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Mechanism of Silicate Binding to the Bacterial Cell Wall in *Bacillus subtilis*

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To investigate the chemical mechanism of silicate binding to the surface of *Bacillus subtilis*, we chemically modified cell wall carboxylates to reverse their charge by the addition of an ethylenediamine ligand. For up to 9 weeks, mixtures of Si, Al-Fe-Si, and Al-Fe-Si plus toxic heavy metals were reacted with these cells for comparison with control cells and abiotic solutions. In general, more Si and less metal were bound to the chemically modified surfaces, thereby showing the importance of an electropositive charge in cell walls for fine-grain silicate mineral development. The predominant reaction for this development was the initial silicate-to-amine complexation in the peptidoglycan of ethylenediamine-modified and control cell walls, although metal ion bridging between electronegative sites and silicate had an additive effect. The binding of silicate to these bacterial surfaces can thus be described as outer sphere complex formation because it occurs through electrostatic interaction.

The surface of the gram-positive bacterium *Bacillus subtilis* has been found to contribute to the formation of fine-grained silicate minerals in laboratory simulations of soil settings (27). Although the binding of metallic cations by different bacteria has been extensively studied (e.g., see references 3–5, 18, 20, and 21), less information is available about the binding of anions and even less about silicate.

The fabric of bacterial cell walls is predominantly electronegative (1, 5, 7, 15, 19), as are most of the superficial layers above them such as capsules or sheaths and some S layers (2, 12). However, in gram-positive organisms at circumneutral pH, these walls also possess a certain number of electropositive amino groups that are available for reaction with soluble anions. In *B. subtilis* walls, these are from the D-alanine residue of the teichoic acid, the amino sugar of the glycan, and the amino function on the diaminopimelic acid from the peptide portion of the peptidoglycan (8). In addition, recent information from *B. subtilis* suggests that, in respiring cells, the proton motive force extrudes H^+ into the wall, which neutralizes many of the electronegative sites (16). These metabolically active cells would then have more accessible electropositive sites within their walls for interaction with dilute environmental anions such as SiO_3^{2-}.

Although infrequent, anion binding to bacterial surfaces has sometimes been seen. For example, the binding of sulfate and carbonate anions can be mediated by calcium bridging, as has been shown during the initiation of gypsum and calcite nucleation on the surface of the cyanobacterium *Synechococcus* (23, 26). Also, the nucleation of sulfides was obtained with copper- or zinc-loaded *B. subtilis* cells in presence of elemental sulfur (6). Mineral nucleation through anion binding to wall-bound cations seems to be one of the processes involved in the formation of minerals by bacterial surfaces. The formation of ternary complexes of this kind is not uncommon in inorganic adsorption at oxide-water interfaces (14, 24). Results from our own previous experiments (27) indicated that silicate nucleation might be occurring in a similar way. However, it is possible that the native positively charged groups of the wall also play a direct role in silicate binding. Certainly, the distribution and abundance of positive charges within the wall has been shown to influence cation binding (7, 9), and it should have an even greater effect on anion interactions.

Because our previous work had shown the surface of *B. subtilis* to be capable of mediating fine-grain silicate production (27) and because the same study could not differentiate between an amine to SiO_3^{2-} or metal bridging to carboxylate mechanism as the nucleation process, in the present study we chemically modified all surface COO^- groups with ethylenediamine so that they became electropositive with an NH_4^+ group. Accordingly, no carboxylates remained available for metal-bridging SiO_3^{2-} and only NH_4^+ groups were exposed for the anion to interact with.

MATERIALS AND METHODS

Growth and harvesting. *B. subtilis* 168 was grown at room temperature in tryptic soy broth supplemented with a 0.5% (wt/vol) of yeast extract (Difco). When the culture reached an exponential phase of growth (optical density at 600 nm (OD_{600}) of approximately 0.2), cells were pelleted by centrifugation (14,000 × *g*) and washed two times with high-resistance ultrapure deionized water (udw) (pH 5.8 to 6.2). After washing, the cells were resuspended in udw to get OD_{600}=0.2.

Chemical modification of wall carboxylate groups. Carboxylate groups of the wall fabric of whole cells were modified to become electropositive by chemical reaction with ethylenediamine (Fisher Scientific Co.) after activation with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma). An aqueous suspension of cells was made 0.5 M with ethylenediamine, and the carbodiimide was added to get a concentration of 0.2 M. The mixture was continuously stirred at room temperature for 6 h under mildly acidic conditions (pH 4.5 to 5.0). After this time, the cells were washed four times with udw, pelleted by centrifugation (14,000 × *g*), and then resuspended in udw to OD_{600}=0.2. Ethylenediamine reacts with the carboxylic groups in the

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TABLE 1. Conditions of the experiments, abbreviations used in the text, and sampling times

System	pH	Bacterial charge	Abbreviation	TIME (days)			Time (wks)			Name
				10	17	21	4	9	14	
Si	5.5	+++	MQ	x[b]	x	x				MQ.Si
		+ and −	BS	x	x	x				BS.Si
		NC[a]	SC	x	x	x				SC.Si
Al-Fe-Si	5.5	+++	MQ				x	x		MQ.AlFeSi
		+ and −	BS				x	x		BS.AlFeSi
		NC	SC				x	x		SC.AlFeSi
Metal-Si	4.5	+++	MQ				x	x		MQ.MSi
		+ and −	BS				x	x	x	BS.MSi
		NC	SC				x	x	x	SC.MSi

[a] NC, no cells.
[b] x, sample.

presence of carbodiimide, making the sites within the walls electropositive (7, 9).

