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Cell Hydrophobicity and Sulfur Adhesion of *Thiobacillus thiooxidans*

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Thiobacillus thiooxidans **cells became more hydrophobic but less adhesive to elemental sulfur in the presence of increasing potassium phosphate concentrations. At a fixed concentration of potassium phosphate, however, there was a peak of both cell hydrophobicity and adhesion to sulfur at around pH 5. Oxidation of sulfur by the cells was affected in a complex manner by the phosphate concentration and pH, although it was inhibited by a high concentration of potassium phosphate.**

Thiobacillus thiooxidans grows on solid elemental sulfur as an energy source and oxidizes it to sulfuric acid. The attack of sulfur particles by these cells is believed to be initiated by the adhesion of the cells to the sulfur surface (10). The problem of bacterial adhesion has been studied intensively in recent years, particularly from the standpoint of cell surface hydrophobicity and hydrophilicity (6). Since elemental sulfur is insoluble in water and soluble only in organic solvents, we expected that an interaction between *T. thiooxidans* cells and sulfur would be hydrophobic. Hydrophobic interactions are known to increase with increasing concentrations of certain salts (salting out) (6); therefore, we have studied the effect of potassium phosphate concentrations, the normal buffer we use for the sulfur oxidation studies, on these cells and their interaction with sulfur. Our experimental results, however, indicate that although *T. thiooxidans* cells become more hydrophobic with increasing concentrations of potassium phosphate, as expected, they become less adhesive to elemental sulfur. The sulfur oxidation rate is also reduced when the potassium phosphate concentration is increased. Recent work on the adhesion of *Thiobacillus ferrooxidans* to mineral surfaces (1, 2, 5) suggests that *T. ferrooxidans*, in contrast to *Escherichia coli*, does not adhere to mineral surfaces by simple hydrophobic interactions. The adhesion could involve hydrophilic interactions in addition to specific iron binding on pyrite or chalcopyrite (5). Thus, our results tend to agree with some of these conclusions, but *T. thiooxidans*, unlike *T. ferrooxidans*, cannot oxidize ferrous iron, and so the mechanism of sulfur oxidation is not likely to involve binding to iron.

T. thiooxidans ATCC 8085 was grown statically for 4 days at 28°C in Starkey's medium 1 adjusted to pH 2.3 with H_2SO_4, with elemental sulfur (BDH; precipitated) spread on the surface (9, 11); the cells were collected after removal of the sulfur by filtration, washed, and suspended in 50 mM potassium phosphate (pH 2.3).

Cell surface hydrophobicity was determined by a modification of the method of Rosenberg et al. (7) used to measure microbial adhesion to hydrocarbons. A 3-ml suspension of cells in the specified buffer was placed in a screw-cap culture tube (Pyrex no. 9825) and was vortexed for 30 s at maximum speed after the addition of 3 ml of *n*-hexadecane (Fisher brand). The emulsion was allowed to separate for 60 s, and the absorbance of the bottom, aqueous phase was measured at 660 nm in a Hewlett-Packard model 8452A diode array spectrophotometer and compared to the value of the original cell suspension.

Cell adhesion to sulfur was determined by a modification of the method of Takakuwa et al. (10). Cells (5 mg [wet weight] of cells unless otherwise indicated) in 10 ml of buffer were shaken for 15 min at 200 rpm (Labline Orbit Shaker) in 50-ml Erlenmeyer flasks. Sulfur (powdered or suspension [32 g per 100 ml of Millipore water containing 500 ppm of Tween 80]) was added, and the mixture was shaken for a further 15 min. The mixture was then filtered through fluted Whatman no. 1 filter paper, and the absorbance of the filtrate at 660 nm was measured as described above and compared to that of the control without sulfur.

Sulfur oxidation was monitored by measuring the oxygen consumption in a Gilson oxygraph with a Clarke oxygen electrode. The 1.2-ml reaction chamber was maintained at 25°C and contained 1 mg (wet weight) of cells and 0.1 ml of sulfur (32 mg) suspension (in 500 ppm of Tween 80) or 100 nmol of sulfur dissolved in dimethyl sulfoxide (DMSO; 5 to 10 μl) in 50 mM potassium phosphate buffer (pH 2.3) unless otherwise specified. The latter form of sulfur was used for stoichiometric oxidation studies (9) and is more convenient for injection into the reaction chamber. Since sulfur is insoluble in aqueous media, it comes out as a finely dispersed suspension. Its oxidation by *T. thiooxidans* cells is very similar to that of sulfur suspended in Tween 80 in most respects, except that the rate of oxidation is normally higher.

