH 8.5. Aliquots (40 ml) were placed into 50-ml centrifuge tubes. A stock solution of methylene blue was added to give total methylene blue concentrations ranging from 0.5 to $7 \mu\text{M}$. The volumes of methylene blue stock solution added ranged from 0.01 to 0.28 ml. Each suspension was then shaken continuously on a rotary shaker (100 rpm). The adsorption kinetics of methylene blue indicated that the adsorption reached equilibrium in 4 h. Each spore suspension was centrifuged for 10 min at $16,000 \times g$, and the supernatant was analyzed to determine the amount of methylene blue remaining. From the amount of methylene blue retained in solution, the quantity adsorbed was determined. To obtain a calibration curve, a blank experiment without spores was also conducted at the same time by using the same concentrations of methylene blue that were used for the spore suspensions. The concentration of methylene blue after adsorption by spores was measured spectrophotometrically at a wavelength of 661 nm. Solutions containing 0.5 to $7 \mu\text{M}$ methylene blue gave A_{661} values of 0.05 to 0.7.

During adsorption of methylene blue it is assumed that complete monolayer

coverage has occurred when the isotherm reaches a plateau. The monolayer adsorption value can be obtained by fitting the data to the Langmuir equation:

$$\Gamma = \frac{K\Gamma_m C_{\text{eq}}}{1 + KC_{\text{eq}}} \quad (3)$$

where Γ is the amount of adsorbed methylene blue, C_{eq} is the equilibrium concentration of methylene blue, K is a coefficient related to adsorption energy (affinity), and Γ_m is the monolayer adsorption capacity. The amount of methylene blue adsorbed as a monolayer can then be related to the spore surface area (S) (in square micrometers) by the following equation:

$$S = \Gamma_m N_A \sigma \quad (4)$$

where Γ_m is the monolayer amount of dye adsorbed (in moles per spore), N_A is Avogadro's number (6.02×10^{23} molecules mol^{-1}), and σ is the area of the methylene blue molecule, which is $0.55 \times 10^{-18} \text{ m}^2$ when maximum monolayer adsorption occurs (25).

Surface site density. Proton exchange was used to measure the surface site density of SG-1 spores (33). Duplicate titrations were performed as follows. A 100-ml spore suspension in 0.01 M NaNO_3 was placed into a 120-ml glass bottle, and nitrogen gas was bubbled through the preparation for 30 min to remove CO_2 prior to titration. The suspension was acidified to pH 3.5 with 0.1 M HNO_3 (standardized; Aldrich), and the suspension was then titrated to pH 8.5 with 0.1 M NaOH (standardized; Aldrich). A preliminary experiment indicated that the spores did not break down at the pH extremes used in the titrations. After 30 min of equilibration with N_2 purged at pH 8.5, spores were removed by filtration by using $0.2\text{-}\mu\text{m}$ -pore-size prewashed 47-mm-diameter Nuclepore polycarbonate membrane filters. The pH of the filtrate did not change after filtration. The filtrate was titrated back to pH 3.5 with 0.1 M HNO_3 . The difference between the number of moles of NaOH (m_{NaOH}) necessary to raise the pH to 8.5 and the number of moles of HNO_3 (m_{HNO_3}) necessary to restore the pH of the filtrate to pH 3.5 is an estimate of the number of moles of surface sites (H^+_{sur}):

$$\text{H}^+_{\text{sur}} = m_{\text{NaOH}} - m_{\text{HNO}_3} \quad (5)$$

This technique may underestimate the absolute concentration of surface sites due to charging effects (6).

Surface charge. The surface charge was measured as a function of pH by acid-base titration (47). A 100-ml spore suspension in 0.01 M NaNO_3 (10^8 spores ml^{-1}) was used, and nitrogen gas ($>99.95\%$ pure) was bubbled through the preparation for 30 min to remove CO_2 prior to titrations. Standardized 0.1 M NaOH was added to the spore suspension to bring the pH up to ~ 8.5 , and the suspension was equilibrated for an additional 20 min. The suspension was then titrated with standardized 0.01, 0.1, and 1.0 M HNO_3 solutions. The points in the titration curve were determined after the pH stabilized (usually after 2 to 4 min). Titrations were duplicated. In this one-sample titration, subtraction of the amount of base added (n_B) (in moles) from the amount of acid added (n_A) (in moles) yielded the net amount (Δn) of base or acid added (in moles):

$$\Delta n = n_A - n_B \quad (6)$$

When Δn is negative, it is the net amount of base added. When Δn is zero, the amount of base added equals the amount of acid added and there is no net addition of acid or base. When Δn is positive, it is the net amount of acid added. An acid-base titration curve was obtained by plotting pH versus net addition (Δn) of acid or base (in moles).

