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Type IV Pili of *Acidithiobacillus ferrooxidans* Are Necessary for Sliding, Twitching Motility, and Adherence

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Abstract We used conventional methods to investigate the mechanism by which *Acidithiobacillus ferrooxidans* colonizes a solid surface by assessing pili-mediated sliding, twitching motility, and adherence. *A. ferrooxidans* slided to form circular oxidized zones around each colony. This suggested that slide motility occurs through pili or flagella, though *A. ferrooxidans* strains ATCC 19859 and ATCC 23270 lack flagella. The results of reverse transcription-PCR demonstrated that the putative major pili gene of *A. ferrooxidans* strains ATCC 19859, ATCC 23270, and BY3 genes were transcribed. Culture of *A. ferrooxidans* between silicone gel and glass led to the production of type IV pili and the formation of rough twitching motility zones. When the bacteria were grown on lean ore cubes, pyrite was colonized readily by *A. ferrooxidans* and there is a correlation between pilus expression and strong attachment. However, non-pili bacteria attached minimally to the mineral surface. The results show a correlation between these functions and pilus expression.

Introduction

Acidithiobacillus ferrooxidans is a Gram-negative, rod-shaped, acidophilic chemolithoautotrophic bacterium that uses CO_2 as a carbon source and obtains its energy for growth from by oxidizing ferrous iron, sulfur, and reduced sulfur compounds [14]. It is used widely in a variety of industrial processes, such as the bioleaching of metals, desulphurization of coal and natural gas, and the decontamination of industrial waste. A chemotactic response of *A. ferrooxidans* toward thiosulfate has been described [2]. Nickel, iron, and copper are attractants and aspartic acid appears to repel *A. ferrooxidans* [1]. However, Valdés et al. [27] has reported that *A. ferrooxidans* ATCC 23270 lacks genes for the formation of flagella and that the signaling transduction system renders it unlikely that there is any chemotactic response by flagella.

The exact mechanism by which *A. ferrooxidans* is able to colonize surfaces has not yet been elucidated. *A. ferrooxidans* can grow on pyrite surfaces, on which it produces an acidic interface between the pyrite and the attached bacteria [16, 18, 24]. *A. ferrooxidans* can attach to pyrite, glass beads, or quartz because the bacteria select minerals by means of extracellular polymeric substances (EPS) [13]. The EPS of *A. ferrooxidans* are composed of neutral sugars, fatty acids, and uronic acids. They mediate attachment to a (metal) sulfide surface, and concentrate iron(II) ions by complexation through uronic acids or other residues at the mineral surface. Cells of *A. ferrooxidans* cover mineral surfaces with a dense biofilm [8, 10, 13, 15]. Farah [7] has reported that the interaction between *A. ferrooxidans* and minerals requires the development of biofilms that is regulated by type AI-1 quorum sensing. Pace et al. [20] have also reported on the shape of cells and biofilm after *A. ferrooxidans* has attached to and colonized pyrite surfaces, using scanning electron microscopy for their observations. Bacteria that can form biofilms organize themselves into communities after attaching to solid surfaces [3, 26, 28]. The expression of a number of cell surface structures and outer membrane proteins, especially those linked to adhesion, aggregation, and colonial morphology, is regulated by

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several phase-variable mechanisms in Gram-negative bacteria [4, 6, 11]. The polar pili of *Pseudomonas aeruginosa* are responsible for its chemotaxis in aqueous environments [5, 19]. The expression of the long polar pili, the adherence factor, mediates its adherence to abiotic surfaces and its twitching motility [9]. Pratt and Kolter [21] have shown that *Escherichia coli* mutants that are unable to form biofilms effectively lack the ability to produce pili and to become motile.