Experimental data were reproducible within a set of experiments with small standard deviations, but the values varied considerably when different batches of cells were used.

Hydrophobicity. The concentration of phosphate buffer clearly influences the distribution of cells between the bottom, aqueous phase and the creamy organic (*n*-hexadecane) phase, as shown in Table 1. Increasing potassium phosphate concentrations decreased the extent of cells remaining in the aqueous phase, indicating an increase in hydrophobicity. The adhesion of a large number of cells to hexadecane droplets was easily observed under a phase-contrast microscope, as described by Rosenberg and Doyle (6), in 0.5 M but not 50 mM potassium phosphate. Ammonium sulfate had a similar effect to potassium phosphate. Ethylene glycol, on the other hand, counteracted the effect of a high (0.5 M) potassium phosphate concentration. The growth phase of the cells had a profound effect on the hydrophobicity test. We found that 4-day-old cells (toward the end of the logarithmic phase) were less hydrophobic than 8-day-old cells in 50 mM buffer but were more sensitive to the buffer concentration and became more hydrophobic than the older cells in 0.5 M potassium phosphate. Therefore, all the following experiments were carried out with cells grown for 4 days.

Hydrophobicity decreased further when the potassium phosphate concentration was reduced below 50 mM, and the plot of

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TABLE 1. Effect of potassium phosphate concentrations on the cell surface hydrophobicity of *T. thiooxidans*[a]

Suspending medium	% of cells remaining in the aqueous layer with cells grown for:	
	4 days	8 days
Potassium phosphate		
50 mM	78	55
0.1 M	37	39
0.5 M	8	25
1.0 M	8	
0.5 M plus ethylene glycol (10%, vol/vol)	58	
Ammonium sulfate, 1.0 M	5	
Growth medium	68	
Water		50

[a] *T. thiooxidans* cells were grown either for 4 or 8 days. The cells were washed in 50 mM potassium phosphate (pH 2.3) and suspended in the pH 2.3 suspending medium as indicated (2.5 mg [wet weight] of cells in 3 ml). Hydrophobicity was determined as described in the text by the *n*-hexadecane extraction method.

the percentage of cells in the aqueous phase against the logarithm of the potassium phosphate concentration was approximately linear, approaching near 100% retention in the aqueous layer at very low phosphate concentrations (Fig. 1A). The effect of pH when using 50 mM potassium phosphate was not as dramatic, dropping from 64% aqueous phase cells at pH 2.3 to 45% at pH 5 to 5.5 and then rising to 58% at pH 7 to 7.5 (Fig. 2A). The effect of potassium phosphate concentration on the cell hydrophobicity was similar at pH 2.3 (Fig. 1A) and 7.5 (data not shown) and was therefore much larger than the effect of pH.

Adhesion to elemental sulfur. The extent of cell adhesion to powdered elemental sulfur was strongly influenced by the phosphate buffer concentration as shown in Table 2. Nearly 70% of the cells were adsorbed onto sulfur at 0.1 mM potassium phosphate, but only 18% were adsorbed at 0.1 M potassium phosphate. Ammonium sulfate also inhibited the cell adhesion. The buffer concentration effect was similar when the detergent Tween 80 was present as a wetting agent. The cell adhesion decreased linearly with the logarithm of buffer concentration up to 0.1 M (Fig. 1B). Thus, *T. thiooxidans* cells adhere to sulfur powder more extensively when their hydrophobicity is reduced, a result that would not be expected if the sulfur-cell interaction were hydrophobic.

