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Enhanced Dissolution of Silicate Minerals by Bacteria at Near-Neutral pH

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Abstract. Previous studies have shown that various microorganisms can enhance the dissolution of silicate minerals at low (<5) or high (>8) pH. However, it was not known if they can have an effect at near-neutral pH. Almost half of 17 isolates examined in this study stimulated bytownite dissolution at near-neutral pH while in a resting state in buffered glucose. Most of the isolates found to stimulate dissolution also oxidized glucose to gluconic acid. More detailed analysis with one of these isolates suggested that this partial oxidation was the predominant, if not sole, mechanism of enhanced dissolution. Enhanced dissolution did not require direct contact between the dissolving mineral and the bacteria. Gluconate-promoted dissolution was also observed with other silicate minerals such as albite, quartz, and kaolinite.

Introduction

Microbial activity in aquifers may enhance the dissolution of rock-forming minerals and thereby affect the porosity and permeability of the aquifer. This possible role of microorganisms was suggested by a recent field study [22] documenting alteration of aquifer minerals near organic-rich patches supporting active microbial metabolism. Another field study in an oil-contaminated aquifer [2] showed an enhancement of silicate mineral dissolution in a zone of enhanced microbial activity due to petroleum biodegradation. The involvement of microorganisms in this rapid dissolution of silicate minerals was further suggested by in situ microcosm experiments at the same site [10]. Etch pits on the mineral grains surfaces, indicative of rapid dissolution, were predominantly located near adhering bacteria.

Laboratory studies provide more conclusive evidence that microorganisms can, in fact, substantially enhance the dissolution of a large array of rock-forming minerals [3,6,13,16,30]. However, the actual mechanism by which microorganisms enhance the dissolution rate of silicate minerals is still unclear. Previous laboratory studies using microorganisms did not separate the possible effect of

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chelating compounds (e.g., organic acids) from other possible dissolution-enhancing processes. Most importantly, pH varied greatly in previous studies, and so the observed enhanced dissolution could have been due to changes in pH rather than the production of chelating compounds such as organic acids. The effect of pH is important. For example, the dissolution rate of aluminosilicates is pH-independent in the pH range of 5–8, but increases greatly outside this range [e.g., 4, 5, 32]. Moreover, since most previous studies examined the dissolution of crushed minerals exposed to microorganisms growing in concentrated media without agitation, it is possible that other microbial processes, besides organic anion and proton production, may have affected dissolution. For example, it is not clear whether extracellular polymer production [18], in addition to organic anions and proton production, also affected dissolution in previous studies.

The purpose of this study was to determine if bacteria can enhance the dissolution of feldspar minerals at near-neutral pH. We used nonproliferating cell suspensions in order to simulate the very low growth rates characteristic of bacteria living in aquifers. We focused on bytownite, a calcium-rich member of the feldspar series, because feldspars are among the most abundant rock-forming minerals in the Earth's crust, second only to quartz, and because calcium-rich feldspars tend to dissolve more rapidly than other feldspars. This relatively fast dissolution rate allows for shorter, more practical experiments. We found that several bacteria substantially increased dissolution of feldspars at neutral pH by excreting gluconate.

Materials and Methods

Bacterial Strains

The bacterial isolates were obtained from the Department of Energy Subsurface Microbial Culture Collection (SMCC) maintained in the laboratory of Dr. D. Balkwill (Florida State University). We also used two commercial strains, *Zooglea ramigera* ATCC 19623 and *Pseudomonas* NCIB 11264. Strains CAP and SLI are subclones of SMCC isolate B0693 and strains CAP^- and SLI^- are nonpolymer-producing variants of strains CAP and SLI, respectively [27]. Strain B0428 was isolated from the Pee Dee formation, strains B0483, B0532, and B0550 from the Black Creek formation, and all other subsurface strains from the Middendorf formation at depths ranging from 181 to 267 m at the Savannah River Site, South Carolina. All strains were routinely cultured in PYG medium (proteose peptone, 5 g; yeast extract, 10 g; glucose, 10 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; $CaCl_2 \cdot 2H_2O$, 0.07 g; in 1 liter of deionized water) and maintained at −70°C in glycerol.