Silicate-metal experiments. Three sets of experiments were conducted with the chemically modified *B. subtilis* cells (MQ).

(i) Experiment 1. An OD_{600}= 0.2 MQ bacterial suspension was mixed 1:1 with an Si solution (as $Na_2SiO_3 \cdot 9H_2O$, pH 5.5; Fisher) to get a final concentration of 0.15 mM H_4SiO_4, using 0.1 M HNO_3 to adjust pH immediately before adding the bacterial cells. Chemically unmodified *B. subtilis* (BS) and inorganic controls (SC) were treated under the same conditions. Duplicate tubes of all experiments were used, and each set was centrifuged (14,000 × *g*) after 10, 17, and 21 days. Experiments could not be incubated longer because bacterial lysis commenced at periods over 30 days if no heavy metal ions (e.g., Fe) were present (10). Pellets were processed for electron microscopy (unstained whole mounts and conventional plastic embeddings with Epon 812) and imaged with a Philips EM300 transmission electron microscope (TEM) operating at 60 kV. Energy-dispersive X-ray spectroscopy (EDS) was conducted with a LINK-Analytical eXL/LZ-5 system connected to a Philips EM400T electron microscope operating at 100 kV. EDS analyses were conducted with a spot size of 400 nm, a beam current of 0.1 μA, and a counting time of 200 s, live time. Supernatants were analyzed for Si by graphite furnace atomic absorption spectrometry (AA) in a Perkin-Elmer 2380 spectrophotometer. This experiment therefore generated three data sets to be read; the chemically modified cells reacted with silicate (MQ.Si), unmodified cells plus Si (BS.Si) as a cell control, and an acellular inorganic solution control (SC.Si) (Table 1).

(ii) Experiment 2. Experiment 2 used an incubation with an Al-Fe-Si solution at pH 5.5 as was used in our previous experimentation (27) so that the final solution contained 5 μM Al, 1 μM Fe, and 0.15 mM H_4SiO_4. Both Al and Fe were added as nitrates (Fisher ultrapure grade). Untreated cells (BS) and inorganic controls (SC) were also established so that this experiment also had three data sets, MQ.AlFeSi, BS.AlFeSi, and SC.AlFeSi (Table 1). Two replicates of each sample were collected at 4 and 9 weeks and were pelleted by centrifugation (14,000 × *g*). The supernatants were analyzed for Si, Al, and Fe by AA, and the pellets were processed for electron microscopy (unstained whole mounts and conventional embeddings).

(iii) Experiment 3. Experiment 3 used an incubation with a metals-Si solution at pH 4.5 as in experiment 2 except 1 μM Pb, Cr, Cu, Cd, Ni, and Zn were added to the 1 μM Fe, 5 μM Al, and 0.15 mM H_4SiO_4 solution. All metals were added as nitrates and Si was added as $Na_2SiO_3 \cdot 9H_2O$ as before (Fluka and Fisher). Untreated *B. subtilis* (BS) and inorganic controls (SC) were also set up. Samples in duplicate were collected as explained in experiment 2, supernatants were analyzed for Pb, Cr, Cu, Cd, Ni, Zn, Fe, Al, and Si by AA, and pellets were processed for electron microscopy. These are the MQ.MSi, BS.MSi, and SC.MSi samples (Table 1).

All experiments were performed at room temperature, and in all cases, acid-leached plasticware was the only material used to ensure no extraneous Si or metal contamination. Five injections per replicate were analyzed by AA following the standard conditions outlined in the service manual for each element. Statistical analyses of variance were conducted on the resulting data, and the lower statistical differences for the means were obtained only when the F-test showed statistical significance at a confidence level higher than 95%.

RESULTS

Incubation with silicon. The amount of Si adsorbed by MQ.Si was significantly greater at the 99% level of confidence than for BS.Si and SC.Si at 10 to 21 days (Fig. 1).

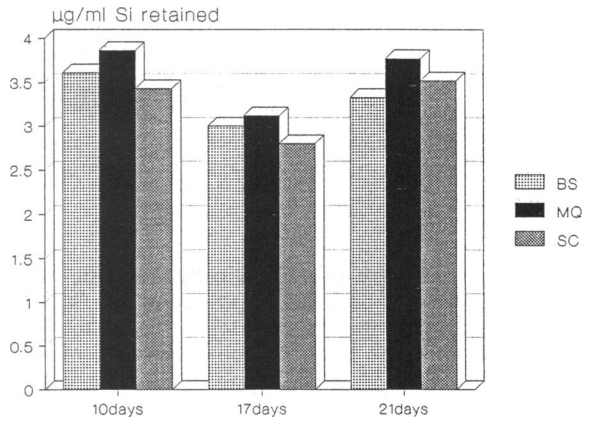


FIG. 1. Si retained by untreated (BS) and chemically modified (MQ) cells and inorganic controls (SC) after incubation with silicate for 10, 17, and 21 days at pH 5.5. Values are the means of two replicates with five injections per sample.

