Culture parameters regulating stalk formation and growth rate of *Gallionella ferruginea*

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(Received 30 May 1990; accepted 5 June 1990)

The growth of *Gallionella ferruginea* in a mineral salts solution with carbon dioxide and iron sulphide was studied by acridine orange stained direct count and most probable number techniques. *G. ferruginea* grew to 2×10^6 cells ml^{-1} with a generation time of 8.3 h under aerobic gradient conditions. The optimum temperature for growth was 20 °C. No growth was obtained under anaerobic conditions, or without carbon dioxide. A method was developed for measuring the length of the stalks formed by *G. ferruginea*. When growing exponentially, the bacterium was free-living, without stalks, and motile with one polar flagellum. A net production of stalk per cell began when the cell number exceeded 6×10^5 ml^{-1} if the pH exceeded 6. This occurred when growth entered the stationary phase. The stalk length increased from 3×10^3 μm ml^{-1} (detection limit) to 1.8×10^8 μm ml^{-1} , during a 400 h growth experiment. There was no stalk formation at growth conditions where ferrous iron was stable, suggesting that stalk formation may be a protection mechanism against an increasing reducing capacity of ferrous iron as it becomes unstable in an environment that becomes oxidized. The results indicate that favourable growth conditions for *G. ferruginea* may be those present in reduced ground waters, rather than those in ditches, drainage tubes, wells, etc., where stalk-forming *G. ferruginea* can usually be found.

Introduction

Gallionella ferruginea is a bean-shaped bacterium that thrives in iron-bearing waters, where it may produce a twisted stalk. The cells are difficult to observe because they are usually mixed with a large amount of iron precipitates and stalks (Kucera & Wolfe, 1957; Wolfe, 1964). The bacterium is laborious to culture – it grows to 2×10^6 cells ml^{-1} in a liquid enrichment medium with inorganic salts and gradients of carbon dioxide, oxygen, ferrous iron and sulphide (Hanert, 1989).

The majority of the work performed on *G. ferruginea* consists of case studies reporting the occurrence of *G. ferruginea* in natural groundwater (e.g. Barbic *et al.*, 1974; Cullimore & McCann, 1977; Hannert, 1970, 1974; Hirsch & Pankratz, 1970; Hässelbarth & Lüdeman, 1972; Ivarson & Sojak, 1978; Pedersen & Hallbeck, 1985; Ridgeway *et al.*, 1981; Wheatley, 1988). A recent study concentrated on the ultrastructure of *G. ferruginea* (Lütters & Hanert, 1989), but most investigations, and also experiments done *in vitro*, have focused on the stalks

(e.g. Balashova, 1967; Hanert, 1967; 1973; Mardanyan & Balashova, 1971; Vatter & Wolfe, 1957). Studies that concentrate on the stalk-forming cells might overlook other phases in the growth cycle. There is still much information lacking on the metabolism of this organism, and on the mechanism behind stalk formation.

In this study we have attempted to identify parameters that regulate growth of, and induce stalk formation by, *G. ferruginea*. We used acridine orange direct counts (AODC) to measure the total number of cells and the most probable number (MPN) technique for the number of viable cells. Growth rates were calculated for growth at different oxygen and carbon dioxide conditions, and at different temperatures. A method was developed for the determination of stalk formation. The influence of oxygen, carbon dioxide, redox potential and pH on stalk development was studied.

Methods

Source of organism. *G. ferruginea* was enriched from a 60 m deep drinking-water well by serial dilution of well water and inoculation in culture tubes, as described below. The cultures were subcultured after

Abbreviations: MSS, mineral salts solution; AODC, acridine orange direct count; MPN, most probable number.

new tenfold dilutions once a week. The total number of cells (AODC) and the viable number of *G. ferruginea* (MPN) in the well water was assayed.

Aerobic gradient conditions. *G. ferruginea* was cultured in the mineral salts solution (MSS) described by Kucera & Wolfe (1957) and modified by Hanert (1981). The MSS consists of 1 g NH_4Cl , 0.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.05 g K_2HPO_4 and 1 litre de-ionized water. It was filtered through a 0.2 μm filter (Nuclepore) before sterilization at 121 °C for 20 min. The MSS was subsequently chilled to 5 °C and infused with CO_2 to pH 4.6–4.8. Sterile screw-capped tubes (180 × 16 mm) were used as culture vessels. These were filled with 9.5 ml MSS and 20 drops of sterile FeS solution, prepared as described by Hanert (1981), were added to give an initial ferrous iron concentration of 0.5 mM.