Silicate Minerals

All minerals were purchased from Wards Natural Science Establishment (Rochester, NY). Except for kaolinite, which was used as received (a fine powder), the minerals were ground and washed as described elsewhere [32]. Samples were also washed in 0.1 m*M* HCl (2 h) to eliminate the initial phase of rapid dissolution. The size fraction between 125 and 250 μm was retained for the present study. Some experiments were carried out with bytownite used in a previous experiment. Before reuse, bytownite was thoroughly washed with deionized water, rinsed in 0.1 m*M* HCl (2 h), and combusted at 550°C for 1 h. Upon reuse, the dissolution rate changed considerably, sometimes increasing up to ten-fold. Thus, to facilitate comparison among treatments tested with different batches of bytownite,

rates of dissolution are usually expressed as the ratio of the observed dissolution for a particular treatment over the dissolution rate of a control run with the same batch of bytownite.

Production of Acid and Exopolymer

Acid production was evaluated by measuring the decrease in pH in 2-day-old cultures grown in a weakly-buffered nutrient medium (pH 6.8) of the following composition (in g liter^{-1}): glucose (36), $MgSO_4 \cdot 7H_2O$ (1), $CaCl_2 \cdot 2H_2O$ (0.14), $Na_2HPO_4 \cdot 7H_2O$ (0.16), KNO_3 (1), Tris · HCl (10 m*M*), yeast extract (0.36), casamino acids (0.36), trace elements solution (0.25 ml; [20]). Aliquots (5 ml) were placed in 16 × 150 mm culture tubes and incubated with shaking. The isolates that lowered the pH below 6.3 were referred to as acid producers.

These acid-producing strains were then tested for acid production in the absence of growth. Overnight cultures in PYG were washed twice in deionized water and resuspended in 40 m*M* glucose, or 40 m*M* glucose-4 m*M* Tris (pH 6.8), at a final cell density of 3 to 4 × 10^8 cells ml^{-1}. The 20-ml washed cell suspensions were incubated for 6 days in 250-ml Erlenmeyer flasks with shaking. Organic acids in the filtered suspensions were determined by ion exclusion chromatography (ICE) using 0.5 m*M* HCl eluant at 0.8 ml/min flow through a Dionex HPICE-AS1 separator column (Dionex Corporation, Sunnyvale, CA). The chromatographic equipment used was a Dionex 2300i series ion chromatograph with sequential conductivity and UV detection. Peaks were identified by coelution of coinjected standards.

We also examined the production of extracellular polymers (exopolymers) by washed PYG-grown cells resuspended in glucose and Tris. Presence of exopolymers was examined in wet mounts, negatively stained with black ink [7], under bright field illumination at a total magnification of ×1250 with an Olympus BH-2 light microscope (Tokyo, Japan). The relatively large proportion of exopolymer-producing strains in our study does not reflect their relative abundance in the SMCC collection, because we had purposely chosen from this collection several strains forming mucoid colonies.

Dissolution in Batch Experiments

The effect of each strain on the dissolution of bytownite was examined by comparing Si release from bytownite exposed to a 1% glucose solution in Tris · HCl (50 m*M*, pH 7.0) with or without added bacteria. In all subsequent experiments with acid-producing strain B0665, PIPES (10 m*M*) was used instead of Tris because the lower pK_a of PIPES affords a larger buffering capacity than Tris. However, PIPES could not be used at concentrations greater than 10 m*M* without impairing its resolution from gluconic acid in ICE runs. Bacteria were prepared by washing twice overnight cultures (grown in PYG) in the buffered glucose solution. Fifty-milliliter aliquots of the washed cell suspension, at a final cell density of 10^8 cells ml^{-1} unless otherwise noted, were transferred to sterile 250-ml polycarbonate bottles in which 1 g of autoclaved mineral had been added. The bottles were then incubated at constant temperature (25°C) and agitation for 5 days unless other wise noted.