The surface charge density (σ_0) (in moles per square meter) can be calculated from the titration curve by the following expression:

$$\sigma_0 = (\Delta n - n_{\text{H}} + n_{\text{OH}})/S \quad (7)$$

where S is the total spore surface area (in square meters) and n_{H} and n_{OH} are the numbers of moles of H^+ and OH^- in the suspension at a measured pH, respectively. A detailed derivation of equation 7 has been given by Schulthess and Sparks (31). The values of n_{H} and n_{OH} are given by the following equations:

$$n_{\text{H}} = V10^{-\text{pH}}/\gamma \quad (8)$$

$$n_{\text{OH}} = V10^{\text{pH}-14}/\gamma \quad (9)$$

where V is the volume of the suspension and γ is the activity coefficient that may be calculated from the Davies equation:

$$\log \gamma = -0.512Z^2 \left(\frac{\sqrt{I}}{1 + \sqrt{I}} - 0.3I \right) \quad (10)$$

where Z is ionic valence and I is ionic strength given by:

$$I = \frac{1}{2} \sum C_i Z_i^2 \quad (11)$$

where C_i is the concentration of the i th ion.

Cu(II) adsorption kinetics. A 100-ml spore suspension in 0.01 M NaNO_3 was placed into a 125-ml flask. The suspension was stirred with a Teflon-coated

magnetic stirrer. A Cu(II) stock solution [$\text{Cu}(\text{NO}_3)_2$] was added to the suspension to start the experiment. Aliquots (2 ml) were removed at various times by using 5-ml syringes with needles. Each sample was filtered through a 0.45- μm -pore-size Gelman type GHP acrodisc filter, and the filtrate was collected in 3-ml Wheaton polypropylene vials, stored in a 4°C cold room, and analyzed within 2 days. A control (blank) experiment indicated that filtration did not affect the Cu concentration in the filtrate.

Cu(II) adsorption isotherm. A plot of the concentration (in moles per liter) of adsorbate in the supernatant solution (or filtrate) versus the amount adsorbed (in moles per gram or moles per square meter) by a solid at a fixed temperature and applied pressure is an adsorption isotherm. If the adsorption of a metal follows the Langmuir equation (equation 3), the adsorption capacity and affinity can be calculated. The Cu(II) adsorption isotherm for SG-1 spores was obtained by the following procedures. A spore suspension in 0.01 M NaNO_3 was adjusted to the desired pH, and 20-ml aliquots were placed in 50-ml centrifuge tubes. A copper stock solution was then added to the suspensions so that the Cu(II) concentrations in the suspensions varied over 2 orders of magnitude (0.3 to 30 μM). Each suspension was shaken to reach equilibrium; a shaking time of 2 h was determined to be appropriate from the adsorption kinetics described above. The suspension was centrifuged and filtered through 0.45- μm -pore-size acrodisc filters.

Distribution coefficients can be used to describe the efficiency of removal of trace elements from solutions by solids. The distribution coefficient (K_d) for Cu(II) sorbed by SG-1 spores may be calculated by the following equation:

$$K_d = \Gamma / C_{\text{eq}} \quad (12)$$

where Γ is the amount of Cu(II) sorbed (in millimoles per gram) and C_{eq} is the equilibrium concentration of Cu(II) in solution (in moles per liter). Thus, K_d has units of milliliters per gram. At low concentrations, K_d is related to K (a measure of affinity) and may be expressed as follows:

$$K_d = \Gamma_m / K \quad (13)$$

where Γ_m and K can be obtained from equation 3.

Cu(II) adsorption as a function of pH. Copper(II) adsorption experiments were carried out in batch systems to determine adsorption edges [percentages of Cu(II) adsorbed as a function of solution pH] (13, 14). Portions (20-ml) of a spore suspension in 0.01 M NaNO_3 were placed in 50-ml centrifuge tubes, and an equal amount of Cu(II) stock solution was added to each tube. After the Cu(II) solution was added, the suspensions were adjusted to the desired pH values by using 0.1 M HNO_3 or 0.1 M NaOH (the total volume was changed by less than 2%). The changes in ionic strength due to acid or base additions were less than 0.003 M. The treated suspensions were shaken for 2 h at 150 rpm. The pH of each suspension was determined with a pH meter and a Ross combination electrode. The samples were centrifuged, the supernatants were filtered, and the filtrates were analyzed for Cu as described below. The exact volume of solution in each tube was calculated from the volume of the suspension, the volume of Cu(II) stock solution added, the volume of acid or base added, and the volume of spores in suspension.