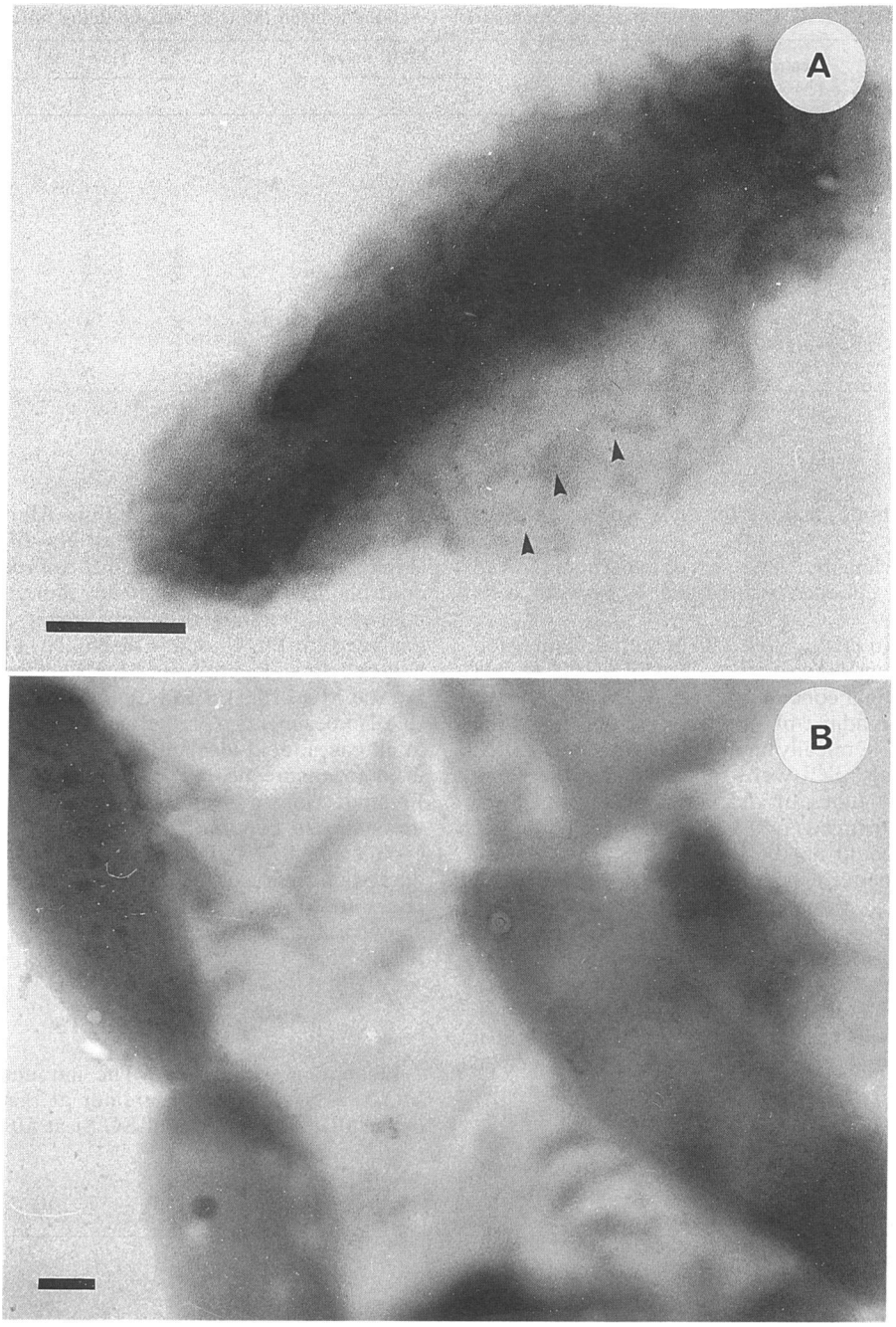


FIG. 2. Mineral precipitates (arrowheads) in whole-mount preparations of the MQ.Si and BS.Si experiments. (A) MQ.Si after 21 days; (B) BS.Si, as a cellular control, after 21 days for comparison. Bar represents 200 nm.

BS.Si and SC.Si were not statistically different from one another. Differences between MQ.Si cells and SC.Si were 0.43, 0.32, and 0.25 mg of Si per liter at 10, 17, and 21 days, respectively, whereas the differences between MQ.Si and BS.Si were 0.65, 0.30, and 1.1 mg of Si more per g (dry weight) of chemically modified cells (Fig. 1).

Electron microscopy showed the presence of numerous small precipitates on the surface of MQ.Si cells together with some bigger crystallites (Fig. 2A). Fewer, if any, crystallites appeared on the surface of the untreated cells (Fig. 2B). Autolytic activity as judged by TEM started to appear in both MQ.Si and BS.Si after 17 days.

Al-Fe-Si system. AA showed a higher adsorption of silicate anions at the 99% confidence level by the MQ cells than either the inorganic (SC) or the bacterial (BS) controls (Fig. 3). Immobilization increased during the 4- to 9-week period in MQ, whereas it did not change over time in either BS or SC controls. In these cells, electron microscopy showed the presence of abundant precipitates around the cell surfaces (Fig. 4). The untreated BS cells possessed smaller granular

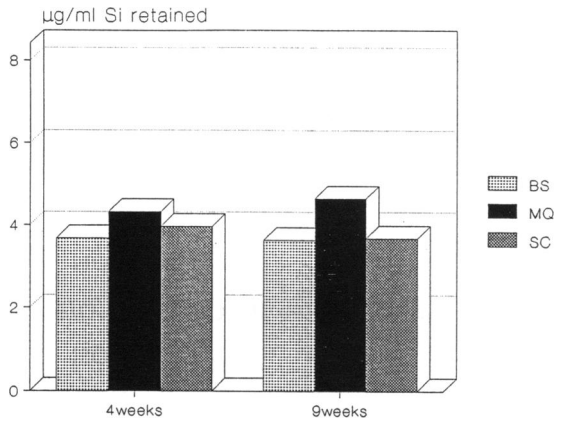


FIG. 3. Amount of Si retained in the Al-Fe-Si system at pH 5.5 after 4 weeks. Values are means of two replicates and five injections per sample.