Anaerobic culture conditions. A methodology similar to that used for cultivation of methanogenic bacteria (Mah & Smith, 1981) was applied. The MSS was cooled under an atmosphere of 90% N_2 and 10% CO_2 (v/v) after sterilization. The medium was subsequently supplied with 0.2 mM- $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ and 30 mM- NaHCO_3 and the pH was adjusted to 6.2 with 20 mM-HCl. The medium was added in 9 ml aliquots to Hungate tubes (150 × 18 mm) with aluminium crimp-sealed butyl rubber stoppers under a 90% N_2 and 10% CO_2 atmosphere. FeS was added as described under aerobic culture conditions. The tubes were inoculated with a hypodermic syringe through the stoppers.

Acridine orange stained direct count. AODC (Hobbie *et al.*, 1977) was used to determine the total number of cells in the culture tubes. Nuclepore filters of 0.2 μm pore size and 13 mm in diameter were used. All solutions were filter sterilized (0.2 μm). The sample was diluted to twice its volume with 0.1% sterile filtered oxalic acid and vigorously shaken. Oxalic acid chelates iron and reduces clogging of the filters by iron precipitates. A portion of the sample was filtered on a pre-stained nuclepore filter at -20 kPa and stained for 6 min with acridine orange. The number of bacteria was counted using blue light (390–490 nm) in an epifluorescence microscope (Olympus BH-2). Between 500 and 600 cells or a minimum of fifteen fields (0.0064 mm² each) were counted on each filter.

Precision of the AODC method. The frequency distribution of the number of cells counted per microscopic field followed a Poisson distribution (the number of fields counted was 405). This means that the precision of the mean of the counted cells is only dependent on the number of cells counted. One filter then predicts the sample mean with a precision of 5% if 400 or more cells are counted (Niemelä, 1983). The standard deviation of the $\log(\text{cells ml}^{-1})$ on 18 replicate AODC from nine tubes ($n=18$) was determined to be 3.5% of a mean of 5.69 $\log(\text{cells ml}^{-1})$ and to be 2.5% of a mean of 6.16 $\log(\text{cells ml}^{-1})$ in a repeat determination.

Most probable number of *G. ferruginea*. An MPN technique was used to assay the number of viable *G. ferruginea*. A dilution series to approximately 1, 1/2 and 1/10 of the inverse of the total number of cells ml^{-1} was made. Ten culture tubes were inoculated from each of these dilutions. The tubes were incubated at 20 °C for 10 d. Tubes with *G. ferruginea* stalks were considered as positive. The MPN was calculated from the equation $\text{MPN} = 2.303/V \cdot \log(n/q)$ (Niemelä, 1983), where V is the volume of the sample (0.5 ml), n is the number of tubes used in one dilution ($n=10$) and q is the number of negative (sterile) tubes in that dilution. The standard deviation for the MPN was approximately 50% of the mean based on the theory of the MPN method.

Measurement of growth. Batches of 30–60 culture tubes were prepared for each experiment. The culture tubes were inoculated with cells from a culture in the late exponential growth phase, 3–4 d old. The starting cell concentration was adjusted to 10^3 cells ml^{-1} and the inoculum

volume was 0.1 ml. The tubes were incubated at 20 °C in air unless otherwise stated. Growth was followed using new tubes on every sampling occasion.

Sampling procedure. The cultures were sampled with a sterile Pasteur pipette, thus leaving the FeS on the bottom of the tube. The cultures in several tubes were pooled before measurements. A minimum of two tubes was used for AODC and MPN, and a minimum of five for stalk measurements.

