The adherence of *Acidiphilium cryptum* to chalcopyrite

By

Blair Heffelfinger

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Department of Microbiology
The University of British Columbia
Vancouver, Canada
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ABSTRACT

Acidiphilium cryptum is a heterotrophic acidophile commonly found in acidic environments and industrial bioleaching operations. Attachment to mineral surfaces may serve to maintain this organism in aqueous environments where it is subject to removal by hydrodynamic forces. Using indirect and direct methods we have looked at the binding of *A.cryptum* to chalcopyrite ($CuFeS_2$) and other mineral ores to determine whether specific adhesins mediate binding. A modified ELISA binding assay (the Ore ELISA) was developed to measure direct adherence. Finely ground chalcopyrite was bound irreversibly to the walls of an ELISA plate, the organisms were added and after incubation and washing, the number of attached bacteria were assessed by reacting with anti-*A.cryptum* antibody followed by goat anti-rabbit IgG conjugated to alkaline phosphatase. This assay was found to be sensitive, rapid and reproducible. The Ore ELISA allowed direct binding measurement in the presence of various inhibitors and provided a rapid screening method for adherence-defective mutants. Adherence was shown to be saturable and increased slightly as pH decreased. A moderate increase in binding affinity was recorded in the presence of monovalent and divalent cations and EDTA. Various bactericidal agents and pentose and hexose sugars had no effect on chalcopyrite attachment. Reducing agents had little effect on cell adherence. A strong increase in

adherence was observed in the presence of surface active agents. Bovine serum albumin and gelatin were both found to markedly reduce mineral surface binding. Competition for attachment sites between *A.cryptum* and the autotrophic acidophile, *Thiobacillus ferrooxidans*, showed that each organism binds to unique sites on the chalcopyrite surface. *A.cryptum* mutant strains displaying reduced adherence to chalcopyrite were shown to lack a 31.6 kDa outer membrane protein.

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Dedicated

to my wife,

Lesley.

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INTRODUCTION

Acidophiles

Acidophiles are unique among microorganisms in that they live at pHs below 5.0. Acidophiles can be divided into two groups; facultative acidophiles and extreme acidophiles. Facultative acidophiles, which are capable of growth at both neutral and acidic pH, include such bacterial species as *Streptococcus*, *Lactobacillus*, and some species of *Escherichia* and *Staphylococcus*. Extreme acidophiles require an acidic environment for growth and will perish at neutral pH. Heterotrophic extreme acidophiles use a variety of carbohydrates and organic acids as both an energy and carbon sources. Examples of heterotrophic acidophiles are *Acidiphilium* spp. and *Thiobacillus acidophilus*. Autolithotrophic extreme acidophiles obtain energy through the oxidation of reduced iron and sulphur compounds and carbon through the fixation of carbon dioxide. Examples of autotrophic acidophiles include *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*, which oxidize reduced sulphur/iron and sulphur respectively (Brock, 1979).

The most common source of acidophilic microorganisms is acid mine drainage (AMD) waters. Since their isolation and postulated involvement in AMD (Colmer, 1950), *Thiobacillus ferrooxidans* has been the subject of considerable environmental research. AMD is characterized by, a pH below 4.5, high ferric iron and sulphate

levels, low concentrations of organic carbon, high levels of aluminum, calcium ions, soluble heavy metals (manganese and lead), and reduced levels of phosphate and nitrogen (Johnson et al., 1979). There are two primary sources of mine waste: (1) Tailings, which are the by-products of mineral ore milling and separation; (2) Overburden, which is the soil and residual ore material left at the mine site. Both forms of mine waste have significant concentrations of reduced sulphur compounds which are oxidized through microbial action to form AMD (Mills, 1985). With the dwindling reserves of high grade ore and an increasing demand for fossil fuels, most future metal-refining and fossil fuel production will involve surface strip mining, resulting in increased water pollution through mine drainage.

The effects of AMD on the environment are well documented (Carpenter, 1925; Campbell and Lind, 1969; Yan, 1979; Johnson et al., 1978; Norris et al., 1981; Olem, 1981). Most reports have studied the physicochemical effect on the environment; the natural flora, plankton, vegetation, invertebrates and fish. The effects of AMD on the microbial community has largely been limited to iron and sulphur oxidizing organisms such as *T.ferrooxidans* and *Thiobacillus thiooxidans* (Brierley, 1978).

There is a growing interest in the effects of AMD on heterotrophic organisms and their contribution, if any, to the rehabilitation of acid mine polluted water. In general most

heterotrophic microbes are sensitive to pH value below 5 or above 9 (Alexander, 1977). In addition, metals such as Cu, Cd, Pb and Zn, which are found in high levels in AMD, are toxic to many microorganisms (Sterritt & Lester, 1980; Zevenhuizen et al., 1979). Though considerable research has questioned the effects of certain aspects of AMD on microorganisms, comparatively little work has addressed the microbial response and succession of events following exposure to AMD. Wassel et al.(1983) studied the water and sediment bacterial communities for stress-related responses to a point source of acid mine drainage. In general, they found that the diversity of the microbial communities was significantly lower at the site receiving AMD as compared to uncontaminated controls; microorganisms at the contaminated sites were more similar to each other than they were to the controls. Mills supported these findings in concluding "that microbial communities developing in extreme environments tend to be generalists, where as those in mesic environments tend to be specialists" (Mills & Mallory, 1987).

Unlike the autotrophic *Thiobacillus* spp., heterotrophic bacteria were long considered transients in acid mine waters (Tuttle et al., 1968). However, specially adapted heterotrophic organisms have been isolated and are capable of growth in AMD polluted waters (Wichlacz et al., 1981; Manning, 1975). AMD is not the only source of acidophilic heterotrophs, they have also been isolated from acidic sewage and soil, and from what was presumed

to be pure cultures of *T.ferrooxidans* (Kishimoto, 1987; Harrison, 1980; Zavarzin, 1972; Johnson & Kelso, 1983). Through DNA homology studies, 25% of 23 stains of *T.ferrooxidans* were found to contain heterotrophic contaminants (Harrison, 1982). One common contaminant was *Thiobacillus acidophilus* which is described by Arkesteyn et al. (1980) as a mixotrophic Thiobacillus species. A second common contaminant contained a DNA G:C content (68-70%) beyond the range of the *Thiobacillus* species. Harrison et al. (1981), proposed a new generic name, *Acidiphilium* gen. nov. (type species: *Acidiphilium cryptum*) and described it as a motile, gram-negative, aerobic, mesophyllic, rod-shaped, heterotrophic acidophile. This new acidophile was non-encapsulated, motile by a polar or two lateral flagellum and unable to oxidize reduced sulphur or iron compounds. *A.cryptum* can utilize various carbohydrates as sole carbon and energy sources. Operational pentose phosphate and Entner-Doudoroff pathways were identified by Shuttleworth et al. (1985). Since its discovery in 1981, four addition species of Acidiphilium have been isolated; *Acidiphilium angustum, Acidiphilium facilis, Acidiphilium rubrum* (Wichlacz, 1986) and *Acidiphilium organovorum* (Lobos, 1986).

It has been suggested that the main cause of variable oxidation rates between different *T.ferrooxidans* cultures (Kelly & Jones, 1978; Chang and Myerson, 1982; Roy et al., 1981; Gormely et al., 1975) is attributed to heterotrophic contaminants. Harrison et

al., suggested that since Acidiphilium and Thiobacillus are so tenaciously associated in cultured isolates, they may cross-feed in the natural environment (Harrison, 1980). The idea of microbial symbiosis between lithotrophs and heterotrophs is not new. It is commonly found, in bioleaching operations, that mixed cultures of bacteria have a higher mineral yield than pure culture of autotrophic organisms (Andrews et al., 1982; Detz, 1979; Hoffman et al., 1981; Dugan, 1978; Andrews et al., 1987; Norris & Kelly, 1978). Many simple organic compounds have been found to inhibit growth and iron and sulphur oxidation by *T.ferrooxidans* (Tuttle & Dugan, 1976; Rao & Berger, 1970). Arkesteyn (1980) demonstrated that inhibition of iron-oxidizing *T.ferrooxidans* by certain organic compounds (eg. 1.0 mM Glucose, ethanol, lactate, succinate, serine and aspartate) was relieved when the mixotrophic *T.acidophilus* was added to the autotrophic *T.ferrooxidans*. A mutualistic relationship is believed to occur between the iron/sulphur oxidizing *T.ferrooxidans* and the strictly sulphur oxidizing *T.thiooxidans*. As iron oxidation proceeds, sulphur layers may coat the ore substrate and inhibit further oxidation. *T.thiooxidans* is believed to oxidize the accumulating sulphur and thus expose more ferrous substrate for *T.ferrooxidans*, however, general agreement on this theory is lacking. Kelly et al. in 1978, described a symbiotic relationship between the iron oxidizing acidophile, *Leptospirillium ferrooxidans* and the sulphur oxidizing *Thiobacillus organoparus* (Kelly et al., 1978).

The mechanisms by which organic compounds inhibit *T.ferrooxidans* is poorly understood. Using electron micrographs, Tuttle et al. (1977), illustrated cell envelope disruption when *T.ferrooxidans* was exposed to organic acids. In 1970, Rao and Berger demonstrated passive permeability to pyruvic acid at low pH, resulting in a decreased intracellular pH. Ingledew proposed that the toxicity of weak acids can be attributed to their accumulation in the cell matrix in response to the pH difference between the cytoplasm and the supporting medium (Ingledew, 1982). The ability of weak acids to permeate membranes requires that the acid be protonated; this, in turn, will lead to acidification of the cell matrix. Alexander et al., supported this prediction by demonstrating that organic acid toxicity is directly related to the pKa values of each acid (Alexander et al., 1987).

The source of the inhibitory organic compounds was investigated by Schnaitman and Lundgren in 1965. Using radioisotopes, it was shown that the majority of the pyruvic acid accumulating in spent media was from CO_2 fixed by *T.ferrooxidans* and subsequently released into the media (Schnaitman & Lundgren, 1965). From these observations, the generally accepted conclusion is that end product inhibition plays an important role in autotrophic acidiphilic growth on mineral substrates.

Contrary to the detrimental effect of acid mine drainage, the

beneficial uses of acidophiles have been practiced for thousands of years. Bioleaching, through the oxidation of metal sulphides, has been successfully applied on an industrial scale to the recovery of copper and uranium from low grade ores and tailings. It has been reported that 15-20% of commercially produced copper in the U.S. is achieved through bioleaching with a definite potential to expand to other ores and ore concentrates (Agate & Khinvasara, 1985).

Over the last three decades, several articles have been written on bioleaching. Bioleaching involves the bacterial oxidation of iron, elemental sulphur and mineral sulphides such as pyrite (FeS_2). This process results in direct and indirect solubilization of metals to be commercially extracted.

The direct mechanism involves the direct biological oxidation of an insoluble divalent metal sulphide to soluble metal and sulphate. It generally believed that direct attack requires bacterial attachment to the mineral since the substrate is insoluble (Bennett & Tributsch, 1978; Berry & Murr, 1978; Tributsh, 1976). Reactions 1 and 2 describe the bacterial oxidation of elemental sulphur and pyrite by the direct mechanism.

(1) $2\ S^0 + 3\ O_2 + 2\ H_2O \rightarrow 2\ H_2SO_4$

(2) $4\ FeS_2 + 15\ O_2 + 2\ H_2O \rightarrow 2\ Fe_2(SO_4)_3 + 2\ H_2SO_4$

Any bivalent metal can be substituted for ferrous sulphide

(FeS_2).

The indirect mechanism of microbial leaching involves three steps.

(a) In the acid environment created by microbial leaching, ferrous sulphate is produced by the chemical oxidation of pyrite (FeS_2) (reaction 3).

(b) Bacterial oxidation of ferrous iron to ferric iron (reaction 4) (Lacey & Lawson, 1970).

(c) Chemical reduction of ferric sulphate through the oxidation of a metal sulphide to regenerate ferrous sulphate (reaction 5) (Singer & Stumm, 1970).

(3) $2\ FeS_2 + 7\ O_2 + 2\ H_2O \longrightarrow 2\ FeSO_4 + 2\ H_2SO_4$

(4) $4\ FeSO_4 + O_2 + 2\ H_2SO_4 \longrightarrow 2\ Fe_2(SO_4)_3 + 2\ H_2O$

(5) $Fe^{3+} + MS \longrightarrow Fe^{2+} + M^{2+} + S^0$

The metal sulphide (MS) oxidized to a soluble ionic form in reaction 5 facilitates the establishment of the ferrous-ferric iron cycle which is characteristic of the indirect mechanism. Bacterial oxidation of ferrous iron to replenish ferric iron levels (reaction 4) is critical for the maintenance of this cycle. If reaction 5 is not allowed to occur, then ferric sulphate will readily react with water to form ferric hydroxide and sulphuric acid. Since all reactions in the indirect mechanism occur in solution, attachment is not considered necessary. Though separate systems for iron and sulphur

oxidation are evident (Beck & Brown, 1968), they appear to function concurrently (Silverman, 1967).

Sulphuric acid is an end product for both reaction systems. The resultant low pH plays an important role in bioleaching. Acidic pH provides selective pressure for the enrichment of acidophilic lithotrophs and heterotrophs. At a low pH, ferrous iron is stable and will not auto-oxidize to ferric iron, therefore, this important substrate remains available to the bacteria. Under low pH conditions, a membrane potential of approximately -10 mV between the acidic environment and the neutral internal pH of *T.ferrooxidans* is generated (Ingledew & Cobley, 1980). This creates an electrochemical proton activity difference of 256 mV which is sufficient for ATP generation, as demonstrated by Ingledew et al.

Various parameters effect bioleaching rates (Lundgren & Silver, 1980). Increasing temperature, within a range (25-45°C), results in not only an increase in chemical reaction rates, but also an increase in bacterial metabolism. The pH of 1.0-2.5 is considered optimal for ferrous iron oxidation. As with most microorganisms, acidophiles require standard mineral salts. In many cases, depending on the ore composition, it may be only necessary to supplement the leaching media with ammonia and phosphate. CO_2 and O_2 availability is directly related to the leaching efficiency. Both gases may become limiting if pulp

densities are too high. The particle size is also an important parameter; decreased particle size causes an increase in surface area resulting in an increased leaching efficiency (Torma, 1977).

Torma and Bosecker addressed the growing interest in commercially applied bio-hydrometallurgy by providing a systematic review of current techniques in mineral leaching by microorganisms. Various methods of industrial bioleaching have been developed; air lift percolators, column leaching, stationary leaching and agitated vat leaching. They found that for best results, the rate of oxygen and carbon dioxide mass transfer must be at a maximum. The authors stress that in order for commercial applicability, an increased understanding of the microbial leach process and the symbiotic growth phenomena is essential (Torma & Bosecker, 1982).

One application of industrial bioleaching which is of growing interest is the desulphurization of coal as a means of curbing sulphur dioxide and nitrogen oxide emissions. Both emissions interact with water molecules in the atmosphere to form sulphuric acid and nitric acid, hence acid rain (LaBastille, 1981). Public concerns of acid rain has encouraged implementation of the Clean Air Act in the U.S. which restricts the sulphur content of combustible coal to 0.7%. Unfortunately, half of the U.S. coal reserves is bituminous in nature with its majority containing greater than 1% sulphur (Mills, 1985). This problem is

compounded by the fact that 47% of the U.S. energy needs result from coal combustion and a predicted 5% annual increase in domestic energy needs is expected for the next several decades (Detz, 1979).

Two common concerns among researchers designing desulphurization treatments are economic feasibility and reduced residence time. Two non-biological approaches are flue gas desulphurization and pressurized fluidized bed combustion, however, high costs and excess waste have limited their usefulness (Detz, 1979). The suppression of pyritic sulphur floatation during coal cleaning is another approach. Boateng and Phillips found that by treating the coal with kerosene and lime, you encourage coal floatation and discourage pyrite floatation (Boateng & Phillips, 1977). Therefore, the sulphur containing pyrite is removed from the coal prior to combustion, variations to this process were attempted by Kempton el al. in 1980 and Townsley et al. in 1987. Both researchers showed that by conditioning the coal with *T.ferrooxidans*, the natural floatability of pyrite was significantly reduced.

Microbial desulphurization through bioleaching is considered the least expensive route to precombustion removal of pyrite from a broad range of coals (Detz, 1979; Dugan, 1986). Huber et al. (1984) provided an analysis of microbial desulphurization of coal. Using a mixed flow reaction followed by a plug flow

reactor configuration, over 90% of the pyrite was removed at an overall 9 day residence time. The kinetics of coal desulphurization was recently reviewed by Andrews et al. (1988). Critical O_2 availability was met through air sparging of slurries up to 50% (Wt/Wt) containing 1% sulphur. Workers found that by recycling cell cultures, a population of heterotrophs established themselves and increased process rates not only by removing *T.ferrooxidans* inhibitors but also by increasing CO_2 levels such that an exogenous source of CO_2 was not necessary.

Questions which have resulted in heated debate among researchers in this field are: Is attachment necessary for mineral leaching, and if so, is the adherence selective and by what mechanism does it occur? Clearly, there is an advantage to the organism to be attached to the mineral surface, since it is then proximal to the insoluble oxidizable substrate.

Early evidence of *T.ferrooxidans* direct interaction with sulphur was provided by Voger (Voger & Umbreit, 1941). Schaeffer demonstrated that *T.thiooxidans* tenaciously binds to sulphur and concluded that attachment is a prerequisite for sulphur oxidation (Schaeffer & Holbert, 1963). The strength and irreversibility of mineral surface binding was further demonstrated by Baldensperger et al. in 1974 with *T.thiooxidans* and *T.denitrificans* and by Myerson and Kline in 1983 with *T.ferrooxidans*. Increasing pH appeared to have no effect on *T.ferrooxidans* desorption nor did

repeat washing (Tuovinen et al., 1983). Mild sonication or treatment with surfactants were also ineffective at removing *T.ferrooxidans* cells bound to chalcopyrite (McGorman et al., 1969).

T.ferrooxidans appears to bind extensively to mineral surfaces. Wakao et al. in 1984 recorded that almost 100% of a *T.ferrooxidans* cell suspension adsorbed to either the pyrite crystals or the walls of the glass tube. McGorman found that over 97% of a cell suspension bound to chalcopyrite (McGorman et al., 1969). Gormely and Duncan reported that 65% of a *T.ferrooxidans* population associated directly with the zinc sulphide substrate (Gormely & Duncan, 1974). The adherence to mineral surfaces occurs quite rapidly. Myerson and Kline observed that over half the cells in a suspension adsorbed to coal particles or glass beads within five minutes (Myerson & Kline, 1983). Twenty-five percent of a *T.ferrooxidans* cell suspension absorbed within five minutes to either pyrite particles, fluor-apatite or glass beads (Tuovinen et al., 1983). Very similar results were recorded by DiSpirito et al. in 1983.