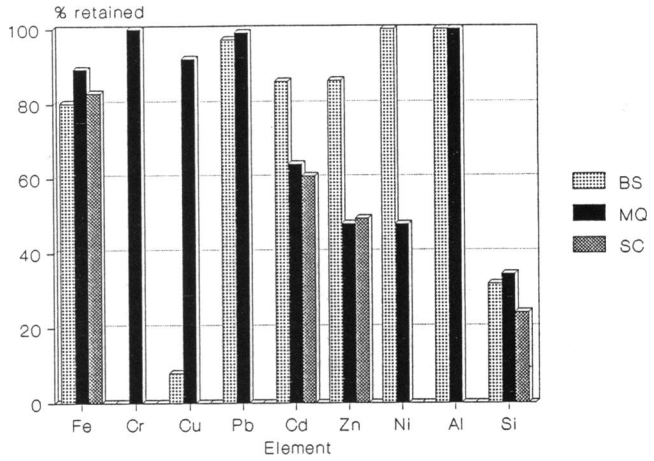


FIG. 5. Amount of metal and Si (expressed as percentage of element) retained in the metal-Si system after 4 weeks. Values are means of two replicates and five injections per sample.

iron precipitates with little Si in their composition. MQ cells immobilized 1.8 (4 weeks) and 2.5 (9 weeks) mg of Si more per g of cells on a dry weight basis than BS cells did over the same period. There was no clear effect on Al and Fe immobilization between MQ and BS after the charge modification.

Metals-Si system. Si retention by MQ cells at 4 weeks was slightly higher than in BS, and in both cases, it was greater than that of the inorganic (SC) controls (Fig. 5); however, Si retention by MQ cells was reduced at 9 weeks (Table 2).

For the metals, Cu adsorption was clearly favored by the increase in positive charges on the bacterial surface (Fig. 5; Table 2); at both sampling times, MQ cells retained almost all the available Cu. Cr immobilization by MQ cells was also high (Table 2), and EDS analysis of the 4-week samples showed an increase in Cr content in individual crystallites from MQ compared with those on BS (Table 3). By comparison, Cd and Zn adsorption were strongly reduced in MQ (99% confidence) compared with BS, and they approached the SC levels (Fig. 5); e.g., at 9 weeks, Zn retention by MQ cells was 8%, whereas it was 72% in BS (Table 2). Pb adsorption was not seriously affected by the modification of cell surface charge at 4 weeks, and although it decreased noticeably at 9 weeks, it maintained higher levels than the inorganic retention (SC). Fe was strongly immobilized in all systems (MQ, BS, and SC), but Al had a stronger tendency to sorb (at least initially) to the cellular systems (MQ and BS) (Table 2; Fig. 5).

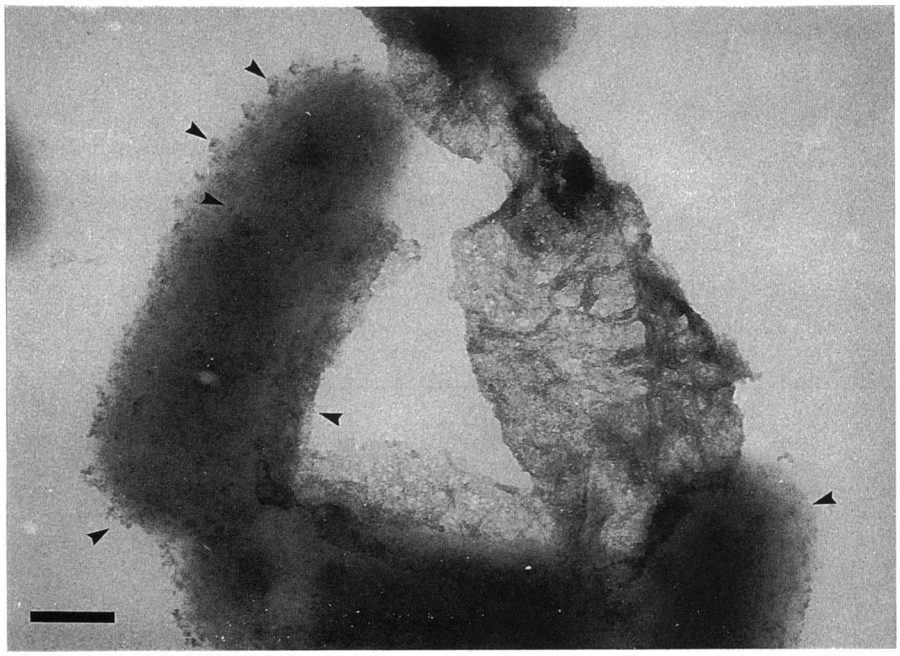


FIG. 4. Whole-mount TEM micrographs of the mineral crystallites (arrowheads) on the MQ samples in the Al-Fe-Si system at 9 weeks. Scale bar represents 200 nm.

TABLE 2. Metal retention in the metals-Si system

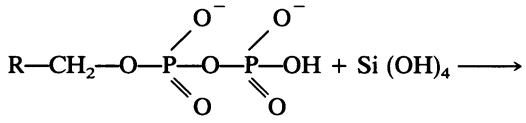
Element	% of element added					
	MQ		BS		SC	
	4 wks	9 wks	4 wks	9 wks	4 wks	9 wks
Si	34	14	32	48	24	54
Fe	89	100	80	75	83	95
Al	96	100	100	83	0	63
Pb	99	62	97	88	0	0
Zn	48	8	86	72	49	37
Cd	64	63	86	69	61	71
Cu	92	100	8	88	0	41
Cr	100	91	0	76	0	72
Ni	48	100	100	81	0	40

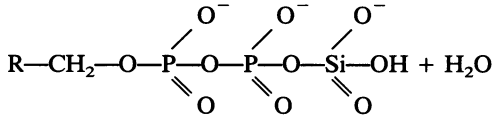
Electron microscopy of whole mounts showed extensive precipitation of metallic silicates on the modified bacterial surfaces (Fig. 6 and 7). These could be granular (Fig. 7A) in which case their metal content was high (Fig. 7B), whereas at other times, they were more crystalline (Fig. 7C) and had lower metal concentrations.

