

Effects of cultivation conditions on acid–base titration properties of *Shewanella putrefaciens*

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Abstract

Acid–base titrations were conducted on pure strain laboratory cultures of the Gram-negative, facultatively aerobic bacterium *Shewanella putrefaciens*, cultivated under a range of conditions. Bacteria used in acid–base titrations were cultivated for periods of 24 to 100 h (exponential growth phase to late stationary phase), in varying media compositions (rich to minimal), and under aerobic and anaerobic conditions. In addition, two mutant strains of *S. putrefaciens* were tested, which are respiration-deficient with respect to U(VI) and Fe(III). Titration data were used to constrain the abundances and proton-exchange properties of bacterial surface functional groups using a discrete multisite surface complexation model. Estimated properties for titratable functional groups at the bacteria–water interface were compared on the basis of cultivation conditions. Results demonstrate that the acid–base behavior of the *S. putrefaciens* surface is largely insensitive to variations in culture age and media composition, yielding values for a wide range of aerobic cultivation conditions that are not significantly different on a statistical basis. Conversely, results for anaerobic growth, in which Fe(III) was supplied as sole terminal electron acceptor, show significantly lower bacterial site densities and differing functional-group acid dissociation constants. It is recommended that different profiles for aerobic and anaerobic Gram-negative bacteria be used in aqueous speciation calculations involving a bacterial component. © 2004 Elsevier B.V. All rights reserved.

Keywords: Bacteria; Titration; *Shewanella putrefaciens*; Surface-complexation; Anaerobic; Growth phase

1. Introduction

Bacteria in surface aquatic environments and in the subsurface influence the chemical speciation of solutes in a variety of ways, not least of which include the coordination of dissolved chemical species to bacterial cell walls. Solute coordinated or adsorbed onto bacteria may undergo accelerated movement through porous media (Yee and Fein,

2002) or may be chemically modified through direct or indirect microbial processes, such as reduction (e.g. Lloyd and Lovley, 2001; Lloyd et al., 2002; Nealson et al., 2002; Nevin and Lovley, 2002; Luu and Ramsay, 2003) and oxidation (Olson, 1991; Sugio et al., 1992; Nordstrom, 2000; Konhauser et al., 2002; Gleisner and Herbert, 2002; Johnson et al., 2002; Nealson et al., 2002; Hamilton, 2003) to yield solid-phase products. Fe(III)-reducing bacteria appear in some settings to depend on coordination of dissolved or colloidal Fe(III) with the bacterial surface as a prerequisite to growth (Nealson et al., 2002; Haas and DiChristina, 2002; Luu and Ramsay, 2003).

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Thus, understanding the acid–base and ion-coordinative properties of the bacterial cell surface becomes an important consideration in efforts to predict the biogeochemical behavior of natural or anthropogenic solutes in aquatic settings.

Recent efforts to quantify the proton- and cation-exchange properties of some common bacteria (e.g. Fein et al., 1997; Fein, 2000; Martinez et al., 2002) have yielded results broadly consistent with expectations based on well-known aspects of bacterial membrane chemistry; i.e. that the outer membrane of a bacterium, albeit complex, comprises a limited and discrete set of biomolecular identities (functional group types) arranged upon an organized matrix of repeating structural elements (the phospholipid bilayer and associated extracellular lipopolysaccharide or peptidoglycan). Fein et al. (1997), Daughney and Fein (1998) and Daughney et al. (1998) have demonstrated that the surface properties of a common aerobic Gram-positive bacterial group, *Bacillus*, may be approximated using a discrete multisite surface-complexation model accounting for both autoionization and solute coordination across a range of pH and ionic strength values. Cox et al. (1999) and Martinez et al. (2002) report similar results for *B. subtilis* and *Escherichia coli*.

These studies indicate that the surfaces of Gram-positive and Gram-negative bacteria may be approximated as possessing at least three discrete site types (carboxyl, phosphoryl, hydroxyl or amine), and possibly also sulfhydryl or phosphodiester groups, each population of sites exhibiting an overall average acid dissociation constant (pK_a) and site density. Most studies have shown that Gram-positive surfaces are best modeled using a three-site approach (e.g. Daughney et al., 1998), however, some studies have reported up to five discrete site populations on Gram-positive and Gram-negative bacteria (e.g. Cox et al., 1999; Martinez et al., 2002). Further work by Fein et al. (2002) has extended the *B. subtilis* surface model to lower pH conditions than previously examined (Fein et al., 1997), and recommends modeling *B. subtilis* as a four-site surface having acidic functionalities with pK_a values ranging from ~ 3 to ~ 9 . The identities of modeled functional groups are not detectable with titration or metal uptake data, however, X-ray absorption spectroscopic (XAS) work (Kelly et al., 2001) is consistent with bulk metal-adsorption data supporting

carboxyl and phosphoryl groups as mainly responsible for Cd and U coordination at the bacterial surface.

Haas et al. (2001) examined a common Gram-negative, facultatively aerobic metal-reducing bacterium (*Shewanella putrefaciens*), and reported titration and metal-adsorption experimental data that were modeled using a discrete multisite approach. In Haas et al. (2001) *S. putrefaciens* surfaces were modeled as having three distinct populations of sites; carboxyl ($pK_a=5.2$), phosphoryl (7.1) and amine (9.4). These results are broadly consistent with findings for aerobic Gram-positive bacteria (e.g. *Bacillus*, *Escherichia*), although site densities and precise values of pK_a vary significantly among the selected bacteria. Further variations in cell surface properties are reported by Daughney et al. (2001), who provide titration data for *B. subtilis* at different stages of growth (exponential-phase, stationary-phase, sporulating). Those authors measured significant differences in surface site density and site-population pK_a values at different stages of *B. subtilis* growth in laboratory culture. These variations in apparent surface properties among common bacterial types at different stages of growth are problematic to constructing an overall generic ‘bacterial component’ in generalized models of solute speciation under natural conditions. Reactive transport models designed to incorporate bacterial influences into a quantitative and predictive depiction of solute fate and transport could most usefully employ a bacterial component that is broadly representative of as many different microbial species as possible. Considering the diversity of microbial species found in a typical aquatic setting (Nealson and Stahl, 1997), one must acknowledge that modeling each individual bacterial strain in a natural consortium is an impractical task. However, it remains yet unclear to what degree the surface properties of bacteria vary. Furthermore, most studies to date have focused on bacteria grown under nutrient-rich conditions in the laboratory, although natural conditions are normally much more nutrient-limited.