Until recently, the majority of the evidence demonstrating direct interaction between microorganisms and mineral surfaces has been from electron microscope studies (Lundgren & Tano, 1978; Tributsch, 1976; Wakao et al., 1984; Bennett & Tributsch, 1978; Gromely et al., 1974; Murr & Berry, 1976; Wiess, 1973; Duncan &

Drummond, 1973; Hiltunen, 1981; Keller & Murr, 1982; Brierley et al., 1973). Scanning electron microscope (SEM) studies (Berry & Murr, 1978; Kelly et al., 1979) suggest that *T.ferrooxidans* attachment occurs selectively to sulphide phases rather than to the silicate matrix of prepared mineral samples. Bennett & Tributsch performed SEM studies on pyrite crystals which were placed in a *T.ferrooxidans* culture for 2 years. They observed etched pits in the shape of bacterial cells which were oriented in a "pearl-like" string. These lines of bacterial corrosion were often intersected at right angles by other corrosion lines, therefore resembling the cubic structure of crystalline pyrite.

Bagdigian and Myerson (1986) demonstrated that *T.ferrooxidans* selectively adsorb to exposed pyrite crystals dispersed throughout the organic coal matrix. Furthermore, preferential attachment was observed along fracture lines and dislocation points of the pyrite crystal. Selective adsorption to pyrite crystals was also demonstrated by Kempton et al. (1980) and Townsley et al. (1987) as a means of desulphurization of coal (previously discussed). According to Weertman and Weertman (1964), stress and strain energies are stored in dislocation sites in the form of crystal formations and lattice vacancies. With this in mind, Andrews found that diffusion of sulphur to dislocation sites of pyrite crystals was significant and as such, it would be advantageous for the bacteria to adsorb to the energetically rich sulphur which accumulates at the dislocation

site (Andrews et al., 1988).

Studies involving surfactants and wetting agents have also produced evidence linking cell-surface interaction with increased rates of iron and sulphur oxidation. It is likely that these substances effect the interaction of bacteria and the mineral surface. According to Kingma et al. (1979), Tween reduces the energy required by the bacterium to overcome the dynamic surface tension between the substrate and the liquid medium, thus facilitating contact between the bacterium and the substratum. Starkey et al. observed an increased sulphur oxidation in shake flask cultures when Fergitol-08 or Tween 80 was added (Starkey, 1956). An enhanced rate and extent of leaching was recorded by Duncan et al. (1964) when either Tween, 20, 40, 60 or 80, or Triton X-100 was added to *T.ferrooxidans* cultures growing on chalcopyrite. Moreover, they found that the Tween to ore ratio was more important, with regards to leaching rates, than the Tween to media ratio, suggesting that Tween acts to increase the contact between the mineral surface and the microorganisms. Aside from increasing selective contact between the bacterium and the substratum (Duncan et al., 1973), Tween was found to increase the rate of contact by eliminating the lag period prior to pyrite oxidation (Roy et al., 1981). Torma et al. (1976), refuted these finding by recording a reduced chalcopyrite oxidation by *T.ferrooxidans* in the presence of surface active agents (Tween 20, 40, 60 and 80) and organic solvents. They explained that the

surface active agents reduced the surface tension and oxygen concentration of the medium resulting in a decreased oxidation rate.

There is evidence suggesting that surface active agents may in fact be endogenously produced within bioleaching systems. Wakao et al. (1983), proposed that the enhanced pyrite oxidation observed in *T.thiooxidans* and *T.ferrooxidans* mixed cultures was due to a surfactant, identified as phosphotidylinositol (Schaeffer & Umbreit, 1963) secreted by *T.thiooxidans*. This surfactant is believed to reduce the *T.ferrooxidans* adherence, therefore allowing it to oxidize iron more readily in suspension. However, in 1961, Jones and Starkey identified a surfactant produced by *T.thiooxidans* which enhanced sulphur oxidation by wetting the substrate and increasing bacterial adherence.

Evidence relating the importance of attachment and substrate oxidation was recently reviewed by Espejo et al. (1987) and Yeh et al. (1987). *T.ferrooxidans* growth on sulphur prills was found to be selective and saturable. Radioisotope studies had shown that only the attached cells were actively dividing. The unattached cells lost viability with a half-life of 3.5 days (Espejo et al., 1987). Yeh et al. (1987) used epifluorescence microscopy to measure mineral adherence and metabolic activity. Through differential fluorescence, they concluded that the cells most actively involved in the leaching process are those attached

to the mineral surface. In general, attachment during bacterial leaching is advantageous for the microorganism. In addition to close proximity of substrate, attached organisms show greater resistance to pH changes, temperature changes and end product toxicity (Karamanev & Nikolov, 1988).

There have been few complete studies on the adherence kinetics of acidophiles. Adsorption to mineral surfaces appears to occur independently of pH, unlike adsorption to glass beads which increased with decreasing pH (Takakuwa et al., 1979; Tuovinen et al., 1983; DiSpirito et al., 1983). Cell viability was not a requirement for adherence (Beck, 1967), however, Tuovinen et al. observed a slight overall reduction in adherence with ultraviolet light treated cells. Adsorption experiments by Badigian et al. (1986) suggests that adsorption occurs through a combination of first order, reversible and second order irreversible kinetics.

Another acidophile of interest concerning mineral surface attachment is the thermophilic lithotroph, *Sulfolobus*. Weiss (1973), through SEM studies, observed *Sulfolobus* adhering to sulphur particles via a pilus and correlated attachment with the number and lengths of the surface pili. By incubating *Sulfolobus* in sulphur, Shivvers et al. (1973) saw a gradual increase in the number of attached organisms. They also correlated increased sulphur oxidation with increased attachment. A comprehensive study of *Sulfolobus* attachment to chalcopyrite drew the following

conclusions (Berry & Murr, 1976):

1). Strong support that direct contact is necessary for chalcopyrite oxidation.
2). Evidence of preferential attachment by *Sulfolobus* to mineral surfaces.
3). Attachment correlated with iron and copper dissolution.
4). Increased surface area corresponds with an increase in cell attachment.
5). Evidence of "biomatter" production at bacterial attachment sites.

The molecular mechanism by which acidophiles attach to mineral surfaces is poorly understood. Takakuwa et al. (1979) postulated the involvement of sulfhydryl-groups in *T.thiooxidans* adherence to sulphur particles since sulfhydryl-binding reagents blocked attachment. This view is disputed by Bryant et al. (1984) who identified a glycocalyx-mediated adsorption of *T.albertis* to sulphur discs. LPS extraction caused reduced adherence to pyrite, sulphur or flouro-apatite, though, it is likely that other surface components would also be removed during the extraction (DeSpirito et al., 1983). In addition to flagella, surface appendages have been reported to exist on *Thiobacillus* spp. (Gonzalez et al., 1987; DiSpirito et al., 1981), however, the function of the appendages has not been determined.

Adherence

Marshall et al. (1980) divides adherence into three separate categories:

A) Specific permanent adhesion. This may also be called active adherence in which the bacteria attaches to a surface by means of complementary surface structures. The majority of this form of adhesion has been described in bacteria-epithelium interactions.

B) Non-specific permanent adhesion, is found in microorganisms which adhere to a variety of surfaces in their natural environment. The surfaces often differ considerably suggesting that a non-specific mechanism such as polymer-bridging may be involved.

C) Temporary adhesion, is observed in gliding bacteria in which contact to a solid surface is necessary for movement.

Adherence is a very complex process and has been the subject of numerous studies. To understand the mechanism of adherence, the researcher must consider charge, wettability, and adsorbed medium components (electrolytes and proteins). Moreover, the observer must not assume that the cell surface physiology of the bacteria remains static throughout the adherence reaction; interaction

with an interface can induce changes in bacterial surfaces (Fletcher et al., 1980).

Adhesion has been described to proceed from a reversible state involving non-specific bonding to an irreversible permanent interaction (Marshall et al., 1971). Doyle et al. (1982) postulated that permanent irreversible adherence is achieved through the additive effect of non-specific interactions after initial contact. The complexity of bacterial adherence is exemplified by Doyle's interpretation of positive cooperativity in *Streptococcus sanguis* binding to hydroxylapatite (Nesbitt et al., 1982), in which the adsorption of one cell encouraged the binding of adjacent cells. He suggests that once a cell binds, pellicle proteins adjacent may change conformation creating new receptors and encouraging the adherence of other cells.

The molecular mechanism of adherence has been divided into four forms:

(1) Chemical bonds which include electrostatic, covalent and hydrogen bonds (Marshall, 1980).

(2) Dipole interactions such as dipole-dipole, dipole-induced dipole and ion-dipole interactions (Heckels et al., 1976; Stotzky, 1980).

(3) Hydrophobic bonding (Doyle et al., 1982; Beachey, 1981).

(4) Lectin-like bonding (Duguid et al., 1966; Cisar et al., 1983).

The first three types of adherence are generalized as being non-specific in nature. Whereas lectin-like bonding is a highly specific form of adherence.

Chemical bonding, dipole-interactions and hydrophobic bonding are considered physicochemical adherence mechanisms and are discussed below. Using well-defined, non-living systems, Rutter & Vincent (1984), attempted to relate studies of particle adhesion to the understanding of microbial adherence to surfaces.

They recognized a limitation of such an approach was that surface charges are not static and are often adaptive. A true physicochemical equilibrium may not be reached because microbial attachment does not occur in a closed system; internal or surface structural changes to the microorganism may occur during the adherence process. They stress that in order to understand the adhesive junction between a cell and substratum, all interactions involving the surfaces and the environment must be considered.

In order for surfaces of similar charge to come into contact with each other, a large free energy barrier must be overcome. They define this free energy based on van der Waals forces and the overlap of the electrical double layer associated with charged groups on each surface. This energy barrier is influenced by the electrolyte concentration in the surrounding medium. As the electrolyte concentration increases, the energy barrier decreases

until a net attraction occurs between surfaces. In cases involving surfaces of opposite charge, the net effect is a strong attraction which is only mildly decreased with increased electrolyte concentration.

The points discussed in the preceding paragraph address long-range forces necessary to bring two surfaces into close proximity. However, short-range forces, in the form of dipole interactions and hydrogen bonding, are necessary to achieve adhesion between the two surfaces. In the case of two hydrophilic surfaces, there is a net increase in energy required to displace the water molecules, resulting in short-range repulsion. The opposite effect occurs when two hydrophobic surfaces interact due to a net decrease in energy required for water displacement.

Macromolecules which layer the surfaces of micro-organisms, can effect microbial adherence: Macromolecules, shed into the environment, can lead to increased solution viscosity and polymer bridge formation. The latter occurs when the macromolecule co-adsorbs onto two separate surfaces. Bridge formation is often enhanced by divalent cations which link polymers through acidic groups.

As more particles of similar surface charge adsorb to a surface, there are lateral interactions which must be considered. The

nature of these forces are dependant on the electrolyte concentration: In low electrolyte concentrations, adsorbing particles tend to repulse one another. However, under high electrolyte concentrations, the lateral forces are attractive. At intermediate electrolyte concentrations a compromise between attractive and repulsive forces is reached. This is defined as the second minimum in which particles are maintained at some distance from the substratum surface by the opposing forces. Second minimum adherence is of low affinity and reversible. High affinity, irreversible adherence is achieved only if the necessary free energy to overcome the primary and lateral forces in available.

Rutter and Vincent summarize by acknowledging that long-range and short-range forces will not completely dictate a particles adhesive behaviour. These forces function to reversibly hold a particle adjacent to a surface, therefore allowing the opportunity for irreversible adsorption. The particle must withstand hydrodynamic and shear forces from bulk medium flow. Ultimately, the particle/surface specificities will draw the final link between a cell and the substratum.

By studying polymer adsorption to surfaces, Robb et al. (1984) predicts the influence of exocellular polymers on bacteria adherence to surfaces. Polymer adsorption to surfaces appears to be irreversible. This is primarily due to the formation of a

large number of relatively weak bonds between the polymer and the substratum. Negatively charged bacteria adhering via polymers to negatively charged surface, illustrates the strength of polymer adsorption.

Polymer active sites responsible for adsorption include positive and negatively charged regions of exocellular polysaccharides and proteins. Since polymers are often highly charged, the ionic strength of the media is an important factor concerning polymer-surface adsorption. The affinity of a cell surface polymers for the solvent is important in preventing the cell from aggregating in the solution and maintaining the polymer in an extended state, thus, available for substratum interaction. Polymer extension from the cell surface is often sufficient enough to contain the net negative charge of the cell wall. As a result, the net charge of the cell is attributed to the polymer itself.

Kjelleberg (1984), through the study of various environmental factors, attempts to link studies on non-biological adhesion to natural microbial ecology. Growing evidence suggests a correlation between bacteria and microzones of increased nutrient concentration.

Most organisms adhere to surfaces by means of exocellular polymers. The composition and quantity of polymer has been linked to environmental conditions. Rosenberg et al. (1983a)

demonstrated that cell starvation strongly decreased the degree of encapsulation of *Acinetobacter calcoacetius* which in turn increased its cell surface hydrophobicity. This change in hydrophobicity markedly effected its adhesive properties as illustrated by a reduced adherence to hydrocarbons.

It is believed that a subtle balance exists between cell surface components which encourage and discourage adherence. The presence of LPS reduced cell surface hydrophobicity of *Salmonella typhimurium* and, as a result, decreased its adherence to the air-water interface (Hermansson et al., 1982). Alternatively, the appearance of fimbriae was found to increase cell surface hydrophobicity.

Adherence specificity has been the subject of increasing debate. For example, Rosenberg et al. (1983b) demonstrated that the oil degrading *Acinetobacter calcoacetius* while adhering to the oil-water interface also readily adhered to tooth surfaces and epithelial cells. Instead of a stereo-specific component responsible for adherence, they argue that these components contribute to an overall hydrophobic cell surface which was in turn responsible for its adherence diversity. In 1973, Marshall and Cruickshank first drew a correlation between cell hydrophobicity and surface adherence. In support of this, Dahlbäck et al. (1981) performed hydrophobicity assays on a broad range of bacterial isolates from both the air-water interface and subsurface water. Their results suggested that isolates with the

highest degree of hydrophobicity were associated with the air-water interface.

Using thermodynamic models, microbial adherence to hydrophobic surfaces is predictably more favourable than adherence to hydrophilic surfaces, provided the surface tension of the liquid medium is higher than that of the bacterial surface. This is the case with most natural environments and is supported by experimental data of other workers (Kjelleberg, 1984).

Various cell surface components have been found to act as adhesins. The abundance and composition of these structures vary markedly between species and within species of microorganisms. The growth conditions and the physiological state of the cell can have a significant effect on the adhesion process. Leech and Heffor (1980) demonstrated an inverse relationship between growth rate and *S.sanguis* deposition on inert polystyrene latex particles. Rosan et al. (1982) further demonstrated that carbohydrate source and growth pH also affects *S.sanguis* adherence.

Extracellular polymers such as lipopolysaccharide (LPS), of gram negative organisms, and lipoteichoic acid (LTA) of gram positive organisms can play a crucial role in cell adherence (Dazzo & Truchet, 1983; Beachey, 1981).

LPS mediated adherence is best demonstrated by the Rhizobium-

legume symbiosis (Dazzo et al., 1982). Specific reversible attachment is a necessary step towards host cell infection and nitrogen-fixing root module formation. The surface components mediating attachment include a multivalent lectin (trifoliin A) on the root hair, and carbohydrate receptors in the fibrillar capsule of the Rhizobium cell surface. Trifoliin A levels on root hairs was found to be regulated by exogenous nitrate supply (Dazzo & Brill, 1978). The levels of the lectin-binding saccharide (quinovosamine) in Rhizobium LPS is growth dependent and increases significantly as cells enter stationary phase (Hrabak et al., 1981).

LPS is highly diverse among gram negative organisms. The LPS molecule consists of three distinct regions; O-polysaccharides, core polysaccharide and the lipid A region. The lipid A region is highly conserved portion of LPS consisting of phosphorylated glucosamines residues in the form of oligosaccharides which are attached to fatty acyl esters, thus providing the hydrophobic, membrane binding, portion of this amphiphilic molecule. The core polysaccharide is also highly conserved and is characterized by aldoheptose and 2-keto-3-deoxyoctonate residues. Ketodeoxyoctonate along with several phosphate residues contribute to a net negative charge in this region of the molecule. The O-polysaccharides are polymers of repeating sequences of two to four mono-saccharides which include a wide range of heptoses and pentoses. The hydrophilic polysaccharide

region of the molecule determines the serotypic differences between strains of a bacterial species (Wicken, 1985).

Less specific adherence by surface polymers is mediated by cell surface S layers or by bacterial glycocalyx. S layers contain a regular array of glycoprotein subunits which are associated with the cell wall via divalent cations. The glycocalyx, or cell capsule, is a highly hydrated polysaccharide matrix which varies in complexity from simple homopolymers to complex heteropolymers. This fibrous matrix may be flexible or rigid and may be shed by the cell or integrated into the outer membrane. The glycocalyx is found in a variety of natural ecosystems and is often lost upon invitro subculturing. During the attachment process the glycocalyx is thought to overcome electrostatic repulsive forces, experienced by the bacteria, by forming a bridge between the cell and the surface. Once contact is established, weaker forces contribute towards an irreversible adherent state (Costerton, 1981). This view has been disputed by Sutherland (1983), suggesting that the glycocalyx may not be involved in initial attachment, but plays a greater role in establishing irreversible attachment.

Prosthecate bacteria, such as *Caulobacter* spp., are characterized by polar extensions, termed stalks. The distal portion of the bacterial stalk contains a holdfast adhesive which is responsible for adherence to surfaces. The life cycle of *Caulobacter* is

separated by a swarmer cell phase and a surface attached phase. The holdfast, which is present during both phases of the dimorphic life cycle, provides a good system for studying the molecular mechanisms of adhesion. Characterization of *Caulobacter* adherence is currently being investigated (Merker & Smit, 1988).

Fimbriae and pili are the most widely studied cell surface appendages. Fimbriae isolated from gram negative organisms are short, straight protein appendages, made up of subunits which vary from 15 to 25 Kd (Jones & Isaacson, 1983). The amino terminus is highly conserved and strongly hydrophobic suggesting a possible association with the outer membrane. They are most prominent in gram negative bacteria and have been implicated in adhesion and cell aggregation. The adherence process has also involved the pilus. Pili, unlike fimbriae, are plasmid-encoded large proteinaceous appendages which facilitate transfer of genetic material between donor and recipient cells. Another cell surface structure involved in adherence are fibrils. Handley et al. (1985) has described fibrils on a number of gram positive and gram negative organisms. Unlike fimbriae, these radiating structures do not have a defined width.

