Attachment of Bacteria to Sulphur in Extreme Environments

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SUMMARY

Sulfolobus attaches to sulphur deposited in acid hot springs by means of pili characterized as follows: (i) generally irregular shape with slight curves and bends; (ii) adhesiveness that enables bacteria to attach to sulphur; (iii) acid-stable, resistant to pH values as low as 2; (iv) heat-stable, resistant to temperatures as high as 75 °C. *Sulfolobus* attached to sulphur in nature and in culture eroded the sulphur crystal where the bacteria were attached. *Sulfolobus* undergoes the following two forms of attachment: (i) to sulphur by pili which separate the bacterium from the sulphur crystal and permit lateral movement of bacteria; (ii) to glass slides by firm adhesion of the wall to the surface of the slide. Attachment to sulphur in flowing springs enables *Sulfolobus* to colonize these low pH (2 to 3) high temperature (70 to 75 °C) habitats.

INTRODUCTION

The distribution of organisms in acid hot springs has been studied by Brock & Darland (1970). Such habitats, designated as 'Sulphataras', contain bacteria of the recently described genus, *Sulfolobus* (Brock, Brock, Belly & Weiss, 1972). These sulphur-oxidizing organisms attach to sulphur deposited in acid hot springs (pH 2 to 3) at temperatures from 70 to 75 °C. Attachment is considered to be a prerequisite for sulphur oxidation (Schaeffer, Holbert & Umbreit, 1963). The mechanism of attachment is related to holdfasts (Hirsch & Pankratz, 1970) rather than surface adhesion (Meadows, 1971) because the bacteria are separated from the sulphur crystal by a short distance. Bacteria attach to surfaces by a variety of holdfasts (Hirsch & Pankratz, 1970), including adhesive pili (Duguid, 1959). These specialized structures appear on *Sulfolobus* in large numbers when organisms are attached to sulphur. Other bacteria that come in contact with sulphur crystals, but are unable to attach, have relatively few pili.

The experiments described in the present paper are concerned with the attachment of *Sulfolobus* to sulphur by means of pili.

METHODS

Cultural methods. Incubations were carried out in a basal salts medium (Brock *et al.* 1972) containing (g/l distilled water): (NH₄)₂SO₄, 1.3; KH₂PO₄, 0.37; MgSO₄·7 H₂O, 0.25; CaCl₂·2H₂O, 0.07. The following trace elements were added to the basal medium (mg/l distilled water): FeCl₃·6H₂O, 20; MnCl₂·4H₂O, 1.8; Na₂B₄O₇·10H₂O, 4.5; ZnSO₄·7H₂O, 0.22; CuCl₂·2H₂O, 0.05; NaMoO₄·2H₂O, 0.03; CoSO₄, 0.01. The pH was adjusted to either 2 or 3 with 5 M-H₂SO₄. Yeast extract (pH 3) was added aseptically from a 10 % (w/v) stock

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to a final concentration of 0.1 % (w/v). When yeast extract was used trace elements were omitted. Sulphur, sterilized by steaming for 1 h on each of three successive days, was added at about 1 % (w/v) to sterile medium. The cultures were incubated at 70 °C.

Environmental methods. Sediment samples were obtained from several acid hot springs in Yellowstone Park, Wyoming, U.S.A., by using a propipette with an inverted sterile 10 ml pipette. The sample consisted almost entirely of elemental sulphur and spring water. The springs were of low pH (2 to 3) and high temperature (70 to 75 °C). Precleaned glass slides were placed directly in springs near the sampling area. Bacteria were allowed to attach for selected periods of time. The slides were then removed, placed in spring water and returned to the laboratory for observation.

Isolation of cultures. Enrichment of *Sulfolobus* cultures was carried out by adding 0.5 ml of a sediment sample to 20 ml basal medium (pH 2), containing sulphur as an energy source. Incubation was carried out at 65 or 70 °C in a water bath until turbidity was observed, usually for a period of 3 to 7 days.

Electron microscopy. An aqueous 1 % (w/v) solution of uranyl acetate was employed for negative staining as previously described (Weiss, 1971). Samples were examined with an Hitachi HU 11C electron microscope operated at 50 kV.

Detection and enumeration of pili. Cultures of *Sulfolobus* 98-3 were grown in basal medium with sulphur plus trace elements or 0.01 % (w/v) yeast extract. After several transfers, cultures were grown to the logarithmic phase (2 to 3×10^8 bacteria/ml) and examined for the presence of pili by negative staining with uranyl acetate. The % piliation and the number of filaments/bacterium were determined by counting in the electron microscope. The magnification was checked with a carbon replica of a diffraction grating (2160 lines/mm) and measurements of pilus diameters were made as previously described (Weiss, 1971).