Formation of stalks. Pieces of polycarbonate tube, 20 mm in height and 19 mm in diameter, were mounted on glass cover-slips with silicon grease. The stalks in 2 ml samples were allowed to sediment on the cover-slips for approximately 24 h. The stalk length was measured by using an inverted microscope (Olympus CK 2) equipped with a drawing arm. Stalks in eight sight fields, each $3.48 \times 10^4 \mu\text{m}^2$, were drawn on paper and measured with a map measurer (Magnet). The standard deviation was approximately 50% of the mean with this procedure. The stalk length per ml sample and per cell was calculated. The sample was concentrated by centrifugation at 100 *g* for 6 min when the stalk length was too low for valid drawing, or diluted with 3% (v/v) Tween-80 when it was too high. With this method a stalk length down to $3 \times 10^3 \mu\text{m ml}^{-1}$ could be estimated.

Temperature. Growth at temperatures from 5 °C to 30 °C, in 5 °C increments, was measured. The culture tubes were incubated in air. If they were placed in water, turbulence from thermal density variations seemed to disturb the iron-oxygen gradient, resulting in low growth. The growth rate, k , h^{-1} ($k = \log_{10}2/\text{generation time}$), for exponential growth was determined for each temperature.

pH and redox measurements. pH was determined with a PHM Autocal pH meter (Radiometer) and a GK2421C combined pH electrode (Radiometer). The measurements were made in the culture tubes before sampling. In some experiments, resazurin was added to a concentration of 2.5 mg l^{-1} to determine whether the redox potential in the tubes was above or below -40 mV. Resazurin is colourless at below -40 mV, and pink at above -40 mV (Mah & Smith, 1981).

Results

Gallionella ferruginea in the well

The total number of cells in the well water from which the enrichment was made amounted to 2.7×10^5 cells ml^{-1} . The MPN value obtained for viable *G. ferruginea* was 3.2×10^4 cells ml^{-1} . There were no stalks in the well water but heavy stalk formation occurred if a water sample was left to stand in contact with air for 24 h or more. Stalks did not appear in closed sample bottles, i.e. without air contact.

Growth under aerobic gradient conditions

The generation time at the optimum growth temperature (20 °C) was 8.3 h (mean of four experiments). The cultures reached stationary phase after 40–70 h and then contained between 1×10^6 and 2.5×10^6 cells ml^{-1} . There was a lag phase if an inoculum older than 3–4 d was used. The total number of cells (AODC) and the number of viable *G. ferruginea* (MPN) followed each

Fig. 1. (a) Growth and stalk formation of *G. ferruginea* under aerobic gradient conditions in MSS with carbon dioxide and FeS. ●, Total number of cells determined by AODC; ▲, viable count of *G. ferruginea* determined by MPN; ■, stalk length. (b) Increase in pH during the growth of *G. ferruginea* in (a).

other for 200 h, during the exponential growth phase and the stationary phase. In late stationary phase (> 200 h) the viability of the culture decreased (Fig. 1a). If the MSS was not infused with carbon dioxide there was no growth. The pH rose from 4.6 to 5.2 after the culture tubes were prepared, and increased during growth, reaching 7 at the end of the experiments (Fig. 1b). The redox potential was below -40 mV until 12–24 h, when the resazurin indicator turned from colourless to pink.

Growth under anaerobic conditions

These experiments were performed to investigate the ability of *G. ferruginea* to grow anaerobically. Because of problems with the stoppers, additional information about the parameters regulating stalk formation was gained. The butyl rubber stoppers first used were of a stiff quality that allowed leakage of oxygen into the cultures after penetration with the hypodermic syringe at inoculation. Cultures incubated with this type of stopper, here called microaerophilic, repeatedly had the same growth rate and reached the same cell number as those incubated under aerobic gradient conditions (Fig. 2). When this experiment was repeated with a new, softer quality of butyl rubber stopper, or when the microaerophilic culture tubes were placed in an anaerobic jar (Oxoid) with a 90% N_2 and 10% CO_2 atmosphere, there was no growth. The pH was 6.4–6.6, and did not change during the anaerobic growth experiments. The redox

Fig. 2. Growth of *G. ferruginea* under microaerophilic conditions in MSS with carbon dioxide, FeS, and a 90% N_2 and 10% CO_2 atmosphere. ●, Total number of cells determined by AODC; ■, stalk length.

Fig. 3. (a) Relation between total number of cells and stalk length per cell in growth experiments with *G. ferruginea* under aerobic gradient conditions. Data from four experiments. (b) Relation between pH and stalk length per cell in growth experiments with *G. ferruginea* under aerobic gradient conditions. Data from three experiments.

potential was below -40 mV throughout the anaerobic and microaerophilic experiments, the resazurin indicator remaining colourless.