In this study the proton-exchange properties of the common Gram-negative, facultatively aerobic, Fe(III) and Mn(IV)-reducing bacterium *S. putrefaciens* are examined as a function of pH, media composition, culture age, strain type, and aerobic/anaerobic conditions during growth. Potentiometric acid–base titrations were conducted using viable bacteria grown

under a range of conditions in the laboratory, and the results of titration studies were modeled using a discrete multisite surface complexation approach. *S. putrefaciens* was selected as a model organism representing a dissimilatory Gram-negative bacterium, capable of anaerobic growth using a wide variety of anoxygenic terminal electron acceptors (e.g. Fe(III), Mn(IV), U(VI), NO₃⁻, SO₃⁻, Cr(VI), and Tc(VII), TMAO, fumarate), yet also capable of aerobic respiration (Winkler et al., 1995; Lloyd et al., 1998; Wade and DiChristina, 2000; Taratus et al., 2000; Madigan et al., 2000).

2. Experimental methods

2.1. Cultivation methods

S. putrefaciens strain 200R (Obuekwe et al., 1981; DiChristina and DeLong, 1994), a spontaneous rifamycin-resistant wild-type strain, was used in this study. In addition, two laboratory mutants of 200R were used in comparison studies. Strain B31 is deficient in the ability to respire using Fe(III) as terminal electron acceptor (TEA), while strain U14 is incapable of growth utilizing millimolar concentrations of dissolved U(VI) as TEA. Strain 200R is capable of utilizing both Fe(III) and U(VI) as TEA (DiChristina and DeLong, 1994; Wade and DiChristina, 2000; DiChristina et al., 2002). Cultures were prepared from frozen glycerol-treated stock maintained at -80 °C, transferred to agar plates of Luria-Bertani (LB) media and cultured at 30 °C aerobically for at least 24 h prior to use. Single colonies from viable, growing LB-agar plates were transferred to sterile liquid LB media (10 g tryptone, 10 g NaCl, 5 g yeast extract per liter) and cultivated in 50-ml volumes aerobically at 30 °C in a heated, shaking environmental chamber for 24 h. These cultures were used as inocula for growth experiments in larger volume media bottles (1:100 inoculum by volume into 1- to 2-l working volumes). For inoculations into anaerobic media, 24-h liquid media cultures were degassed with N₂ prior to inoculation.

Experimental cultures were cultivated for 24, 48, 72, and 100 h in LB media. Cultures were also grown in a semi-defined medium (Obuekwe and Westlake, 1982), and a defined minimal basal salts

medium (Lovley, 1993) for 48 h. Cultures were also grown under anaerobic conditions, in media amended with ferric citrate as sole terminal electron acceptor. Anaerobic media were prepared aerobically, then transferred to a Coy[®] glove-box anaerobic chamber and degassed under an 85% N₂/5% CO₂/10% H₂ atmosphere for 24 h prior to inoculation. All media were prepared according to previously published recipes and either autoclaved or filter-sterilized, as appropriate.

Cell densities at harvest were measured using UV-visible spectrophotometry (absorbency at 600 nm). At harvest, cells were treated with 25 µg/l chloramphenicol to arrest growth and protein synthesis. Bacteria were then cleansed of soluble media components and extracellular metabolites using a repeated centrifugation/resuspension protocol (Haas et al., 2001) yielding approximately 10–25 ml of concentrated bacteria, in a solution of 0.1 M NaCl, per liter of original culture. Bacteria grown in ferric-citrate media were decanted under anoxic conditions into 250-ml capped centrifuge tubes and transferred out of the anaerobic chamber for centrifugation. To prevent green rust formation caused by oxygen leakage during transit, EDTA was introduced into media in an equimolar concentration with initially available Fe(III) (i.e. 50 mM). The added EDTA chelated Fe(II) and prevented the precipitation of green rust. Final suspensions were maintained at 4 °C for no longer than 1 week prior to use in titration studies. Cell density in some cleansed cell suspensions was measured by acridine-orange direct counting (AODC) (Lovley and Phillips, 1988) with an epifluorescence microscope (Nikon[®] daiphot 300). Replicate measurements (not shown) indicated that analytical uncertainties on AODC estimates of cell concentration were approximately 10% (1σ).

Cell wet mass (g bacteria per liter of solution) was measured for cleansed bacterial suspensions. Wet cell mass was measured by pipetting a known volume of concentrated bacterial stock into a 1.5-ml centrifuge tube, centrifuging the suspension for 20 min at 5000 × g, then decanting and centrifugally removing excess water from the pelletized cell mass. Cell mass was obtained by subtracting the mass of each individual empty 1.5-ml tube from the mass of the same tube containing the pelletized cell mass. Fivefold replicate measurements were made for each harvest. Results were highly reproducible. Combined replicate

measurements for all harvests (not shown) yielded analytical uncertainties on cell mass of approximately 5% (1σ). This method may overestimate the mass of each bacterium slightly due to the presence of water retained by surface tension with pelletized bacteria. However, 1.5-ml tubes after water removal were free of observable supernatant, therefore the amount of overestimation in per-cell mass is expected to be minimal. Wet bacterial mass was measured using this approach for all cultures in this study, therefore any bias in measured masses is expected to be systematic, yielding values that are at least internally consistent.

Combining mass data with AODC direct counts of bacterial density in stock solutions yielded values for average individual bacterial mass (bacteria/g). An overall average value of 7.3×10^{12} ($\pm 2 \times 10^{12}$) cells/g was obtained.

2.2. Potentiometric titrations

Acid–base titrations were performed using an automated potentiometric titrator (Mettler Toledo® DL-58) at 25 °C, using methods as reported in Haas et al. (2001). Titrate solutions were prepared by diluting concentrated stocks of bacteria with 0.1 M NaCl to achieve the desired cell density. Thirty milliliters of 0.1 M NaCl was placed in polypropylene vessels, secured to the burette assembly, and sparged with N₂ gas for at least 30 min before experiments. Fresh titration grade NaOH (0.1 M) and HCl (0.1 M) (Titristar) standard solutions were calibrated against potassium hydrogen phthalate (for NaOH) and TRIS (for HCl). Before each experiment, bacterial stocks were added to nitrogen-sparged 0.1 M NaCl and allowed to equilibrate for 15 min. During experiments a positive pressure of N₂ was maintained in the vessels. N₂ was not bubbled through the solution after addition of bacteria, to avoid bubble-flotation of bacteria from the solution to the upper walls of the vessel. Titrations at pH values above 10 or below 4 were avoided to minimize hysteretic effects resulting from cell lysis, protein denaturing, or biomolecule elution. Titrations were done by instrumental addition of titrant, following measurement of pH stability below a drift threshold of ~ 0.001 pH units/10 s (0.1 mV/10 s). Titrant volumes were accounted for in calculating dilution factors, and were less than 2% of initial titrate solution volume for all titrations in this study.

