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The polar polysaccharide capsule of *Hyphomonas adhaerens* MHS-3 has a strong affinity for gold

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Select groups of bacteria, including prothescate species, have an unusual capacity to sequester gold and bioconcentrate it to very high levels. *Hyphomonas adhaerens* MHS-3 (MHS-3) is one such species, as demonstrated by Energy Dispersive Spectroscopy. Transmission electron microscopy revealed that the binding site was specific on the polar polysaccharide capsule. A capsuleless mutant and periodate-treated wild type did not sequester gold. The gold may interact with the same sites in the capsule that naturally adhere MHS-3 to surfaces in the marine environment. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 1–4.

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Introduction

It is well documented that bacteria bind metals and are involved in the formation of different types of minerals [7]. It has also previously been reported that microorganisms can bind gold [3,26]. The thermophilic cyanobacterium, *Thermothrix thiopora*, has been shown to accumulate silver and gold in thermal springs [4]. *Bacillus cereus* spores, exposed to $AuCl_4^-$ solutions, accumulated thick coats of metallic gold [4]. Gold specimens from alluvial strata in the Quebrada Grande Mining Zone of Venezuela are proposed to have been formed by gold accumulation on the surface of the fossil budding bacterium, *Chrysoagyrtus venzuelaensis* ichnogen. nov., ichnosp. nov. [3].

With regard to placer gold, morphology alone is not considered adequate evidence of bacterial involvement in gold formation, since artifacts, resembling budding bacteria, can be formed in placer gold amalgams by nitric acid leaching [28]. Even considering this caveat, it has been demonstrated that prosthecate *Pedomicrobium*-like bacteria are involved in the formation of placer gold in South Africa [10] and Alaska [27]. Furthermore, gold-encrusted, hollow, filamentous specimens of *C. venezuelaensis* were clearly in evidence, even though the specimens were gently cleaned with hydrogen peroxide only when necessary in order to preclude artifact development [3]. Also, the formation of placer gold by bacteria has been conclusively demonstrated *in vitro* [25]. It is thought that gold binds to the surface of the cells, forming a metallic monolayer, which is an attractive surface for further deposition of gold [1].

Bacteria appear to have different types of mechanisms capable of immobilizing gold. Several microorganisms have been reported to have an energy-dependent gold uptake mechanism [26]. This process is dependent upon the proton motive force at the cell membrane and can be disrupted by uncoupling agents such as sodium azide [26]. Southam and Beveridge [25] produced placer gold *in vitro*, using *B. subtilis* that was washed and resuspended in distilled water, minimizing the contribution of bacterial metabolism to gold immobilization.

Another mechanism for gold binding involves bacterial extracellular polysaccharides (exopolysaccharides, EPS [5]). Bischoff *et al.* [3] reported gold-coated structures between filaments of *C. venezuelaensis* reminiscent of extracellular polysaccharide threads. The adhesive polysaccharide holdfast of 26 different strains of *Caulobacter* bind colloidal gold with high affinity [18], and without regard of the material used to coat the gold particles. Competition experiments between colloidal gold and lectins, known to bind the holdfast, indicated that colloidal gold bound preferentially [18].

The genus *Hyphomonas* is comprised of Gram-negative marine bacteria, which reproduce by budding from the tip of a prosthecum. Its members have a biphasic life cycle: one phase being sessile and the other free-swimming (flagellated swarmer cell [19]). *Hyphomonas* spp. have been observed as primary colonizers of surfaces in the marine environment [2,29] and in areas adjacent to hydrothermal vents [14]. *Hyphomonas adhaerens* strain MHS-3 (MHS-3; ATCC 43965) was recently characterized [29]. It produces a polar polysaccharide capsule [21,22,24]. Here we show that the capsule of MHS-3 strongly sequesters colloidal gold.

Materials and methods

Bacterial strains, media and chemicals

H. adhaerens MHS-3 was isolated from shallow water sediments in Puget Sound, WA, by J. Smit, and kindly given to R. Weiner. A reduced adhesion (rad) strain, which does not produce a polar polysaccharide capsule [24], was also used. These strains were cultured in Marine Broth 2216 (MB; 37.4 g/l) (Difco Laboratories, Detroit, MI) at 25°C.

Electron microscopy supplies were purchased from Electron Microscopy Sciences (Fort Washington, PA) or Sigma Chemical (St. Louis, MO). Other chemicals and supplies were obtained from VWR Scientific (Bridgeport, NJ). Copper grids (200 or 400 mesh)

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were used for electron microscopy; all were coated with collodion, and then coated with carbon using a MED 10 Deposition System (Balzers Union, Fürstentum, Liechtenstein).