As mentioned above, *A. ferrooxidans* is used widely in ore enrichment and the exact mechanism by which it colonizes surfaces is not fully understood in this context. There have been no reports on the pili-mediated sliding, twitching motilities, and growth of *A. ferrooxidans* on lean pyrite surfaces. It is difficult to knock out genes in *A. ferrooxidans* since they are acidophilic chemolithoautotrophic bacterium and organic compound is toxic for them. The regular molecular techniques are not suitable for them. For these reasons, we decided to investigate the mechanism by which this bacterium colonizes solid surfaces. Using conventional methods to assess slide motility, and transmission electron microscopy (TEM) and scanning electron microscopy (SEM), we showed that *A. ferrooxidans* can colonize a solid surface by pili-mediated sliding, twitching motility, and adherence.

Materials and Methods

Bacterial Strains and Culture Media

In the experiments, we used the following strains of bacteria: *A. ferrooxidans* strain ATCC19859, ATCC23270, DX, DBS, TKY, and BY3. The strains were cultured, as described previously [16, 23].

Sliding Motility

Slide plates that were composed of 0.5% agarose (semi-solid) and 9K nutrient medium that contained $FeSO_4{\cdot}7H_2O$ were prepared and dried overnight at room temperature, as described by Rashid and Kornberg [22]. The suspension volume of 3 µl *A. ferrooxidans* bacterial cells (10^8 ml^{-1}) were point-inoculated onto the slide plates with a sterile inoculator and were then incubated at 30°C for 72 h. After incubation, a yellow slide motility zone was observed and a sample from its edge was selected for examination under TEM.

Twitching Motility

Twitching motility was first assessed under silicone gel, because agarose can be degraded to glucose, which can be toxic for *A. ferrooxidans*. Silicone gel plates that contained the volume of 3 ml 9K nutrient medium that contained $FeSO_4{\cdot}7H_2O$ were prepared, as described by Hu et al. [12]. Cells were inoculated as described by Rashid and Kornberg [22]. Upon completion of 24–96 h incubation at 30°C, a hazy growth zone was observed at the interface between the thin silicone gel layer and the glass surface of the Petri dish. The silicone gel layer was first removed by a scraper and the unattached cells were removed by washing the plate with a gentle stream of distilled water. After the unattached cells had been removed, the twitching motility of the attached bacteria on the glass surface of the Petri dish was observed as a clear zone. A sample from the edge of this zone was then selected for examination under TEM.

Transmission Electron Microscopy (TEM)

Cells that were selected of *A. ferrooxidans* from sliding motility and twitching motility experiments were suspended into 9K nutrient medium that contained $FeSO_4{\cdot}7H_2O$. A Formvar-coated copper grid was floated on a drop of the bacterial suspension for about 45 s, rinsed in a drop of water, and then stained with 2% aqueous solution of phosphotungstic acid for 15 s [5]. The specimens were then observed using TEM (JEM-1230; Japan Electron Company).

Scanning Electron Microscopy (SEM)

Acidithiobacillus ferrooxidans was cultured, as described by Mielke et al. [16]. Culture samples were taken and prepared using the method described by Pace et al. [20]. Lean ore cubes (1 cm^3) whose constituents were 0.66% Ni, 0.51% Cu, 0.018% Co, 10.45% Fe, 2.51% S, 27.8% MgO, 2.57% CaO, 35.95% SiO_2, 3.43% Al_2O_3 were obtained from Jinchuan Company, GanSu Province, China.

Transcript Analysis of Pili Genes

Primers were designed by using Primer Primier 5.0 according as AFE-0970 and AFE-0971 (putative major type IV pilin gene in *A. ferrooxidans ATCC 23270* (TIGR)) and then synthesized by TaKaRa Biotech (TaKaRa) (forward, 5′-CTCACCGGCGGCTATCAGA-3′; reverse, 5′-CACATCCCGTTGCCTTGC-3′), and amplified regions of approximately 251 nucleotides of the putative major type IV pilin gene of *A. ferrooxidans*. Total cellular RNA was isolated using the H.Q.&Q.Total RNA Kit II (U-GENE Biotech) according to the manufacturer's protocol. The RNA samples were treated with RNase-free DNase I (TAKARA) and used with BioRT One Step RT-PCR Kit (Bioer), under the conditions suggested by the manufacturer. The amplicons were analyzed by 1.5% agarose gel

electrophoresis. The nature of the amplicons was confirmed by automated DNA sequencing (TAKARA).

