SHORT COMMUNICATION

Detection of Heterotrophic Contaminants in Cultures of *Thiobacillus ferrooxidans* and Their Elimination by Subculturing in Media Containing Copper Sulphate

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Of the four cultures of *Thiobacillus ferrooxidans* held by the National Collections of Industrial and Marine Bacteria, U.K., two were found to be contaminated with acidophilic heterotrophic bacteria. One of the contaminated cultures was the type strain (NCIB 8455) of *T. ferrooxidans*; the equivalent culture from the American Type Culture Collection (ATCC 23270) was found not to be contaminated. Heterotrophic contaminants could be subcultured along with *T. ferrooxidans* in media containing ferrous iron, but devoid of organic material, and remained viable in mixed cultures for periods of at least one year. Subculturing in media containing high concentrations of copper ions resulted in the elimination of the heterotrophic bacteria.

INTRODUCTION

Thiobacillus ferrooxidans, a chemolithotrophic bacterium characterized by its oxidation of ferrous iron at low pH, is generally grown in synthetic media devoid of organic materials (Leathen *et al.*, 1951; Silverman & Lundgren, 1959). It was reported that *T. ferrooxidans* was capable of utilizing glucose, and that repeated subculturing in a glucose/basal salts medium resulted in the loss of ability to oxidize iron (Tabita & Lundgren, 1971). It is probable that this behaviour was due to the presence of contaminant bacteria. Arkesteyn & de Bont (1980) found that the facultative autotroph *Thiobacillus acidophilus* was present in numbers about equal to those of *T. ferrooxidans* ATCC 13598 in cultures growing in medium containing ferrous sulphate. Harrison *et al.* (1980) also showed that several cultures of *T. ferrooxidans* contained a phenotypically unrelated obligate heterotroph, and obtained pure *T. ferrooxidans* cultures by serial dilution, since the autotroph was found to outnumber the heterotroph. Mackintosh (1978) observed, however, that mixed cultures of acidophilic bacteria were still present after numerous subcultures in medium containing ferrous sulphate. *Thiobacillus ferrooxidans* cultures might therefore be expected to remain contaminated over an extended period.

Harrison (1981) characterized an acidophilic heterotrophic bacterium isolated from a culture of *T. ferrooxidans* ATCC 13598 and named it *Acidophilium cryptum*. The isolate was obligately acidophilic and failed to grow on elemental sulphur or ferrous iron. Growth of *A. cryptum* was inhibited by sodium acetate (at 0.01%, w/v) or glucose (above 0.1%, w/v).

The National Collections of Industrial and Marine Bacteria (NCIMB), Aberdeen, U.K. (which has taken over functions of the National Collection of Industrial Bacteria; NCIB) maintain, at the time of writing, four strains of *T. ferrooxidans*: NCIB 8455 (ATCC 23270) which is the type strain, NCIB 9490, NCIB 10435 and NCIB 11335. In the work described in the present paper, these cultures were tested for the presence of heterotrophic contaminants. ATCC 23270, which to the authors' knowledge has not been reported as having been tested for contamination, was also examined.

A reliable and relatively rapid method for eradicating heterotrophic contaminants from *T. ferrooxidans* cultures is also described.

METHODS

Bacteria. Cultures of *Thiobacillus ferrooxidans* (strains NCIB 8455, 9490 and 10435) were obtained from the NCIB on three separate occasions (January and April 1981, and June 1982). Strain NCIB 11335 was obtained from the NCIMB on one occasion (February 1983). The ATCC equivalent of the type strain (ATCC 23270) was obtained during February 1983 directly from the American Type Culture Collection. Freeze-dried cultures were revived in the medium described by Leathen *et al.* (1951) (ferrous iron concentration 200 mg l⁻¹) and thereafter maintained in either a modified version of this medium (designated L+; ferrous iron concentration 400 mg l⁻¹) or in the 9K medium (ferrous iron concentration 9 g l⁻¹) of Silverman & Lundgren (1959). *Thiobacillus ferrooxidans* numbers were estimated using a Most Probable Number technique in L+ or 9K media. All cultures were grown at 30 °C.