Dissolved Si was assayed colorimetrically with the ammonium molybdate reagent [9] on a Technicon Autoanalyzer II (Technicon Instrument Corp., Terrytown, NY). Absorbance was corrected for the turbidity caused by the bacteria. Use of glassware was always avoided in these experiments in order to minimize the amount of dissolved Si present at the onset of the experiment. Dissolved Si content just before addition of the silicate mineral was always measured and subtracted from all subsequent measurements. All experiments were run in triplicate unless otherwise noted. Total cell abundance was determined with epifluorescence microscopy of acridine orange-stained samples [11].

Dissolution Rate in Flow-Through Reactors

Mixed-bed flow-through reactors [32] were used to measure the steady-state dissolution rate of bytownite at various gluconate concentrations. Deionized water (pH 6.0) with 0.0, 0.25, 0.5, 1.0, and

2.0 m*M* gluconate (Na salt) was pumped through the columns at 6 ml h^{-1}. A recycle loop, with a flow rate of 2 ml min^{-1}, ensured a vigorous mixing of the bytownite grains. Samples of the effluent stream were collected daily for dissolved Si analysis. After an initial decline, Si concentration usually stabilized after three days. Rates of dissolution (mol of Si m^{-2} s^{-1}) were computed by multiplying the steady-state Si concentration in the effluent (from which the inlet Si concentration had been subtracted) by the flow rate, and dividing it by the initial specific surface area of the bytownite (0.297 m^2 g^{-1}). This surface area was determined by krypton gas adsorption [17] with a Quantasorb instrument (Quantachrome Corp., Syosset, NY).

Results

Effect of Bacterial Isolates on Bytownite Dissolution

Seventeen bacterial strains, of which fifteen had been isolated from subsurface sediments, were screened for their effect on bytownite dissolution. Washed cultures of the various strains were added to ground bytownite suspended in a buffered glucose solution. The increase of dissolved Si in the aqueous phase was used to monitor the dissolution of the mineral. After a few days, several strains increased dissolution more than ten-fold relative to an abiotic control and, at the same time, decreased the pH to less than 5. Since we wanted to examine the dissolution process at near-neutral pH, these data are not shown. Considering only those experimental runs for which pH remained near 7.0, we found seven strains that significantly increased the release of Si relative to an uninoculated control. These strains enhanced dissolution 1.1- to 1.4-fold over the control in 5-day incubations (Table 1); this table includes only those data referring to experiments for which the pH, initially near 7.0, did not drop below 6.5. Larger effects were observed when the experiments were conducted in two steps to allow a significant production of acid while maintaining a neutral pH during bytownite dissolution (see below).

Our initial working hypothesis was that bacterially-produced low-molecular-weight organic acids and high-molecular-weight polysaccharides affect dissolution. Therefore the different strains were examined for production of these compounds. Five strains produced acids under the conditions prevailing in the dissolution experiments. All five acid-producing strains significantly enhanced dissolution (Table 1). Seven strains deposited insoluble polymers outside the cell wall. Only one of these exopolymer producers affected the dissolution rate, i.e., the one strain, B0577, that produced both acids and exopolymers. Of the remaining six strains that produced neither acids nor exopolymers, two enhanced significantly bytownite dissolution (Table 1).

The acids produced by the bacteria were examined by ion chromatography. Washed suspensions of the different acid-producing strains were resuspended in a weakly-buffered glucose solution and incubated on a shaker table for six days. At that time, pH values were between 3.0 and 3.9. All strains produced only one major organic acid, gluconate, in the supernatant (Table 2). In addition to gluconate, much smaller amounts of other acids were produced. The strain B0577 produced traces of lactate; *Z. ramigera* produced traces of lactate and formate; and the strains B0665, C0564, and CAP^- produced lactate, formate, and unidentified 2-keto-acid. Since the strain B0665 was a particularly active gluconate producer, it was used in subsequent experiments.