Dissolved Cu analysis. Dissolved Cu concentrations were measured by graphite furnace atomic absorption spectrophotometry performed with a Perkin-Elmer model Zeeman 5000 spectrophotometer equipped with a model HGA 400 programmable graphite furnace and a model AS 40 autosampler. Standard metal solutions were prepared from SPEX plasma standards and contained 0.01 M NaNO_3 . The external standard calibration method was used in the measurements. Calibration curves were obtained by using three standards that covered the concentrations of the samples. Three absorbance values were averaged to obtain values for standards. The standard calibration was repeated every 10 samples. The Zeeman effect background correction was used to significantly improve the signal-to-noise ratio. Samples were analyzed with pyrolytically coated graphite tubes equipped with L'vov platforms. The graphite furnace system was set up with a sample volume of 20 μl and six steps consisting of time-temperature sequences. All of the samples (in polypropylene autosampler cups) were analyzed in duplicate.

RESULTS

Spore surface area. The specific surface area of freeze-dried SG-1 spores determined with a BET surface area analyzer was 6.86 $\text{m}^2 \text{g}^{-1}$, which is similar to the surface area (7 $\text{m}^2 \text{g}^{-1}$) obtained for *Bacillus subtilis* spores (23) and slightly higher than the value (5.04 $\text{m}^2 \text{g}^{-1}$) reported by Berlin et al. (5), who used the same technique.

The individual spore sizes, as determined by scanning electron microscopy, were 1.26 ± 0.13 by 0.60 ± 0.09 μm . The spore surface area calculated from the spore dimensions was 2.02 μm^2 per spore, and the spore volume was 0.237 μm^3 per spore. The ratio of surface area to spore volume was 8.5. The number of spores per gram of dried spores was calculated by

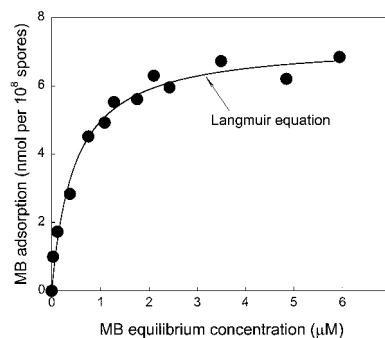


FIG. 2. Methylene blue adsorption isotherm obtained with SG-1 spores (10^7 spores ml^{-1} , pH 8.5). The curve was obtained by fitting the data to the Langmuir equation. MB, methylene blue.

using a previously published value for average spore density of 1.36 g cm^{-3} (5). The value obtained was 3.103×10^{12} spores g^{-1} . From the values presented above, the specific surface area of SG-1 spores was calculated to be 6.27 $\text{m}^2 \text{g}^{-1}$, which is comparable to the value determined with the BET surface area analyzer. A similar value (6 $\text{m}^2 \text{g}^{-1}$) was also calculated for *Bacillus* spores by Neihof et al. (23). Since the specific surface area was small, we postulated that there is no external porosity and the spore surface is smooth (5).

Figure 2 shows a plot of the amounts of methylene blue adsorbed by SG-1 spores versus the equilibrium concentrations of methylene blue. The surface area of spores was calculated from the monolayer adsorption value that was obtained by fitting the data to the Langmuir equation (equation 3). The methylene blue monolayer adsorption capacity was estimated to be 7.27 nmol per 10^8 spores (Fig. 2). As described previously, when the maximum adsorption of methylene blue by clay minerals is reached, the surface area of a methylene blue molecule is 0.55 nm^2 (25). If it is assumed that this value is applicable to the spore-water system, the spore surface area is 24.1 μm^2 per spore, which corresponds to a specific surface area of 74.7 m^2 per g of dry weight and a ratio of spore surface area to spore volume of ~ 100 . Because we believe that the wet spore surface area measured by methylene blue adsorption is the best estimate of the true spore surface area (see below), this value was used for all calculations of site density, charge density, and adsorption capacity presented in this paper.