Scanning electron microscopy. Cultures were cooled to 23 °C and 10 % (w/v) glutaraldehyde in 0.1 M-cacodylate-HCl (pH 7) was added to a final concentration of 2 % (w/v) glutaraldehyde. The culture was fixed 2 h at 23 °C, washed three times in fresh cacodylate buffer by decanting and the sulphur crystals were checked for attachment of bacteria with the phase microscope. After the addition of 0.2 % (v/v) Photoflow 200 (Eastman Kodak, Rochester, 3, New York, U.S.A.) to the preparation, a small amount of sulphur was placed on the surface of the specimen holder and dried at 50 °C. The sample was then attached to a rotary turntable (Denton Shadowing Accessories, Cherry Hill, New York, U.S.A.) and shadowed with gold after being coated with carbon. A Cambridge Stereoscan Electron Microscope Mark II (Cambridge Instrument Co. Ltd, London) was used at an accelerating voltage of 20 kV with a specimen angle of 37°. Photographs were taken on Polaroid film (Polaroid Corporation, Cambridge, Massachusetts, U.S.A.).

RESULTS

Sulphur in acid thermal habitats arises as the result of spontaneous oxidation of H_2S . Sulphur deposits in the outflow channel of small springs or is mixed freely in large bubbling pools. When samples from bubbling pools were observed by light microscopy, the structure of the prismatic needles was evident and organisms were not attached to the crystal surface. In contrast, sulphur obtained from flowing springs contained large numbers of *Sulfolobus* (Fig. 1a). On many crystals the bacteria were so numerous that the oxidation of sulphur had completely eroded the crystalline structure. However, in some instances fewer bacteria were attached and the crystal faces were clearly evident (Fig. 1b). On crystals with large numbers of bacteria (Fig. 1a) it was apparent that not all the attached bacteria were in

Fig. 1. *Sulfolobus* attached to sulphur in nature. (a) Large clusters of cells forming several layers have eroded the crystal structure; (b) individual cells are separated from the sulphur crystal by a short distance; the crystal faces are shown.

direct contact with the crystal surface, since the organisms were arranged along the crystal as an aggregate, several layers thick. When individual bacteria were observed, they appeared to be separated from the sulphur crystal by a short distance. This separation is shown for several bacteria in Fig. 1(b). The stability of the bacteria-crystal contact can be tested by moving the fine adjustment of the oil-immersion lens up and down, an action that decreases and increases pressure on the coverslip. During this procedure, the bacteria remain firmly attached to the sulphur, but show significant lateral movement. In contrast, bacteria in the same habitat that attach to glass slides apparently adhere to the surface of the slide and do not show lateral movement in response to differences in pressure on the coverslip.

Observations of pili. Several aquatic habitats of *Sulfolobus* were selected for direct electron microscopic examination of bacteria to obtain structural information on the mechanism of attachment to sulphur. The results of these studies show that when *Sulfolobus* was attached to sulphur deposited in flowing springs, bacteria were particularly enriched with pili. On one bacterium large numbers of these filaments were spread out from the surface, forming small clusters of slightly curved or bent filaments (Fig. 2a). Pili on cells attached to sulphur also formed in larger single clusters (Fig. 2b). The length of these pili was usually 1 to 2 μm although occasionally pili up to 4 μm in length were observed. Measurements made on negatively stained preparations of bacteria attached to sulphur in nature and in culture show that *Sulfolobus* pili have a diameter of 4.94 ± 0.27 nm.

Phase-microscope observations showed that cells in bubbling pools rarely attached to sulphur. Correspondingly, negatively stained preparations of these bacteria did not contain pili.

Attachment to sulphur. Several obligate autotrophs were isolated from flowing springs. Initially these isolates readily attached to sulphur and when examined in the electron microscope the cells displayed large numbers of pili. Upon subsequent transfer in basal medium with sulphur and trace elements, the bacteria lost their ability to attach to sulphur.

Fig. 2. Pili of *Sulfolobus* on bacteria attached to sulphur in a flowing acid hot spring, pH 2.3, 75 °C. (a) Small clusters of slightly curved and bent pili; (b) single culster of pili.