Duguid et al. (1955) first identified the non-flagellar, polar and peritricious, short filamentous appendages in *Escherichia coli*. These surface structures, designated antigen K-88 and K-99

have been shown to mediate *E.coli* diarrhoeal disease in piglets and calves, respectively (Orskov et al., 1961; Orskov et al., 1975). It is believed these antigens play a role in attachment leading to virulence since infectivity is inhibited by adhesin specific antisera or purified fimbriae. In addition, non-fimbriated strains of *E.coli* were found to be non-virulent. Since K88 and K99 were shown to be plasmid encoded, they have been categorized as pili instead of fimbriae (Isaacson, 1984). This phenomenon has been observed in other gram negative organisms. Pili-mediated attachment of pathogenic *Neisseria* spp. to mucosal surfaces has been demonstrated (Stephens, 1984). Of all the adherence mechanisms discussed, this is an example of the most specialized form since a specific interaction between the pili and the receptor molecule on the intestinal epithelium is necessary for attachment.

A number of different techniques have been employed in the study of microbial adherence. These methods can be divided into two general approaches: A) Characterization of the adherence reaction. B) Isolation and identification of the adhesin.

Adherence reaction characterization can involve chemical or enzymatic modification of the cell surface. For example, protein-mediated adherence can be eliminated by treating the cells with a protease (Weerkamp et al., 1980). Similarly, sialic acid residue removal by neuraminidase was shown to effect

Streptococcus sanguis interaction with salivary proteins (McBride et al., 1977; Levine et al., 1978). Chemical treatment of cells can also markedly effect adherence. For example, treatment with reducing agents implied the involvement of sulfhydryl groups in the adherence of *Sulfolobus* to sulphur (Wiess, 1973).

Competitive inhibition studies using receptor analogues to characterize adherence is a common approach. Carbohydrate-sensitive adherence has been described in oral bacteria by Duguid et al. (1966). Competitive binding experiments between similar or different cell types has been a useful tool in studying adherence. Staat et al. (1984), used competitive binding between radioactive and non-radioactive cells to distinguish specific from non-specific adherence.

Adsorption isotherms have become a useful tool for studying adherence specificity and kinetics. The binding isotherm is obtained by plotting the number of free cells (U) versus the number of bound cells (B) at equilibrium in a given system. Information about the adherence kinetics can be obtained using the Langmuir equation; $U/B = K/N + (1/N)U$, where K is the dissociation constant and N is the maximum number of binding sites. By plotting U/B verses U, an estimation of the dissociation constant and the affinity constant, (1/K from the x intercept), can be determined. Though the coefficient of variation is high using these isotherms, some useful information

regarding cell-surface interactions can be obtained (Gibbons et al., 1976).

The more difficult method of studying adherence is through the isolation and identification of the cell adhesin. A popular approach used by many workers is the isolation of non-adherent mutants. The cell surface protein responsible for adherence can in some cases, be identified by comparing the outer membrane profiles of the mutant and the wild-type (Fives-Taylor et al., 1985; Gaastra et al., 1982). Mutants lacking a surface adhesin can then be used to adsorb wild-type antisera in order to purify monospecific polyclonal antisera to the adhesin molecule (Fachon-Kalweit et al., 1985; Cisar et al., 1983). The adhesin-specific antisera is useful in adhesin localization studies through immunogold techniques and in adherence inhibition studies.

Several researchers have addressed the question whether attached bacteria contribute to the survival of free-living bacteria. It is generally accepted that in a nutrient depleted aquatic environment, organic substrates tend to accumulate at liquid interfaces. However, in natural aquatic environments, only a small percent of the total surface area is colonized by bacteria (Hoppe, 1984). Khailov and co-workers (1970) made three observations which could explain this environmental enigma:

1) High molecular weight macromolecules which are prevalent

in natural aquatic systems and display high degrees of surface activity, are almost exclusively adsorbed to surfaces.

2) Attached bacteria exhibit higher extracellular enzymatic capacities necessary for macromolecule degradation than their free-living counterparts.

3) Attached bacteria do not immediately consume their products resulting from macromolecular degradation.

They interpret these findings to mean that attached bacteria benefit the free-living bacteria by degrading the high molecular weight macromolecules and make available soluble organic substrates.

I will now review the current methods of measuring microbial adherence to mineral surfaces (Van Es & Meyer-Reil, 1982). Most investigators have measured cell adherence indirectly and determined the concentration of unbound cells by correlating cell numbers with various biomass or metabolic indicators. Examples of biomass measurement indicators are:

1) Protein concentration (Myerson et al., 1983; DiSpirito et al., 1983).
2) Optical density measurements at 660 nm (Kakakuwa et al., 1979).

3) Organic nitrogen concentration (Gormely et al., 1974).

4) Adenosine triphosphate (ATP) content (Tuovinen & Sormuneu, 1979).

Bioactivity indicators used to measure adherence are:

1) Radioactive carbon fixation (Brierley, 1977).

2) Radioactive phosphate incorporation (McCready & LeGallais 1984).

The major drawback to indirect techniques is that one cannot distinguish the binding of one cell type from another. In addition, microscopic mineral fines, which interfere with many biomass assays, are difficult to remove from unbound cells left in suspension. Protein and nitrogen concentration assays restrict the possible uses of certain protein or nitrogen-containing adherence inhibitors. Radioactive isotope studies have problems of decay quenching during scintillation counting. ATP assay techniques have encountered difficulties with ATP extraction and measurement in the presence of high levels of iron compounds and heavy metals in the samples.

The introduction of fluorescent-antibody (FA) techniques allowed direct observation of bound cells (Apel et al., 1976; Muyzer et al., 1987). In addition, fluorescent-antibody microscopy can identify specific cell strains, providing the antibody used is specific. The FAINT technique (Baker & Mills, 1982), which is a

modified version of the FA technique, allows the measurement of actively growing cells through the biological reduction of INT (2-(p-iodophenyl)-3-(p-nitrophenyl)-5 phenyl tetrazolium chloride) to a pigmented formazan compound which accumulates in the cell. This type of physiological information can also be obtained through epifluorescence microscopy (EFM) using acridine orange (Yeh et al., 1987) which, depending on the activity of the cell, will fluoresce different colours. However, conflicting results have been experienced by the latter two techniques; both authors attributed these inconsistencies to the unknown effects of low pH. Though these direct microscopic techniques can be informative, FAINT and EFM are labour intensive. In addition, background fluorescence through non-specific adsorption by the antibody to the mineral surface, can interfere with adherence estimations.

In light of the drawback discussed regarding current adherence assays, I have developed a non-isotopic, specific, sensitive approach to measuring microbial adherence to mineral surfaces.

Growing evidence supports the importance of substrate attachment in industrial applications of acidophilic microorganisms; however, a thorough analysis of acidophile attachment mechanisms is lacking. Given the importance of heterotrophic acidophiles and their symbiotic relationship with autotrophic acidophiles, I have limited my study primarily to the organism, *Acidiphilium*

cryptum. The purpose of this report then is to provide a comprehensive study of heterotrophic acidophile adherence to mineral surfaces by characterizing the adherence reaction and studying adherence-defective mutants.

MATERIALS AND METHODS

Bacteria

The bacterial strains used in this study were *A.cryptum*, ATCC 33463, and *T.ferrooxidans*, ATCC 23270. Both cultures were obtained from American Type Culture Collection.

Media and stock maintenance

A.cryptum was cultivated on a medium containing the following per litre: $(NH_4)_2SO_4$, 2 gm; KCl, 0.1 gm; K_2HPO_4, 0.25 gm; $MgSO_4$-$7H_2O$, 0.25 gm; $Ca(NO_3)_2$-$4H_2O$, 0.01 gm; dehydrated yeast extract (Difco laboratories, Detroit,Michigan), 0.1 gm; D-dextrose, 1.0 gm. This complete media will be referred to as MSGYE medium for the rest of the text. Media without the organic ingredients was designated basal mineral salts. The basal mineral salts was pH adjusted to 3.2 with 1 N H_2SO_4. To prepare MSGYE media, double strength basal mineral salts (pH 3.2) and double strength glucose-yeast extract solutions were autoclaved separately at 15 lb/in^2 for 20 min and then aseptically mixed after cooling. For solid media, 15 gm/litre of agar was added to the glucose-yeast extract solution prior to autoclaving. Small, white, convex, round colonies could be detected within three days of growth at 30° C. *A.cryptum* was stored on MSGYE agar slants at 4° C for one month periods between transfers. Stock cultures of *A.cryptum* were maintained at -70° C in a basal mineral salts solution containing 10% glycerol (pH 3.2). Late log-phase growing cells

were harvested from a 3 day culture incubated at 30° C. Plate-grown cells were gently removed from the agar surface and washed once in 1/2 concentration basal mineral salts (1/2 MS), pH 3.2 which will be referred to as adherence buffer.

T.ferrooxidans was grown and maintained on 100:10 solid media as described by Schrader and Holmes (1988). Exponentially growing cells were harvested from a 7 day culture grown at 30° C and washed once in adherence buffer. Cell suspension concentrations were determined using a Petroff-Hausser counting chamber and phase-contrast microscopy.

Buffers

The adherence buffer consisted of a 1/2 dilution of the basal mineral salts which was adjusted to pH 3.2 with 1 N H_2SO_4. For inhibition assays, the various putative inhibitors were added to the adherence buffer. Ore ELISA buffers, PBS/Tween and PBS 1% BSA, were each pH adjusted to 5.0 with 1 N HCl.

Bio-Rad Adherence Assay

A known number of cells were resuspended in adherence buffer. 1.5 ml of the cell suspension was added to microfuge tubes containing pre-measured amounts of mineral ore. This mixture was agitated through continuous inversion by the Labquake (Labindustries, Calif.) at 20° C for the duration of the incubation period. The ore was removed from solution by low

speed centrifugation (5800xg) for 20 seconds. To control for false positive cell loss during this step, "spun" and "non-spun" cell suspension controls were performed. A 0.9 ml volume of the supernatant, containing the unbound cells left in suspension, was removed to a new microfuge tube. The unbound cells were harvested by high speed centrifugation (13,000xg) for 10 min. The supernatant was discarded and the cell pellet was resuspended in distilled water. The cells were then transferred to glass tubes and boiled for 10 min to lyse the cells. When the tubes were cool, 0.2 ml of Bio-Rad protein assay concentrate (Bio-Rad Laboratories, Richmond, CA.) was added to each tube and protein concentration was measured at A_{595}. In order to calculate adherence, the following equation was used:

$$\% \text{ Adherence} = [\ 1 - (\ \text{test} / \text{cell spun control}\ )] \times 100$$

The number of cells bound was determined by multiplying the % adherence by the number of cells originally added to the assay tube.

Ore ELISA Adherence Assay

This assay procedure is a modified version of the Enzyme-Linked Immunoadsorbent Assay (ELISA), by Engvall (1972). To prevent cell adsorption to preparative materials, all glassware was siliconized with Surfa Sil (Pierce Chem. Co., Rockford, Il.). Mineral ore samples were provided by British Columbia Research

Inst. and are described in Table I. The ore samples were washed, desiccated and finely ground to an average particle size of 1.5 μm. A coat of silicone-based glue (Dow Corning corp., Midland, MI.) was applied to each well of a flat-bottom microtiter plate (Gibco, Canada Inc.). Each well was then coated with prepared mineral ore and allowed to dry overnight (Fig. 5). Unbound ore was removed and the plate was thoroughly rinsed with distilled water to remove loosely bound particles. The plates were then dried at 37° C for one hour.

A known number of cells were resuspended in adherence buffer. 300 μl of cell suspension was added to each well and incubated on a rotary shaker (100 rpm) at 20° C. After the desired period of

Table I. Title and description of mineral ore.

Source	Geological constituent
Newmont mine	chalcopyrite/pyrite
Gibraltar mine	chalcopyrite/pyrite
Empire mine	pyrite
Cambell Red lake	pyrite/arsenopyrite

incubation, the unbound cells were removed and each well was rinsed twice with adherence buffer. 400 µl of blocking solution (PBS/1% BSA, pH 5.0) was added to each well and incubated for one hour at 37° C. After the blocking solution was removed, the plate was rinsed twice with washing buffer (PBS/Tween, pH 5.0). The primary antibody was diluted in PBS/1% BSA (pH 5.0) and 300 µl was added to each well and incubated overnight at 4° C. The primary antibody was removed and the plate was washed 3 times with washing buffer (the last wash was allowed to sit for 10 min). The secondary antibody solution contained alkaline phosphatase conjugated goat anti-rabbit IgG (Helix Biotech ltd.) diluted in PBS/1% BSA (pH 5.0). 300 µl of secondary antibody solution was added to each well and incubated at 37° C for one hour. The secondary antibody was removed and the plates were washed 3 times with washing buffer (the last wash was again allowed to sit for 10 min). The plates were developed by adding 300 µl of alkaline phosphatase substrate (Sigma Chemical Co., St.Louis, Mo.) to each well and incubating at 37° C in the dark. Upon colour development, 100 µl of spent alkaline phosphatase substrate was removed to a second microtiter plate for adsorbance reading at 405 nm.

In order to calibrate the A_{405} values to bacteria per millilitre, specific activity measurements were performed for each assay run. The antibody reactivity was determined by measuring the A_{405} values of known cell concentrations, in 1 ml volumes, under

identical conditions as the Ore ELISA assay. This assay was performed in microfuge tubes to allow the centrifugation of the cells between each assay step.

Isolation of adherence variants

Ore adherence variants were obtained through nitrosoguanidine (NG) mutagenesis (Adelberg, 1965) followed by several enrichment steps. The Ore ELISA (as described above) was used to screen the enriched cell population for adherence-defective mutants.

A survival curve was determined with a constant concentration of NG and different time exposures. Exponentially growing cells were harvested washed and resuspended in citrate buffer to an OD_{660} of 0.5. NG was added to a final concentration of 50 μg/ml and this was incubated at 37° C. At 20 min intervals cells were removed, washed and resuspended in phosphate buffer and plated out on MSGYE media to determine the number of colony forming units (CFU).

Mutagenized cells were incubated for 24 hrs in MSGYE liquid media. The cells were washed and resuspended in adherence buffer prior to enrichment. Each enrichment procedure consisted of two adsorption steps. The adsorption step involved aseptically mixing 20 mls of the cell suspension (approx. $1x10^9$ cells per ml) with 1 gm of sterile Newmont mineral ore. The mixture was agitated through continuous inversion in a labquake for 20 min at

20° C. After incubation the ore was removed by differential centrifugation (2 min at 500xg) and the supernatant was recovered. After repeating once, the unbound cells were resuspended in MSGYE media and grown for three days at which time the next enrichment was performed. When enrichment was completed the cells were plated and examined macroscopically and microscopically. Selected cells were screened for adherence ability using the Ore ELISA assay, the Bio-Rad assay and a second indirect method described by Takakuwa et al. (1979) in which biomass is measured by OD_{660}.

Cell envelope protein preparation

Cells were washed in adherence buffer and resuspended in a buffer containing 0.05 M sodium phosphate, 0.15 M sodium chloride and 0.01 M EDTA adjusted to pH 7.4 (Boyd & McBride, 1984). The cell suspension was sonicated (Branson Sonic Power Co., Danbury, Conn.) 3 times for 40 sec on pulsed mode followed by 10 sec on continuous mode. The mixture was then centrifuged at 10,000xg for 20 min to remove unbroken cells. The supernatant was recovered and centrifuged at 80,000xg for 2 hours. The pellet of cell envelope protein (CEP) was resuspended in distilled water and its concentration was determined by the Bio-Rad protein assay.

Cell envelope solubilization

Proteins were solubilized from the CEP preparation using various

detergents; 0.1% Chaps, 0.1% Triton X-100, 1 M lithium chloride, 8 M urea, 5 M sodium thiocyanate, 6 M guanidine HCl, 0.1 M EDTA, 2.0% SDS or a combination of 8 M urea, 6 M guanidine HCl and 0.1 M EDTA. 50 μg of CEP was added to 2 ml of each of the detergents. This mixture was incubated for 2 hours at 37° C and centrifuged for 2 hours at 80,000xg. The supernatant was dialysed overnight at 4° C against distilled water. A sample of CEP preparation was treated with distilled water and after centrifugation, the supernatant and pellet were separated to act as negative and positive controls respectively.

Inhibition assay

Cells were pretreated with various agents and tested, by the Ore ELISA, to determine their effect on cell adherence. These agents included; polysaccharides, proteins, monovalent and divalent cations, reducing agents, bactericidal agents, metal chelating agents and surface active agents. Cells were washed and resuspended, to a concentration of $1x10^9$ cells per ml, in adherence buffer containing one of the agents. The bacteria were incubated for 30 min at 20° C before dispensing 300 μl to each Ore ELISA plate well.

Cell modification

Cells were subjected to enzymatic, physical and heat treatment to investigate their effect on adherence to mineral. The cells were washed and resuspended, to a concentration of $1x10^9$ cells per ml,

in each treatment buffer. Enzymatic treatment buffer (50 mM Tris, pH 7.2) contained either; 50 μg/ml phospholipase C, 50 μg/ml proteinase K, or 100 μg/ml mixed glycosidase. Phospholipase C buffer was supplemented with 1 mM $CaCl_2$. For enzymatic treatment, cells were incubated for 1 hr at 37° C. Heat treatment involved incubation for; 60 min at 50° C, 10 min at 70° C or 5 min at 100° C, in 50 mM Tris buffer, pH 7.2. Physical treatment involved vortexing cells with a magnetic stir bar for 30 min in 50 mM Tris, pH 7.2 at 20° C. After treatment, cells were washed and resuspended in adherence buffer for assay.

Competition assay

Competitive binding assays were preformed with immunologically distinct cell types. The two cell types were mixed prior to adding them to the Ore ELISA plate. One cell type was maintained at a constant concentration while the other cell type was added in increasing concentrations. The primary antibody used during the Ore ELISA assay was directed towards the cells which were kept at constant concentration.

Hydrophobicity assay

Bacterial surface hydrophobicity was determined by measuring the number of bacteria adhering to hexadecane (Rosenberg et al., 1980; Ganeshkumar,1985). Cells were washed and resuspended in adherence buffer to an absorbance at A_{660} of 0.5. A volume of 0.1 ml hexadecane was added to 3.0 ml of cell suspension in a 18x150

mm test tube. The suspension was mixed on a vortex mixer for 60 sec at 10 sec intervals and allowed to stand for 20 min before reading. The cells remaining in the aqueous phase were carefully removed and A_{660} values were determined. The test samples were compared to control samples which were not treated with hexadecane.