3. Results

3.1. Potentiometric titrations

Titration curves for suspensions of *S. putrefaciens* in 0.1 M NaCl are shown in Figs. 1 and 2. These figures display measured pH (x-axis) versus the net concentration of added protons during the titration (y-axis) normalized to wet cell mass (mmol H⁺ added/g bacteria). Values are normalized to cell mass to facilitate direct visual comparisons among results at different cell densities. The net concentration of H⁺ added is calculated from titration data according to the relationship

$$[\text{H}^+]_{\text{added}} - [\text{OH}^-]_{\text{added}} = [\text{H}^+]_{\text{net added}} \quad (1)$$

where $[\text{H}^+]_{\text{added}}$ and $[\text{OH}^-]_{\text{added}}$ are the known amounts of NaOH or HCl supplied at each step of the titration.

In Figs. 1 and 2 each titration of *S. putrefaciens* is displayed alongside a model titration curve for pure water (blank) absent additional buffering agents. In comparison with water, 200R cells strongly buffer pH in both acidic and basic pH regions. These results are consistent with those of Haas et al. (2001), who also report buffering of solution pH by *S. putrefaciens* 200R at high and low pH conditions. The effect of the bacteria in water absent NaOH or HCl titrants is to buffer pH near a value of ~ 6.5 to ~ 6.8 , with immersion pH being essentially invariant at higher bacterial concentrations. Bacterial concentrations (LB-grown cultures) during titrations varied from approximately 1.3×10^{11} ($\pm 0.1 \times 10^{11}$) cells/ml (100 h) to 1.8×10^{11} ($\pm 0.2 \times 10^{11}$) cells/ml (48 h), representing a total bacterial mass during 200R (LB media) experiments of 17.7–25.5 (± 1) g bacteria/l.

Fig. 1 depicts titrations of 200R cultivated in LB media for 24, 48, 72 and 100 h, respectively. Each curve represents a titration of *S. putrefaciens* from separately grown batches inoculated from separately cultivated plate cultures. Titration curves of 24-, 48-, 72- and 100-h batches of 200R show similar overall shapes. Fig. 2 illustrates titration data for 48-h harvests of: 200R grown on Westlake (WL) media (Obuekwe and Westlake, 1982) under aerobic and anaerobic conditions, 200R grown on *Geobacter* freshwater (Gf) media (Lovley, 1993) under aerobic and anaero-

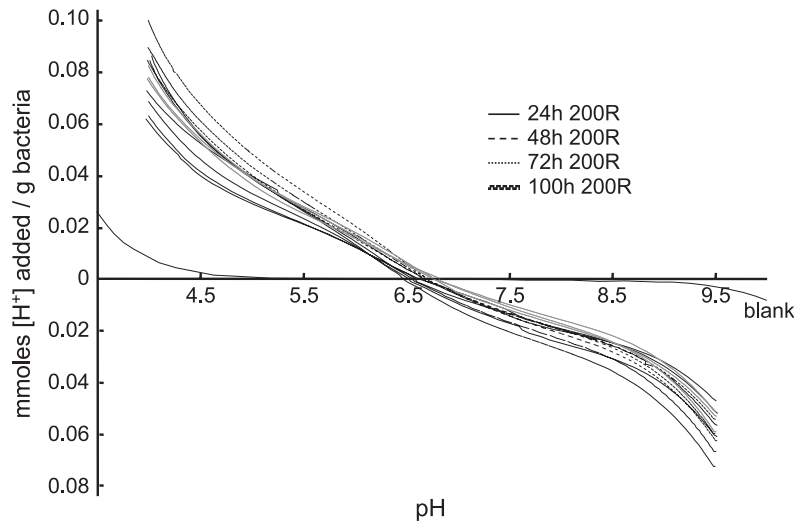


Fig. 1. Results of separate acid–base titrations of *S. putrefaciens* 200R grown in LB media under aerobic conditions for 24 to 100 h. Titration data are shown as smoothed lines for clarity. The blank represents a calculated titration curve for 0.1 M NaCl alone.

bic conditions, and B31 and U14 strains grown aerobically in LB media.

3.2. Modeling of titration data

Titration data were used to constrain the proton-exchange properties and concentrations of functional groups that contribute to buffering in the measured pH range. To facilitate the extraction of pK_a values and

site concentrations from the experimental data, the computer code FITEQL (Westall, 1982) was used in concert with experimental data to constrain best-fit approximations of the titration data in terms of different stoichiometric scenarios.

A constant-capacitance model (Stumm and Morgan, 1996) was used to depict reactions at the bacteria–water interface. The CC model was selected because it is a relatively simple surface-complexation

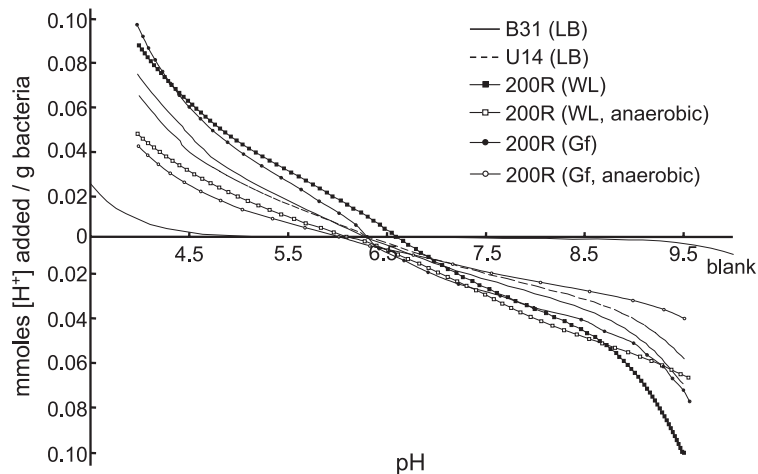


Fig. 2. Results of separate acid–base titrations of *S. putrefaciens* 200R grown aerobically in WL and Gf media, grown anaerobically on WL and Gf media, and strains U14 and B31 grown aerobically in LB media, all at 48-h incubation time. Titration data are shown as smoothed lines for clarity. The blank represents a calculated titration curve for 0.1 M NaCl alone.

treatment, requiring only one fit parameter (capacitance of the electrical double layer). The CC model was also selected to provide consistency with previous work involving this bacterium (Haas et al., 2001) and other bacteria (e.g. see review of Fein, 2000).