Table 1. Release of Si from bytownite exposed to washed bacterial suspensions[a]

Strain	Production of: exopolymer	Production of: acid	Cell Density ($\times 10^8$ cells ml^{-1})	$[Si]/[Si]_{without\ cells}$[b] [mean ± SD (n)]
B0665	−	+	1.0	1.20 ± 0.04 (3)**
CO564	−	+	1.0	1.19 ± 0.11 (5)**
			5.0	1.32 ± 0.03 (2)**
CAP[c]	−	+	1.0	1.53 ± 0.42 (2)
			5.0	1.21 ± 0.09 (3)*
Z. ramigera	−	+	1.0	1.17 ± 0.04 (3)**
ATCC19623			5.0	1.42 ± 0.01 (2)**
BO577	+	+	2.0	1.19 ± 0.03 (2)*
CAP[c]	+	−	1.0	1.08 ± 0.11 (3)
SLI[c]	+	−	1.0	0.96 ± 0.03 (2)
BO428	+	−	1.0	1.21 ± 0.18 (3)
BO483	+	−	1.0	1.05 ± 0.06 (3)
BO550	+	−	1.0	0.80 ± 0.26 (3)
BO649	+	−	1.0	1.05 ± 0.08 (3)
Pseudomonas NCIB11264	−	−	1.0	1.05 ± 0.07 (3)
SLI^{-}[c]	−	−	5.0	0.98 ± 0.18 (2)
CO484	−	−	1.0	1.17 ± 0.03 (3)**
CO528	−	−	1.0	1.15 ± 0.06 (3)*
BO532	−	−	1.0	1.03 ± 0.13 (3)
BO724	−	−	1.0	1.23 ± 0.15 (3)

[a]Dissolved Si was assayed after a 5-day incubation (2-day incubation for strains CAP^{-}, SLI, and BO577) in 50 m*M* Tris-55 m*M* glucose. The data include only experimental runs in which the pH, initially near 7.0, did not decrease to less than 6.5

[b]**,Significantly greater than 1.00 by Student's *t*-test ($P < 0.01$); *, significantly greater than 1.00 by Student's *t*-test ($P < 0.05$)

[c]Strains CAP and SLI are subclones of isolate BO693. Strains CAP^{-} and SLI^{-} are their respective nonmucoid variants

Table 2. Acid production by nonproliferating cell suspensions after 6 days in 40 m*M* glucose with shaking

Bacterial strain	Final pH	Final concentration (m*M*) Gluconate	Lactate	Formate	2-ketoacids
B0577	3.5	1.5	0.16	−[a]	−
B0665	3.0	6.0	0.26	0.18	0.35
CO564	3.1	8.2	0.12	0.12	0.42
CAP^{-}	3.9	3.0	0.16	0.05	0.66
Z. ramigera	3.4	1.4	0.09	0.13	−

[a]Not detected (<10 μ*M*)

Time Course of Bytownite Dissolution

Bytownite dissolution with and without the strain B0665 was measured daily, along with pH and the production of gluconate. The release of Si from bytownite was much higher with B0665 than in the abiotic control (Fig. 1A). Maximum dissolu-