Surface protein preparation (SPP)

Cells were washed and resuspended, to a concentration of $5x10^9$ cells per ml, in a buffer containing; 20 mM Tris, 150 mM NaCl, 10 mM $MgCl_2$, pH 7.2. This solution was mixed with a magnetic stir bar for 30 min at 20° C. The cells were removed by centrifugation and the supernatant recovered. Forty percent ammonium sulphate was added to the supernatant and slowly stirred overnight at 4° C. The precipitate was pelleted (80,000xg for 2 hrs) and resuspended in distilled water. The preparation was exhaustively dialysed against distilled water at 4° C and frozen at -20° C.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE, as described by Laemmli (1970), was performed with a 10% or 12% polyacrylamide separating gel and a 3% stacking gel. The samples were boiled for 10 min in SDS-βME before adding approximately 0.3 μg of protein per well. The gels were stained for protein with silver nitrate as described by Oakley (1980). The molecular weight standards used were myosin (200,000),

phosphorylase B (97,400), bovine serum albumin (68,000), ovalbumin (43,000), α-chymotrypsinogen (25,700), β-lactoglobulin (18,400) and lysozyme (14,300).

Preparation of IgG

Antisera to wild-type *A.cryptum* and wild-type *T.ferrooxidans* cells was prepared in female New Zealand white rabbits. Cells were washed in distilled water and resuspended to a final concentration of $1x10^9$ cells per ml in complete Freund's adjuvant. One ml of this mixture was injected intramuscularly on day 1. Cell injections were repeated in incomplete Freund's adjuvant on days 7 and 14. The rabbits were bled on day 21 and immunoglobulin G (IgG) was obtained by purification on a Protein A-Sepharose CL4B (Sigma) column. Unbound serum constituents were removed from the column with borate buffer (0.1 M borate, 0.5 M sodium chloride, pH 8.4) until the eluate was protein free. The bound IgG was eluted with glycine buffer (0.1 M glycine, 0.5 M sodium chloride, pH 2.5). The eluate was neutralized with dilute NaOH and stored at -20° C. This antibody preparation will be referred to as the primary antibody through the rest of the text.

Adsorption of IgG

Monospecific polyclonal antiserum was prepared by adsorbing anti-whole cell antisera to adherence-defective variant cells. Cells were washed and resuspended in PBS (pH 7.2) to a concentration of $5x10^9$ cells per ml. 60 μl of IgG was added to 6 mls of cell

suspension and incubated overnight on a labquake at 4° C. The cells were removed by centrifugation and the supernatant was recovered. Adsorption was repeated four times.

Western blot

Western blot analysis was performed on samples that were electrophoresed in 10% and 12% SDS-PAGE. The gel was equilibrated in transfer buffer (14.4 gm/l glycine, 3.0 gm/l Tris, 200 ml/l methanol, pH 8.6) and placed on nitrocellulose paper which had been wetted by immersion in transfer buffer. The gel and nitrocellulose were sandwiched between two stacks of 3M Whatman filter paper which was also wetted with transfer buffer. This package was placed in a holding cassette and immersed in a Bio-Rad Trans-blot cell containing transfer buffer. To transfer the proteins to the nitrocellulose, a current of 25V was applied overnight followed by a current of 60V for 2 hrs.

The nitrocellulose containing the transferred proteins was immersed in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) containing 3% bovine serum albumin (BSA) for 30 min to block all remaining binding sites. The nitrocellulose was incubated for 2 hrs in primary antibody (see the last two sections) diluted in TBS-1% BSA. Unbound antibody was removed by washing twice in TTBS (20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5). The nitrocellulose was then incubated for 2 hrs in goat anti-rabbit IgG conjugated to horse radish peroxidase (GAR-HRP) diluted in

TBS-1% BSA. Unbound conjugate antibody was removed by two washes in TTBS. Bio-Rad GAR-HRP substrate was added and colour development was terminated by immersing the nitrocellulose in distilled water.

Immunogold bead labelling

Cells were washed and resuspended in PBS to an A_{660} of 0.5. Monospecific antibody (see "Adsorption of IgG" section) was added to the cell suspension and mixed in a labquake at 4° C for 4 hrs. Cells were washed twice and resuspended in 200 μl PBS. 15 μl of anti-rabbit gold bead IgG (5 nm) was added and incubated overnight at 4° C in a labquake. The cells were then washed three times to remove non-specific labelling.

Electron microscopy

Cells washed in PBS were negatively stained with 5% uranyl acetate in 70% alcohol. These were then observed under a Philips EM 300 electron microscope.

SPP inhibition assay

Inhibition of cell adherence was performed using the SPP. Ten micrograms of SPP in distilled water, from either mutant-15 or the parental strain was added to each well and incubated for 30 min at 20° C. After rinsing the plates with adherence buffer, $1x10^9$ cells per ml of the parent strain were added to each well and the Ore ELISA was performed. The antibody control wells were

treated with SPP but did not receive any cells.

Antibody inhibition assay

Monospecific antibody was used to inhibit *A.cryptum* adherence to chalcopyrite. Parental strain cells ($1x10^9$ cells/ml) were incubated overnight at 4° C in PBS, pH 5.0, with either; monospecific antibody or non-immune serum. Cells with adsorbed antibody were washed with and assayed in adherence buffer, pH 5.0.

RESULTS

Bio-Rad assay for measuring *A.cryptum* adherence to chalcopyrite

The Bio-Rad adherence assay was developed to characterize *A.cryptum* adherence to chalcopyrite ore. The design is similar to current indirect methods of measuring microbial adherence which rely on protein measurement as an indicator of biomass. However, it was determined that protein measurement using the Bio-Rad assay was faster and more sensitive than the modified Lowry procedure (Lowry & Rosebrough, 1951).

Some characteristics of *A.cryptum* adherence to chalcopyrite are described in Figures 1 through 4. Unless indicated otherwise, each Bio-Rad assay was performed using $1x10^8$ *A.cryptum* cells per ml and 50 mg of Newmont ore. The organisms were incubated with the ore for 20 min. The adsorption isotherm (Fig. 1) shows a linear relationship between cells added and cells bound until saturation was reached. Saturation occured when $1.2x10^8$ cells were mixed with 50 mg of ore.

Figure 2 demonstrates the relationship between the amount of Newmont ore and the number of cells bound. 75 mg of ore bound the maximum number of cells. At this quantity of ore,

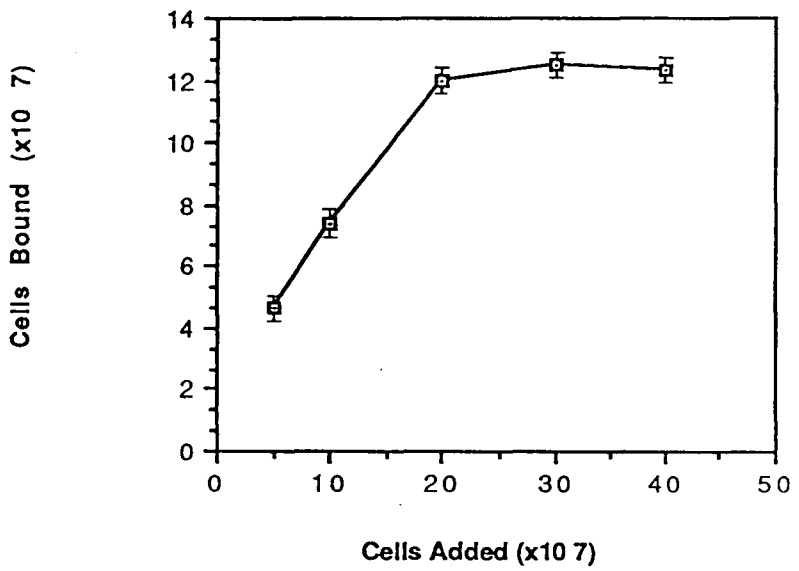


Figure 1. Bio-Rad assay illustrating the adsorption of *A.cryptum* to chalcopyrite. Cell were incubated for 20 min with 50 mg of Newmont ore.

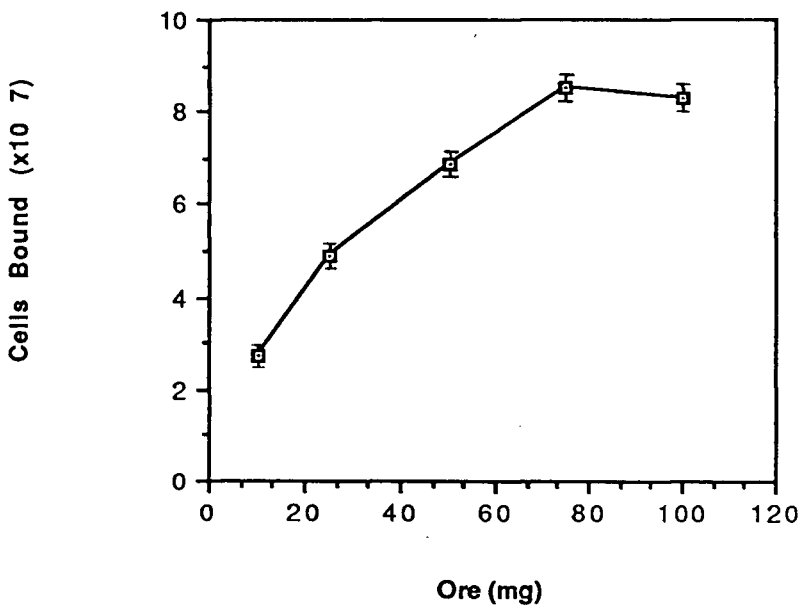


Figure 2. Bio-Rad assay demonstrating the relationship between the number of cells bound and the amount of Newmont ore added. A concentration of $1x10^8$ *A.cryptum* cells were added and incubated for 20 min.

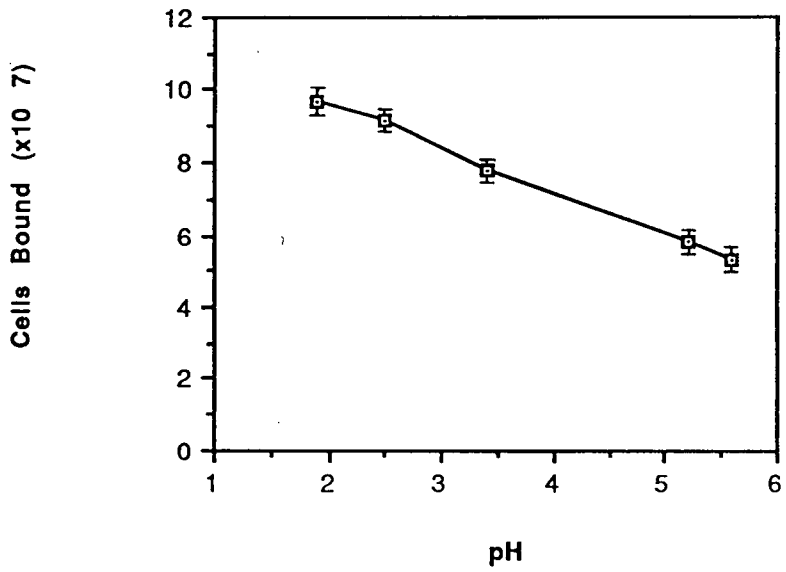


Figure 3. Bio-Rad assay illustrating cell adsorption as a function of adherence buffer pH. $1x10^8$ cells were added and incubated for 20 min with 50 mg of Newmont ore.

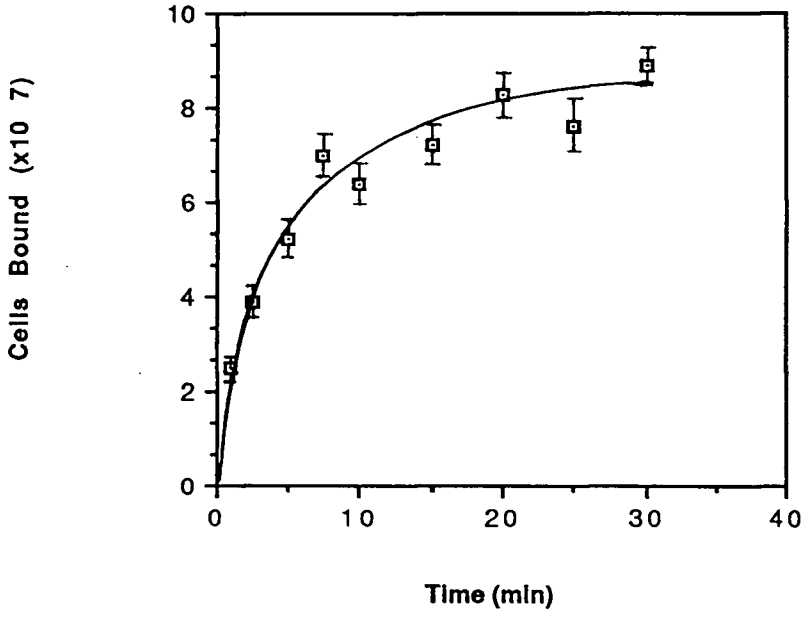


Figure 4. Bio-Rad assay illustrating the relationship between incubation time and the number of *A.cryptum* cells bound. $1x10^8$ cells were added to 50 mg of Newmont ore.

approximately 15% of the $1x10^8$ cells available for attachment, remained unbound. No increase in binding was observed when the quantity of ore was increased to 100 mg.

The effect of pH on *A.cryptum* adherence to chalcopyrite was investigated. The pH range tested corresponds with that in which *A.cryptum* cells are viable. An inverse relationship between adherence buffer pH and affinity to chalcopyrite was recorded (Fig. 3).

Figure 4 illustrates the effect of incubation time on the number of cells bound to chalcopyrite. Binding was rapid and complete in 20 to 30 min.

The age of the *A.cryptum* culture had little effect on cell adherence to chalcopyrite. Maximum adherence was measured at 4 days. This level of adherence was maintained through to a 10 day culture at which time cells began to self-aggregate.

There were a number of drawbacks encountered using the Bio-Rad assay for cell adherence: An initial problem was the separation of ore fines from suspended cells. Adequate centrifugation to remove mineral fines, also removed unattached cells from suspension. Conversely, less stringent centrifugation left fines and their attached bacteria in suspension. The loss of freely suspended cells was evaluated by the "spun" and "non-spun"

controls, and the removal of ore fines in suspension was measured by adsorbance. A compromise between these opposing factors was reached with a 20 sec spin at 5800xg. A second problem was the adsorption of bacterial protein to the glass tubes during the assay which resulted in an over-estimation of bound cells. Finally, the Bio-Rad assay was not a convenient means for screening large numbers of potential adherence-defective mutants. These concerns provided a rationale for investigating an alternative approach for measuring adherence.

Development of the Ore ELISA

The assay is based on the same principle as the standard ELISA except that a layer of mineral ore is glued to the well surfaces of a flat-bottom microtiter plate. The ore acts as the receptor for bacterial cells (Fig. 5).

Ore attachment to the ELISA plate was stable provided the plate was used within three days following preparation. It was found that after three days, ore particles would become detached. To ensure that the wells were thoroughly coated, each plate was inspected under a dissecting microscope for voids in the mineral surface.

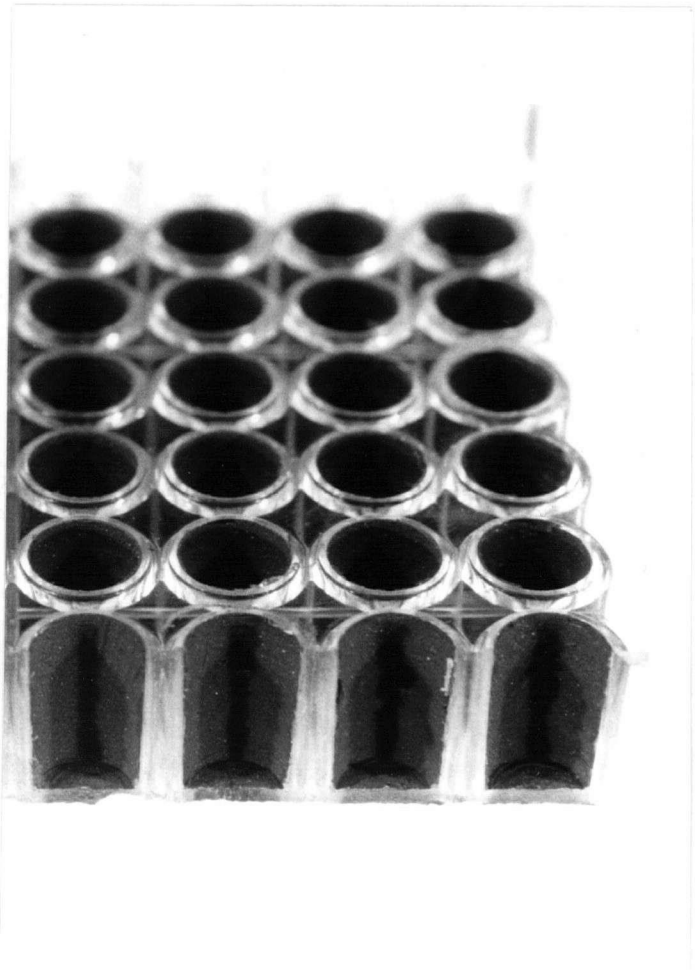


Figure 5. Cross-section of an Ore ELISA plate.

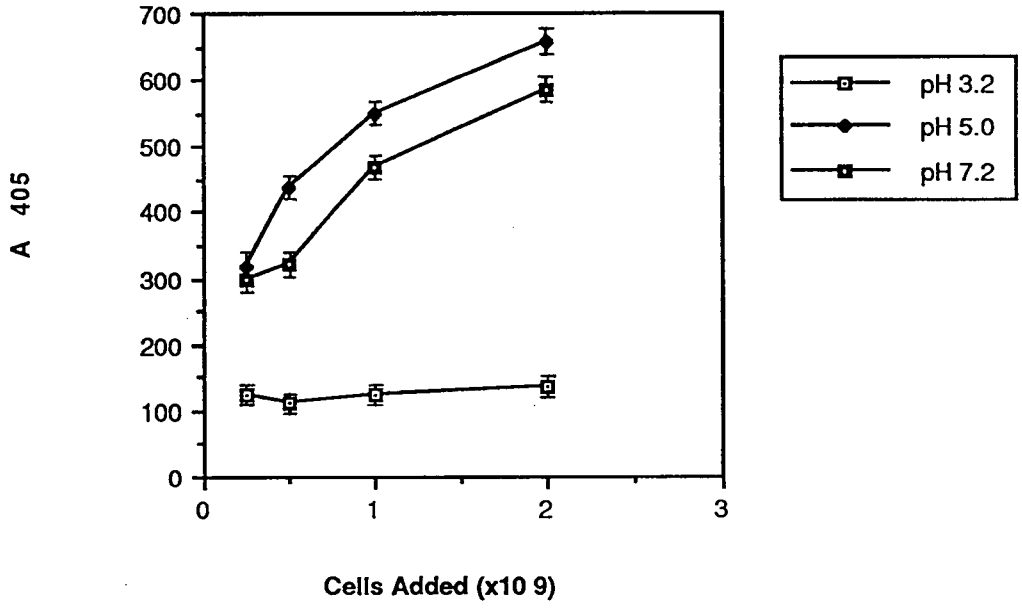


Figure 6. Effect of assay pH on adsorption at 405 nm.