Results

Sliding Motility

When *A. ferrooxidans* was cultivated on semisolid agarose plates, we observed zones around the bacterial colonies that were yellow due to iron oxidation (Fig. 1). Pili or flagella of cells of sliding motility were confirmed by TEM (Fig. 2), as TEM may confirm the presence of pili or flagella in strains that demonstrate sliding motility. Pili were observed for *A. ferrooxidans* strains ATCC19859 and ATCC23270, but no flagella were seen, and BY3 produced not only pili but also flagella (Fig. 2a, b, d). However, the DX, DBS, and TKY strains of *A. ferrooxidans* failed to produce pili but had flagella (Fig. 2c). The size (diameter) of the oxidized zones suggested the occurrence of slide motility on the part of the *A. ferrooxidans* colonies. Slide zones of *A. ferrooxidans* strains DBS, DX, and TKY were smaller than those of the other strains of the bacillus. There was some dispersion of the bacterial cells from the point of inoculation, but concentric slide motility rings were not observed.

Twitching Motility

The zones of twitching motility for *A. ferrooxidans* strains ATCC19859, ATCC23270, and BY3 at the glass and silicone gel interface were bigger and denser than those of the other bacilli (Fig. 3). When the silicone gel was removed from the Petri dish and the exposed glass surface was rinsed gently with distilled water, the water removed the unattachment cells of *A. ferrooxidans* easily. However, *A. ferrooxidans* in the twitching zone remained firmly attached to the glass surface. The firmly piliated cells were observed by TEM. Cells micrographs of *A. ferrooxidans* in the twitching zone were similar to those of *A. ferrooxidans* in Fig. 2 (data not show). Furthermore, we observed striking differences between the patterns in which *A. ferrooxidans* strains ATCC19859, ATCC23270, DX, DBS, TKY, and BY3 adhered to the glass plates to form twitching zone. *A. ferrooxidans* strains ATCC19859, ATCC23270, and BY3 produced a thick twitching zone, which indicated that bacteria in the twitching zone became attached and reproduced. However, a few cells of *A. ferrooxidans* strains DX, DBS, and TKY adhered to the glass surface to form small-size twitching zone.

TEM

TEM of cells that were sampled directly from the bacterial suspension of 9K nutrient medium revealed that *A. ferrooxidans* strains ATCC19859, ATCC23270, and BY3 produced pilus but ATCC19859 and ATCC23270 failed to produce flagella, and BY3 produced not only pili but also flagella (Fig. 2a, b, d), as the cells were surrounded by pili fragments. Pili were especially in the cell aggregates. Bundles formed by the entwinement of numerous pili, some larger than flagella, were observed frequently. Some of the cells were aggregated by pili (Fig. 2a). However, the DX, DBS, and TKY strains of *A. ferrooxidans* failed to produce pili but had flagella (Fig. 2c.). Micrographs of *A. ferrooxidans* strains DX and TKY were similar to those of *A. ferrooxidans* strain DBS (data not shown).

SEM

A scanning electron micrograph of bacterial colonies of *A. ferrooxidans* strains ATCC19859, BY3, and DX on a