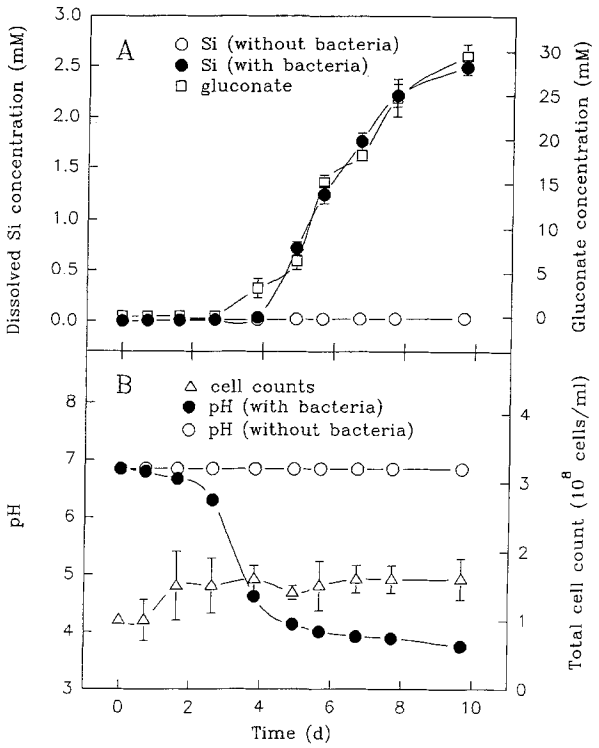

Fig. 1. Bytownite dissolution, gluconate production **(A)**, cell abundance, and pH **(B)** during a 10-day incubation of bytownite in a washed cell suspension of B0665. *Bars* represent two standard deviations (n = 3).

tion rates, computed from the slope of the Si concentration vs time, were 1.21 and 0.0062 μmol Si g^{-1} hr^{-1} (or 113×10^{-11} and 0.58×10^{-11} mol m^{-2} s^{-1}) with and without added bacteria, respectively. The bacteria had no detectable effect on dissolution during the first three days of the incubation period. After day 7, as dissolved Si neared 2 m*M*, the net rate of Si release started to level off. The accumulation of dissolved Si closely paralleled the partial oxidation of glucose to gluconate (Fig. 1A), and also paralleled the decrease in pH from 5.0 to 3.8 (Fig. 1B). In addition to gluconate, lactate and formate were also detected, although these two acids did not exceed 0.2 m*M* except at day 5 when lactate suddenly peaked at 1.6 m*M* and formate at 0.3 m*M* (data not shown).

Mechanism of Enhanced Dissolution

Because both pH and organic acid concentration varied, we cannot discriminate between proton-enhanced dissolution and the gluconate-enhanced dissolution in the time course experiment described above. To separate these two mechanisms of enhanced dissolution, a washed culture of B0665 was incubated without bytownite in a buffered glucose solution. The cells were then removed and the spent medium, containing gluconate and other compounds, was neutralized and added to bytownite. The spent medium enhanced the mineral dissolution rate three-fold over the control (Table 3). A very similar effect was observed when the cells, instead of

Table 3. Dissolution of bytownite mixed with various components of a washed cell suspension of strain BO665

Treatment	Final pH	Si released (μM)
Control	7.1	26.8 ± 3.0
Live[a]	4.2	110.0 ± 3.2
Killed[b]	6.8	75.7 ± 2.5
Spent medium[c]	6.9	80.0 ± 3.6
Gluconate (11 nM)	7.1	80.0 ± 5.6

[a] Washed B0665 cells (10^8 ml^{-1}) incubated for 5 days in 1% glucose-10 mM PIPES with shaking, neutralized, and added to bytownite

[b] Same as "Live" but killed with $HgSO_4$ (30 mg l^{-1}) just before adding bytownite

[c] Same as "Live" except cells were removed by centrifugation

being removed, were poisoned by addition of mercuric sulphate before exposure to bytownite (Table 3). The pH did not drift substantially from neutrality in these treatments, and therefore pH effects can be ruled out. However, in the absence of cell inactivation or removal, the pH value dropped again, and a slightly greater dissolution rate followed (Table 3). This experiment also indicates that the enhanced dissolution mediated by B0665 occurs independently of direct contact between bacteria and mineral surfaces, and that the compound responsible for the enhancement is in the spent medium.

In order to assess the relative importance of gluconate vs other components of the spent medium, we tested the effect of gluconate with the same molarity, 11 mM, and pH as the spent medium. This synthetic gluconate solution enhanced the rate of bytownite dissolution the same as the spent medium (Table 3), which indicates that the effect of the spent medium can be attributed to the action of gluconate.

Dissolution Rate at Steady-State

Gluconate concentrations as high as 11 mM are unlikely to occur in natural systems. The effect of lower gluconate concentrations on the rate of bytownite dissolution was therefore investigated. Various concentrations of gluconate were pumped for several days through columns containing bytownite until dissolved Si in the effluent stream reached a constant concentration. In contrast with previous experiments performed in closed batch vessels, the rate of dissolution in a flow-through system can be accurately determined because it eventually reaches a steady-state. The dissolution rates determined in this manner increased linearly as the gluconate concentration was progressively raised from 0.0 to 2.0 mM (Fig. 2). Gluconate at 1.0 mM enhanced the rate of Si release 1.7-fold. In contrast, 1.0 mM oxalate enhanced Si release 7.3-fold [32].