In 50 mM potassium phosphate buffer at pH 2.3, *T. thiooxidans* cell adhesion increased linearly when the amount of powdered sulfur was increased from 2 to 6 g at a cell concentration of 20 mg (wet weight) of cells in the 10-ml assay system or when the cell concentration was raised from 2 to 6 mg (wet weight) of cells with a fixed sulfur concentration of 1 g in 10 ml. These conditions, however, were extreme, and at lower cell concentrations (e.g., 5 mg per 10 ml) or lower sulfur concentrations (e.g., 0.5 g per 10 ml), normal saturation-type kinetics was observed. By considering the cell adhesion to sulfur as being equivalent to a ligand binding to an enzyme and by using a Scatchard plot (3, 8) of [bound cell]/[free cell] against [bound cell], linear curves for 0.4 and 0.5 g of sulfur with an approximate binding capacity of 2 and 4 mg (wet weight) of cells and a dissociation constant of 2 and 1.5 mg of cells, respectively, were obtained (Fig. 3). These values apply only to the lower concentrations of sulfur (0.4 to 0.5 g per 10 ml) and cells (2 to 5 mg per 10 ml) and fluctuate among different batches of cells. Treatment of the data by assuming the Langmuir adsorption isotherm (5) and use of double-reciprocal plots yielded a dissociation constant of 2.9 mg of cells with 0.4 g of sulfur but much higher values with more sulfur. Obviously, these methods are unsatisfactory when the concentrations of both free cells and free cell-binding sites of sulfur are substantially affected. The graphical method of determining the total enzyme concentration and the K_m when the concentration of the enzyme-substrate complex is high enough to reduce substantially the concentrations of free enzyme and free substrate, i.e., the Dixon plot (3, 8), has been applied to the cell binding data in Fig. 3. The binding capacity of around 2 and 4 mg of cells and the dissociation constant of 1.8 and 1.3 mg of cells obtained (plot not shown) are close to the Scatchard plot results.

The effect of pH on cell adhesion to sulfur was even less dramatic than its effect on cell hydrophobicity, but the slightly higher value at pH 4.5 to 5.0 (Fig. 2B) corresponded approximately to the pH where the hydrophobicity of cells was the highest (pH 5 to 5.5 [Fig. 2A]). The adhesion, however, increased at pH 2.3 and pH 7, where hydrophobicity decreased. The effect of pH is considered much smaller than that of the potassium phosphate concentration (Fig. 1).

Takakuwa et al. (10) reported the inhibition of cell adhesion

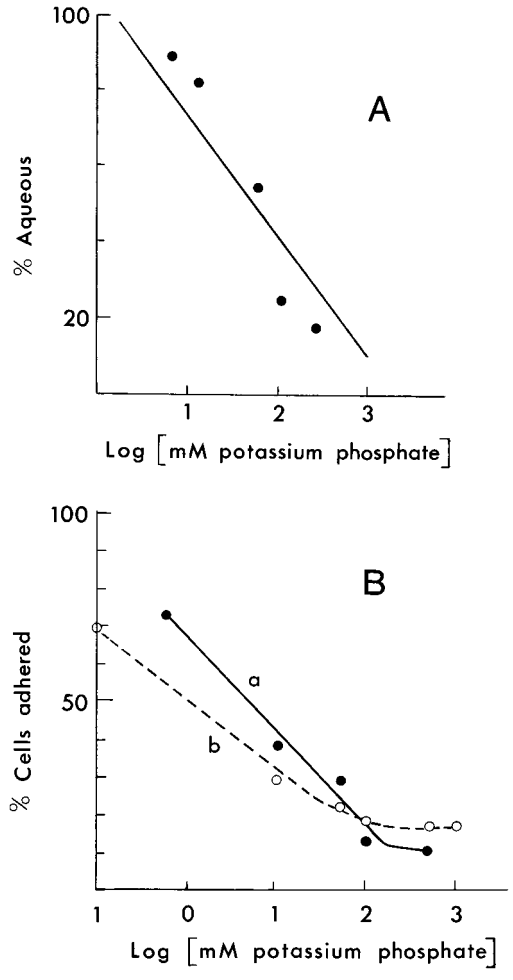

FIG. 1. Effect of potassium phosphate concentrations on hydrophobicity (A) and sulfur adhesion (B) of *T. thiooxidans* cells. (A) The percentage of cells remaining in the aqueous layer at pH 2.3 was determined as in Table 1. (B) Cell adhesion to sulfur at pH 2.3 was determined with 5 mg (wet weight) of cells with 0.32 g (curve a) or 0.4 g (curve b) of sulfur with (curve a) or without (curve b) Tween 80.