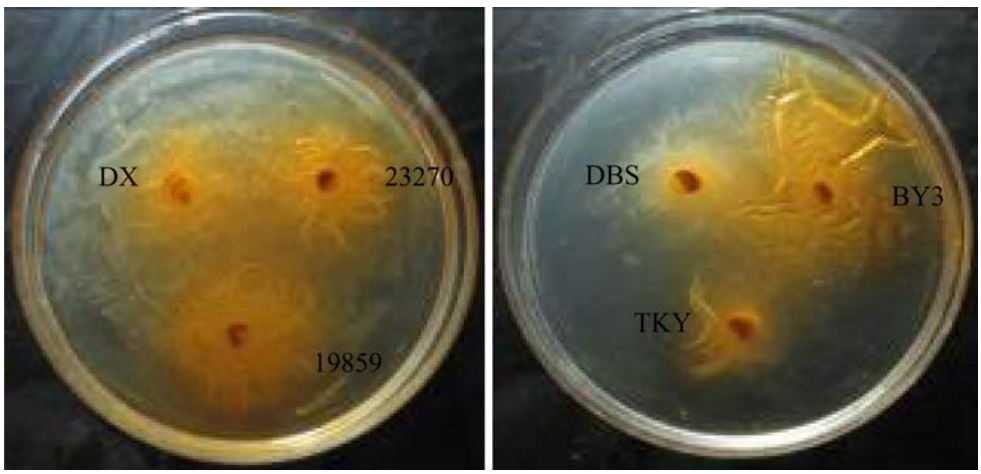

Fig. 1 Sliding motility of *A. ferrooxidans*. Bacteria were cultured on slide plates that were composed of 0.5% agarose (semisolid) and 9K nutrient medium that contained $FeSO_4 \cdot 7H_2O$. The suspension volume of 3 µl *A. ferrooxidans* bacterial cells (10^8 ml^{-1}) were point-inoculated onto the slide plates with a sterile inoculator and were incubated at 30°C for 72 h. The plates were examined after 48 h