Stalk formation

Stalk formation in aerobic gradient cultures started at or below the detection limit of $3 \times 10^3 \mu\text{m ml}^{-1}$ and increased to $1.2 \times 10^6 \mu\text{m ml}^{-1}$ when the cultures entered the stationary phase. During the following 400 h, the

Fig. 4. The growth rate (k) of *G. ferruginea* under aerobic culture conditions in the temperature range between 5 and 30 °C.

stalk length increased to $1.8 \times 10^8 \mu\text{m ml}^{-1}$ (Fig. 1*a*). There was no stalk formation in the cultures under microaerophilic conditions (Fig. 2).

The mean stalk length per cell was $3.0 \mu\text{m}$ when the cell numbers were below $6 \times 10^5 \text{ ml}^{-1}$, i.e. when the culture was in the exponential growth phase (Fig. 3*a*). When the cell numbers increased above $6 \times 10^5 \text{ ml}^{-1}$, i.e. when the cultures entered the stationary phase, the mean stalk length increased from $3.0 \mu\text{m}$ per cell to $34 \mu\text{m}$ per cell (values from four experiments). The maximum value measured was $60 \mu\text{m}$ per cell. The mean stalk length per cell was $4.2 \mu\text{m}$ at pH below 6 and increased to $33 \mu\text{m}$ at pH above 6 (Fig. 3*b*) (values from three experiments).

Temperature

Fig. 4 shows growth rates at different temperatures. The optimum growth temperature was 20 °C, the maximum temperature for growth was below 30 °C and growth still occurred at 5 °C.

Discussion

G. ferruginea grew to $2 \times 10^6 \text{ cells ml}^{-1}$ with a generation time of 8.3 h under aerobic gradient conditions. The optimum temperature for growth was 20 °C. Microaerophilic growth conditions resulted in the same generation time and the same number of cells in stationary phase. No growth was obtained under anaerobic conditions. The pH in the aerobic gradient cultures rose from 5.2 to 7.0 during a 400 h growth experiment but remained at 6.4–6.6 in the microaerophilic cultures. The stalk length increased from $3 \times 10^3 \mu\text{m ml}^{-1}$ (detection limit) to $1.8 \times 10^8 \mu\text{m ml}^{-1}$ under aerobic gradient conditions. A net production of stalks per cell began when the cell number exceeded $6 \times 10^5 \text{ ml}^{-1}$ but there was no stalk

formation when pH was below 6 or under microaerophilic conditions. The MPN measurements showed that the viability of the culture was highest at approximately one week, after which it decreased (Fig. 1*a*). Therefore, the optimum time to inoculate a new culture was after one week of growth of the previous one.

The enumeration technique made it possible to calculate an *in vitro* growth rate for our strain of *G. ferruginea*. The mean generation time of 8.3 h is long compared to that of many heterotrophic bacteria growing under optimal growth conditions. As there was no organic carbon source in the culture medium, the bacterium had to synthesize all cell components from carbon dioxide; such use of carbon dioxide was postulated by Hanert (1989). It is also noteworthy that the cell number never exceeded $2.5 \times 10^6 \text{ ml}^{-1}$, under either aerobic gradient conditions or microaerophilic conditions. We have not been able to identify any limiting factor, but the factor regulating stalk formation can be excluded because of the absence of stalks during growth under microaerophilic conditions. The experiments revealed that *G. ferruginea* is a microaerophilic organism that cannot grow anaerobically. This corresponds with the observations of Kucera & Wolfe (1957), who found that *G. ferruginea* did not form stalk colonies in a nitrogen atmosphere with carbon dioxide added.

The temperature experiments showed that our strain of *G. ferruginea* can be characterized as a psychrotrophic bacterium, that grows well at low temperatures, 5–10 °C, in relation to its optimum temperature, 20 °C (Fig. 4). Temperature studies have been made previously but these focused on stalk elongation or stalk colony formation on the tube walls. Hanert (1973) measured stalk elongation in natural water at 14 °C and 8.5 °C; the higher temperature gave the higher rate of stalk formation. Kucera & Wolfe (1957) observed that stalk colonies appeared earliest at 25 °C; colony formation was slightly slower at 20 °C, very slow at 12 °C and no colony formation was obtained above 30 °C.