Gold labelling of capsular EPS

A drop of MHS-3 mid-log culture was placed on a collodion-coated copper grid, incubated at room temperature for 1 min, blocked with a solution of 5% bovine serum albumin (BSA; in 0.1 M PBS) for 5–10 min, incubated with 20 nm colloidal gold particles (Sigma), diluted 1:10 in 5% BSA for 30 min, then rinsed with distilled water five times. The grids were observed with a JEM-100CX II transmission electron microscope (JEOL, Tokyo, Japan). MHS-3, fixed with glutaraldehyde, and MHS-3 rad were also used in these experiments.

Lectin–ferritin labelling of capsular EPS

Ferritin-labelled *Bauhinia purpurea* lectin (BPA-ferritin) (EY Laboratories, San Mateo, CA) was used to visualize the capsular EPS of MHS-3. BPA lectin has high affinity for the MHS-3 polysaccharide capsule [22]. These experiments were carried out as described above, substituting colloidal gold with BPA-ferritin (1:10 dilution of BPA-ferritin in 5% BSA) and incubating the suspension for 10–15 min. The same experiment was carried out using plain ferritin as a negative binding control.

Properties of colloidal gold–MHS-3 interaction

Gold–BPA complexes have been used to label the MHS-3 polysaccharide capsule [22,24]. Both BPA lectin and gold interact with the MHS-3 polysaccharide capsule. To elucidate the true nature of the BPA–Au interaction with the MHS-3 capsule, *N*-acetylgalactosamine, the sugar for which the lectin has highest affinity [30], was added (2 mg/ml) as a competitive inhibitor together with the BPA–Au (10 nm Au particles). The minimum inhibitory concentration of *N*-acetylgalactosamine, which was demonstrated to prevent binding of BPA to the MHS-3 capsule, is 50 μg/ml [21].

Several blocking agents were also tested to prevent the gold binding to the MHS-3 capsular polysaccharide: BSA, gelatin, casein, tryptone, Tween 20, and skim milk (all solutions were prepared 5% in 1× PBS). One-milliliter aliquots of MHS-3 from mid-log phase cultures were mixed with 50 μl of Pronase solution (20 mg/ml stock; final concentration 1 mg/ml) or periodic acid (0.2 M final concentration), as described previously [22], prior to exposure to colloidal gold.

Energy dispersive X-ray analysis (EDAX)

MHS-3 biofilms were grown on carbon planchets (EY Laboratories) at 25°C for 1 week. The biofilms were rinsed with 10 mM $CaCl_2$ (pH 4.0), and treated with 2.5 mM chloroauric acid ($HAuCl_4$) (10 mM $CaCl_2$, pH 4.0) for 30 min, fixed with 1% glutaraldehyde, and washed three times with the $CaCl_2$ solution. Planchets were dehydrated by subsequent 10-min incubations in a graded series of ethanol solutions (75%, 90%, 100%, 100%, and 100%). The planchets were then critical-point-dried in a Denton DCP-1 critical point drying apparatus (Denton Vacuum, Moorestown, NJ) and subjected to Energy Dispersive Spectrometry (EDS; EDAX Int. Spectrophotometer, 1820D 20 kV, KLA-Tenor/Amray, Bedford, MA). Biofilms were counted for 300 s to determine elemental metal concentrations.

Results and discussion

EDS was used to investigate the ability of MHS-3 to bind gold. Gold comprised an average of 58.2±2.27% wt/wt (standard error, $n=3$) of the elemental metals found in MHS-3 biofilms incubated in a 2.5-mM solution of chloroauric acid. The bioconcentration factor for gold in MHS-3 biofilms was calculated by dividing the percent weight of gold in the biofilm (58.2%) by the percent weight of gold in the 2.5 mM $HAuCl_4$ solution (0.049%). MHS-3 capsule concentrated gold 1187 times that of the chloroauric acid solution. The predominant metals found in control samples were iron (23.25±11.45%), phosphorus (21.0±2.1%), and calcium (9.1±0.3%, $n=2$).

These results showed that MHS-3 EPS has the ability to sequester cationic gold, possibly because of the interactions of the negatively charged EPS with positively charged metal ions [15]. In the environment, gold may be present either as Au (III) species, Au (I) species, or it may be elemental, depending on dissolved oxygen and pH, among other factors. Cations bind to electron-donating groups [8] and carboxyl moieties. Phosphate and sulfate groups may also contribute to a polymer's net negative charge [13]. An example of the interaction between divalent cations and acidic EPS is that of Ca^{2+} and the glucuronate residues of alginate [5]. Alternatively, it is possible that gold binds to a site, yet to be discovered, that renders the EPS adhesive to marine substrata.

Colloidal gold adhered to the polar polysaccharide capsule of *Hyphomonas* MHS-3 (Figure 1), but not to the capsuleless MHS-3 rad cells. Glutaraldehyde fixed MHS-3-bound colloidal gold as well as untreated cells. The lectin, BPA, also bound to the MHS-3 capsule, whether it was conjugated to ferritin (BPA-ferritin; Figure 2) or to another marker. Ferritin alone did not adhere to the capsule.