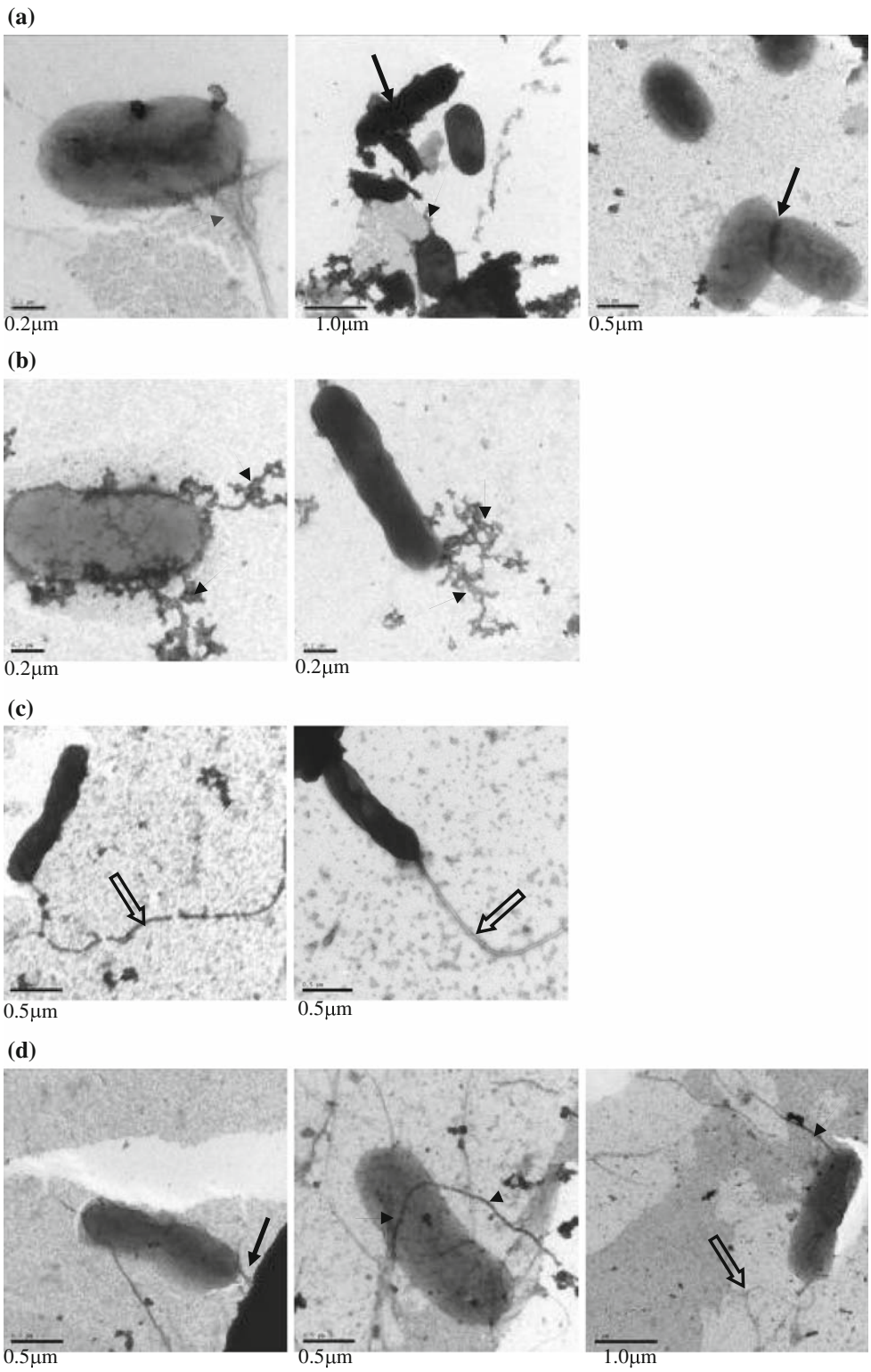

Fig. 2 Transmission electron micrographs of *A. ferrooxidans* pili. **a**, **b**, **c**, and **d** display *A. ferrooxidans* strains ATCC19859, ATCC23270, DBS, and BY3, respectively. The first micrograph of *A. ferrooxidans* strains ATCC19859, ATCC23270, DBS, and BY3 is from the sliding motility experiment, and others from the twitching motility experiment. The *thin black arrowheads* indicate pili, the *thick black arrowheads* indicate aggregated cells, and the *void arrowheads* indicate flagella

pyrite surface after oxalic acid washing is shown in Fig. 4b, c, d (micrographs of the colonies of *A. ferrooxidans* strains ATCC23270 are similar to those of BY3 and micrographs of the other *A. ferrooxidans* strains are similar to those of DX; data not shown). Many cells of *A. ferrooxidans* strains ATCC19859, 23270, and BY3 attach to the mineral surface, but hardly any cells of *A. ferrooxidans* strains DX, DBS, and TKY. The individual bacterial cells were elongated and ellipsoid and were attached strongly to the pyrite surface. Each bacterial cell had begun

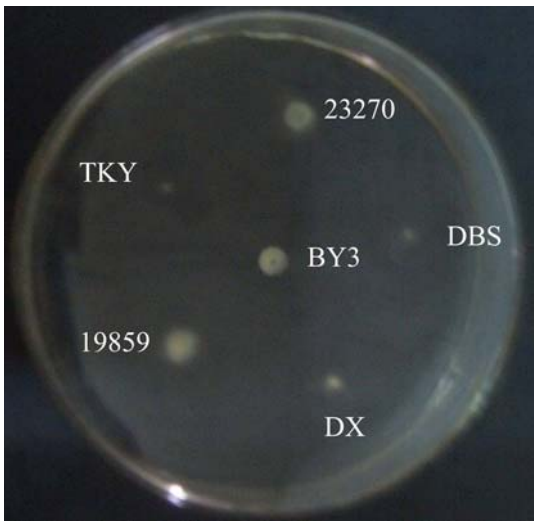

Fig. 3 Expansion of twitching motility zones of *A. ferrooxidans*

to oxidize the pyrite immediately below its cell body. A pit just below the bacterial cell formed after its removal from the pyrite surface. Furthermore, the bacterial cell seemed to sink into the pyrite surface as its own etch pit expanded. The sizes and shapes of these corrosion pits could be seen after removal of the bacteria (Fig. 4a). Numerous small ellipsoidal etch pits were also visible and are presumed to have been due to oxidation of the underlying pyrite by *A. ferrooxidans*.