Previous studies have shown that bacterial surfaces may be approximated as comprising three (Fein et al., 1997; Daughney and Fein, 1998; Haas et al., 2001), four (Fein et al., 2002), or five (Cox et al., 1999; Martinez et al., 2002) distinct type-populations of proton-exchanging functional groups. In this study stoichiometric scenarios involving up to four sites, including Lewis-acid and Lewis-base stoichiometries, were considered in modeling the experimental titration data. In all cases (i.e. for all sets of experimental data) the best-fitting solution involved a three-site model. A close fit was obtained using a three-site model involving two Lewis-acid sites and one Lewis-base site. This depiction of the bacterial surface necessitates that the outer membrane exhibits a net positive charge up to a pH of ~ 9 . However, electrophoretic mobility measurements of whole bacteria typically show that the overall electrostatic charge of the outer cell surface is negative under all but highly acidic pH conditions (Rodriguez and Armstrong, 2004; Buszewski et al., 2003; Sokolov et al., 2001). This observation argues against a strong positive-charge contribution from amine groups at the outer surface.

An alternative to the inclusion of a base functional group in modeling the *S. putrefaciens* titration data is to consider the donation of base cations from the bacterial surface to the bulk solution, or rather the exchange of aqueous protons for surface-bound or

structural cations from macromolecules at the bacterial/aqueous interface. The immediate effect of base cation donation would be to buffer solution pH to higher values than would be the case where only proton exchange occurs between the microbial surface and the bulk solution. In the present study, chelating agents were avoided during normal preparation and cleansing of harvested bacteria, and apparent base behavior of the bacterial surface was observed. In several previous studies (Fein et al., 1997; Daughney et al., 1998) chelating agents (i.e. EDTA) were used during bacterial preparation, and no base behavior of the bacterial surface was observed. One expected outcome of treating bacteria with a strong chelating agent is the removal of cations from exchangeable sites. To test this hypothesis, in this study 200R bacteria grown aerobically on WL media were pretreated with EDTA, and titration results for these bacteria were compared with a control titration of an untreated aliquot of the same 200R batch. EDTA treatment involved exposure of the bacteria to a solution containing 0.1 M NaCl and 1 mM EDTA for 1 h, followed by a conventional triplicate wash procedure in 0.1 M NaCl. The results of these titrations are shown in Fig. 3.

EDTA pretreatment apparently stripped exchangeable cations from the bacterial surface, minimizing the capacity of the surface to donate base cations under acidic pH conditions. To compensate for the effect of cation efflux under acidic conditions, titration data in this study were corrected on the basis of the EDTA pretreatment results, such that the pH of zero net added H^+ corresponded with that observed following

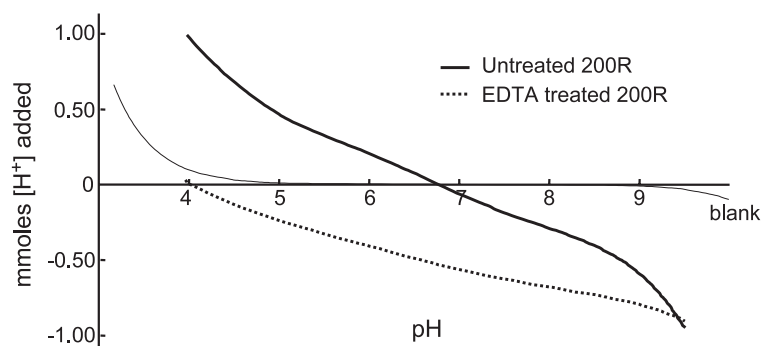


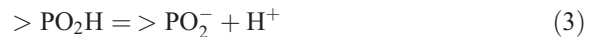
Fig. 3. Results of acid–base titration of *S. putrefaciens* 200R grown aerobically for 48 h on WL media. The dashed line shows data for 200R pretreated with 1 mM EDTA for 1 h, followed by washing. The solid line shows data for untreated 200R.

EDTA pretreatment (~ 4.0). Corrected titration data were used in all subsequent modeling calculations. In all cases an optimal fit to corrected titration data was obtained using a three Lewis-acid site model. Calculated results for uncorrected (two Lewis-acid and one Lewis-base sites) and corrected (three Lewis-acid) data were closely similar with respect to both predicted pK_a values (proton-leaving reactions) and site densities, indicating that both depictions adequately approximate the effects of H^+ exchange at the cell–aqueous interface. The three Lewis-acid site model was adopted in this study to provide consistency with previously published electrophoretic mobility data.

Models involving one to two Lewis-acid sites failed to provide an acceptable fit to the experimental data, yielding both high values for the calculated sum of squares of residuals function (wsos/df), and a poor visual fit to the experimental data. Similarly, models involving (1) one site, (2) one Lewis-base site and one Lewis-acid site, (3) more than one Lewis-base site, or (4) more than three sites overall failed to approximate the experimental data or failed to converge. Models involving four or more sites failed to converge, indicating that additional sites beyond three are (1) not required to model the data, (2) do not contribute significant buffering capacity, or (3) cannot be effectively constrained with the available data. The latter alternative is an important possibility, because any buffering at a pH less than ~ 4 would not have been measured in this study. Titrations below pH 4 were avoided to minimize disruption of cell membranes or elution of acid-soluble membrane proteins.

The model results of this study are broadly consistent with previous work characterizing the titration behavior of *S. putrefaciens* (Haas et al., 2001), the Gram-positive bacteria *B. subtilis* and *B. licheniformis* (e.g. Daughney and Fein, 1998), and Gram-negative Enterobacteriaceae (Ngwenya et al., 2003), in terms of a three-site model. Table 1 summarizes best-fit FITEQL model results for titrations involving all of the growth conditions examined in this study. Results for each conditions category (i.e. 24, 48 h, etc.) are shown alongside average and standard deviation values for that category of experiment. Table 1 lists pK_a values for the three Lewis-acid sites predicted by FITEQL calculations. Values of pK_a fall into three ranges; 3.8–4.5, 6.0–6.3, and 7.9–9.1. These pK_a ranges are consistent with those reported for other bacterial species,

including *S. putrefaciens*, and are also consistent with a model of carboxyl, phosphoryl, and hydroxyl (or phenolic) groups fulfilling an important role in proton exchange reactions at the bacterial surface. Values of pK_a for postulated carboxyl, phosphoryl and hydroxyl groups at the *S. putrefaciens* surface correspond with mass-law expressions of the form



where “>” for each functionality represents the locus of attachment of each functional group to an appropriate macromolecular component of the bacterial surface. The mass law relation for each of reactions (2)–(4) can be summarized according to

$$K = \frac{[> \text{COO}^-][\text{H}^+]}{[> \text{COOH}]} \quad (5)$$

for (e.g.) a carboxyl site deprotonation reaction, where K represents the apparent equilibrium constant of the Lewis-acid dissociation reaction. The values of pK_a for reactions (2)–(4) were determined according to the conventional expression