The adherence buffer, with only trace levels of salts and a pH of 3.2, was designed to mimic the environment of this acidophilic organism. An adherence buffer pH of 3.2 was chosen because *A.cryptum* growth was highest in media adjusted to this pH.

A number of parameters which could effect the assay were investigated. Assay pH was an initial concern, since *A.cryptum* is an extreme acidophile and adherence to chalcopyrite was shown to be inversely related to the pH (Fig. 3), it was necessary that the assay be performed at an acidic pH. However, it was found that antibody reactivity was reduced at lower pHs. To address this problem, the assay was performed at various assay pHs: The adherence buffer for each assay was maintained at 3.2. However, the assay pH, which includes all other buffers of the assay (antibody buffer, washing buffer etc.) was varied. The results of this experiment are shown in Figure 6. An assay pH of 5.0 generated the highest OD_{405} values. Our interpretation of these results was that at pH 5.0 there was a balance between the desorption of cells and the maintenance of enzyme and antibody reactivity.

A 1/1000 dilution of primary antibody stock solution was found to provide sufficient A_{405} readings and yielded minimal background adsorption. *A.cryptum* did not possess an extracellular alkaline phosphatase capable of reacting with the assay substrate.

A.cryptum showed strong affinity for the plastic of the ELISA plate and moderate affinity for a glue coated ELISA plate. Therefore it was necessary to reduce background binding to non-mineral surfaces. This was accomplished by extensive washing with PBS/Tween. Adherence to ore coated plates, glue coated plates and untreated plastic plates was reduced by 5%, 65% and 82% respectively by washing with PBS/Tween. This suggests that the cells were interacting with the ore as opposed to the glue or the plate.

Initial trials of the Ore ELISA produced substantial background readings due to non-specific adsorption of the antibody to the mineral ore substrate. Table II illustrates the procedures employed to reduce the background levels; these primarily involved altering the washing or blocking steps. A six percent reduction in background was achieved by introducing a blocking step prior to adding the primary antibody. Increasing the concentration of BSA or using other protein blocking agents (data not included) during the blocking step had little effect on furthering background reduction. The most effective assay condition for maintaining low background levels was a 1 hr block with 1% BSA and a for 10 min wash with PBS/Tween. This procedure reduced background levels from 32% to 12%.

To relate A_{405} readings to the number of cells, the reactivity of the cells with antibody was assessed by performing the tube assay

concurrently with each experiment. This procedure served to standardize A_{405} assay readings such that adherence comparisons between different cell types, species and strains could be made. For an assay to be useful, it must be specific, reproducable and sensitive. Table III indicates that the assay is dependent upon having a specific antibody and that non-specific adsorption of antibody to ore is minimal. Reliability was assessed by measuring the variability of readings between and within assay plates. Each experiment performed on the Ore ELISA was run in triplicate. Readings from different wells on the same plate varied between 3% and 7%. Assay sensitivity was assessed by measuring A_{405} at various cell concentrations. A 100% increase over background was observed with a minimum of $1x10^5$ cells.

The Ore ELISA was applied to adherence kinetic studies and the results were compared to the Bio-Rad assay results. The adsorption isotherms for the two assays both displayed binding saturation (Fig. 1 & 7). Binding saturation for the Ore ELISA was found to be $1.4x10^8$ cells which is similar to the $1.2x10^8$ cells recorded for the Bio-Rad assay. The effect of pH on adherence as measured by the Ore ELISA was less marked than that shown by the Bio-Rad assay (Fig. 3 & 8).

Table II. Effect of Tween and BSA on background-reduction in the Ore ELISA assay.

Assay conditions		A_{405}		
BSA block	Tween wash	Ab control[1]	Test[2]	Background[3]
-	-	.580	1.807	32
-	+	.456	1.683	27
1%	+	.335	1.562	21
2%	+	.331	1.400	23
3%	+	.293	1.367	22
1%	+[4]	.155	1.294	12

1. Assay performed without cells added.
2. Assay performed with $1x10^9$ cells added.
3. Background was calculated as the percent of antibody control relative to the test.
4. The final PBS/Tween wash was for 10 min.

Table III. Immunological specificity of the Ore ELISA assay.

Assay Condition	A_{405}
Without cells added	0.144
Without anti-A.cryptum Ab	0.078
Without secondary Ab	0.027
Without alkaline phosphatase substrate	0.005
Complete assay with anti-A.cryptum Ab	1.350*
Complete assay with anti-T.ferrooxidans Ab	0.101

* A_{405} of 1.350 represents 1.0×10^8 cells bound.

Cells bound rapidly, adherence was essentially complete in 10 min (Fig. 9). The flat plateau of Figure 10 would suggest that cells adhere in a monolayer and do not aggregate. This was supported by microscopic studies in which cell aggregation was not observed.

Adherence to different mineral ore substrates was investigated. The greatest number of *A.cryptum* cells bound to the Cambell Red lake ore sample with twenty five percent fewer binding to the Empire mines ore sample (Fig. 10). Attempts at using elemental sulphur as the receptor proved unsuccessful as the sulphur did not form a stable homogenous layer in the microtiter plate.

Adherence inhibition studies

The inhibitory effect of various agents was investigated, in an attempt to identify the mechanism of adherence. These agents were present in the adherence buffer during the binding reaction. The adherence recorded in Tables IV through IX was calculated as a percent, assuming that the number of cells bound in the control represents 100%. N-acetyl glucosamine, N-acetyl galactosamine and a number of other sugars tested had no effect on *A.cryptum*

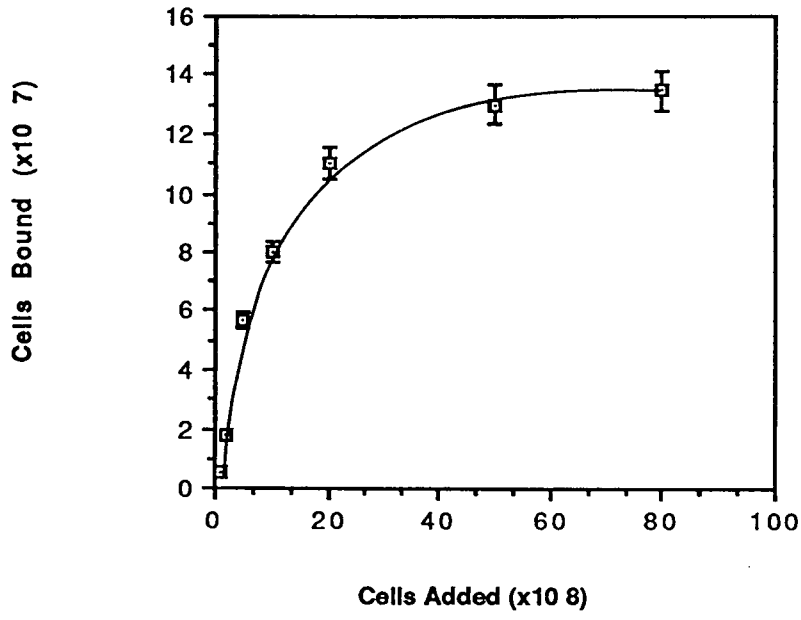


Figure 7. Ore ELISA measuring the adsorption isotherm for *A.cryptum* binding to chalcopyrite.

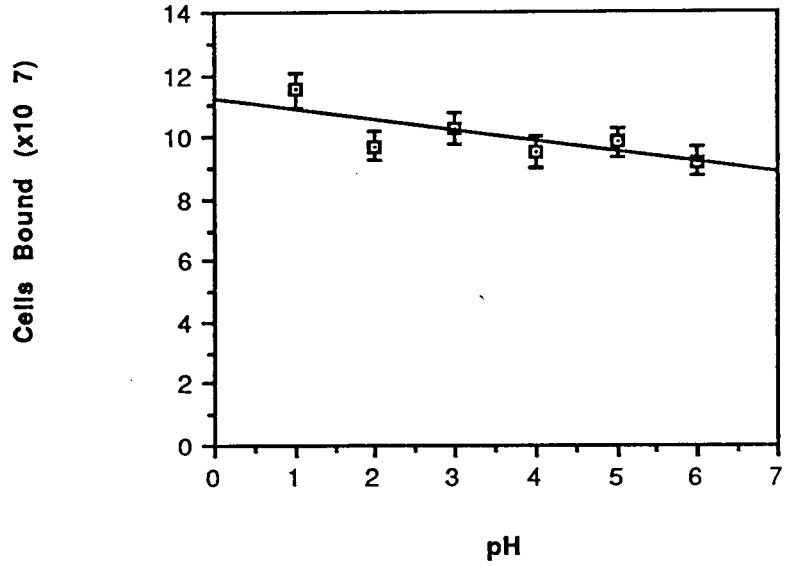


Figure 8. Ore ELISA demonstrating the effect of adherence buffer pH on *A.cryptum* binding to chalcopyrite.

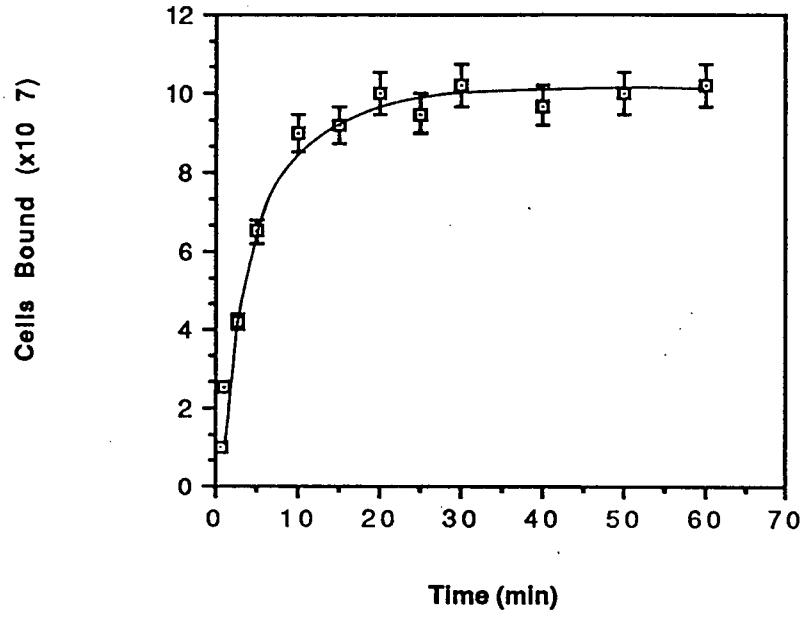


Figure 9. Relationship between length of incubation and adherence as measured in the Ore ELISA assay.

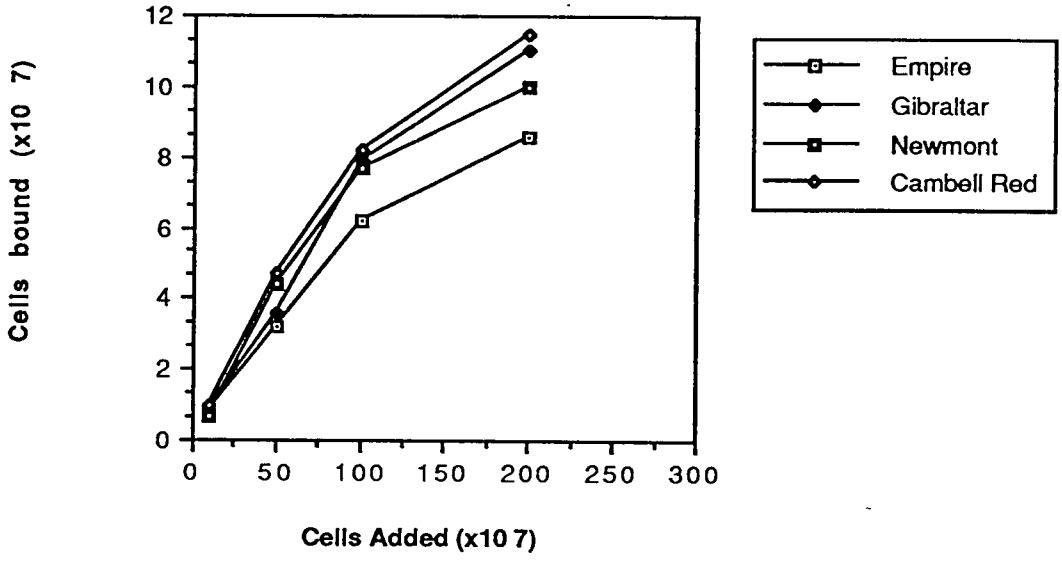


Figure 10. A comparison of *A.cryptum* adherence to different minerals.

adherence to chalcopyrite (Table IV). On the other hand, both BSA and gelatin inhibited cell binding to the ore (Table V). For example, 0.1 mg/ml of BSA or gelatin reduced cell adherence by 25% and 67% respectively. Though this form of inhibition appeared non-specific in nature, the effect of gelatin was greater than that of BSA. That is, a 10 fold increase in BSA was necessary to achieve an equivalent level of inhibition.

In the presence of monovalent and divalent cations, *A.cryptum* adherence to chalcopyrite was enhanced (Table VI). However, this phenomenon was only observed at high ion concentrations. Surface active agents greatly enhanced the adherence of *A.cryptum* to chalcopyrite (Table VII). For example, Triton X-100 at a 0.01% concentration increased cell adherence by 310%.

The effect of chelating agents, bactericidal agents and reducing agents are summarized in Table VIII. The cell suspension was incubated with the chemical agent for 30 min at 20° C and then added to the Ore ELISA plate. Elevated adherence was recorded in the presence of the chelating agent, EDTA. 2-mercaptoethanol and DTT increased and decreased adherence respectively. The sulphur binding agents, iodoacetamide and mercuric chloride, had little effect on adherence. The respiratory poisons, sodium azide and sodium cyanide had a minimal effect on adherence suggesting that cell viability was not necessary for adherence (Table VIII).

Table IV. The effect of polysaccharides on *A.cryptum* adherence to chalcopyrite.

	Conc. (mM)	Adherence[1]
Control[2]		100
Glucose	10	113
	100	110
Galactose	10	88
	100	99
Lactose	10	93
	100	100
Mannose	10	111
	100	104
Ribose	10	101
	100	73
N-Acetyl Glc	10	108
	100	116
N-Acetyl Gal	10	99
	100	109

1. Percent of cells bound relative to the control.
2. Cells incubated in adherence buffer.

Table V. The effect of protein on *A.cryptum* adherence to chalcopyrite.

	Conc. (mg/ml)	Adherence[1]
Control[2]		100
BSA	0.01	93
	0.1	75
	1	37
Gelatin	0.01	73
	0.1	33
	1	11

1. Percent of cells bound relative to the control.
2. Cells incubated in adherence buffer.

Table VI. The effect of monovalent and divalent cations on *A.cryptum* adherence to chalcopyrite.

	Conc. (mM)	Adherence[1]
Control[2]		100
NaCl	10	114
	100	111
	500	156
$MgSO_4$	5	105
	20	112
	100	120
$CaCl_2$	5	109
	20	114
	100	129

1. Percent of cells bound relative to the control
2. Cells incubated in adherence buffer.

Table VII. The effect of surface active agents on *A.cryptum* adherence to chalcopyrite.

Agent	Percent	Adherence[1]
Control[2]		100
Tween 20	0.001	123
	0.01	216
	0.1	214
Triton X-100	0.001	123
	0.01	310
	0.1	290

1. Percent of cells bound relative to the control.
2. Cells incubated in adherence buffer.

Table VIII. The effect of reducing agents, bactericidal agents and metal chelating agents on *A.cryptum* adherence to chalcopyrite.

Agent	Conc. (mM)	Adherence[1]
Control[2]		100
Iodoacetamide	1.0	116
Mercuric Chloride	1.0	104
2-Mercaptoethanol	1.0	148
Dithiothreitol	1.0	74
Sodium azide	2.0	93
Sodium ferricyanate	0.1	23
Sodium cyanide	2.0	121
EDTA	2.0	153

1. Percent of cells bound relative to the control.
2. Cells incubated in adherence buffer.

Sodium ferricyanate, unlike the other bactericidal agents, strongly reduced cell adherence. It should be noted that in the presence of sodium ferricyanate, the solution turned a vivid blue colour. This was probably due to the chemical leaching of copper from chalcopyrite by ferricyanate.

In an attempt to determine if inhibitors of binding were acting on the cells or the ore, experiments were run in which the inhibitor was preincubated for 30 min with either the ore or the cells and then removed prior to the assay (Table IX, column 1 & 2). The results were compared to assays in which the inhibitor was present during the assay (Table IX, column 3). EDTA pretreatment of either the ore or the cells had no effect on adherence but if present during the adherence reaction, binding was increased. Sodium ferricyanate seems to act primarily at the cell surface, however, an accumulative effect may be necessary to reduce adherence to 46% (Table IX, column 3). Dithiothreitol pretreatment had little effect on cell attachment to chalcopyrite. However, if DTT was present during the adherence reaction, binding was decreased. Similar to the effects of EDTA, the presence of 2-mercaptoethanol in the adherence buffer was necessary for the observed increase in binding.

The effect of cell surface modification on adherence was investigated. Phospholipase C, proteinase K and mixed glycosidase had no effect on *A.cryptum* adherence to chalcopyrite.

Table IX. The effect of ore or cell pretreatment with reducing agents, bactericidal agents and metal chelating agents on *A.cryptum* adherence to chalcopyrite.

	Pretreatment of ore	Pretreatment of cells	Cells in treatment buffer
Control[1]	100	100	100
2-Mercaptoethanol	98	79	152
Dithiothreitol	90	90	64
Sodium ferricyanate	83	72	46
Sodium cyanide	79	88	104
EDTA	96	96	124

All values calculated as a percent of cells bound relative to the control.

1. Cells incubated in adherence buffer.

The effect of heat could not be assessed as the cells formed aggregates when heated. Cells which had been mixed vigorously with a stir bar retained the ability to bind to chalcopyrite.