Transcript Analysis of Pili Genes

Reverse transcription-PCR (RT-PCR) analysis of the putative major type IV pilin gene (251 bp) (AFE-0970 and AFE-0971) of *A. ferrooxidans* demonstrated that *A. ferrooxidans* strains ATCC19859, ATCC23270, and BY3 were transcribed (Fig. 5, lanes 1–6). However, the DX, DBS, and TKY strains of *A. ferrooxidans* failed to transcribe the type IV pilin gene (Fig. 5, lanes 1–6). Amplicons of *A. ferrooxidans* strains ATCC19859, ATCC23270, and BY3 were same by automated DNA sequencing and BLAST analysis. The Genbank accession number of the amplicons is GQ409499.

Discussion

Slide Motility

Sliding motility is bacterium sliding or gliding on semisolid surface by flagella or pili swaying, whereas twitching motility is bacterium moving on solid surface by pili contracting or extending. The slide motilities of *A. ferrooxidans* that we observed in our investigation are similar to the swarm motilities of *Leptospirillum ferrooxidans*, which have been already been described [1]. However, the slide and swarm motilities of *A. ferrooxidans* have not been reported. The diameters of the slide zones that surrounded the colonies of *A. ferrooxidans* suggested that *A. ferrooxidans* was capable of slide motility when cultured on semisolid agarose plates, though *A. ferrooxidans* strain ATCC23270 lacks genes for the formation of flagella [27] and *A. ferrooxidans* strain ATCC 19859 and ATCC23270 lacks flagella. The results of RT-PCR also demonstrated that the putative major pili gene of *A. ferrooxidans* strains ATCC 19859, ATCC 23270, and BY3 genes were transcribed. The slide motility of *A. ferrooxidans* is accomplished by its flagella (*A. ferrooxidans* strain DX, TKY, BY3, and DBS) or pili (*A. ferrooxidans* strain ATCC 19859, ATCC23270, and BY3) and is important for its survival. However, chemotaxis in some *A. ferrooxidans* strains seemed to be impaired, because there was some dispersion of cells from the point of inoculation without concentric slide motility rings being formed. The slide motilities of *A. ferrooxidans* have been implicated in the movement of *A. ferrooxidans* on semisolid surfaces by its flagella or pili. However, sliding motility of *P. aeruginosa* and *Legionella pneumophila* is non-pilus-mediated [17, 25]

Twitching Motility and Adherence

The pili apparatus is anchored in the periplasm and outer membrane of Gram-negative bacteria [4, 6, 11]. Twitching motility has been implicated in the movement of *A. ferrooxidans* on abiotic surfaces and in the formation of microcolonies by its pili. The sliding, twitching motility, and high adherence of *A. ferrooxidans* suggested the existence of a strong pili substrate bond, though Harneita et al. considered the attachment of *A. ferrooxidans* to minerals by means of EPS [8, 10, 13, 15]. However, we found that there were a lot of pili. *A. ferrooxidans* strains ATCC19859, ATCC23270, and BY3 produced a very large number of pili, but strains DX, DBS, and TKY produced none. The results of RT-PCR also demonstrated that the putative major pili gene of *A. ferrooxidans* strains ATCC 19859, ATCC 23270, and BY3 genes were transcribed. The pili and twitching zones that were produced by strains ATCC19859, ATCC23270, and BY3 may have accounted for the strong bacterial adherence to a solid surface (Fig. 2a, b, d). *A. ferrooxidans* strains ATCC19859, ATCC23270 and BY3 produced dense twitching zones whose density may have affected their ability to sense neighboring cells, because twitching motility requires cell to cell contact. The long pili of *P. aeruginosa* mediate its adherence to abiotic surfaces and its twitching motility [4]. In the study reported herein, we found that *A. ferrooxidans*