Some bacteria can collect gold by an energy-dependent gold uptake mechanism [26]. However, this is probably not the mechanism by which MHS-3 sequesters gold because glutaraldehyde-fixed cells bind colloidal gold just as well as live cells. Another mechanism is through interactions between microbial polysaccharides and gold, including either trapping or chemical binding. Three lines of evidence suggest that this mechanism of action applies to MHS-3: (a) the colloidal gold particles adhere only to the prosthecate cell where the polar polysaccharide capsule is produced [22,24]; (b) periodate oxidation of the polysaccharide

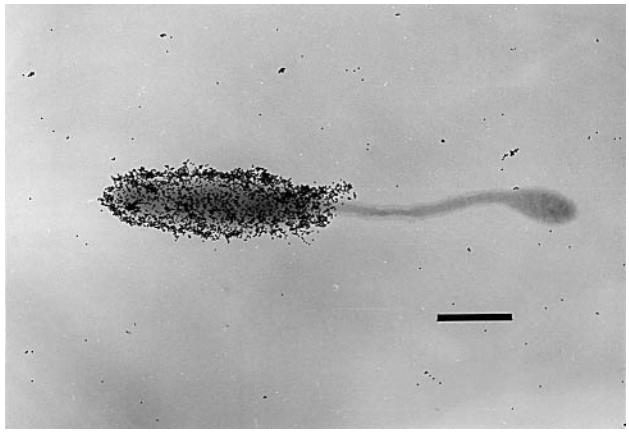

Figure 1 Binding of colloidal gold to the *Hyphomonas* MHS-3 polar polysaccharide capsule. Following incubation in 20 nm colloidal gold particles in 5% BSA, only the main body (capsulated section) of the prosthecate cell is bound by gold. Scale bar = 1 μm.

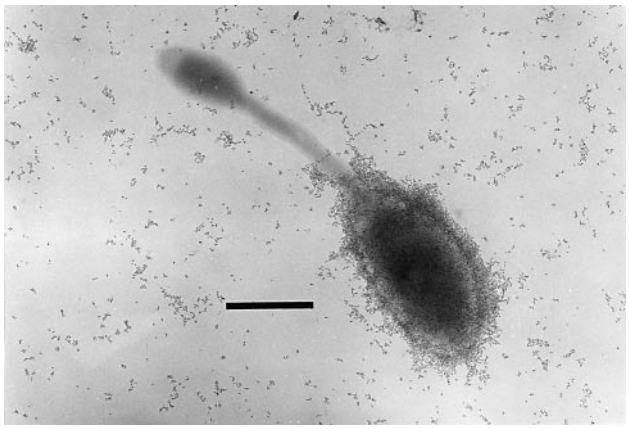

Figure 2 Visualization of *Hyphomonas* MHS-3 polar polysaccharide capsule with ferritin-labelled *Bau. purpurea* lectin (BPA-ferritin). Only the main body of the prosthecate cell is bound by the lectin. Scale bar = 1 μm.

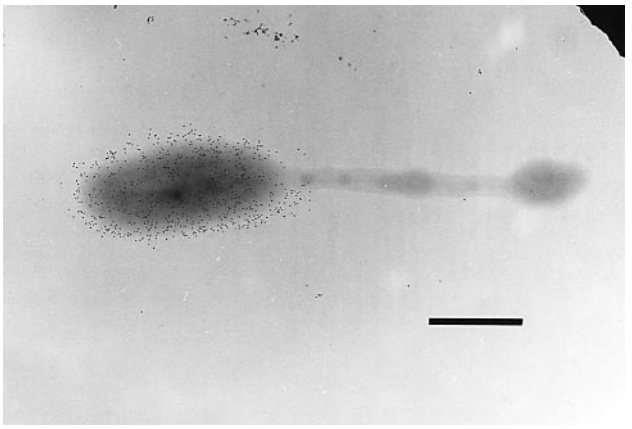

Figure 3 Gold binds to MHS-3 polar polysaccharide capsule. The addition of *N*-acetylgalactosamine as a competitive inhibitor failed to prevent the labeling of the polar capsule with gold-labeled *Bau. purpurea* lectin (BPA–Au), demonstrating that gold (10 nm particles) directly binds to the polysaccharide. Scale bar = 1 μm.

destroys the gold-binding properties of the capsule (This treatment coincidentally hinders the adhesion of the MHS-3 cells to surfaces [22].); and (c) the rad strain of MHS-3, which does not produce the polar polysaccharide capsule, does not adhere colloidal gold.