Competition studies

A.cryptum and *T.ferrooxidans* are closely linked in nature and are believed to exist symbiotically. Of interest was whether these two acidophiles compete for binding sites on chalcopyrite. This type of experiment was possible using the Ore ELISA assay because the two species cell types are immunologically distinct and do not coaggregate as shown by microscopic studies. Table III, in addition to immunofluorescence studies, demonstrates that antisera to *A.cryptum* and *T.ferrooxidans* are not cross-reactive.

The competition experiment was designed to maintain one species at a constant saturating concentration and increase the concentration of the second species. This protocol would provide comparative information about the nature and specificity of adherence of the two cell types. Saturation was achieved with $3x10^9$ *A.cryptum* cell/ml (Fig. 7) and $2x10^9$ *T.ferrooxidans* cell/ml (Fig. 11). Figure 12 (a) demonstrates that a 20 fold excess of *A.cryptum* cells did not interfere with *T.ferrooxidans* binding to chalcopyrite. A similar result was recorded for the binding of

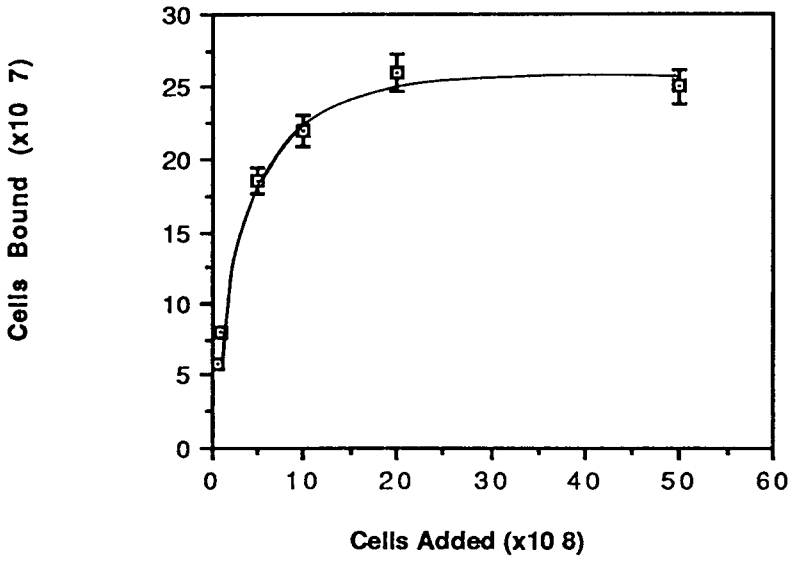
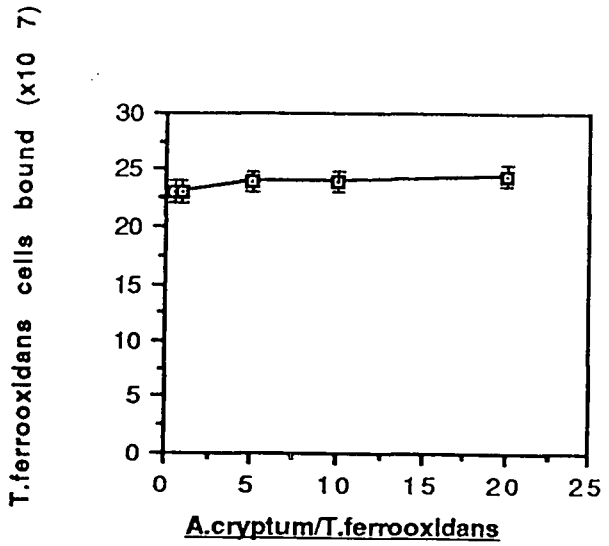


Figure 11. Ore ELISA measuring the binding of *T.ferrooxidans* to chalcopyrite.

a)



b)

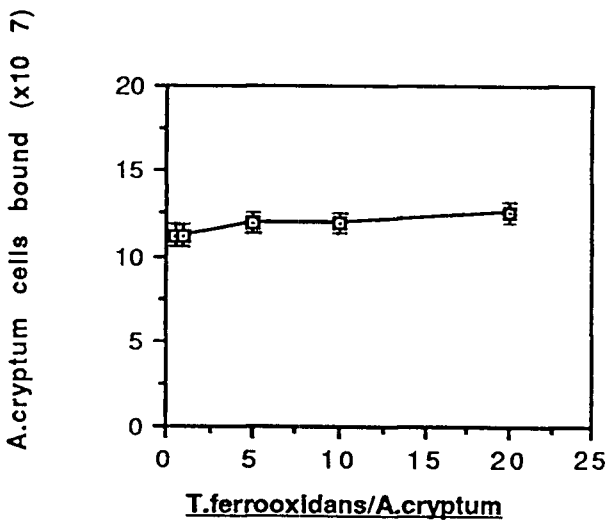


Figure 12. Competition between *A.cryptum* and *T.ferrooxidans* for binding sites on chalcopyrite:
a) Increasing numbers of *A.cryptum* cells were added to a constant number of *T.ferrooxidans* cells ($2x10^9$ cells/ml).
b) Increasing numbers of *T.ferrooxidans* cells were added to a constant number of *A.cryptum* cells ($3x10^9$ cells/ml).

A.cryptum to chalcopyrite in the presence of an excess of *T.ferrooxidans* (Fig. 12 (b)). It was concluded that the binding sites for each organism are unique and distinct.

Isolation of adherence-defective variants

In order to identify the adhesin responsible for attachment to mineral surfaces, *A.cryptum* cells were mutagenized, enriched and screened for adherence-defective mutants. Each of three mutagenesis experiments performed used a starting concentration of $3x10^8$ cells per ml and 50 μg/ml of nitrosoguanidine. The first mutagenesis was aimed at a 50% kill according to the survival curve (Fig. 13). This required a 47 min exposure to the mutagen and was followed by a series of 6 enrichment procedures. In a second and third experiment cells were exposed to NG for 80 min and enriched for non-adherent cells. Cells were enriched 6 and 14 times following second and third enrichments respectively. In these experiments, enrichment involved mixing mutagenized cells with ore for 20 min and then culturing cells which did not settle with the ore. The first two attempts at obtaining non-adherent mutants were unsuccessful. Assuming that binding may involve more than one type of receptor-adhesin interaction, it was decided to modify the third mutagenesis enrichment procedure in an attempt to differentiate between weak and strong binding

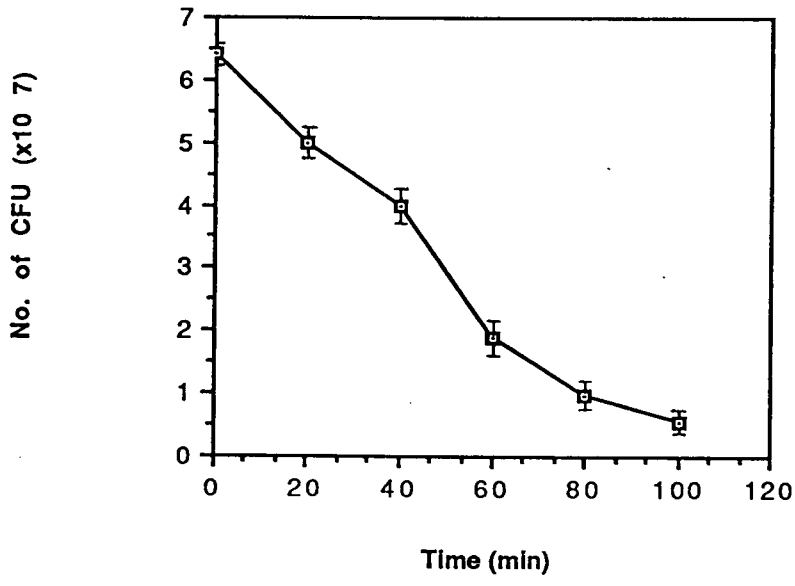


Figure 13. Nitrosoguanidine survival curve.

cells. Instead of a 20 min incubation with 1 gm of Newmont ore, the cell suspension was incubated with either 25 mg of ore for 20 min, or 100 mg of ore for 10 sec. The modified enrichments would select for binding strength and binding speed respectively. These enrichments were performed 6 times, the cells remaining in suspension were plated and colonies screened for their ability to bind to chalcopyrite.

Isolates were screened by the Ore ELISA and recorded as the percent of adherent cells relative to the number of parent cells bound. Indirect assays confirmed the Ore ELISA screening results. Prior to screening, the cell concentration of the mutant and parent strains was adjusted to $1x10^9$ cells per ml. Each mutant was tested for antibody reactivity by the tube assay to ensure that mutagenesis did not create quantitative changes in the reactivity of the cells to the antibody. Several of the mutants proved to be self-aggregating and were not further studied.

Table X lists several of the mutants isolated. A strictly non-adherent mutant was not obtained. Most mutants displayed a 41-61% decrease in ability to adhere to chalcopyrite. However, mutant-4 showed an increase in the ability to bind to chalcopyrite. Mutants -30, -39, -76, and -90 were isolated from

Table X. Characteristics of adherence-defective mutants.

Strain	Adherence[1]	Hydrophobicity[2]	Self-aggregation
Parental	100	86	-
4	128	85	-
11	40	83	-
12	51	78	-
15	51	89	-
19	43	65	+
23	40	69	+
30	52	77	-
39	58	48	+
76	59	58	+
90	39	40	+

1. Percent of cells bound relative to the control.
2. Percent of cells remaining in the aqueous phase.

the first mutagenesis. The remaining mutants were isolated from the third mutagenesis. Phenotype reversion was not detected in any of the isolates.

Analysis of adherence-defective mutants

Many studies have correlated cell surface hydrophobicity with adherence to mineral surfaces and to interfaces in the environment. However, in these studies, a relationship between cell surface hydrophobicity and the ability to bind to ore was not observed. For example, mutant-90 is hydrophobic and mutant-15 is hydrophilic yet both are defective in their ability to adhere to chalcopyrite. Hydrophobic cells appeared to self-aggregate.

Many microorganisms attach to surfaces via polar or lateral flagella. This did not appear to be the case with *A.cryptum* since the adherence-defective mutants possessed flagella and are motile.

Western immunoblot analysis of whole cell lysates showed that a 31.6 kDa antigen present in the parent strain (Fig. 14, Lane A), was absent from the adherence-defective mutants (Fig. 14, Lanes D, E, & F). Mutant-4 (Fig. 14, Lane C) which showed enhanced

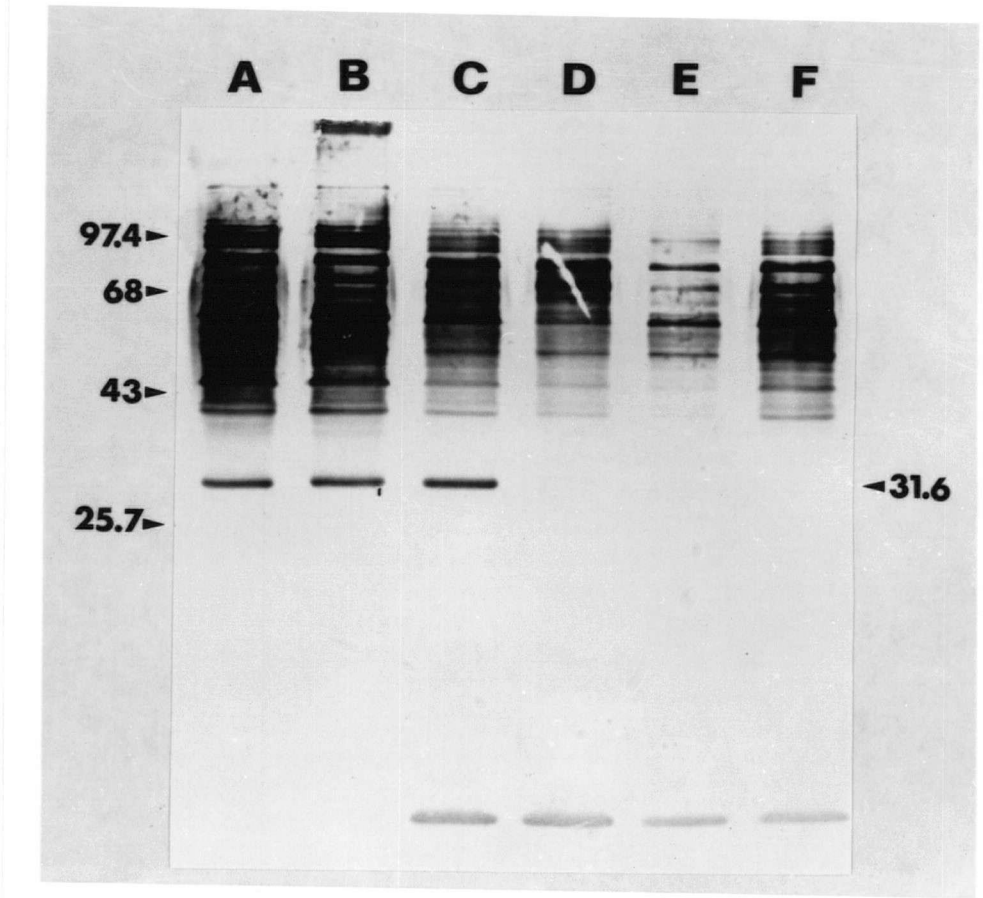


Figure 14. Western blot of a 10% SDS-PAGE developed with anti-*A.cryptum* antibody. Whole cell lysates of: Lane A and B, parental *A.cryptum*. Lane C, mutant-4 which showed enhanced adherence ability. Lanes D, E, and F are adherence-defective mutants-11,-12, and -15 respectively. Samples in lanes A, C-F, were heated to 100° C in SDS prior to electrophoresis. The sample in lane B was incubated in SDS at 20° C prior to electrophoresis.

adherence ability, also contained the 31.6 kDa antigen. Boiling the sample had no effect on the migration pattern of the 31.6 kDa antigen (Fig. 14, Lane A & B). An additional low molecular weight antigen, common to all mutants, was identified in Figure 14. The antigen migration profile of each mutant closely resembles the parental strain, suggesting that the mutants are not contaminants. This was supported by microscopic and macroscopic comparisons. The 31.6 kDa protein was absent in all the adherence-defective mutants tested. The protein was present in parental cells harvested from liquid and solid media. Cells from either medium had equivalent ore adherence abilities.

Localization of the 31.6 kDa protein

Since the 31.6 kDa protein was absent in adherence-defective strains, and present in parental and adherence-enhanced strains, it was postulated that the protein might be involved in the binding of *A.cryptum* to chalcopyrite. Therefore, attempts were made to localize the 31.6 kDa protein. On the assumption that loosely associated cell surface structures could be removed by gentle means, we vortexed cells with a magnetic stir bar and analysed the material solubilized by this process (surface protein preparation).

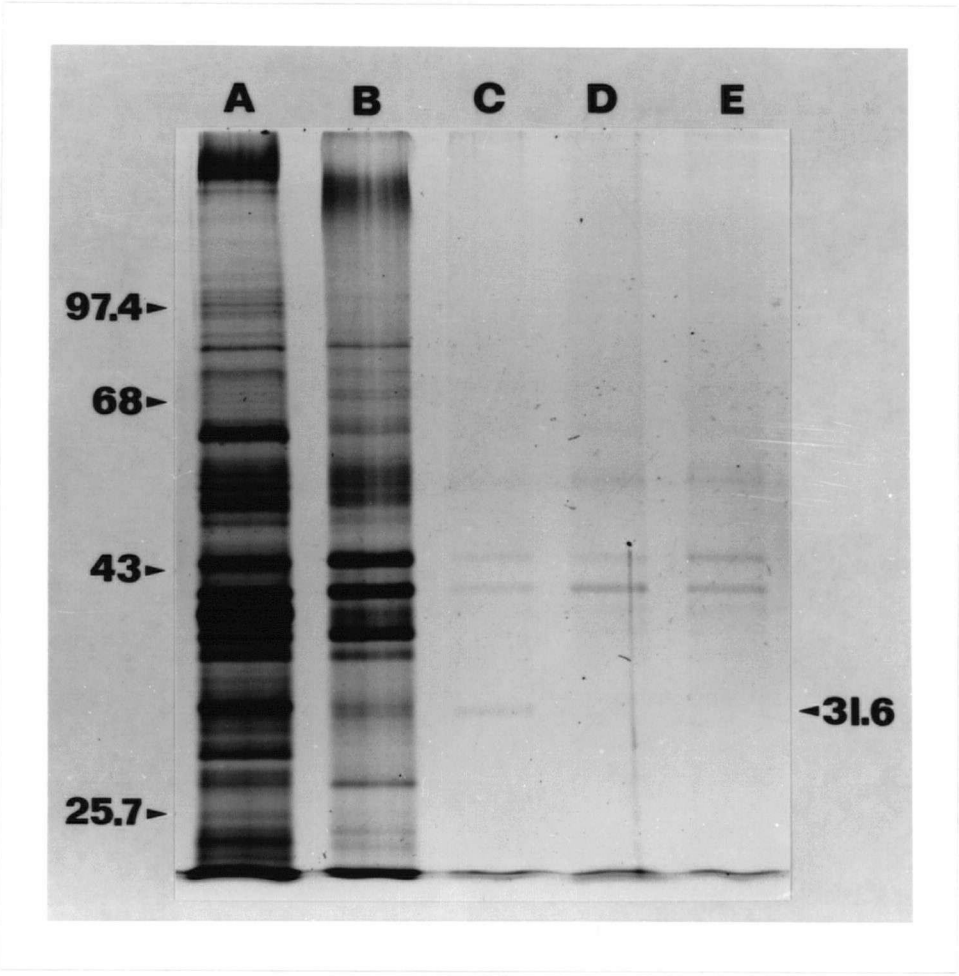


Figure 15. SDS-PAGE of surface protein preparations. Lane A, whole cell lysate of parental *A.cryptum*. Lane B, cell envelope protein preparation of parental *A.cryptum*. Lane C, D, and E, surface protein preparations of parental *A.cryptum*, mutant-12, and mutant-15 respectively. Lane A contains 0.3 g of protein. Lane B contains 0.2 μg of protein. Lanes C, D, and E each contain 0.05 μg of protein.