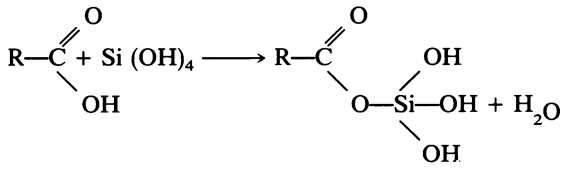
Thin sections extended the whole-mount electron microscopy observations and proved that the mineralization occurred entirely around the cell periphery at the level of the cell wall (Fig. 8). These minerals consisted of crystallites with a high Si content which were most extensive at the 14-week period.

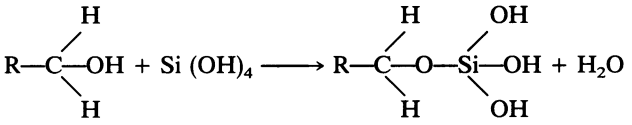
DISCUSSION

Silica binding to organic compounds can occur by several different mechanisms. Krumbein and Werner (17) suggested three possible linkage patterns through phosphoryl, carboxyl or hydroxyl complexation:









Another mechanism, the polymerization of soluble Al-Fe hydroxy species in the presence of soluble silica, has been suggested as explaining the occurrence of polycations and amorphous silica complexes which are sometimes found in soils (25). That is,

$$(Al,Fe)_n(OH)_{3n-x}^{x^+} + xSi(OH)_4 \rightarrow$$

$$(Al,Fe)_n(OH)_{3n-x}[OSi(OH)_3]_x + xH^+$$

This reaction would result in a reaction mixture pH decrease that was not found in previous experiments conducted with Si and Al-Fe in the presence of bacteria (27). Other studies with oxide surfaces suggested that either cations or anions may bind to surface hydroxyl groups at the water-oxide interface (14).

The observation of silicate binding and the production of metallic silicates by bacterial surfaces with soil-simulating concentrations (27) suggested that at least two different mechanisms might be participating in the process of bacterially mediated silicate formation, (i) the binding of SiO_3^{2-} to positively charged groups of the wall (amine groups from either the D-alanines of the teichoic acid or the diaminopimelic acid from the peptide portion of peptidoglycan and the amino sugars of the glycan) or (ii) the binding of SiO_3^{2-} through cationic bridging by preexisting wall-bound metallic cations. The former would be facilitated by the proton motive force in respiring cells (28) and a low pH in a medium with nonrespiring cells; in both cases, H^+ would reduce the number of negatively charged carboxyl radicals and, consequently, the electrostatic repulsive forces for anions. The metasilicate anion would then form outer sphere complexes since electrostatic interactions are mostly involved.

TABLE 3. Composition of individual crystallites found on the bacterial surfaces of MQ.MSi 4-week samples obtained by EDS

Sample	% Wt of:									Morphology
	Fe	Si	Al	P	Cd	Ni	Cr	Zn	Pb	
Whole mount	10.0	70.3	6.9	4.7			2.3	4.7	1.0	See Fig. 6A
	10.0	71.4	7.3	1.1	2.9	1.1	1.6	2.3	2.1	See Fig. 6C
	8.8			85.7		2.7	0.6	2.1		Small precipitate
	2.4		1.7	6.1		1.1		88.9		Small precipitate
	9.7	1.9	34.8	31.7	2.3		13.3	0.4	5.8	Granular
	6.8	4.7	39.6	24.1		1.5	14.8	1.5	7.0	Granular
	13.7	36.1	18.5		5.1	5.4	1.4	3.5	16.5	Crystalline
Thin section	5.4	21.7	22.0	38.6	2.1	0.2	6.9	2.4	0.6	Mineralized wall
	4.5	39.9	28.6	15.5	0.3		8.2	1.3	1.7	Mineralized wall
	2.9	80.5	4.4	10.1	0.3		0.4	1.3	0.1	Layered