$$pK_a = -\log K \quad (6)$$

and in FITEQL modeling calculations are adjusted to account for electrostatic effects at the bacteria–water interface according to the expression

$$K = K_{\text{int}} \exp\left(-\frac{\Delta Z F \Psi_o}{RT}\right) \quad (7)$$

where K_{int} stands for the intrinsic equilibrium constant for the reaction at a condition of zero net surface charge and zero surface concentration of the adsorbing ion (H^+), ΔZ represents the change in charge of the adsorbing ion, F is the Faraday constant, Ψ_o stands for the surface electrical potential, R is the gas constant and T is the temperature in Kelvin (Stumm and Morgan, 1996). In the CC model adopted in this study Ψ_o is calculated assuming a constant electrostatic capacitance of the electrical double layer (EDL) adjacent to the surface, and is evaluated according to the relation

$$C = \frac{\sigma}{\Psi_o} \quad (8)$$

Table 1
 Tabulation of FITEQL estimates of bacterial surface functional group properties

Culture	$pK_{a>COOH}$	$pK_{a>PO_2H}$	$pK_{a>NH_2}$	$p[>COOH]$	$p[>PO_2H]$	$p[>OH]$	wsos/df
<i>200R, 24 h, LB</i>							
$A_{600}=0.738$	4.45	6.35	8.95	4.35	4.43	4.37	1.235
$A_{600}=0.812$	4.53	6.40	8.86	4.32	4.48	4.31	2.447
$A_{600}=0.859$	4.54	6.28	8.85	4.29	4.39	4.21	1.017
Mean	4.51	6.34	8.89	4.32	4.43	4.30	
%Stdev	1.1	1.0	0.6	0.7	1.0	1.8	
<i>200R, 48 h, LB</i>							
$A_{600}=1.024$	4.64	6.34	9.06	4.29	4.39	4.19	0.879
$A_{600}=1.058$	4.57	6.22	8.90	4.16	4.43	4.01	1.624
$A_{600}=0.952$	4.58	6.24	8.92	4.14	4.39	3.96	3.419
Mean	4.60	6.27	8.96	4.20	4.41	4.05	
%Stdev	0.8	1.0	1.0	2.0	2.0	2.9	
<i>200R, 72 h, LB</i>							
$A_{600}=1.161$	4.59	6.22	8.99	4.09	4.40	3.97	1.229
$A_{600}=1.171$	4.63	6.36	8.99	4.14	4.48	4.11	1.659
$A_{600}=1.233$	4.64	6.28	9.06	4.12	4.46	3.95	1.100
Mean	4.62	6.29	9.01	4.12	4.44	4.01	
%Stdev	0.6	1.1	0.4	0.7	1.0	2.2	
<i>200R, 100 h, LB</i>							
$A_{600}=1.104$	4.59	6.26	8.78	4.23	4.48	4.23	0.989
$A_{600}=1.161$	4.61	6.34	8.89	4.20	4.53	4.21	0.924
$A_{600}=1.120$	4.62	6.47	9.11	4.17	4.48	3.91	1.006
Mean	4.61	6.36	8.93	4.20	4.49	4.11	
%Stdev	0.3	1.7	1.9	0.7	0.6	4.3	
<i>200R, 48 h, WL</i>							
$A_{600}=0.519$	4.46	6.20	9.05	4.19	4.31	3.91	1.213
$A_{600}=0.235$	4.53	6.52	9.10	4.19	4.37	3.83	1.912
$A_{600}=0.264$	4.52	6.20	8.79	4.18	4.25	3.82	0.946
Mean	4.50	6.31	8.98	4.19	4.31	3.85	
%Stdev	0.8	2.9	1.9	2.8	1.4	1.3	
<i>200R, 48 h, Gf</i>							
$A_{600}=0.051$	4.48	6.46	8.98	4.02	4.44	4.19	1.238
$A_{600}=0.074$	3.75	6.07	8.68	4.50	4.50	4.80	0.341
Mean	4.12	6.27	8.83	4.26	4.47	4.49	
%Stdev	13.	4.4	2.4	8.0	1.0	9.7	
<i>200R, 28h anaerobic, WL (ferric-citrate)</i>							
$A_{600}=n/a$	4.34	6.61	8.51	4.57	4.62	4.90	2.101
$A_{600}=n/a$	4.29	6.48	8.28	4.51	4.61	4.77	1.613
Mean	4.32	6.55	8.40	4.54	4.61	4.83	
%Stdev	0.8	1.4	1.9	1.0	0.2	1.9	
<i>200R, 28h anaerobic, Gf (ferric-citrate)</i>							
$A_{600}=n/a$	3.99	5.99	7.86	4.46	4.63	4.80	0.326
<i>B31, 48 h, LB</i>							
$A_{600}=0.380$	4.55	6.45	8.98	4.14	4.52	4.01	0.974

(continued on next page)

Table 1 (continued)

Culture	$pK_{a>COOH}$	$pK_{a>PO_2H}$	$pK_{a>NH_2}$	$p[>COOH]$	$p[>PO_2H]$	$p[>OH]$	wsos/df
U14, 48 h, LB							
$A_{600}=0.357$	4.52	6.48	9.11	4.22	4.53	4.11	1.222

pK_a represents the $-\log$ of the acid dissociation constant for each site type, while $p[]$ for each site type represents the $-\log$ of the site concentration in mol/g bacteria. Wsos/df refers to the weighted sum of squares of residuals function returned by FITEQL computations.

where C stands for the capacitance of the EDL in F/m^2 , and σ represents the surface charge. C is effectively a fit parameter. In Haas et al. (2001) a value of $1.0 F/m^2$ was used for *S. putrefaciens* in 0.1 M NaCl, in accordance with the recommendation of Sahai and Sverjensky (1997) for the EDL capacitance in NaCl electrolyte. In this study, a value of $6.0 F/m^2$ was adopted for all calculations. It was found in this study that the results of FITEQL calculations are not strongly sensitive to C , however, an average value of $6.0 F/m^2$ returned minimal wsos/df values for the experimental data. A specific surface area for *S. putrefaciens* of $55 m^2/g$ bacteria (Haas et al., 2001) was used for all calculations in this study. This value is calculated based on a representative geometric surface area of *S. putrefaciens* determined using dry cell dimensions obtained from SEM imagery (Haas et al., 2001), along with BET data comparing wet and dry bacterial surface areas (He and Tebo, 1998).