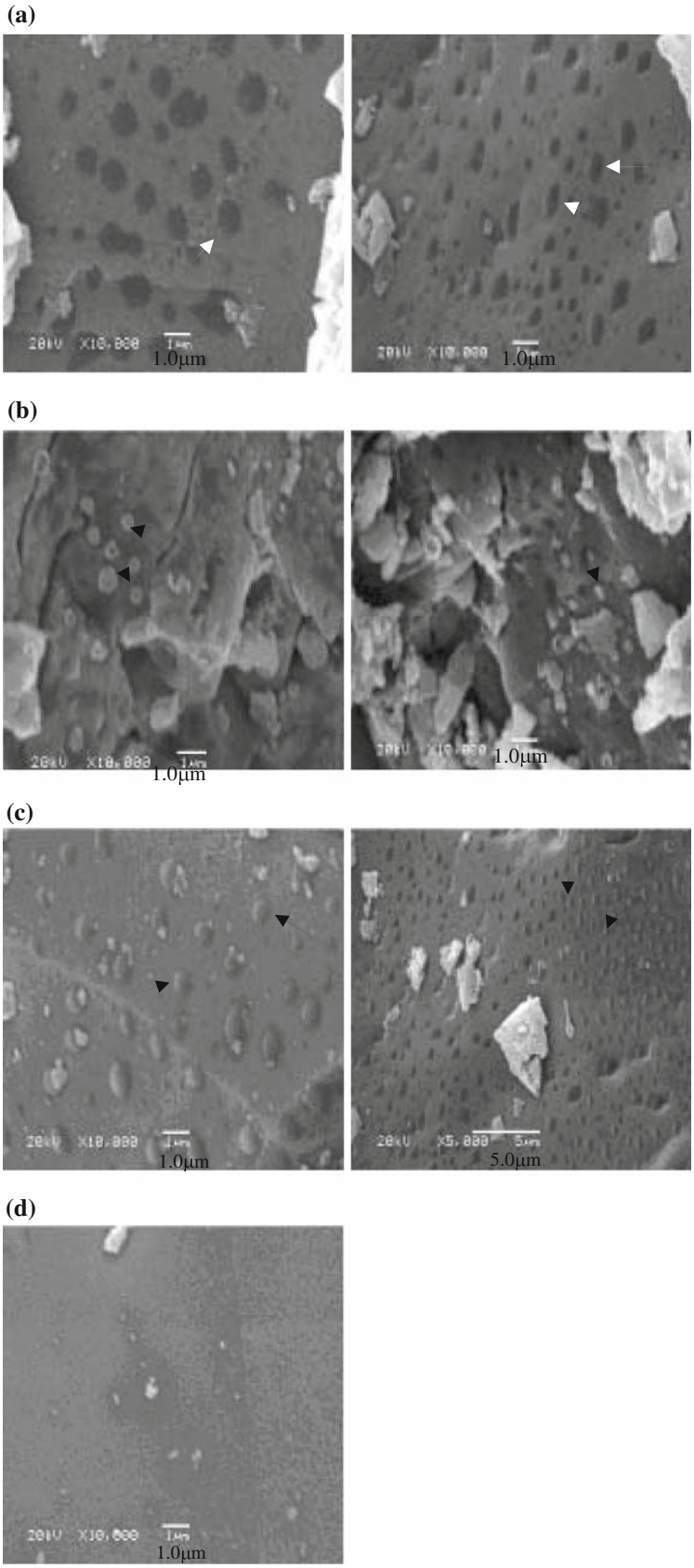

Fig. 4 Scanning electron micrographs of the surface of a pyrite cube that was colonized by *A. ferrooxidans*. The bacteria were allowed to grow on the pyrite surfaces for up to 1 month at room temperature. After incubation, the ore cubes to which the bacteria had become attached were rinsed in sterile water and treated with 1% oxalic acid (w/v) for 1 h, in order to remove any secondary minerals from the surfaces of the cubes. After drying, the ore cubes were divided into two groups. The surfaces of the first group of cubes were examined under a scanning electron microscope, without any further treatment. The second group of cubes was treated with 1‰ sodium dodecyl sulfate (SDS) at room temperature for 5 min, then cleaned in anhydrous ethanol to remove all bacteria and other organic material. They were then washed in deionised water, before being observed using SEM. **a**, **b**, **c**, and **d** show, respectively, bacterial cells removed, *A. ferrooxidans* strain ATCC19859, *A. ferrooxidans* strain BY3, and DX. The *white arrowheads* point to etch pits and the *black arrowheads* to bacterial cells