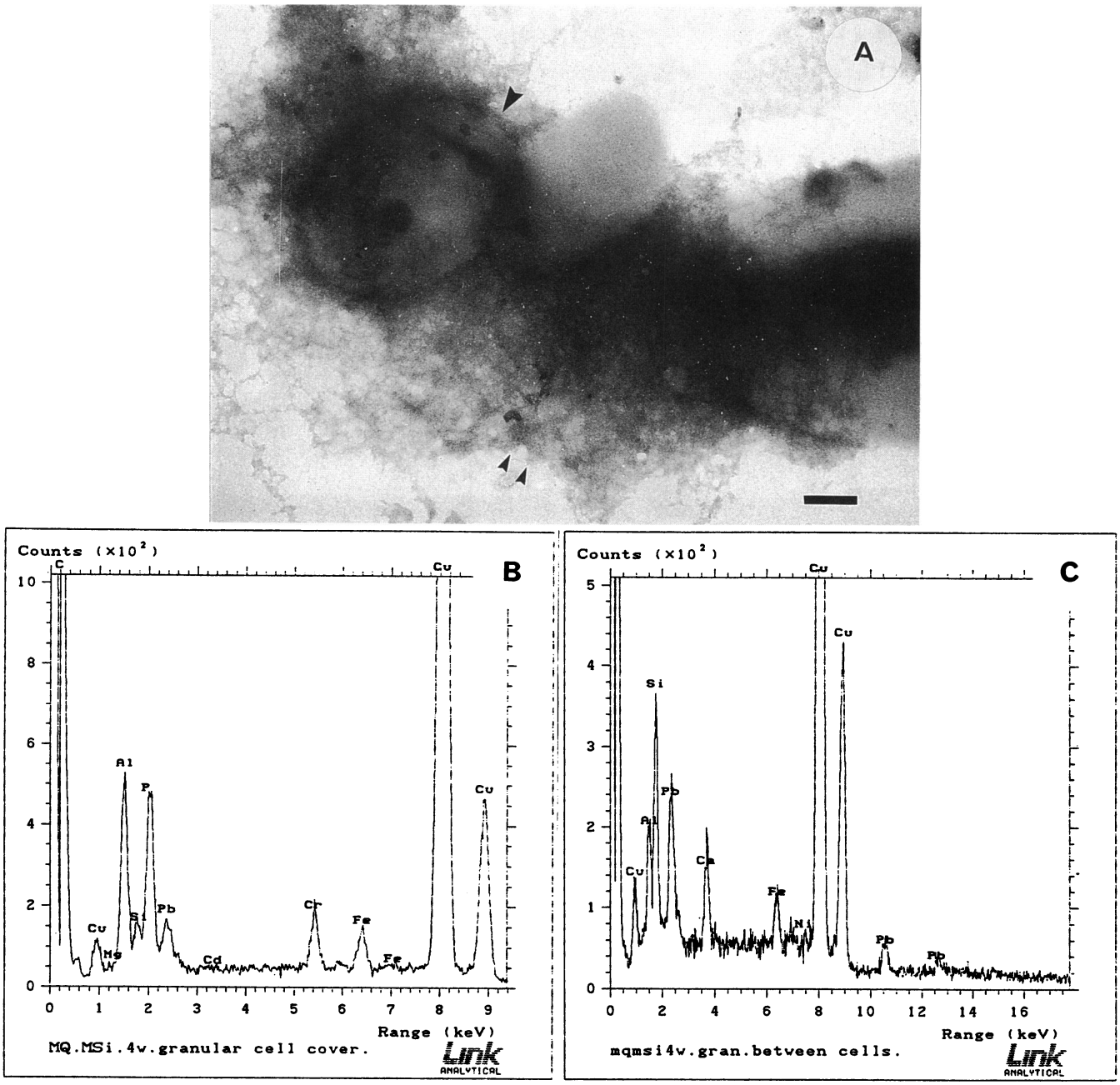


FIG. 6. Whole-mount TEM micrographs of the mineral forms observed on the MQ samples at 4 weeks in the metals-Si system. (A) Mineral morphology (scale bar represents 200 nm). (B) EDS spectrum of the cell surface (large arrowhead in panel A). (C) EDS spectrum of a region away from the cells (small arrowhead in panel A). EDS-Cu peaks correspond to the Cu grid for TEM; P and Ca peaks correspond to the cell wall and Al, Si, Pb, Cr, Fe, and Ni correspond to the mineral phase.

The objective of the present work was to give some insight into the mechanism of silicate binding to bacterial surfaces. Cells whose —COO^- groups were converted into electropositive groups by the addition of ethylenediamine were used to monitor the binding of silicate anions in progressively more complicated systems: silicate alone, silicate with Al and Fe, and finally, silicate, Al, and Fe combined with different heavy metals (Pb, Cd, Cr, Cu, Zn, and Ni). When Si is the only element added, cell lysis will occur after 30 days (10); for this reason, samples were observed only over a 3-week period. All other experiments contained heavy metals which inactivate autolysins, and these experiments were allowed to proceed for 4 and 9 weeks.

The amount of silicate that was adsorbed in the incubations with Si or in the Al-Fe-Si system showed that positively charged *B. subtilis* (MQ) has the capacity to bind a greater amount of silicate than the untreated cells (BS) or the inorganic controls (SC) (Fig. 1 and 3). The MQ samples adsorbed 1.1 mg more Si per g of cells (dry weight) after only 21 days than those with untreated surfaces, and 1.8 and 2.5