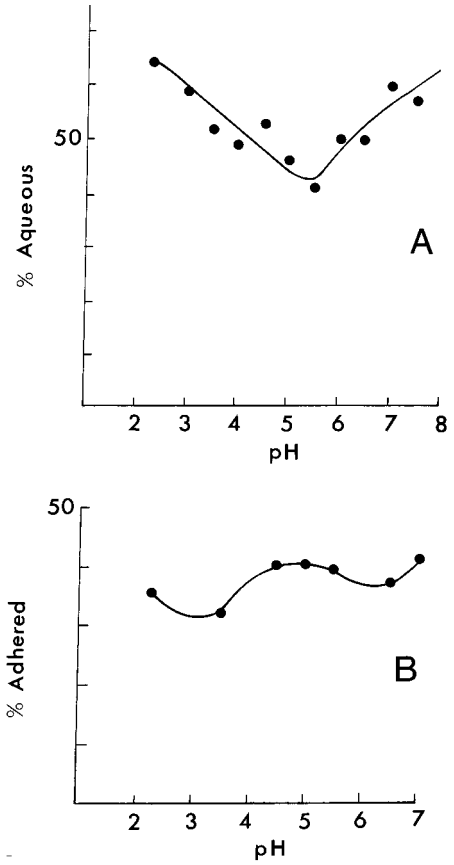

FIG. 2. Effect of pH on hydrophobicity (A) and sulfur adhesion (B) of *T. thiooxidans* cells. (A) Hydrophobicity was determined, as in Fig. 1, in 50 mM potassium phosphate. (B) Cell adhesion to sulfur was determined with 5 mg (wet weight) of cells with 0.4 g of sulfur in 50 mM potassium phosphate.

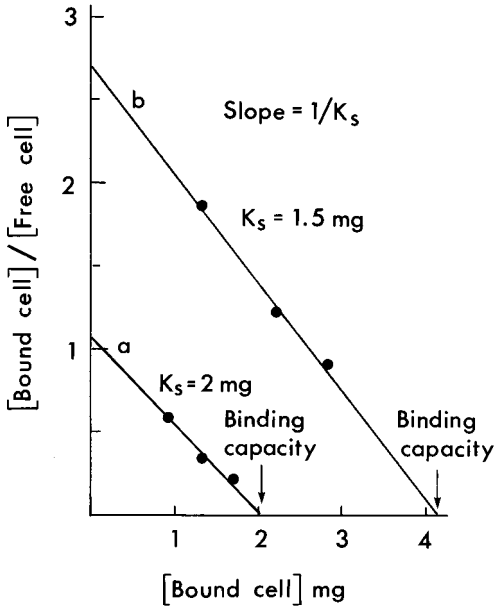

FIG. 3. Scatchard plot of *T. thiooxidans* cell binding to sulfur. Cell adhesion to sulfur was determined in 50 mM potassium phosphate at pH 2.3 with 0.4 g (curve a) or 0.5 g (curve b) of sulfur. Two different batches of cells were used to generate curves a and b.

to sulfur by inhibitors of sulfur oxidation. Thiol agents, iodoacetate (1 mM), *p*-chloromercuribenzoate (0.1 mM), *N*-ethylmaleimide (1 mM), and a protonophore, 2,4-dinitrophenol (0.1 mM), had no appreciable effect on the cell adhesion to sulfur, and the last three did not affect the hydrophobicity of the cells.

Sulfur oxidation. If the adhesion of cells to sulfur is the first step in the oxidation of sulfur, the concentration of potassium phosphate should affect the rate of sulfur oxidation by *T. thiooxidans* cells. Oxidation with suspended sulfur at pH 2.3 is inhibited (rate of oxidation, 2 nmol of O_2 consumed per min) at a very high (0.5 M) potassium phosphate concentration compared to an intermediate (50 mM to 0.1 M) concentration (28 nmol/min), but at a very low (1 mM) concentration, it is also inhibited (14 nmol/min). Therefore, it is obvious that the rate of sulfur oxidation is governed by factors in addition to just the adhesion of cells to sulfur. The effect of pH on the sulfur oxidation is shown in Fig. 4. The oxidation is inhibited when the potassium phosphate concentration is raised from 0.1

TABLE 2. Effect of potassium phosphate concentrations on the *T. thiooxidans* cell adhesion to elemental sulfur[a]

Potassium phosphate concn	Cells adhered	
	%	Wet weight (mg)
0.1 mM	69	3.45
10 mM	29	1.45
50 mM	22	1.10
0.1 M	18	0.90
0.5 M	17	0.85
1.0 M	17	0.85

[a] *T. thiooxidans* cells (5 mg [wet weight]) were shaken in 9 ml of potassium phosphate buffer (pH 2.3) at different concentrations with 0.4 g of powdered sulfur before filtration to remove the sulfur to determine the cells remaining as described in the text.