Spore surface charge. The surface exchange capacity of SG-1 spores as determined by the proton exchange technique was 30.6 $\mu\text{mol m}^{-2}$ (2.29 mmol per g of dry spores), which is equivalent to 18.3 sites nm^{-2} . The acid-base titration data for the spore suspension in 0.01 M NaNO_3 are presented in Fig. 3. The surface charge density (σ_0) as calculated by equation 5 is shown in Fig. 4. The zero point of charge (ZPC) is pH 4.5, which is identical to the ZPC of the cell wall of a gram-positive bacterium (28). Nearly all Fe and Al (hydr)oxides have ZPC values of pH >8 , while the ZPC of birnessite ($\delta\text{-MnO}_2$) is approximately pH 2.2 and the ZPC of kaolinite is pH 4.6 (47). Powder materials with low ZPCs should exhibit high capacities for binding metals at low pH values.

At pH >4.5 , negatively charged sites predominate (σ_0 is negative), and the maximum σ_0 is 27.3 $\mu\text{mol m}^{-2}$ (2.04 mmol g^{-1}) at pH 8.59, which is comparable to the surface exchange capacity (30.6 $\mu\text{mol m}^{-2}$ at pH 8.50) determined by the proton exchange method. At pH <4.5 , the net surface charge is positive, but the positive charge density at pH 2.5, 5 $\mu\text{mol m}^{-2}$ (0.37 mmol g^{-1}), is much lower than the negative charge density, indicating that fewer positively charged sites exist on the

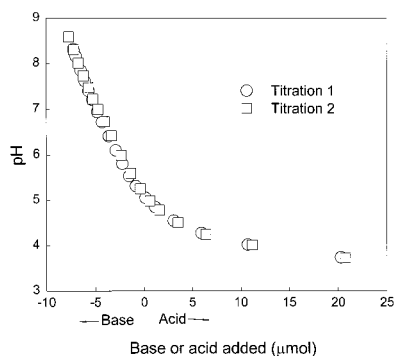


FIG. 3. Acid-base titration of a spore suspension (10^8 spores ml^{-1} , 0.01 M NaNO_3).

spore surface. Plette et al. (28) reported that the σ_0 for a soil bacterium changed from $+0.25$ to -0.75 mmol per g of cell walls in the pH range from 3 to 10.

Mineral ions may leach from spores during acid-base titration processes, particularly at low pH. Mineral leaching should not have interfered with the results of surface site density determinations since we used the back-titration method, which takes into account all ions, such as leached metals that consume H^+ ions (31). On the other hand, since back-titration was not used for surface charge determinations, mineral leaching may affect the surface charge results via metal leaching at low pH or through precipitation and hydrolysis of the leached metals at high pH. However, because the concentrations of metal leached over the pH range used in the experiments were low, the released mineral ions should not have significantly influenced the surface charge results reported here.

Cu(II) adsorption by spores. The Cu(II) adsorption reaction with spores was rapid, and more than 60% of the Cu(II) was sequestered from the solution at pH 7.2 within the first 1 min (Fig. 5). In many biosorption systems, most of the metal sorption occurs within 5 to 15 min after solid-liquid contact (44). The Cu(II) adsorption curve for glutaraldehyde-treated spores leveled off within 10 min, whereas Cu(II) adsorption by the untreated spores reached its highest value in minutes and then decreased gradually with time. The difference in Cu(II) adsorption kinetics between the treated and untreated spores may be attributed to spore germination because it was found that the concentrations of dissolved Mn and Zn increased with time in the untreated spore suspensions but not the glutaraldehyde-fixed spore suspensions (data not shown). Although release of Mn and Zn is not the best indicator of germination, there was greater release of these metals and lower adsorption

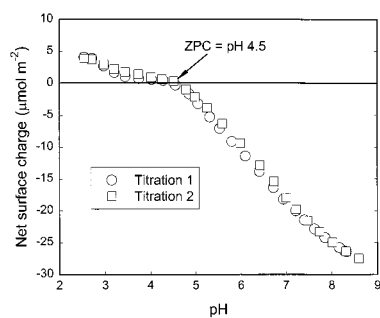


FIG. 4. Net surface charge of SG-1 spores as a function of pH. SG-1 spores have a ZPC of pH 4.5.