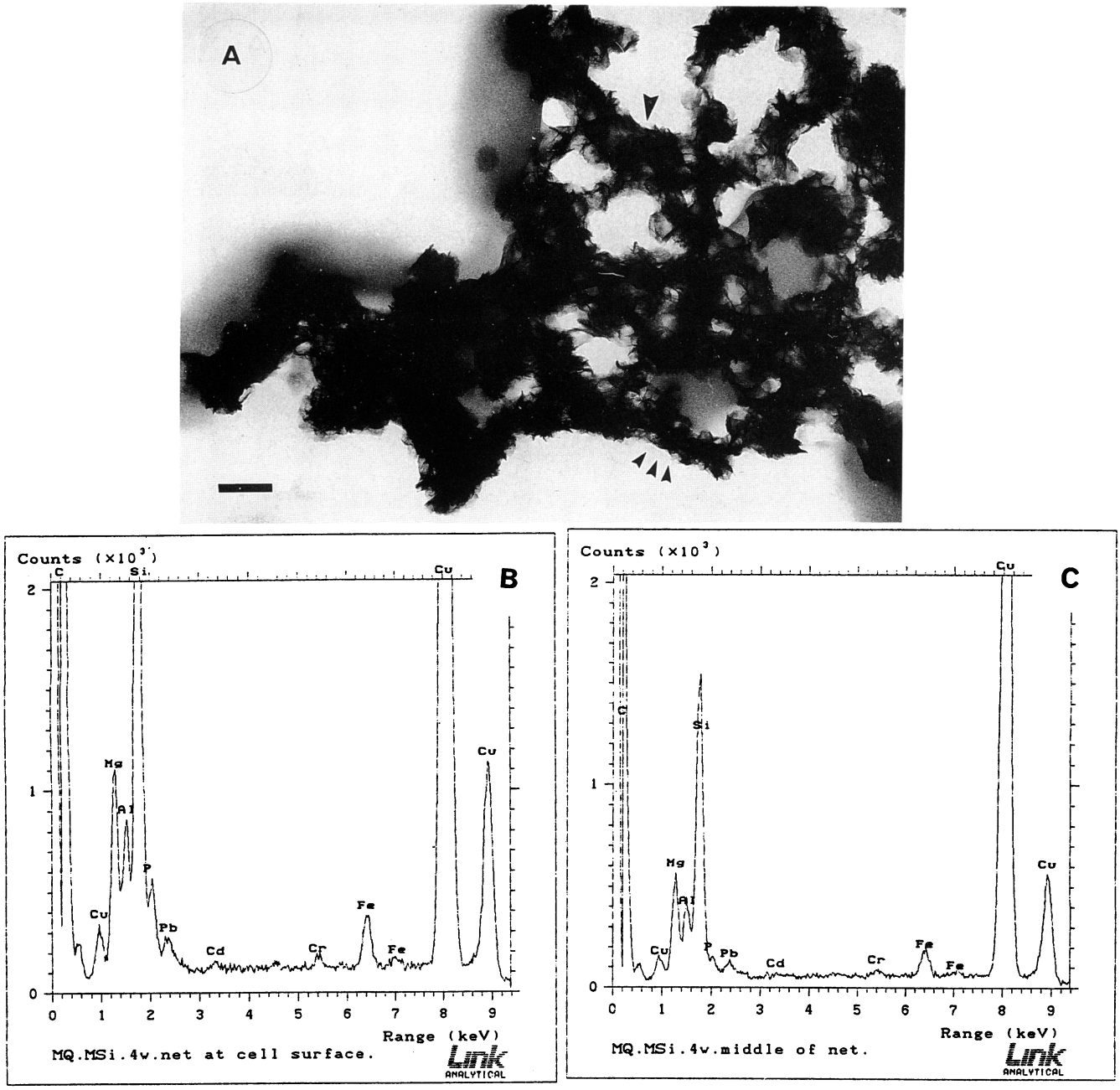


FIG. 7. Morphology of the minerals on the MQ surfaces at 4 weeks (metals-Si system). (A) Bacterially associated mineral forms (scale bar represents 200 nm). (B) Representative EDS spectrum of the minerals at the cell surface (small arrowhead in panel A). (C) EDS spectrum of those precipitates with more crystalline morphology (large arrowhead in panel A). Cu peaks are due to the Cu grid used for TEM, P and Mg to the cell wall, and Al, Si, Pb, Cd, Cr, and Fe to the experimentally obtained mineral forms.

mg/g at 4 and 9 weeks, respectively, when Al and Fe were present. These results suggest that the positive charges on the surface were participating directly in the binding of silicate anions.

When cations such as heavy metals were added, the adsorption of Si was slightly higher in the positively charged than in the untreated cells at 4 weeks (Fig. 5), but it was lower at 9 weeks (Table 2). Heavy-metal cations can act as bridges for anion binding by formation of ternary surface complexes, a process that is not unusual for a variety of solid-liquid interfaces under inorganic conditions (22). Yet, the additional positive charge did not improve silicate adsorption when the cationic metals were present. Possibly, this was because metal retention (especially that of Zn and Cd) was reduced by the increase in positive charges within the wall and metal bridging was discouraged. The presence of amino groups has previously been found to negatively affect metal-wall interactions (7, 9). This same observation