Dissolution of Other Silicate Minerals

Beside bytownite, B0665 also enhanced the dissolution rate of other silicate minerals. After six days, the bacteria increased Si-release from quartz 1.7-fold, from

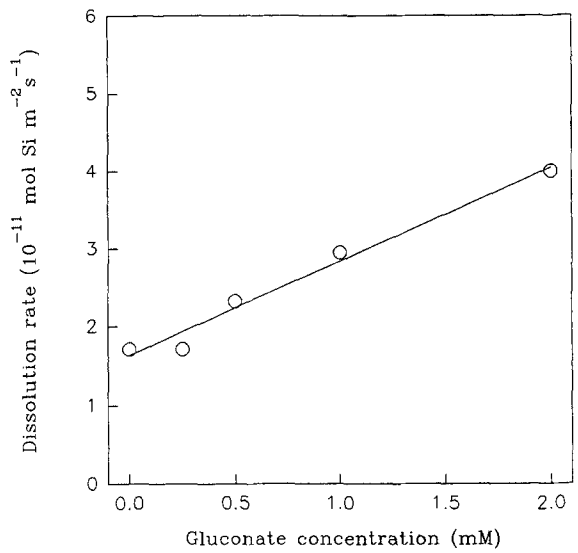

Fig. 2. Bytownite dissolution rate as a function of gluconate concentration at pH 6.

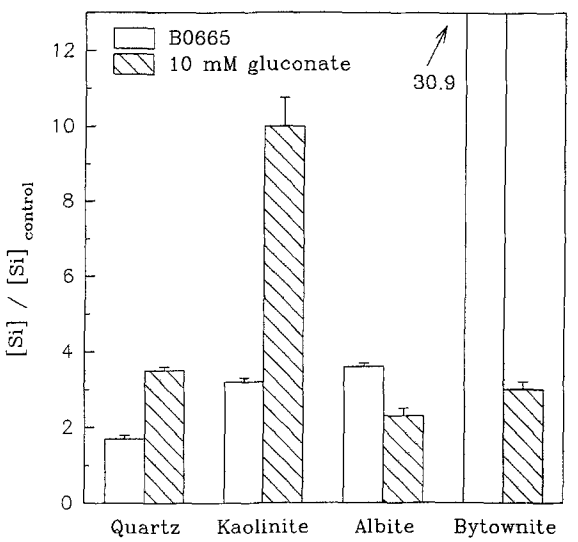

Fig. 3. Dissolution of various silicate minerals after 6 days in a washed cell suspension of strain B0665 (final pH was 3.8 and final gluconate concentration was 11 m*M*). The bacteria-promoted dissolution is compared with gluconate-promoted dissolution (10 m*M*; pH 6.9). *Bars* represent one standard deviation (n = 3).

albite 3.6-fold, and from kaolinite 3.2-fold compared to an abiotic control (Fig. 3). Bytownite dissolution, which is comparatively more sensitive to low pH values, increased 31-fold. As mentioned above in the case of bytownite, both proton-promoted dissolution (final pH was 3.8) and gluconate-promoted dissolution (final gluconate concentration was 11 m*M*) could have contributed to the enhanced dissolution of albite and kaolinite by strain B0665. Quartz, on the other hand, is not susceptible to proton-promoted dissolution above pH 3 [4,5].

To separate these two effects, dissolution rates in 10 m*M* gluconate at neutral pH was examined. The effect of gluconate was similar for the various minerals,

ranging from a 2.3-fold increase for albite to a 3.5-fold increase for quartz (Fig. 3). Dissolution of kaolinite was increased ten-fold.