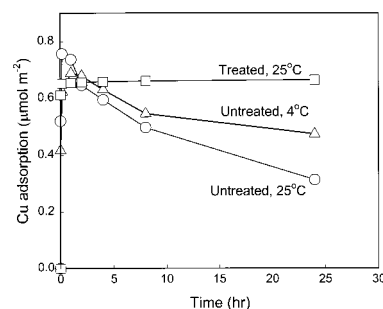


FIG. 5. Kinetics of adsorption of Cu(II) by SG-1 spores that were treated with 2% glutaraldehyde or not treated at 4 and 25°C [10^8 spores ml^{-1} , 2 mM Cu(II) , 0.01 M NaNO_3 , pH 7.2].

of Cu(II) by the spores at room temperature than by the spores at 4°C (Fig. 5). Based on these results, glutaraldehyde-fixed spores and a 2-h equilibrium time were used for the subsequent experiments in this study.

The isotherm of Cu(II) adsorption by spores was characterized by a large increase in the amount of adsorbed Cu(II) with increasing Cu(II) concentration at low equilibrium concentrations and leveling off or saturation at higher concentrations (Fig. 6). This L-type curve indicates that the spores had a high affinity for Cu(II). The Cu(II) adsorption affinity coefficient (K) and adsorption capacity (Γ_m) of the spores were obtained by fitting the experimental data to the Langmuir equation (equation 3). The K value was found to be 2.08×10^6 liters mol^{-1} . This affinity value is 2 to 4 orders of magnitude higher than the affinities of Cu(II) determined for a variety of biomasses, such as fungi, bacteria, and marine algae (20), or for an alginate gel (17). The Cu(II) adsorption capacity at pH 7.0 was $10.77 \mu\text{mol m}^{-2}$, equivalent to 0.829 mmol per g of dried spores, which is the same order of magnitude as the Cu(II) adsorption capacities of various biomasses, such as bacteria, algae, fungi (20). Pirszel et al. (26) reported that the ion-exchange capacities were $0.825 \text{ mmol g of dry weight}^{-1}$ for cells for *Anacystis nidulans* (a cyanobacterium), $0.205 \text{ mmol g}^{-1}$ for *Synechocystis aquatilis* (a cyanobacterium), $0.260 \text{ mmol g}^{-1}$ for *Stichococcus bacillaris* (a green alga), and $0.041 \text{ mmol g}^{-1}$ for *Vaucheria sp.* (a macroalga). The capacity at pH 7.0 is one-half of the charge density of SG-1 spores (Fig. 5). This might indicate that Cu(II) is adsorbed on the spore surface by the bidentate mechanism; i.e., one Cu(II) ion shares two surface sites on SG-1 spores. It is also possible, however, that Cu(II) binds to the spore surface in nonbidentate modes because Cu(II)-binding sites might differ from sites to which H^+

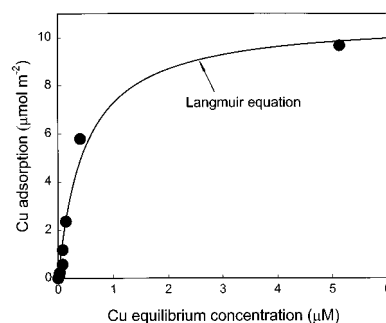


FIG. 6. Cu(II) adsorption isotherm obtained with SG-1 spores (10^8 spores ml^{-1} , 0.01 M NaNO_3 , pH 7.0). The curve was obtained by fitting the data to the Langmuir equation.

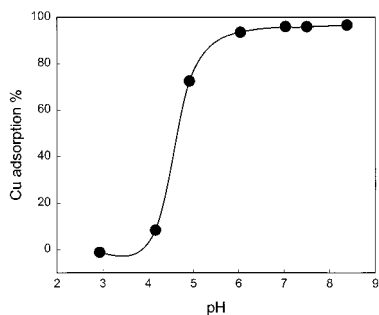


FIG. 7. Cu(II) adsorption by SG-1 spores as a function of pH [10^8 spores ml^{-1} , 0.01 M NaNO_3 , 2 μM Cu(II)]. The adsorption value for 100% adsorption is $1.5 \mu\text{mol m}^{-2}$.

ions bind, as described for Pb(II) sorption onto Al and Fe oxides by Bargar et al. (2, 3).

The percentage of Cu(II) adsorption was lower at lower pH values and increased as the pH increased (Fig. 7). Copper adsorption was zero around pH 3 and rapidly increased to $\sim 100\%$ at pH 6. This adsorption behavior is similar to metal adsorption by microbial biomasses (44) and metal oxides (35).