FITEQL estimates of surface functional group properties show that pK_a values and site concentrations are highly reproducible for all titrations of LB-grown 200R at 24, 48, 72 and 100 h. Interexperimental percent standard deviations on postulated carboxyl pK_a values are generally small, with a minimum of 0.3% (100 h) and a maximum of 1.0% (24 h). Interexperimental percent standard deviations are similarly small for phosphoryl (1–1.7%) and hydroxyl (0.4–1.9%) sites. Larger interexperimental variation is observed with respect to predicted site concentrations. Postulated carboxyl site concentrations ($\log[mol/g]$) vary by 0.7–2.0% (one standard deviation), while postulated phosphoryl concentrations vary by 0.6–2.0%, and postulated hydroxyl concentrations by 1.8–4.3%. pK_a values and log site concentrations for WL-grown 200R, Gf-grown 200R, anaerobically grown 200R, and LB-grown B31 and U14 are broadly similar to those estimated for LB-grown 200R, although some variations are evident, and will be discussed in the next sections.

4. Discussion

4.1. Effects of culture age on bacterial surface properties

The results of FITEQL calculations involving LB-grown 200R cultivated to 24, 48, 72 and 100 h illustrate how culture age affects the concentrations and dissociation equilibria of titratable functional groups at the bacterial surface. Fig. 4 illustrates the dependence of culture age on site density in mmol sites/g bacteria, and site density in 10^6 sites per bacterium. Site densities are presented in Fig. 4 in terms of both site concentrations per unit bacterial mass and per individual (average) bacterium. This is done because two separate measurements of bacterial density in aqueous suspensions were made in this study; total bacterial mass was determined by weighing centrifuged wet cell populations, and bacterial population density (in most cultures) by AODC direct counting. The second procedure yields a direct assessment of the number of bacteria per unit volume of solution, and when combined with experimentally determined and computationally constrained values for titratable functional group concentrations per unit volume of solution, may be used to calculate the average density of functional groups per average individual bacterium. This measure of site concentration is potentially more useful when attempting to model field conditions in which bacterial mass is unknown but average microbial population density is estimable through, for example, turbidity tests, direct counting, most-probable number assessment, or plate colony counts (Fig. 5).

Site concentrations are also provided in Fig. 4 in terms of mmol of sites per gram of bacteria (wet mass). This formulation of site density is provided to allow for easy incorporation of model-derived bacterial surface properties into preexisting computational models (speciation codes, reactive transport algorithms, etc.) that parameterize colloid phase concentration in terms of mass per unit volume of solution.

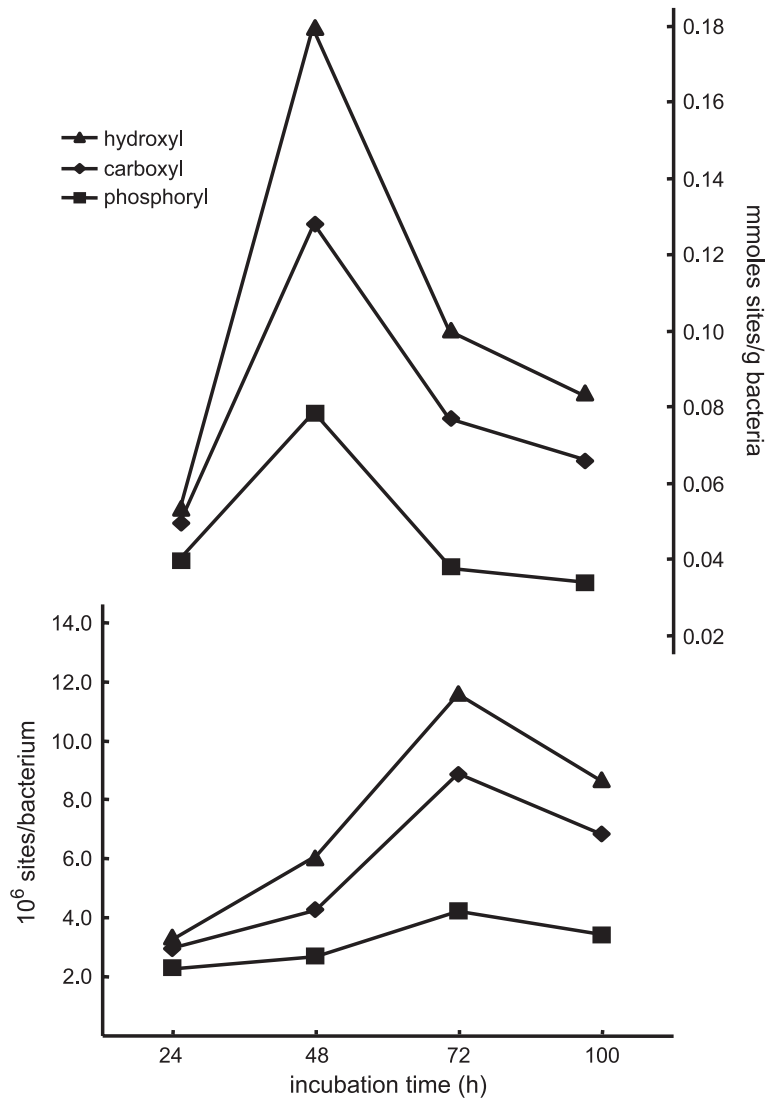


Fig. 4. FITEQL model calculation results estimating (upper y-axis) molar concentrations of titratable sites per gram of bacteria (mmol/g), and (lower y-axis) 10^6 sites per average individual bacterium, plotted as a function of incubation time in hours.

Average pK_a values do not appreciably change as a function of culture age, up to 100-h incubation time. Postulated carboxyl group pK_a values vary by 1.1% (1σ), while phosphoryl pK_a values vary by $\sim 0.7\%$ and hydroxyl pK_a values by only $\sim 0.6\%$. A Student's t -test analysis shows that the replicate carboxyl, phosphoryl and hydroxyl pK_a values, for 24- to 100-h incubation of 200R in LB media, are not statistically different within a 95% confidence interval (data not shown). Thus, culture age does not appear

to exert a significant influence on the acid dissociation properties of modeled titratable functional groups on *S. putrefaciens*.