Discussion

Several laboratory studies have suggested that acid-producing microorganisms can enhance the dissolution of various types of minerals. However, none of these studies identified unequivocally the mechanism underlying the process of enhanced dissolution. For example, the proposed effect of excreted chelating compounds (organic acids) has never been assessed independently of extremely low pH and often not independently of low redox potential. Our data singled out the partial oxidation of glucose to organic acids as the predominant mechanism by which the nonproliferating bacteria used in our study enhanced feldspar dissolution at neutral pH.

The putative effect of organic acids on bytownite dissolution was investigated in some detail with the strain B0665, an active gluconate producer. The stimulatory effect of this bacterium on bytownite dissolution required some aspect of its metabolism, since the mere presence of bacterial cells had no detectable effect on dissolution during the first three days of incubation. Moreover, the mechanism of dissolution enhancement did not require direct contact between cells and mineral surfaces, because the stimulatory effect of a 5-day-old washed suspension was only slightly less after removal of the cells; the slightly lesser effect was probably caused by the absence of further acid production after cell removal. These two observations suggest that B0665 enhanced dissolution by excreting a compound into the surrounding medium. A comparison of the effects of gluconate, spent medium, and live culture suggested that the production of gluconate was the sole mechanism by which strain B0665 enhanced bytownite dissolution in the experiments conducted at neutral pH. This conclusion probably applies to the other four acid-producing isolates, since all produced mainly gluconic acid.

Production of organic acids is not limited to anaerobic metabolism. Even under oxic conditions, many bacteria excrete organic acids [14,15,16,23,24,25,30,31], the type of acid produced usually being a function of the nature of the limiting nutrient [23,24]. This incomplete oxidation of a carbon substrate in an oxic environment has been referred to as overflow metabolism [24]. It should be noted that various physiological advantages have been postulated for bacteria producing these different acids under specific growth limitations [24]. For example, the production of gluconate (or 2-ketogluconate), which seems common to many bacteria [14,15,16,23,31], may be used to generate energy at a high rate [24].

Other common by-products of overflow metabolism include, e.g., pyruvate, acetate, or 2-ketoglutarate [23,24]. These metabolites can also enhance dissolution of silicate minerals [32]. Therefore, gluconate is obviously not the only microbial metabolite that may have an impact on the dissolution rate of silicate minerals.

Previous reports on organic acid production by bacteria under aerobic conditions focused on rapidly-growing cells. For example, Wanner [28; translated in 29] found that the production of acetate in batch cultures of *Klebsiella pneumoniae* took place only during the exponential phase of growth. In contrast, the present study indicates that bacteria can excrete organic acids at a fairly high rate, 6×10^{-14}

mole cell^{-1} d^{-1}, in the absence of significant growth. This observation suggests that bacteria, which are growing very slowly if at all in soils and aquifers, may produce organic acids after exposure to a carbon source. The starvation regime characteristic of oligotrophic environments may, in fact, induce the release of incompletely oxidized organic compounds [1].

Bacterial metabolism appears to be involved in dissolving rock-forming minerals in aquifers but the actual mechanism is still unclear, especially at near-neutral pH [10]. Our results confirm that bacterial can dissolve rock-forming minerals near pH 7.0 and further suggest that chelating compounds (organic acids), rather than exopolymers, are involved. The dissolution-enhancing properties of many organic acids have already been well established in the geochemical literature [e.g., 12, 19, 32]. These studies, together with the data presented here, suggest, however, that the concentration of organic acids in terrestrial environments is too low to account for the observed enhancement of dissolution. Indeed, maximum concentrations of organic acids in terrestrial environments reach only a few tenths millimolar in uncontaminated aquifers [21] and about one millimolar in oil-contaminated aquifers [2]. However, it is expected that high concentrations of organic acids may build up around bacteria adhering to a mineral surface [8], in effect creating micro-reaction zones. The existence of such micro-reaction zones is supported by microscopic analysis of eroded minerals [10,26]. Thus, even when the concentration of dissolution-enhancing compounds in the bulk liquid phase is very low, dissolution may nonetheless be governed by biological processes in micro-reaction zones around attached colonies. More work is needed, however, to fully appreciate the relative importance of these restricted reaction zones.

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