Using the Cu(II) adsorption isotherm data obtained at pH 7 (Fig. 6) and equation 12, we obtained K_d values ranging from 1.4×10^5 to $1.3 \times 10^6 \text{ ml g}^{-1}$ (average, $7.3 \times 10^5 \text{ ml g}^{-1}$). These values are 2 to 4 orders of magnitude higher than those obtained for most inorganic materials. It is important to note that K_d values vary with pH. From the Cu(II) sorption data obtained at different pH values (Fig. 7) we calculated that the K_d values were between $3.1 \times 10^4 \text{ ml g}^{-1}$ at pH 4.16 and $1.0 \times 10^7 \text{ ml g}^{-1}$ at pH 8.38 and increased with solution pH. The K_d value calculated by equation 13 by using the Γ_m and K values obtained at pH 7 (Fig. 6) was $1.6 \times 10^6 \text{ ml g}^{-1}$, which was in reasonable agreement with the values calculated by equation 12.

DISCUSSION

Spore surface area. The surface area of a solid is more important than the mass for understanding and interpreting the ion sorption properties of the solid. There are two categories of methods: (i) physical determination of the size and morphology of solid particles and (ii) measurement of the adsorption of gas or solute molecules having known dimensions and interpretation of the resulting data with a particular adsorption model. Both of these types of methods were used to measure the specific surface area of SG-1 spores in this study.

It is relatively easy to obtain the geometric surface area of spores because the number of spores in a suspension can be counted easily and spores have nearly uniform size and shape. It should be noted that geometric surface area is calculated with the assumption that spores are nonporous. N_2 gas adsorption (the BET method) is the most popular technique for measuring the specific surface areas of powder samples. The BET method has some serious limitations, however. For example, the structure and surface characteristics of solids, particularly biological materials, can change significantly in the drying step required in the BET method. For SG-1 spores, the surface area determined by the BET method was low and very similar to the geometric surface area. This similarity indicates that either the spores were smooth (nonporous) or the drying step altered the surface structure of the spores.

To test this hypothesis, a dye adsorption method, in which the drying step was avoided, was used to measure the surface

area of SG-1 spores. Dyes with well-characterized dimensions have been used by many investigators to measure the specific surface areas of porous oxide materials (43), clay minerals (25), activated carbon (24), and sludge (1, 34). The spore surface area determined by the methylene blue adsorption method was 11-fold higher than spore surface areas determined by the BET technique and the spore dimension measurement technique. A similar specific surface area ($74 \text{ m}^2 \text{ g}^{-1}$) for spores was reported by Neihof et al. (23), who used a solvent replacement procedure. These authors washed spores six times with absolute ethanol and then four times with dry pentane and measured the surface area of the treated spores with the BET technique. Neihof et al. (23) indicated that although the water in spores was removed by the solvent replacement procedure, considerable porosity was retained. This was verified by the fact that the surface area decreased after the solvent-replaced spores were rewetted and dried.

In this study, the dye methylene blue was used to measure the specific surface area of SG-1 spores. The dimensions of methylene blue have been quantified very well, and the orientation of adsorbed methylene blue molecules has been described well (25). The large surface area determined by the methylene blue adsorption method suggests that in the water-wet state, a spore is swollen and there is a water-filled porous structure. When the spore is dried in air or even freeze-dried, this porous structure collapses, resulting in a much smaller surface area. Even molecules as small as nitrogen are unable to get in. We believe that measurements obtained by using methylene blue better reflect the real surface area of the spore material because drying is not required.

Dyes other than methylene blue have also been used to measure the surface areas of powder materials. Andreadakis (1) found that the specific surface area of an activated sludge determined by adsorption of lissamine scarlet 4R was 1 or 2 orders of magnitude greater than the geometric surface area of the sludge. Depending on the assumptions concerning the amount of absorbed dye and the orientation of the molecules used, the specific surface area varied between 60 and $189 \text{ m}^2 \text{ g}^{-1}$, suggesting that the activated sludge was porous (1). Sorensen and Wakeman (34) believed that the surface area of activated sludge measured by rhodamine B adsorption was a realistic measure of the solid-liquid interfacial area.