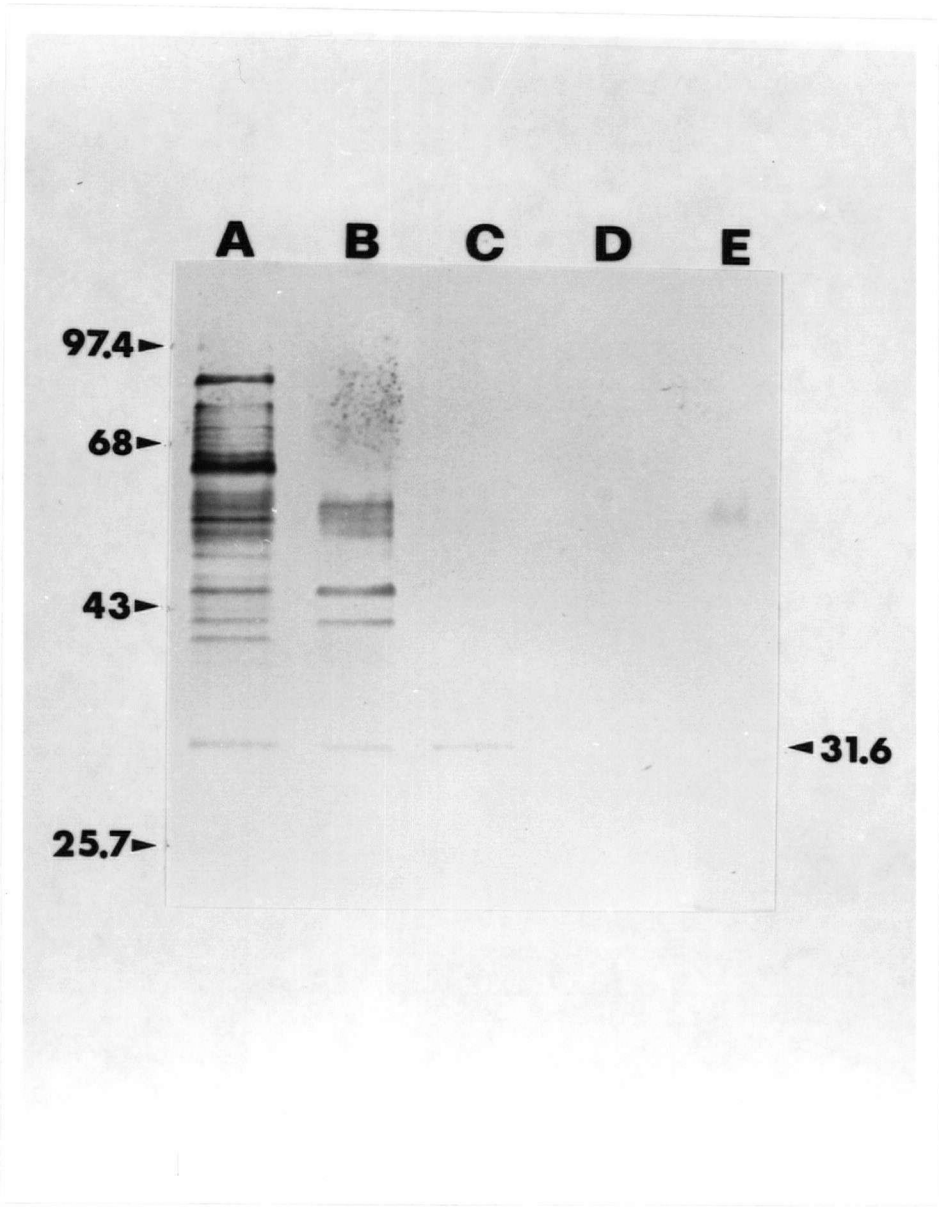


Figure 16. Western blot of a 12% SDS-PAGE developed with anti-*A.cryptum* antibody. Lane A, whole cell lysate of parental *A.cryptum*. Lane B, cell envelope protein preparation of parental *A.cryptum*. Lane C, D, and E, surface protein preparations of parental *A.cryptum*, mutant-12, and mutant-15 respectively.

Figure 15 is a silver stained SDS-PAGE comparing the migration profile of; parental whole cell lysate (Lane A), parental cell envelope protein preparation (Lane B) and parental surface protein preparation (Lane C). The absence of the 31.6 kDa protein is illustrated in the surface protein preparations of mutants-12 and -15, lanes D and E respectively. Figure 16 is a western immunoblot of Figure 15 and further suggests that the 31.6 kDa protein is localized at the cell surface of the parental strain and is absent in the mutant strains. The varying intensity of the 31.6 kDa protein between preparations in Figure 15 was disturbing. Though figure 16 shows similar intensities of banding suggesting similar antigen concentrations, a distinct reduction in banding intensity is observed in figure 15. There are two possible explanations for this discrepancy. The titer of antibody specific to the 31.6 kDa protein may be limited in the anti-*A.cryptum* antisera and therefore would not reflect antigen concentration differences during western immunoblot analysis. An alternative explanation is the presence of a similar sized protein in the whole cell lysate with was removed during the cell envelope protein preparation.

A monospecific polyclonal antibody preparation was developed in order to localize the 31.6 kDa protein through immunogold labelling. Antisera to the parental strain of *A.cryptum* was thoroughly adsorbed by the mutant-15 to generate the monospecific antibody preparation illustrated in Figure 17. Several

conclusions can be made from this figure: The adsorption procedures generated antibody with a single specificity. Further evidence was provided to suggest that similar concentrations of the 31.6 kDa antigen exist in whole cell lysates and cell envelope preparations of the parental strain (Fig. 17, Lanes A & B). The mono-specificity of the adsorbed antibody suggests that mutant-15 and the parental strains differ only in the 31.6 kDa protein.

Mutant-15 and the parental strain were immunogold labelled using the antibody preparation specific to the 31.6 kDa protein. Figure 18 shows an even distribution of gold beads indicating that the antigen was widely distributed over the surface of the parental strain. This was compared to mutant-15 labelled with the monospecific antibody in Figure 19 (a), and the parental strain labelled with non-immune serum in Figure 19 (b). The intensity of gold bead labelling in Figure 18 was not consistent with all parental cells observed; some contained very few gold beads while others showed excessive labelling. This may be indicative of phase variation in the protein expression of the 31.6 kDa protein.

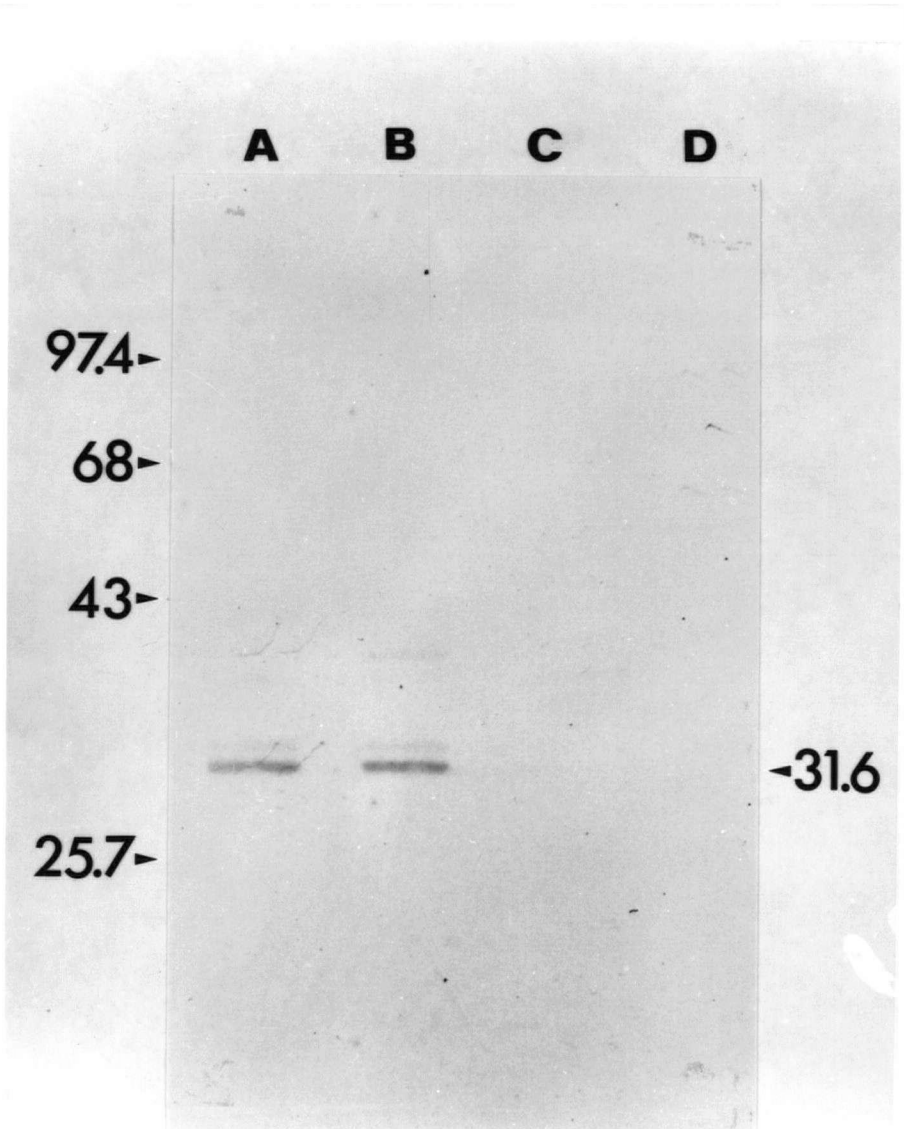


Figure 17. Western blot of a 12% SDS-PAGE developed with the monospecific anti-31.6 kDa protein polyclonal antibody preparation. Lane A, cell envelope protein preparation of parental *A.cryptum*. Lane B, whole cell lysate of parental *A.cryptum*. Lane C, and D, cell envelope protein preparation and whole cell lysate of mutant-15 respectively.

Variations in the stringency of washing during immunogold labelling generated marked differences in the numbers of gold beads located on the cell surface. Gentle cell resuspension using a pasteur pipette resulted in consistent labelling as shown in Figure 18. However, more vigorous washing in a vortex mixer generated a less evenly distributed pattern of gold beads as illustrated in Figure 20. In this case, the 31.6 kDa protein appeared to form aggregates on and away from the parental cell surfaces as indicated by the clumps of beads (Fig. 20, arrow). The apparent loose association of the 31.6 kDa protein with the cell surface was also shown in Figure 21. In this case the gold beads appeared to be deposited in the shape of an *A.cryptum* cell suggesting that the 31.6 kDa protein was adsorbed to the electron microscope grid and left behind after the cells were sheared away.

Having established that the 31.6 kDa protein was associated with the cell envelope, solubilization studies were performed in an attempt to purify the protein. Cell envelope protein preparations were treated with detergents and differentially centrifuged to separate the solubilized protein from the remaining envelope-bound proteins. The solubilized proteins were subjected to western immunoblot analysis. Only very faint

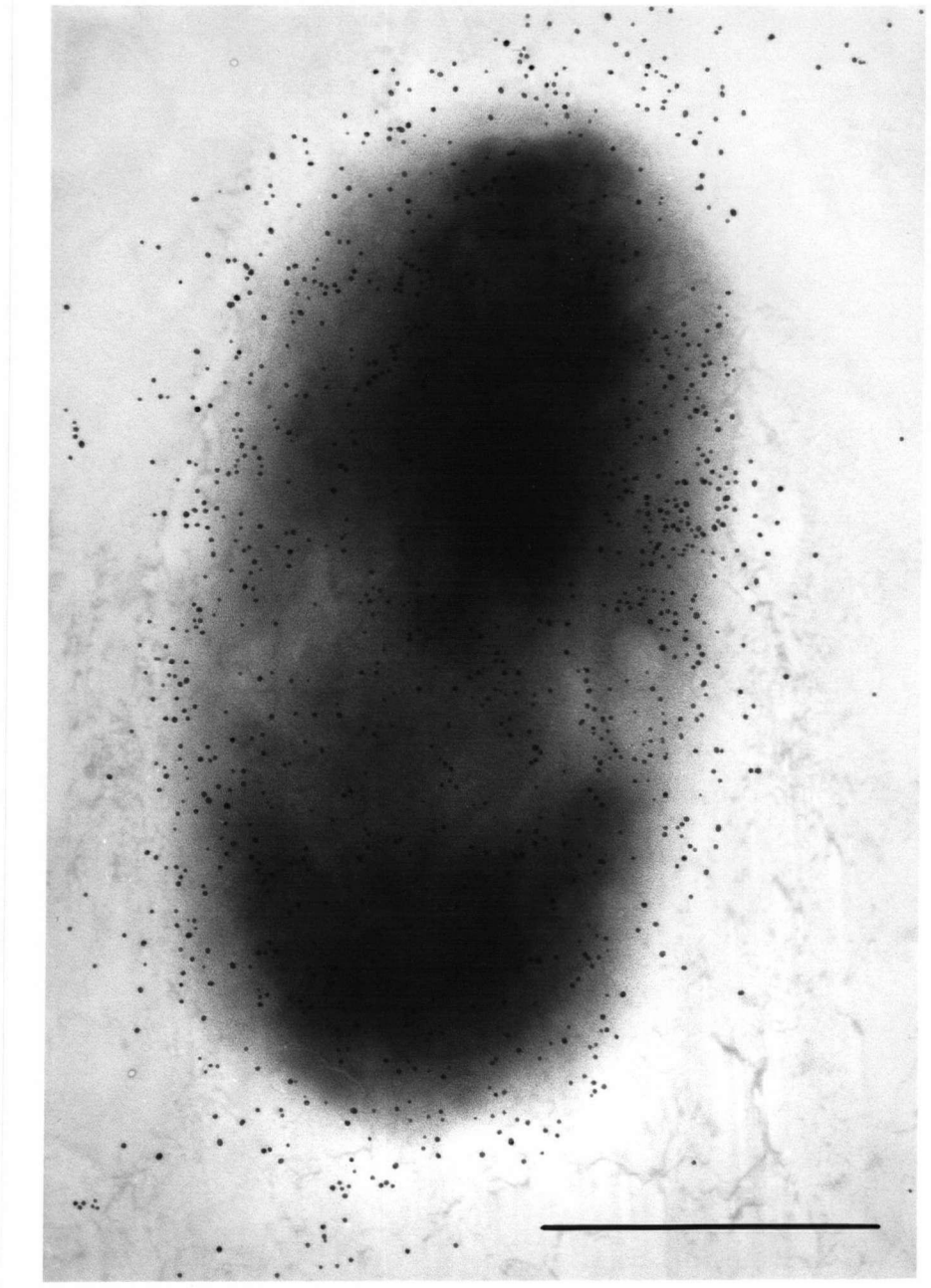


Figure 18. Immunogold bead labelling of parental *A.cryptum* cells with anti-31.6 kDa antibody followed by uranyl acetate negative staining. Bar = 0.5 μm.

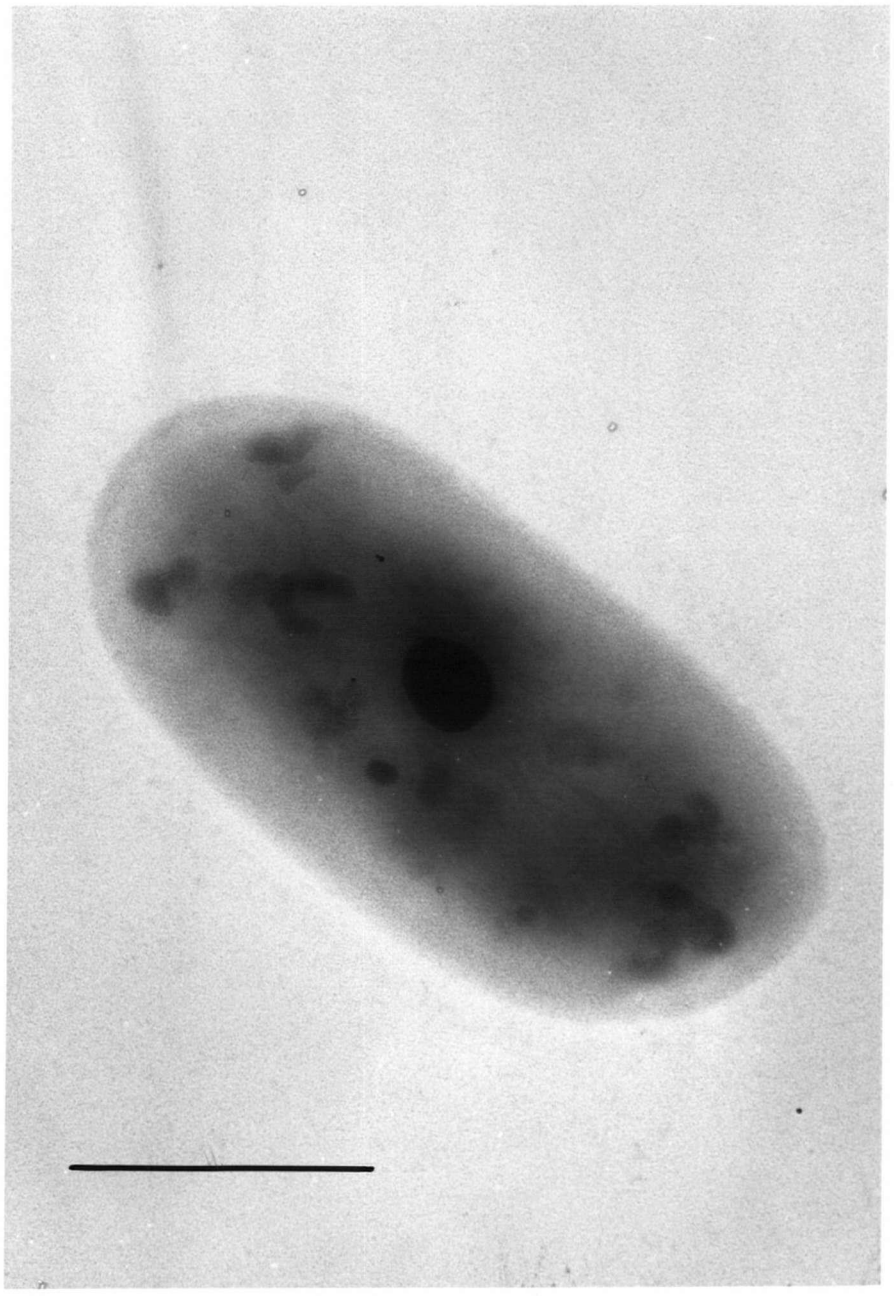


Figure 19 (a). Immunogold bead labelling of mutant-15 with anti-31.6 kDa polyclonal antibody followed by uranyl acetate negative staining. Bar = 0.5 μm.