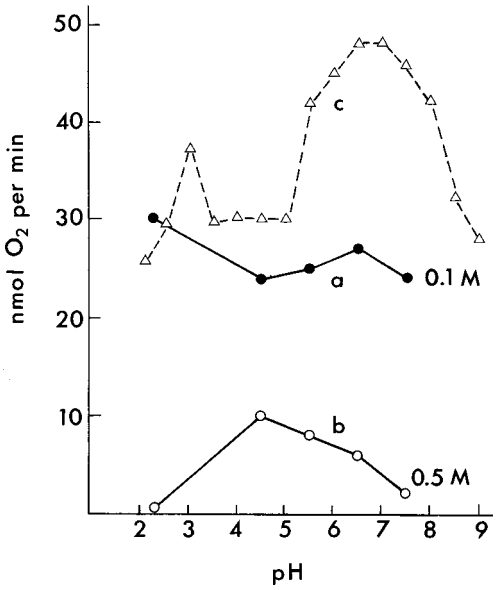

FIG. 4. Effect of pH on sulfur oxidation by *T. thiooxidans*. Potassium phosphate buffer at 0.1 M (curve a) or 0.5 M (curve b) was used with 1 mg (wet weight) of cells and 100 nmol of sulfur dissolved in DMSO. Curve c indicates the results of a separate control experiment in 50 mM potassium phosphate with 2.5 mg (wet weight) of cells and 100 mg of suspended sulfur.

to 0.5 M at any pH (curves a and b), but the inhibition is smallest at pH 4 to 5, because the rate in 0.1 M potassium phosphate has a minimum and that in 0.5 M potassium phosphate has a maximum around the pH. That is close to the pH range with increased hydrophobicity and sulfur adhesion (Fig. 2). Curve c in Fig. 4 is an example of suspended sulfur oxidation activity of *T. thiooxidans* in 50 mM potassium phosphate with two pH optima, pH 2.5 to 3 and pH 6.5 to 7.0. A similar pH curve with a trough between pH 4 and 5 is observed with other strains of *T. thiooxidans* and also with sulfur supplied as a solution in DMSO. A similar pH profile has also been reported for sulfur oxidation by sulfur-grown *T. ferrooxidans* (4). The trough between pH 4 and 5 is the pH range at which the peaks in hydrophobicity (Fig. 2A) and cell adhesion (Fig. 2B) occur. Increased cell hydrophobicity and decreased cell adhesion at high potassium phosphate concentrations (Fig. 1) are consistent with inhibition of sulfur oxidation by 0.5 M pH 2.3 potassium phosphate if hydrophilic interaction between the cells and sulfur is important in the oxidation of sulfur. The peak in hydrophobicity and cell adhesion (Fig. 2) and the trough in sulfur oxidation (Fig. 4, curves a and c) at pH 4 to 5 may imply the existence of some cell adhesion to sulfur mediated by hydrophobic interaction, which leads to the inhibition of sulfur oxidation by hydrophilic interaction. If so, curve b in Fig. 4 may indicate the oxidation of sulfur by hydrophobic interaction in 0.5 M potassium phosphate, where the hydrophilic interaction is largely suppressed. An extended lag period (5 to 10 min or longer) before the start of oxygen consumption and a low sulfur oxidation rate suggest slow interaction-oxidation processes.

These experiments show the significance of hydrophilic interaction between *T. thiooxidans* cells and elemental sulfur when the potassium phosphate concentration is varied; however, when the concentration is fixed, the effect of pH suggests that hydrophobic interaction also plays a role.

We are currently studying the effects of different anions and cations and osmotic pressure on the oxidation of sulfur to elucidate the detailed mechanism more clearly.

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