In contrast, site concentrations appear to vary somewhat as 200R cultures mature. Fig. 4 illustrates variations in site concentrations of postulated carboxyl, phosphoryl and hydroxyl groups as culture age increases. Unlike pK_a values, site concentrations appear to vary systematically. Molar carboxyl, phosphoryl and hydroxyl site concentrations increase up to 48 h, after

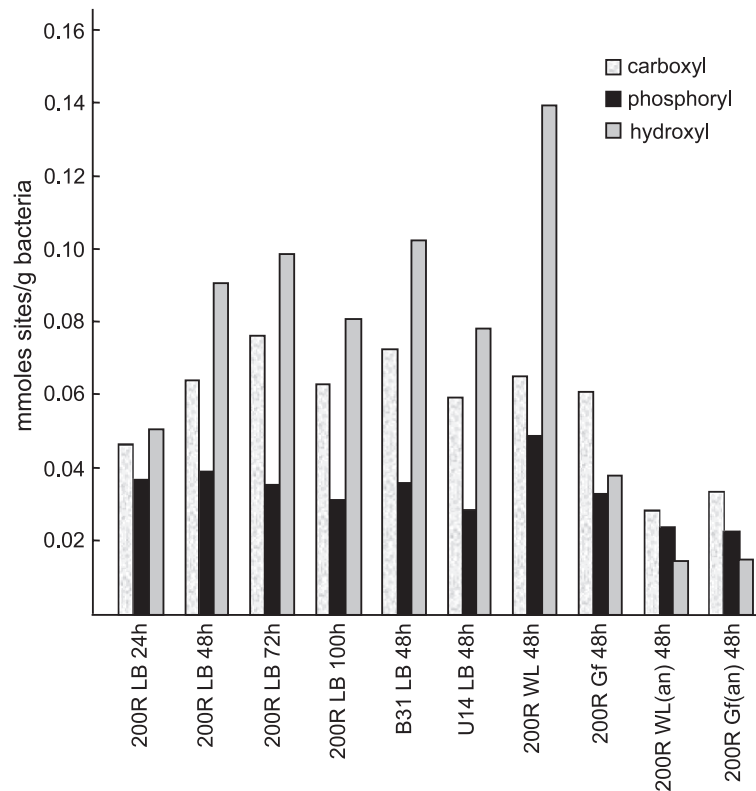


Fig. 5. FITEQL model results estimating average molar concentrations of titratable sites per gram of bacteria (mmol/g) for bacteria at each different cultivation condition in this study.

which molar site concentration diminishes to values that are equivalent or slightly higher than 24 h values. In contrast, the number of sites predicted on each bacterium increases through 72 h, and only slightly decreases at the maximum cultivation time of 100 h. These trends suggest that the bacteria undergo progressive changes during culture maturation and senescence that translate into observable trends in titratable functional group properties on the cell surface or within the readily exchangeable intramembrane region.

One potential explanation for the trends shown in Fig. 4 could involve the relative availability of nutrients through the course of batch cultivation. Early in the experiments (24 h) nutrient availability remains high, but at longer cultivation times nutrients become progressively limiting. Under conditions of increasing nutrient deprivation, bacteria will tend to initiate the production of reserve polymers such as polysaccharides, polyphosphates, and acquisition proteins (Madi-gan et al., 2000). One effect of reserve polymer or

sheath formation at the outer membrane could be an increase in the absolute density of titratable functional groups, particularly carboxyl and phosphoryl groups associated with reserve macromolecules. At the same time, nutrient limitation will tend to limit the size of bacterial cells. Thus, at longer incubation times it is expected that bacteria would express more functional groups (more sites/cell), but would be individually smaller (lower mol sites/g).

Although bacterial site density appears to vary systematically as a function of incubation time, the magnitude by which this property varies is fairly small for cultures grown in LB media. Molar (per g bacteria) site concentrations for postulated carboxyl, phosphoryl and hydroxyl groups vary from 24 to 100 h by 44% (% standard deviation), 47% and 54%, respectively. The magnitude of variability in site concentrations is significantly greater than that exhibited by pK_a values, indicating that although site abundances change as the bacterial culture matures, site identity

remains essentially unchanged. Furthermore, the magnitude of change in site concentrations appears to be sufficiently small that average site concentration values could reasonably be used in bulk speciation estimates involving natural microbial populations. A Student's *t*-test analysis of values for 24-, 48-, 72- and 100-h incubations shows that, within a 95% confidence interval, molar site concentrations per gram of bacteria are statistically identical. This is also true for estimated site concentrations per bacterium.

Small variations in site concentrations and pK_a values for aerobically grown bacteria in LB media give an interesting comparison with the results of Daughney et al. (2001), who report data measuring titration and metal adsorption properties of the Gram-positive bacterium *B. subtilis* as a function of growth phase. Daughney et al. (2001) compare *B. subtilis* titration properties at exponential, stationary and sporulated growth phases, and find significantly higher titratable site concentrations in bacteria harvested at exponential phase, compared with bacteria harvested at stationary or sporulated phases. The present study also finds relatively higher molar site concentrations in late exponential phase (48 h), and lower at stationary and decline phases (72–100 h). Nonetheless, the present study indicates that observed variations in site density, at least for *S. putrefaciens*, do not represent statistically significant variances that would tend to require different surface property assessments for bacteria at different phases of growth.

4.2. Effects of media composition

S. putrefaciens 200R was cultivated in this study using rich media (LB) under aerobic conditions, and two different minimal growth media (WL and Gf) under aerobic and anaerobic conditions. The intent of this exercise was to ascertain the variability in cell surface properties under conditions of nutrient paucity that are reflective of conditions found in most geologic settings. Incubations (48 h) of 200R in WL and Gf media are compared in Fig. 2, in terms of mol sites/g bacteria. Model results for these titrations are tabulated in Table 1.

Bacteria grown using LB and WL media do not differ significantly in either pK_a values or site densities per gram wet weight, although molar site concentrations appear slightly higher for WL-grown 200R. A

Student's *t*-test (data not shown) indicates that all three pK_a values and the predicted carboxyl site density (mol/g bacteria) are statistically similar (95% confidence level) for LB-grown and WL-grown bacteria. Phosphoryl and hydroxyl site densities are statistically higher on WL-grown bacteria, relative to LB-grown bacteria. This statistical difference disappears at a 99% confidence level. A higher phosphoryl site density on bacteria grown under nutrient-limiting conditions may signify more efficient scavenging of P under the imposed conditions, and this result would not be unexpected.

Bacteria grown in Gf media under aerobic conditions appear to exhibit overall site concentrations and pK_a values that are similar to LB- and WL-grown *S. putrefaciens*. Concentrations of postulated hydroxyl groups are statistically lower than for LB and WL-grown bacteria at the 95% confidence interval, though not at a 99% confidence interval. Site pK_a values are statistically similar for LB and Gf-grown bacteria, save for carboxyl groups, which for Gf-grown bacteria are slightly more acidic (~ 0.3 log unit). Overall, the imposition of more severe growth limitations, which may more closely model the oligotrophic conditions found under most natural conditions, does not appear to result in a substantial shift in bacterial surface properties for *S. putrefaciens*. These results imply that measurements of titratable functionalities on bacteria grown under rich conditions may not significantly misrepresent the behavior of bacterial surfaces within an environmental context.