Colloidal gold has been shown to be bound by the holdfasts of marine bacteria, such as *C. crescentus* [18]. Like the MHS-3 polar capsule, the *Caulobacter* holdfast adheres the organisms polarly to surfaces. Thus, there may be a correlation between adhesive holdfasts and the ability to adhere to colloidal gold particles, although the nature of this relationship is not yet clear.

Interestingly, the holdfast polysaccharides of *Caulobacter* spp. [20] and the MHS-3 capsule are all acidic EPS [23]. Furthermore, MHS-3 polysaccharide contains the amino sugar, *N*-acetylgalactosamine [23]. Fifteen of 16 marine species and 6 of 10 freshwater species of *Caulobacter* contain the amino sugar, *N*-acetylglucosamine [18]. Thus, it is conceivable that acidic groups and amino sugars may be characteristic of a class of adhesive polysaccharides with affinity for gold. It is hypothesized that the gold bound to the same sites in the capsule that naturally adhere *Hyphomonas* MHS-3 to surfaces in the marine environment [16] and gold–EPS binding could be an important clue in determining why the MHS-3 capsule and some caulobacter holdfasts are adhesive.

Pretreatments with protease, *N*-acetylgalactosamine (Figure 3), BSA, gelatin, casein, tryptone, and Tween 20 failed to block adherence of colloidal gold to the MHS-3 capsule. Yet, significantly, gold adherence was blocked in MHS-3 that had been pre-treated with periodate, a polysaccharide oxidizer (Figure 4), and by skim milk.

Although polycationic ferritin binds to the capsule of MHS-3 very well, evidencing the negative charge of the polysaccharide [22], plain ferritin does not. Therefore, BPA-ferritin labels the MHS-3 capsule (Figure 2) because of lectin–polysaccharide interactions. *N*-acetylgalactosamine failed to inhibit binding of BPA–Au to the MHS-3 capsule (Figure 3), even when the sugar is added at a concentration 40 times higher than that necessary to inhibit the binding of BPA lectin to the MHS-3 polysaccharide [21]. This suggests that for the BPA–Au complex, it is the colloidal gold particle itself that chemically binds to, or is trapped by, the polar capsule.

Gold is present in seawater in a wide range of concentrations, especially in chloride form [6]. Gold has been found in abundance in hydrothermal vent settings in both chimneys and hydrothermal fluids [11,12], and has been suggested as a possible tracer of hydrothermal activity in ocean bottom waters [9]. Gold is soluble in hydrothermal fluids at high temperatures (>350°C) and its solubility decreases as the fluid cools (seawater mixing), resulting in the deposition of gold sulfides, which are carried into the water column as a buoyant hydrothermal plume containing most of the gold arriving at the seafloor [11].

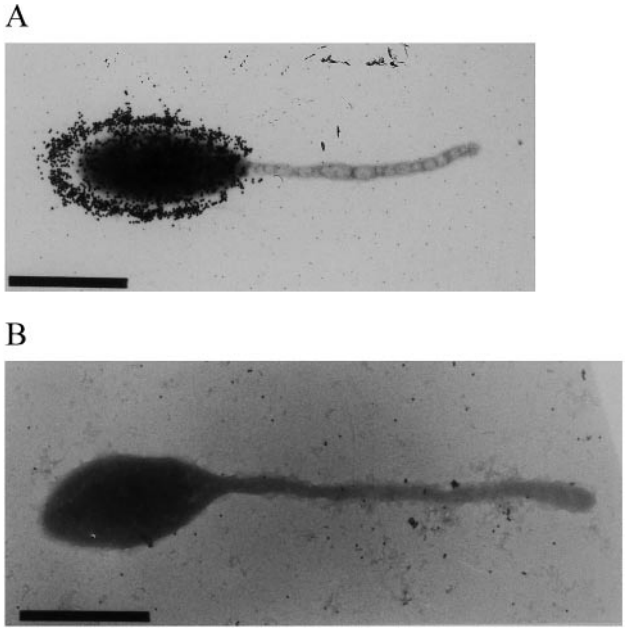

Figure 4 *Hyphomonas* MHS-3 holdfast EPS binds colloidal gold particles. MHS-3 was treated with 0.2 M periodic acid for 30 min, incubated in a 1:1 mixture of 20 nm colloidal gold particles and 2% BSA/PBS. Control cells were treated with PBS. (A) The EPS of MHS-3 binds 20 nm colloidal gold particles. (B) MHS-3, treated with 0.2 M periodic acid, did not bind colloidal gold. Scale bar = 1 μm.

It has been suggested that the polar capsules and holdfasts of hyphomicrobia facilitate the release of swarmer progeny away from the surface, without trapping them in the biofilm matrix. However, it has also been suggested that hyphomicrobia synthesize prosthecae to maintain contact with the aqueous phase, as manganese deposits develop around the attached main cell bodies [17]. The polar production of the MHS-3 capsule could serve a similar purpose, preventing the total encrustation of the cell when it is growing in metal-rich environments.

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