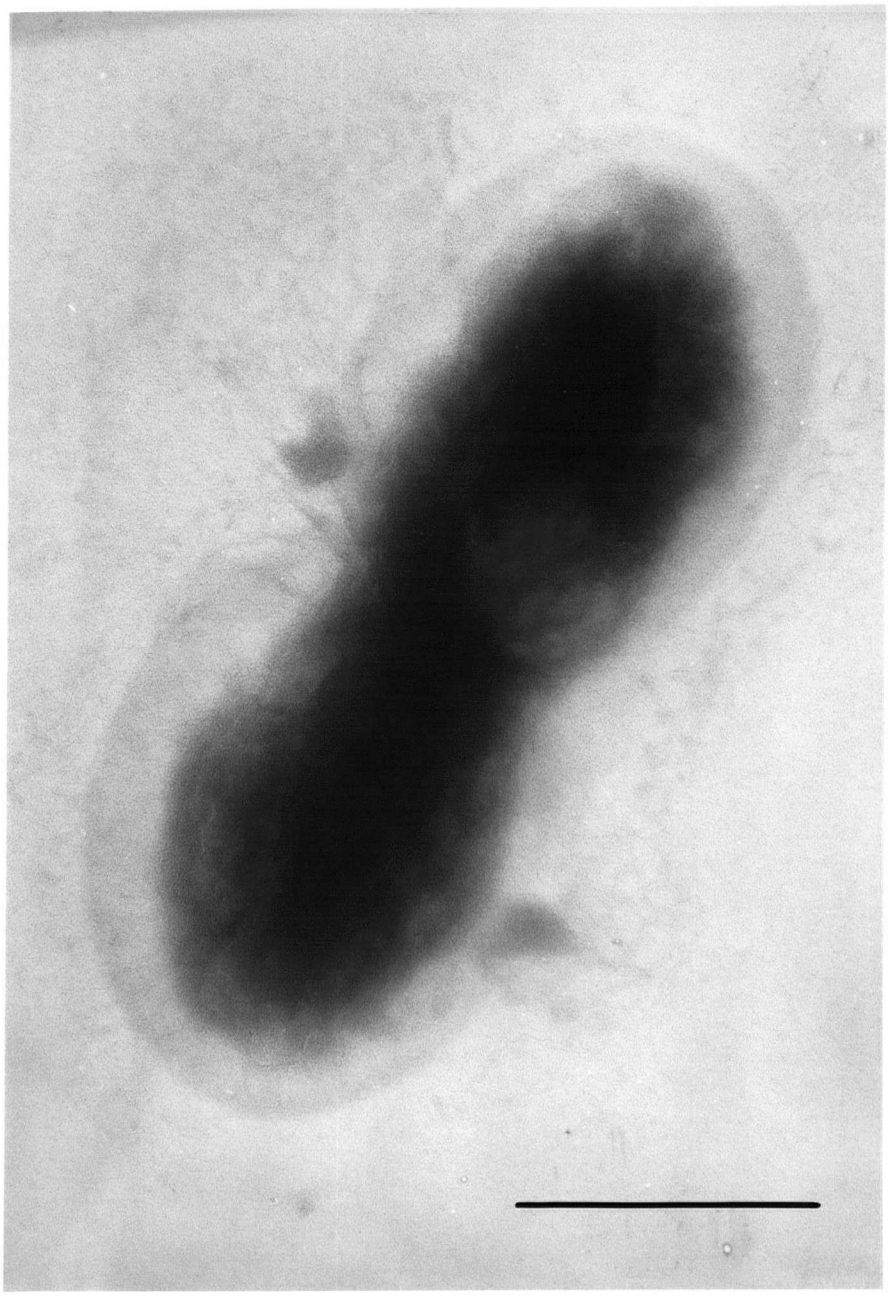


Figure 19 (b). Immunogold bead labelling of parental *A.cryptum* with non-immune serum followed by uranyl acetate negative staining. Bar = 1 μm.

Figure 20. Immunogold bead labelling of parental *A.cryptum* cells with anti-31.6 kDa polyclonal antibody followed by uranyl acetate negative staining. Cells were mixed vigorously during the washing steps. The arrow indicates 31.6 kDa protein aggregates at the cell surface. Bar = 1 μm.

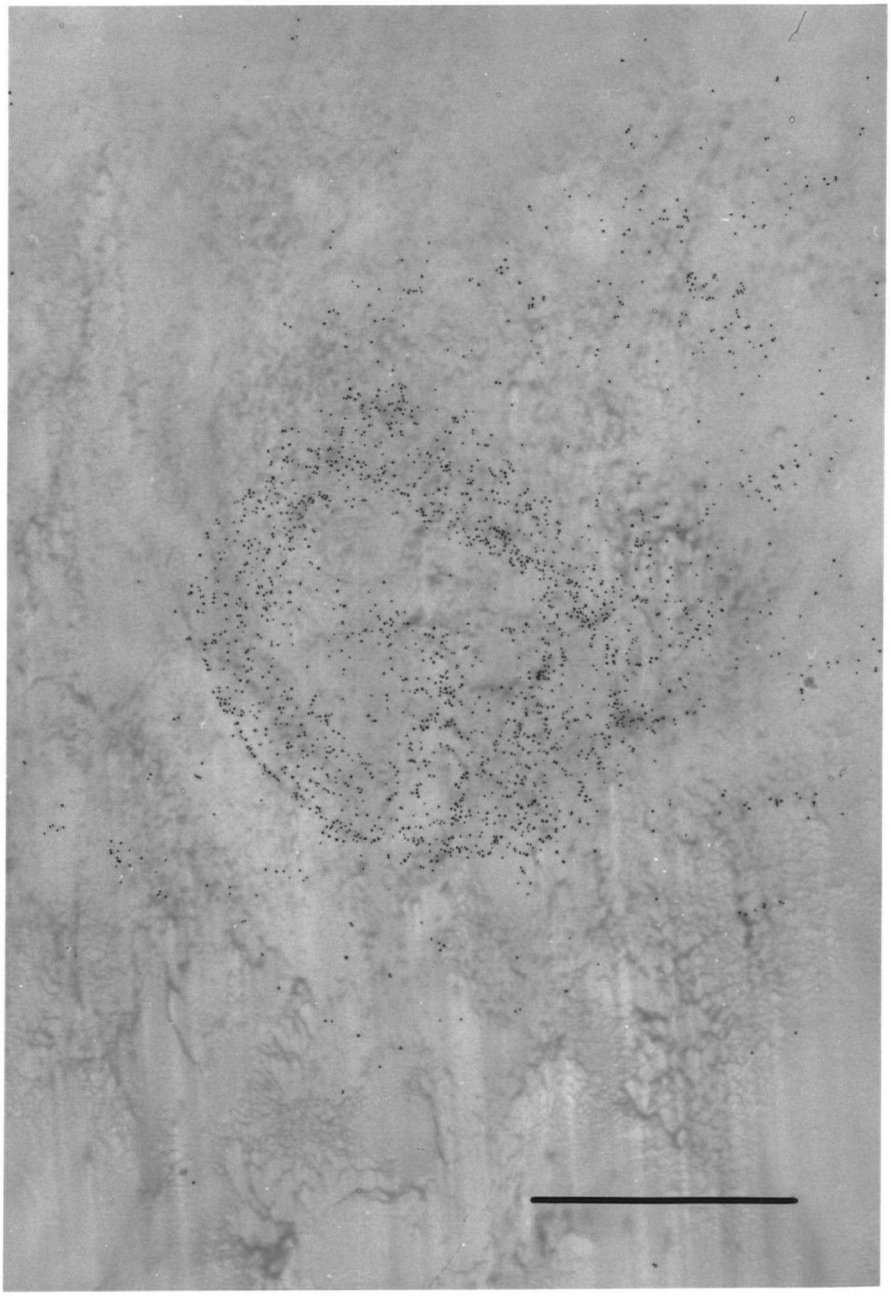


Figure 21. Immunogold bead labelling of a parental strain *A.cryptum* cellprint with anti-31.6 kDa polyclonal antibody. Bar = 1 μm.

banding was observed through treatment with guanidine HCl, urea or combined guanidine HCl-urea-EDTA and as such, was not considered an effective approach to purification.

Inhibition assays were attempted using the monospecific antibody or the 31.6 kDa surface protein preparation but the results were inconclusive. After treating the cells with the monospecificantibody, the cells formed large aggregates. Treating the plates with the surface protein preparations resulted in high background readings and as such limited the usefulness of this technique.

DISCUSSION

The purpose of this research was to establish techniques for studying acidophile adherence to mineral surfaces. This is the first study on the adherence properties of the acidophilic heterotroph, *A.cryptum*. The results clearly demonstrate that this organism binds to chalcopyrite and pyrite. Binding was demonstrated by assays which measured cell protein and reactivity with a specific antibody. The acidophilic heterotrophs are thought to be important ecological partners in *T.ferrooxidans* colonization of mineral surfaces. The heterotrophs consume organic acids and other microbial metabolites which are toxic to the nutritionally fastidious autotrophs. This type of interdependence would benefit from a close association of the organisms. The data presented here shows that *A.cryptum* has the capacity to bind to sulphur containing minerals and therefore would be in a position to enhance the growth of *T.ferrooxidans*.

A concern when comparing the Bio-Rad assay and the Ore ELISA was the difference in required cells necessary for saturation. A 10 fold increase in cells added to the Ore ELISA over cells added to the Bio-Rad assay was necessary to reach an equivalent saturation point. That is, while 60% of the cells added to the Bio-Rad assay had bound to the chalcopyrite, only 6% of the cells added were bound in the Ore ELISA. This difference in results is likely due to the substantial reduction in mineral surface area

available for cell adherence in the Ore ELISA as compared to the Bio-Rad assay. Approximately 18 mg of mineral ore is bound to each ELISA well, of which a portion of this, that is involved with the ore-glue interface, would not have been available for cell attachment.

When converted to numbers of cells bound per milligram of ore, the values recorded between assays were similar. However the Bio-Rad assay usually measured a slightly higher number of bound cells than the Ore ELISA. This variation can be explained by the inherent differences between the assays. The Bio-Rad assay separates bound cells from unbound cells through differential centrifugation. It is likely that during centrifugation, some unbound cells as well as weakly bound cells are removed from suspension with the pelleting ore particles. The Ore ELISA, with its several washing steps, has greater stringency and detects only tenaciously bound cells. As a result, the Bio-Rad assay will record a higher number of cells bound.

The Ore ELISA and Bio-Rad assay generated very similar results on the kinetics of *A.cryptum* adherence to chalcopyrite. Both binding isotherms suggest that each cell adheres independently to form a cell monolayer. Adherence occurs rapidly and is complete by 20 to 25 min which compares well with data for *T.ferrooxidans* (Myerson & Kline, 1983; Tuovinen et al., 1983).

Studies have shown that the relationship between pH and cell adherence depends on the substrate. Tuovinen (1983) and DiSpirito (1983) found that adherence to glass beads increased with decreasing pH but adsorption to mineral surfaces was independent of pH. The latter is comparable to the Ore ELISA results which showed little relationship between chalcopyrite binding and pH. The Bio-Rad assay results compared more closely to the glass bead adsorption. These results could be explained by the fact that the mineral is the only surface exposed in the Ore ELISA where as the mineral and the polypropylene microfuge tube wall are exposed to the cell in the Bio-Rad assay. Therefore, attachment to polypropylene like attachment to glass beads, may be pH dependant.

The effect of surface active agents on increasing the binding of *A.cryptum* to chalcopyrite was similar to that reported for *T.ferrooxidans* (Duncan et al., 1964; Kingma et al., 1979; Starkey, 1956). An explanation for this observation is that the surfactant reduces the dynamic surface tension enabling *A.cryptum* to be more readily adsorbed to the ore surface. In these studies both *A.cryptum* and *T.ferrooxidans* gave similar results.

Prior to this report the use of purified proteins (ie. BSA, gelatin or SPP) in acidophile adherence inhibition studies had not been investigated. Both BSA and gelatin strongly inhibit cell adherence to chalcopyrite. These proteins probably act by

blocking binding sites on the mineral surface. This also appeared to be a generalized phenomenon occurring in both *A.cryptum* and *T.ferrooxidans* attachment to ore. This finding could have a serious impact on industrial bioleaching processes. That is, as the age and density of a culture increases, the concentration of secreted proteins or other macromolecules will increase. Therefore, ironically, the crucial act of adherence may be inhibited by macromolecules released from *A.cryptum* and *T.ferrooxidans*. The inhibitory effects of BSA on cell adherence to surfaces is not unique to *A.cryptum*. For example, Gibbons (1972) has shown that the adherence of several species of oral bacteria to hydroxyapatite beads is inhibited by BSA.

The exact mechanism by which *A.cryptum* attaches to chalcopyrite is still unclear. A lectin-like interaction was not detected given the limited number of sugars investigated. Supplemental studies on polysaccharide mediated adherence will verify this finding. Many putative inhibitors enhanced cell adherence. The increased adherence recorded with monovalent cations is likely due to the reduced lateral repulsive forces experienced by adsorbing particles in concentrated electrolyte solutions (Rutter and Vincent, 1984). Divalent cations and chelating agents probably act in the same way in causing elevated cell adherence to chalcopyrite. Both chemical agents can mediate cell aggregation by linking charged groups on two bacterial cell surfaces.

It doesn't appear as though cell viability is necessary for chalcopyrite adherence since cell adherence to chalcopyrite was maintained in the presence of bactericidal agents. Many researchers are still divided on this question (Beck, 1967; Tuovinen et al., 1983). Reducing agents appear to have little effect on cell adherence. The effect of sulphur binding agents is inconclusive and should be viewed with caution due to the instability of these agents at such a low pH.

Hydrophobic interactions are considered to be an essential aspect in bacterial adherence to surfaces and interfaces (Marshall & Cruickshank, 1973; Dahlbäck et al., 1981). Stenström (1989) drew a correlation between cell surface hydrophobicity of various *Salmonella typhimurium* strains and adherence to the minerals; quartz, albite, feldspar, and magnetite.

From this study the role of hydrophobicity in the attachment of *A.cryptum* to mineral surfaces is not clear. Enhanced adherence was observed under conditions of high ionic concentration or low pH (high proton concentration) suggesting that hydrophobicity may play a role in mineral adherence. However, this conclusion was not supported by our cell surface hydrophobicity studies. That is, while some adherence-defective strains of *A.cryptum* demonstrated increased cell surface hydrophobicity, other adherence-defective strains showed increased hydrophilicity.

Direct comparison of attachment of different bacterial species was achieved through competitive inhibition studies. It was shown that *A.cryptum* and *T.ferrooxidans* do not compete for attachment to chalcopyrite. The results suggest that each bacterial species has unique attachment sites on the chalcopyrite mineral surface. This finding is of great significance in the study of microbial adherence to mineral surfaces. It provides the first documented evidence to suggest that bacterial attachment to mineral surfaces is a specific process that is unique among different strains of bacteria. In addition, the lack of competitive binding between *A.cryptum* and *T.ferrooxidans* to chalcopyrite implies that the mechanisms of attachment for each cell type may differ.

From an ecological stand point, non-competitive attachment to mineral surfaces would benefit both *A.cryptum* and *T.ferrooxidans*. This would allow the establishment of a close physical association between the two organisms necessary for cross-feeding. In addition, the establishment and proliferation of either organism would not be restricted by attachment site constraints due to binding competition between strains.

Several lines of evidence suggest that a 31.6 kDa protein may be involved in the adherence of *A.cryptum* to chalcopyrite. The 31.6 kDa protein does not appear to be associated with the cells as typical fimbriae-like structures. That is, visible surface

processes were not evident in negative stained micrographs or immunogold labelled micrographs. Heat treatment or treatment with reducing agents prior to electrophoresis did not generate detectable subunits of this protein. The 31.6 kDa protein appears to be associated with the outer membrane and can be removed from the surface of cells by physical means. In addition, this protein may be at least, in part, hydrophobic in nature. This was presumed because the partially purified preparation (SPP) of this protein was impossible to solubilize in distilled water, and immunogold labelling indicated that the protein formed aggregates when dislodged from the cell surface.

Conclusive evidence verifying that the 31.6 kDa protein mediates mineral surface adherence is still lacking. However, several observations were made which would suggest that this protein is involved in attachment: Figure 14 and the antibody adsorption studies show that this is the only protein missing in adherence-defective mutants. A mutant showing enhanced ore adherence contained the 31.6 kDa protein. The "footprint" left by an adherent cell in Figure 21 suggests that the protein may be involved in attachment. This observation is similar to what was described by Marshall (1971) who showed that mechanical shearing of bacteria from surfaces produced a polymer footprint. This argument is further supported by the fact that the only effective inhibitor of chalcopyrite binding by *A.cryptum* was protein. Convincing evidence to demonstrate that the 31.6 kDa protein is

directly involved in cell adherence would be through inhibition studies using purified protein or monospecific antibody to the putative adhesin. Both of these experiments were attempted with limited success due to complications arising from the type assays used. Further research in this area would help to elucidate the role of the 31.6 kDa protein in cell adherence.

Attempts to identify the structural association of this protein with the cell surface through cell treatment studies were inconclusive. If the 31.6 kDa protein is involved in mineral attachment, proteinase K or physically treated cells should have displayed reduced chalcopyrite adherence. The results recorded, however, are not uncommon since many cell surface proteins are inaccessible or resistant to exogenous enzymes. In addition, physical treatment of the cells is not likely to remove all surface components and as such may not effect cell adherence. For example, treatments which removed the haemagglutonin from *Streptococcus sanguis* cell surfaces did not reduce the binding ability of the treated cells (McBride, pers. comm.).

It should be noted that mechanisms other than the 31.6 kDa protein may be involved in ore adherence. The inability to generate non-adherent mutants after exhaustive enrichments, suggests that the other adherence factors, necessary for cell viability, may be involved.

The limitations of the Bio-Rad assay prompted the development of

the Ore ELISA. The Ore ELISA can provide adherence measurements of specific cell types such that competition for binding sites between two cell types, can be performed. The mineral substrate for adherence is immobilized to the ELISA plate, therefore, interference by mineral fines is eliminated. This assay is particularly adapted to characterization studies: The potential for multiple runs of a given test condition, provides statistically significant and reliable results. A variety of potential inhibitors can be screened with little affect on assay fidelity. When preparing the plates, various mineral substrates can be substituted for comparative analysis. In addition, this assay is a powerful tool for screening adherence-defective mutants. An added feature of this assay is that if colour development exceeds the detectable A_{405} range, the plates can be emptied, new substrate added and incubation shortened until desirable A_{405} values can be read.

In summary, this study demonstrates that *A.cryptum* adherence to chalcopyrite is rapid, saturating, and tenacious. Surface active agents and chelating agents increased adherence. Binding was inhibited by a strong oxidizing agent and by proteins, suggesting that reduced metals and polypeptides may be involved in adherence. This was supported by studies which show the absence of a 31.6 kDa cell surface protein on adherence-defective mutants.

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