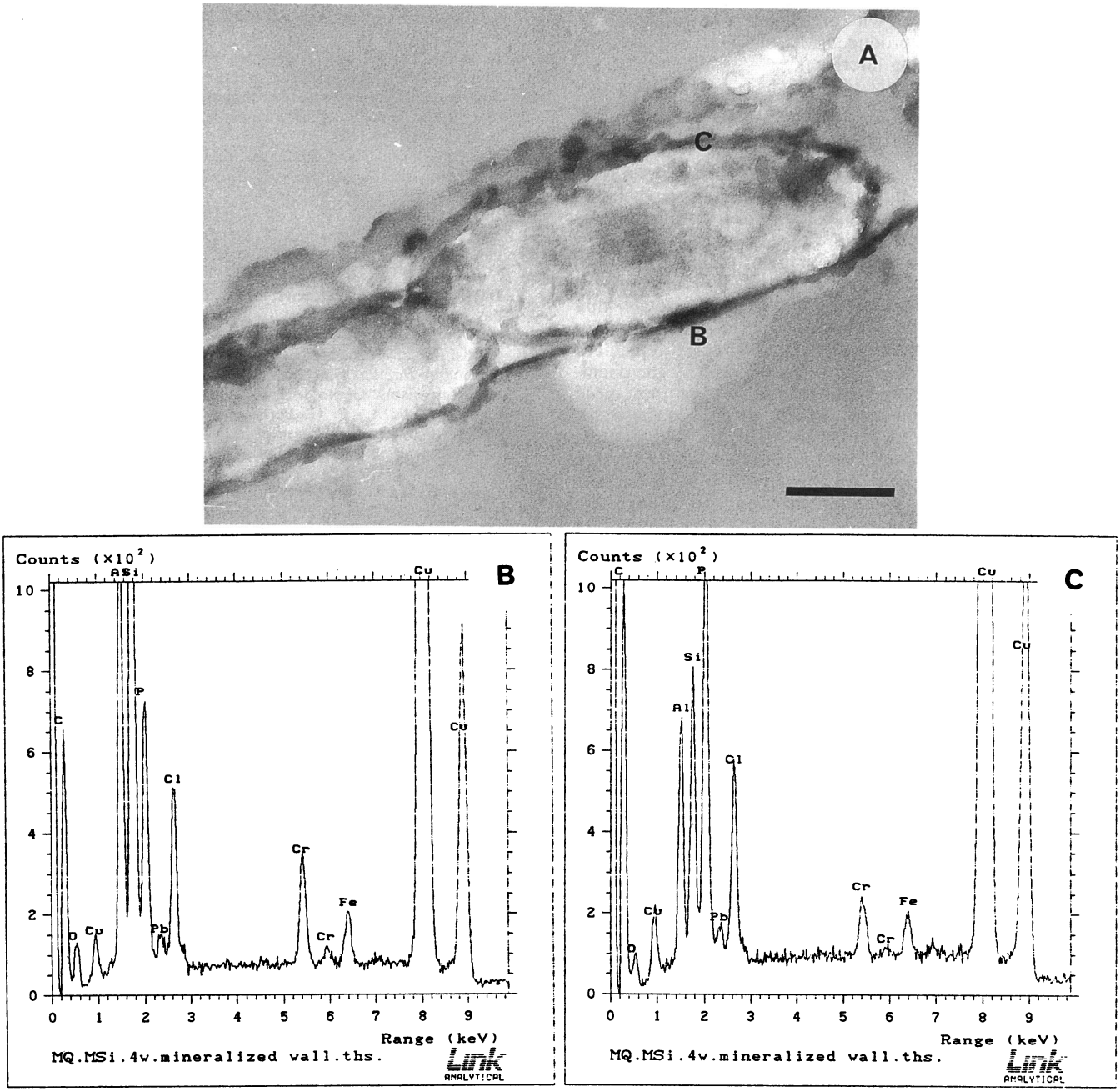


FIG. 8. Micrograph of a thin section of the MQ cells in the metals-Si system after 4 weeks. (A) General appearance of the cells (bar represents 200 nm). (B and C) EDS spectra of two different regions of mineralized wall, as marked in panel A by the letters B and C, respectively. Cu peaks are from the Cu grid used for TEM, Cl is from the Epon resin used for embedding, P is from the cell wall components, and Al, Si, Pb, Cr, and Fe are from the experimentally obtained mineral phases.

was made when Fe-pretreated cells were exposed to silicate under acidic conditions (27). Accordingly, the binding of silicates (i.e., SiO_3^{2-} metasilicate, SiO_4^{4-} orthosilicate, or polymerized forms that are favored by acidity [13]) is most favored by electropositive sites on the cell surface. For *B. subtilis*, these are the amino functions within the peptidoglycan (7, 9), and an increase in the number of these functions increases silicate binding and mineral development. But even then, metal bridging still had some influence, albeit a more minor one. Presumably, the metal cation influence on silicate binding depends on a delicate balance between available electronegative sites (COO^-, PO_4^{3-}, or OH^-) for metals and bridging, and electropositive sites (NH_4^+) for silicate, the latter being the more dominating nucleation site for outer sphere complexation. In cell surfaces with a predominance of electronegative sites, such as the untreated BS control, the amine sites would rapidly be filled by silicate and the metal-bridging mechanism would be left to proceed.

Overall, natural surfaces with greater electropositivity, similar to our artificially produced MQ surfaces, would make better silicate-generating interfaces.

During silicate mineralization, it was possible that heavy metal sorption would be profoundly affected. Clay-like materials have good capacity for metal binding, but this capacity is actually surpassed by that of bacterial walls (11, 29). Therefore, it was important to also look at metal sorption during our study. As stated previously, the overwhelming tendency of increased electropositivity in the walls was for metal sorption to decrease (Fig. 5; Table 2). There were also some unexpected results. For example, increased electropositivity had little effect on Pb at 4 weeks but caused a decrease in binding at 9 weeks. Unlike other metals, thin sections showed both a wall and cytoplasmic location of Pb at 4 weeks; at 9 weeks only the cytoplasmic Pb remained. Although Zn and Cd were negatively effected by the chemical treatment of the cell surface (suggesting that these metals were mainly bound to carboxyl groups on the wall), Cu had a high affinity for positively charged amino groups, and this is in agreement with the previous work of Beveridge and Murray (7). Cr showed the same behavior as Cu in these experiments. Initially, Ni immobilization was strongly reduced, but it increased considerably from 4 to 9 weeks. It is possible that Ni is later bound to phosphate groups of teichoic acid which are still present on the cell wall.

Under acidic conditions in natural open systems, where runoff water drains freely, rock weathering is intensified. First, the loss of easily soluble elements (Ca, Mg, Na, K, CO_3^{2-}, etc.) from the rock minerals occurs, and in time, the more resilient elements such as Si, Al, and Fe will be sequentially mobilized. Minor constituents, such as heavy metals, will also be solubilized. These are the conditions under which our bacterial cells proved to be most efficient at forming metallic silicates on their surfaces, drastically reducing the level of toxic metals in solution. Clearly, acidic conditions are not amenable for the growth of all bacteria and may even induce death, but even then, their surfaces remain powerful sorption agents (28). Because bacteria are ubiquitous to soils and sediments and because they have presumably been there since the advent of life, we believe them to be powerful global agents which help promote the genesis of fine-grained metal and silicate minerals.

ACKNOWLEDGMENTS

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