When these bacteria were again examined in the electron microscope, pili were no longer observed. The response of *Sulfolobus* 98-3, a facultative autotroph (Brock *et al.* 1972), was similar to this. Bacteria grown in basal medium plus 0.1 % yeast extract initially attached to sulphur and showed an abundance of pili similar to those presented in Fig. 2. After a few transfers in basal medium with sulphur and trace elements the bacteria lacked pili and were unable to attach to sulphur. The addition of 0.01 % yeast extract to this culture or to obligate autotrophs did not enable cells to attach to sulphur. However, when *Sulfolobus* 98-3 was allowed to grow in basal salts plus sulphur and 0.01 % yeast extract, the bacteria regained the ability to attach to sulphur after one or two transfers. The effect of yeast extract

Fig. 3. Number of pili/bacterium on 100 piliated bacteria with 89% piliation. (a) Bacteria grown in basal medium with sulphur and 0.01% yeast extract; bacteria attach to sulphur; (b) bacteria grown in basal medium with sulphur; bacteria do not attach to sulphur.

Fig. 4. Individual or groups of bacteria attached to sulphur. Erosion of the sulphur is evident at the site of attachment.

on the occurrence of pili was determined by observing negatively stained cells in the electron microscope. The number of pili on bacteria grown on sulphur with and without yeast extract is shown in Fig. 3. About 89% of the cells in the culture supplemented with yeast extract were piliated. Although the same value was obtained for autotrophically grown cells, significant differences were noted in both the number of pili/bacterium and the length of

pili. Most bacteria able to attach to sulphur had from 1 to 3 pili; about 20 % had more than 3; less than 10 % had more than 5. Bacteria unable to attach to sulphur were characterized by a sharp decrease in the number of pili/bacterium. Most bacteria had one or two pili; about 25 % had more than two. Only 8 % had over three pili/bacterium. In agreement with these results, a corresponding decrease in the length of pili was also observed. Pili on bacteria attached to sulphur were from 1 to 2 μm long, whereas pili on bacteria unable to attach were usually less than 0.25 μm in length.

Scanning electron microscopy. Bacteria grown on sulphur in the presence of 0.01 % yeast extract were attached to sulphur. These bacteria were fixed as described and observed in the scanning electron microscope (Fig. 4). Individual groups of bacteria can be recognized by the three-dimensional structure of the cellular lobes seen in the scanning electron micrograph. Areas on the sulphur crystal have been eroded by the growth of *Sulfolobus*. These carved-out areas appear near bacteria attached to the sulphur crystal and probably correspond to the eroded portion of the crystal seen in the phase microscope (Fig. 1a). In the scanning electron microscope, direct contact between the pili and the sulphur crystal was not observed, since the size of these filaments is well below the resolution of the instrument.

DISCUSSION

Sulfolobus attaches to sulphur by means of a new type of pili characterized by a number of unusual properties: (i) generally irregular shape with slight curves and bends; (ii) adhesiveness that enables cells to attach to sulphur; (iii) acid-stable, resistant to pH values as low as 2; (iv) heat-stable, resistant to temperatures as high as 75 °C. These characteristics distinguish *Sulfolobus* pili from those of other bacteria (Brinton, 1965).

The relation between the attachment of bacteria to sulphur and sulphur-oxidation is different for *Sulfolobus* and *Thiobacillus*. Schaeffer *et al.* (1963) concluded that attachment enabled *Thiobacillus* to oxidize sulphur; it has been suggested that a 'wetting' agent enables these bacteria to attach to sulphur (Jones & Starkey, 1961). The 'wetting' of sulphur occurs naturally in low pH, high-temperature habitats and attachment of *Sulfolobus* to sulphur is not a prerequisite for sulphur oxidation by bacteria in culture. In contrast, attachment of cells to sulphur in flowing springs enables *Sulfolobus* to colonize these habitats.

Sulfolobus apparently undergoes the following two forms of attachment: (i) to sulphur by means of pili which separate the bacteria from the sulphur crystal and permit lateral movement of organisms; (ii) to glass slides and presumably other silicious material in acid hot springs by a firm adhesion of the wall to the glass surface. The stability of the two forms of attachment corresponds to reversible and irreversible attachment which have been described previously (Marshall, Stout & Mitchell, 1971).

Finally, observations of organisms attached to sulphur (Fig. 4) provide additional insight into the way in which bacteria colonize micro-environments. The method of scanning electron microscopy could probably be applied much more widely in microbiological studies, especially with those problems concerning the growth and attachment of micro-organisms in nature.

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