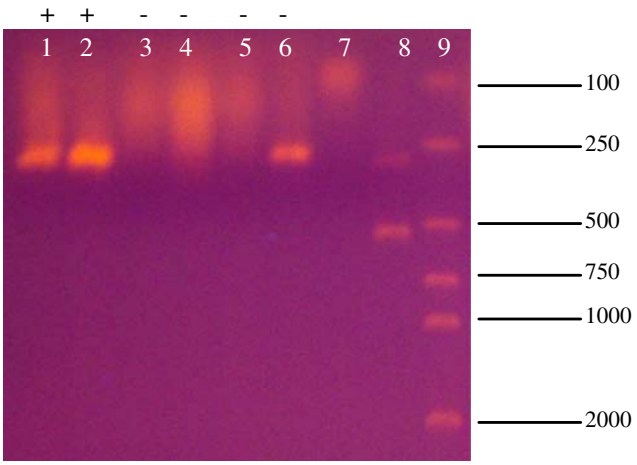

Fig. 5 PCR analysis of cDNA from reverse transcribed total RNA of *A. ferrooxidans* (*lanes* 1–9 display *A. ferrooxidans* strains ATCC19859, ATCC23270, DX, DBS, TKY, BY3, negative control, positive control, and DNA marker, respectively). The negative control is distilled water, and the positive control is BioRT RNA control (it was reverse transcribed as 500 bp DNA segment. "+" and "−" indicates to transcribe and not to transcribe, respectively

strains ATCC19859, ATCC23270, and BY3 attached to glass surfaces in surface pellicles. Although the environmental signals that can be detected by the twitching motility signal transduction system are still undefined, Rashid and Kornberg [22] have suggested that the pili might be the sensory organelle for detecting neighbouring cells and type IV pili-mediated twitching motility. The zone of adherent cells appeared to enlarge at the rate at which the substrate was consumed and in proportion to the intensity of twitching motility. *A. ferrooxidans* was attracted strongly to solid surfaces, such as glass, and pyrite, by type IV pili. The results also established that pili were required by *A. ferrooxidans* for their attachment.

Growth of *A. ferrooxidans* on the Surface of Lean Ore

We observed that pyrite was colonized readily by *A. ferrooxidans*, and the cells of *A. ferrooxidans* strains 19859, 23270, and BY3 attached strongly to the mineral surface through pili. However, *A. ferrooxidans* strains DX, DBS, and TKY cells attach minimally to the mineral surface because they lack pili. This shows that pili can mediate the attachment of bacteria to the mineral surface. Initial oxidation of the underlying substrate released secondary minerals that helped to bind the bacterial cells to the pyrite surface. The bacterial cells were attached strongly to the pyrite surface and oxidized the pyrite material below the cell body. This resulted in the formation of a pit just below the cell where pyrite had been removed and the accumulation of secondary minerals that surrounded or partially encapsulated the bacterial cell. The cell itself may have begun to sink into the pyrite surface, because it expanded its own etch pit. It is believed that the bacterial interface with the pyrite substrate is fully or partially sealed by these secondary minerals. Large colonies of these bacteria may grow and these colonies may release acids into the surrounding environment [13]. This acidic environment further enhances the biodegradation of the pyrite, which results in pits eventually being etched on the pyrite surface, with a size and shape that are comparable to those of the bacterial cells themselves.

In conclusion, the results established that pili were required by *A. ferrooxidans* for their sliding, twitching motility, and attachment. *A. ferrooxidans* can colonize pyrite surfaces readily and form a strong bond between the cell and the pyrite substrate. These bacteria bio-oxidize the underlying pyrite. This process creates etch pits in the pyrite.

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