The increase in pH and redox potential during aerobic gradient growth was due to the gas exchange of carbon dioxide from the culture to the gas phase and of oxygen from the gas phase to the culture medium. The decrease in carbon dioxide increased the pH which, together with introduction of oxygen, increased the redox potential. As the redox potential rises, ferrous iron becomes increasingly unstable and oxidizes to ferric iron, which in turn immediately precipitates as $\text{FeO}(\text{OH})$ due to the extremely low solubility for ferric hydroxy complexes at neutral or higher pH (Stumm & Morgan, 1981). A stability diagram (E_h -pH diagram) for iron compounds (Garrels & Christ, 1965) shows that as pH goes above 6 and redox potential above 0 mV, ferrous iron becomes unstable. These were the conditions in the aerobic

cultures when *G. ferruginea* began forming stalks (Fig. 3b). *G. ferruginea* grew well under microaerophilic conditions but in contrast to the aerobic cultures did not produce any stalks. These cultures were closed systems with a nitrogen and carbon dioxide atmosphere, a pH buffered to about 6.5 and a redox potential below -40 mV. In addition, the hydrogen carbonate in the buffer can act as a chelating agent for ferrous iron. Ferrous iron is stable in this environment (Garrels & Christ, 1965).

Most of the literature describes *G. ferruginea* solely as a stalk-forming bacterium (e.g. Ghiorse, 1984; Hanert, 1981, 1989). We have found that the bacterium is free-living without stalks, and motile with one polar flagellum (Lutters & Hanert, 1988), when growing exponentially or under reducing conditions (less than -40 mV). Stalk formation started when the pH rose above 6 and the culture entered the stationary phase under aerobic growth conditions. A number of suggestions as to the purpose of stalk formation have been made. Hanert (1973) suggested that the stalks make it possible for the bacterium to escape from masses of precipitated iron. The cell attaches to a surface and starts to produce a stalk while it rotates. Stalk formation can be compared with the formation of sheets by *Leptothrix ochracea*; the sheets are thought to be produced as a residue for oxidized iron and manganese (Ghiorse, 1986). Ghiorse (1984) discusses the possibility that extracellular structures of iron and manganese bacteria may be some form of defence against oxygen toxicity (e.g. formation of H_2O_2). We believe that stalk formation by *G. ferruginea* may be a protective mechanism against the increasing reducing capacity of ferrous iron as it becomes unstable in an environment that becomes oxidized.

Speculation on similarities between our culture conditions and a natural environment may give some clues to the ecology of *G. ferruginea*. The aerobic culture is an environment rapidly changing from reduced to oxidized conditions. It simulates upwelling reduced ferrous ground water with free-living *G. ferruginea* cells, reaching an oxidized atmosphere in ditches, drainage tubes or wells. The ferrous iron becomes unstable and begins to oxidize and this induces stalk formation. The microaerophilic culture mimics a stable ground-water zone where ferrous iron is stable with a low content of oxygen, balanced between strictly anaerobic and aerobic environments – therefore there is no stalk formation.

In the well from which our strain of *G. ferruginea* was isolated, the cells of *G. ferruginea* were free-living and there were no stalks. Oxygen could not be detected (by the Winkler titration method with Mn), the pH was 6.8 and there was a slight smell of sulphide from the well water. When oxygen was allowed to dissolve in the water, stalk formation began. Favourable growth condi-

tions for *G. ferruginea* may be those present in reduced (less than 40 mV) ground waters, as in the well where we found free-living cells, rather than those in ditches, drainage tubes, wells, etc., where stalk-forming *G. ferruginea* usually can be found. The stalks may be a survival structure, to protect cells awaiting more favourable growth conditions, e.g. rain after a dry period when the saturated ground-water zone will be lowered.

The skilful technical assistance of Susanne Ekendahl is acknowledged. We are indebted to Amanda Goodman for valuable comments on the manuscript. This work was supported by Carl Tryggers Foundation for Scientific Research and the Swedish Natural Science Research Council, B-BU 8466-102.

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