Spore surface charge. Surface charge and site density may be experimentally measured by acid-base titration (H^+ exchange), tritium ($^3\text{H}_2\text{O}$) exchange, or other ion (e.g., Ca, Mg, F^- , and PO_4^{3-}) adsorption techniques. Site density estimates obtained with different methods typically differ by factors of 2 to 3 and sometimes more (9). Acid-base titration is widely used for measuring the surface charge of powder materials and bacterial cells (28, 31) and is the simplest technique since only the pH must be measured. The surface site density measured by acid-base titration depends to some extent on ionic strength. A low ionic strength results in underestimates of surface site density because the surface sites are not totally ionized. In this study, we used an ionic strength of 0.01 M, which is between the ionic strengths of freshwater and seawater.

The surface site density of SG-1 spores ($18.3 \text{ sites nm}^{-2}$) is on the same order of magnitude as the surface site densities of most mineral colloids, such as Fe, Mn, and Al (hydr)oxides (2 to 20 sites nm^{-2}) (6, 36). Theoretically, the maximum surface site density for oxides is $39 \mu\text{mol m}^{-2}$ ($23.3 \text{ sites nm}^{-2}$) if the radius of H^+ is 0.145 nm and surface groups are located on two layers (36). The site density of SG-1 spores ($2.29 \text{ mmol g of spores}^{-1}$) is similar to the previously reported values for cell walls of other bacteria and algae, which range between ~ 1 and $2.5 \text{ mmol g of cell wall}^{-1}$ (28).

While spores clearly differ from vegetative cells in structure and heat and chemical resistance, our results suggest that similar surface functional groups (i.e., carboxylate, phosphate, and amino groups) are present. The carboxylate and phosphate groups carry negative charges that allow the cells to be potent scavengers of cations. In gram-positive bacteria, the carboxylate and phosphate groups are primarily associated with the peptidoglycan; the peptidoglycan backbone is rich in carboxylate groups, and the associated teichoic acids are rich in phosphate groups (32). In gram-negative bacteria, the carboxylate and phosphate groups are primarily in the outer membrane lipopolysaccharide (32). In both types of bacteria, amino groups are associated with the peptidoglycan or other proteins on the cell surface.

In SG-1 spores, the surface charge is primarily associated with the outermost layer of the spore, the exosporium, which in *Bacillus cereus* is composed mainly of protein, polysaccharide, and lipids (21). The surface charge density (absolute value) (strictly speaking, it should be called the net surface charge density) increases linearly from pH 4.5 to 7.5 and seems to level off at pH values greater than 8 (Fig. 5). This leveling off at higher pH values suggests that the spore surface carries phosphate groups which have pK_2 (the second dissociation constant of phosphoric acid) values of 7 to 8 (16). The negative charge between pH 4.5 and 6 could develop from carboxylate groups that have pK values of 4 to 5 (16, 27). The net positive charge at pH values of less than 4.5 is probably due to amino groups on the SG-1 spore surface. Our results are consistent with previous reports. The electrophoretic mobility of spores of three *Bacillus* species suggested that in these spores carboxylate groups are the principal surface charge groups and there are some phosphate and amino functional groups (8). It should be noted that macroscopic analyses based on titration curves are indirect methods for examining surface functional groups. Direct spectroscopic techniques, such as Fourier transformed infrared spectroscopy and X-ray adsorption fine-structure spectroscopy, should be employed to identify the surface functional groups on SG-1 spores. Results obtained by using Fourier transformed infrared spectroscopy suggest that carboxylate is the most important surface functional group for a variety of biomasses, including algae, diatoms, fungi, seaweed, and terrestrial plants (11, 12, 18). Other functional groups, such as amino and sulfonate groups, have also been identified.

The mechanism of surface charge development for SG-1 spores and other biomasses (i.e., protonation or deprotonation) is similar to the mechanism of surface charge development for metal (hydr)oxides, but the surface groups are different. Metal (hydr)oxides have amphoteric surfaces that may be charged negatively or positively at the same site, depending on the pH, whereas SG-1 spores are zwitterionic surfaces, like latexes, which carry carboxylate, sulfonate, and amino surface groups (15). The ZPC of SG-1 spores determined in this study is pH 4.5. This means that at pH 4.5 the net surface charge is zero and the positive charge of the spore surface equals the negative charge. When the pH is greater than the ZPC, there is a net negative charge, which largely results from deprotonation of the carboxylate and phosphate groups. When the pH is less than the ZPC, protonation of all three major groups (carboxylate, phosphate, and amino groups) results in a net positive charge.