4.3. Aerobic versus anaerobic growth

S. putrefaciens 200R was grown in both WL and Gf media under anaerobic conditions, where ferric citrate was supplied as sole terminal electron acceptor. Anaerobically grown bacteria were harvested, cleansed of growth media, and titrated using methods described above. Anaerobically grown 200R exhibit significantly lower site densities, and significantly different pK_a values, in comparison with LB-grown, aerobically grown *S. putrefaciens*. Molar site concentrations are significantly lower for anaerobically grown cultures (95% confidence interval), though *S. putrefaciens* grown anaerobically on Gf and WL media display properties broadly similar to each other. Site pK_a values also differ between aerobic and anaerobic

cultures; pK_a values for anaerobically grown 200R, on both WL and Gf media, differed even at the 99% confidence interval from LB-grown bacteria. The average anaerobic phosphoryl pK_a value is more acidic than LB-grown bacteria, though anaerobic values exhibit a broad range. Carboxyl and hydroxyl pK_a values for anaerobic cultures were more consistently acidic than those of aerobic cultures.

Under natural oligotrophic conditions in suboxic and anoxic waters, facultatively anaerobic Gram-negative bacteria exemplified by *S. putrefaciens* could exist under conditions not dissimilar to those obtained during anaerobic/Gf-media experiments. Under these conditions, *S. putrefaciens* would be expected to exhibit substantially lower surface site concentrations than in rich, aerobic media, and therefore the ultimate capacity, but not necessarily the affinity, for bacterial adsorption of metals may be consequently lower than predictions based on aerobic/LB-media experiments might suggest. The results presented here suggest that bacteria under anaerobic conditions in limiting media may in fact be fairly poor adsorptive agents, at least in comparison with expectations based on bacteria grown in enriched media, and considering only the bulk density of available sites. The data presented in this study do not address the relative per-site metal adsorption affinity of bacteria grown under aerobic versus anaerobic conditions. Bacteria grown under oligotrophic conditions might exhibit stronger binding affinities for cationic solutes, relative to bacteria from a more permissive environment. Further work is required to assess this possibility.

4.4. Mutant strains

To address the question of genetic and phenotypic variability in bacterial surface properties, two mutant strains of *S. putrefaciens* were examined in this study. Results of titration studies on mutant strains U14 and B31, strains incapable of dissimilatory U(VI) reduction and Fe(III) reduction, respectively, indicate that the genetic components separating these mutant strains from the wild-type do not significantly impact the properties of titratable functional groups on the bacterial surface. Molar site concentrations and carboxyl pK_a values on U14 and B31 strains are statistically similar at the 95% confidence interval. Phosphoryl and hydroxyl pK_a values differ from

200R at the 95% confidence interval, but not at 99%. The proton-exchange properties of 200R, U14 and B31 are effectively indistinguishable.

5. Conclusions

Titration studies involving *S. putrefaciens* cultivated for different times, using rich and restricted growth media, under aerobic and anaerobic conditions, and using two mutant strains of the wild-type, demonstrate that cultivation parameters can significantly influence bacterial pH-buffering properties. Aerobic growth in rich, restrictive and intermediate media yielded broadly similar results over a wide range of cultivation times, from 24 to 100 h. Although discernible trends occurred in postulated site concentrations as a function of culture age, the modeled values for aerobic cultures of *S. putrefaciens* were statistically similar at a 95–99% confidence level, regardless of either media composition or incubation time. This result indicates that studies examining the surface properties of bacteria similar to *S. putrefaciens* may yield quantitatively comparable results even if interexperimental growth conditions are not duplicated. Two mutant strains, B31 (incapable of dissimilatory Fe(III) reduction) and U14 (incapable of dissimilatory U(VI) reduction at mM concentrations), also exhibited pH-buffering properties that were effectively identical to the wild type.

In contrast, 200R grown anaerobically (with Fe(III) as sole TEA), under moderate (WL) and restrictive (Gf) nutrient conditions, exhibited markedly fewer titratable sites per gram of bacteria. Anaerobic 200R was modeled as having significantly fewer sites per gram of bacteria than if prepared under any other set of conditions in this study. An expectation arising from these findings is that natural bacteria in the subsurface—typically characterized by limiting nutritional constraints—may normally exhibit a smaller number of charged surface sites than might be expected based on studies of aerobically cultured bacteria. Fewer titratable functional groups may mean fewer available sites for ion adsorption, suggesting that estimates of the adsorption capacity of bacteria in the subsurface—based on laboratory studies of bacteria grown under optimal circumstances—may be insufficiently conservative. Further experimental work is needed to quan-

Table 2

Recommended values for titratable surface functional groups on aerobically and anaerobically grown *S. putrefaciens*

Model organism	$pK_{a>COOH}$	$pK_{a>PO_2H}$	$pK_{a>OH}$	$p[>COOH]$	$p[>PO_2H]$	$p[>OH]$	$10^6[>COOH]/$ bact	$10^6[>PO_2H]/$ bact	$10^6[>OH]/$ bact
<i>S. putrefaciens</i> , aerobic	4.47	6.33	8.95	4.21	4.43	4.10	5.12	3.08	6.50
<i>S. putrefaciens</i> , anaerobic	4.21	6.36	8.22	4.51	4.62	4.82	2.54	1.98	1.24

pK_a represents the $-\log$ of the acid dissociation constant for each site type, while $p[]$ for each site type represents the $-\log$ of the site concentration in mol/g bacteria. $10^6[]/\text{bact}$ signifies the concentration of each site type (in units of 10^6) per average individual bacterium.

tify the ion adsorption potential of bacteria grown under anaerobic and limiting conditions.

On the basis of the results presented in this paper, it is recommended that separate estimates of site density be adopted in estimating the distribution of aqueous chemical species in the presence of bacteria, based on an expectation of bacterial type and growth conditions. The results presented herein demonstrate that the Gram-negative bacterium *S. putrefaciens* displays quite different proton-exchange properties under aerobic and anaerobic conditions, and that these differences are substantially greater than those engendered by differing media formulations or culture age. In modeling the effects of bacterial surfaces on chemical speciation under aerobic to microaerophilic conditions in an environmental setting, a set of values representing an average of aerobically grown cultures should be used. Such values for an average aerobic Gram-negative bacterium, based on *S. putrefaciens* as a model organism, are shown in Table 2. Values obtained from anaerobic experiments provide an alternative Gram-negative model, also listed in Table 2, which would be more appropriate to oligotrophic anaerobic conditions.

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