Cu(II) adsorption by spores. The adsorption of Cu(II) by SG-1 spores was extremely rapid. Because of the fast kinetics of the Cu(II) sorption reaction, it is believed that the overall reaction rate is controlled by mass transfer of Cu(II) ions to the reactive sites of SG-1 spores. The Cu(II) adsorption kinetics are different in spores treated with glutaraldehyde and

spores not treated with glutaraldehyde. We believe that this difference is due to spore activation and germination in experiments performed with untreated spores because, consistent with germination, the untreated spores released Mn and Zn, whereas the fixed spores did not. Although glutaraldehyde treatment might alter the surface properties of SG-1 spores by reducing the numbers of charged groups on the surface, this alteration seems small since the initial (≤ 4 -h) Cu(II) adsorption capacity was nearly the same in the treated and untreated spores (Fig. 5).

The L-curve isotherm, into which Cu(II) adsorption by SG-1 spores falls, is characterized by an initial slope that does not increase with the concentration of adsorbate in solution. This type of isotherm is the result of a high relative affinity of the solid particles for the adsorbate at low surface coverage coupled with a decreasing amount of adsorbing surface remaining as the surface excess of adsorbate increases. By fitting experimental data to the Langmuir equation we obtained the values for two parameters, K and Γ_m . The parameter K is related to adsorption energy and determines the magnitude of the initial slope of the isotherm. The magnitude of the slope indicates the affinity of an adsorbate for an adsorbent. The Cu(II) adsorption by SG-1 spores in this study (Fig. 7) showed a relatively high affinity since at low Cu(II) concentrations Cu(II) was almost totally adsorbed by SG-1 spores, as demonstrated by a high K value (2.08×10^6 liters mol^{-1}).

The K value obtained from the Langmuir equation is the inverse of the K_m value determined from the Lineweaver-Burk or Eadie-Hofstee plot that was described by Lee and Tebo (19) for Co(II) oxidation by SG-1 spores. The K_m values for Co(II) oxidation obtained from the Eadie-Hofstee plot range from 3.3×10^{-8} to 5.2×10^{-6} mol liter $^{-1}$, corresponding to a range of K values between 1.92×10^5 and 3.03×10^7 liters mol^{-1} . Therefore, the K value obtained in this study for Cu(II) adsorption by SG-1 spores falls in the range of the K values obtained for Co(II) oxidation (19), indicating that SG-1 spores have similarly high affinities for both Co(II) and Cu(II).

SG-1 spores have proved to be an excellent model system for studying oxidative precipitation reactions, such as Mn(II) and Co(II) oxidation (19, 29, 41). Although the biochemical mechanism of metal oxidation has not been fully elucidated, copper has been shown to be important, both from genetic investigations that have implicated a multicopper oxidase-like protein in Mn(II) oxidation and because Cu(II) stimulates Mn(II) oxidation (45). While the results presented here are not conclusive proof, the high affinity and binding capacity for Cu(II) may be related to the use of Cu(II) as a cofactor for Mn(II) oxidation.

SG-1 spores are an attractive system for possible use in metal removal and recovery applications (38). They actively oxidize Mn(II) and Co(II) over a wide range of environmental conditions and are naturally resistant to a variety of physical and chemical stresses which may be encountered in a waste stream or polluted environment, and their growth does not have to be sustained. SG-1 is capable of binding or oxidizing metals both directly on the spore surface. This study shows that SG-1 spores have both a high affinity and a high capacity for Cu(II). Thus, SG-1 spores have a metal removal capability that exploits both active metal precipitation and passive adsorption processes, and the spores may be well-suited for solving problems where mixed metals occur.

ACKNOWLEDGMENTS

This research was funded in part by grant MCB94-07776 from The National Science Foundation, by grant NA36RG0537 from the National Sea Grant College Program (National Oceanic and Atmo-

spheric Administration, U.S. Department of Commerce; project R/CZ-123 of the California Sea Grant College), and by a grant from the California State Resources Agency. L.M.H. received partial support as a postdoctoral trainee from the University of California Toxic Substances Research and Teaching Program.

We appreciate two elaborate anonymous reviews and most useful criticisms. We thank John Bargar at Stanford University and Margo Haygood and Karen Casciotti at the Scripps Institution of Oceanography for critically reviewing an early draft of the manuscript. Laboratory assistance rendered by Deeanne Edwards, Karen Casciotti, Ron Caspi, Chris Francis, Larry Knight, and Donna Givens is greatly appreciated.

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