Test for heterotrophic contamination. Acidified mannitol-TSB contained (g l⁻¹): MgSO₄ · 7H₂O, 0.5; (NH₄)₂SO₄, 0.1; KH₂PO₄, 0.05; KCl, 0.05; Ca(NO₃)₂, 0.01; mannitol, 1.0; tryptone soya broth (TSB, Oxoid), 0.1; agar (bacteriological, Oxoid) or agarose (BDH), 12.0. The pH of the medium (excluding agar or agarose) was adjusted to 3.5 with H₂SO₄ prior to autoclaving (120 °C, 15 min). The gelling solution (4.5%, w/v) was autoclaved separately to avoid hydrolysis, and the two solutions combined when cool. Agarose was used because Wichlacz & Unz (1981) found that acidophilic heterotrophic bacteria from mine waters would only grow on solid media if this gelling agent was used. Nutrient agar (Oxoid) was used in some experiments.

Samples (0.2 ml) of growing cultures were plated on solid media, if necessary after serial dilution using sterile tap water adjusted to pH 3.0 with H₂SO₄.

Purification of cultures. Two methods were used to eradicate heterotrophic contaminants from *T. ferrooxidans* cultures: (a) serial dilution as described by Harrison *et al.* (1980); (b) growing cultures in L+ or 9K media containing high concentrations of copper ions; an 0.8 M solution of copper (II) sulphate (CuSO₄ · 5H₂O) was separately sterilized and appropriate volumes added to L+ or 9K media to give final concentrations of between 1 and 5 g Cu²⁺ l⁻¹.

RESULTS

Identification of contaminated cultures

On all occasions when cultures were obtained from the NCIB, strains NCIB 8455 and NCIB 10435 were found to be contaminated, whilst NCIB 9490 contained no detectable heterotrophic contaminants. NCIB 11335 and ATCC 23270 (each tested once only) also appeared to be free of heterotrophic contaminants.

Colonies of heterotrophic bacteria developed on acidified mannitol-TSB but not on nutrient agar. Numbers and proportions of autotrophic and heterotrophic bacteria varied with the culture and growth medium. NCIB 8455 contained about 10⁶ heterotrophic bacteria and 5 × 10⁷ *T. ferrooxidans* cells ml⁻¹ when grown in L+ medium, whilst NCIB 10435 contained about 10⁷ heterotrophic bacteria and 5 × 10⁷ *T. ferrooxidans* cells ml⁻¹ when grown in L+ medium, and 10⁸ heterotrophic bacteria and 10⁹ *T. ferrooxidans* cells ml⁻¹ when grown in 9K medium. Three heterotrophic bacteria (84/A, 84/B and 84/C) were isolated from NCIB 8455, and two (10/A and 10/B) from NCIB 10435. Isolates 84/B and 84/C tended to be diluted out on subculturing in L+ or 9K media, whilst 84/A grew in L+ medium (mixed cultures with *T. ferrooxidans*) but tended to die out in 9K medium within 17 d.

There was no increase in numbers of heterotrophic bacteria when agarose, as opposed to agar, was used in acidified mannitol-TSB solid media.

Stability of mixed cultures

Cultures in L+ or 9K media were stored in the dark at room temperature for up to one year. They were analysed periodically for numbers of viable *T. ferrooxidans* and heterotrophs. Mixed cultures (NCIB 8455 and 10435) in L+ medium were found to contain viable autotrophic and heterotrophic bacteria throughout this time; numbers of heterotrophic bacteria after one year being around 1 to 5% of those counted in ten-day-old cultures. In 9K cultures, however, *T. ferrooxidans* appeared to lose viability after 8 months in the case of NCIB 8455 and 9490, but NCIB 10435 did not.

Purification of cultures

Initial subculturing in 9K medium containing $1 \text{ g Cu}^{2+} \text{ l}^{-1}$ resulted in the eradication of detectable heterotrophic bacteria from NCIB 8455, but not from NCIB 10435. Further subculturing in up to $5 \text{ g Cu}^{2+} \text{ l}^{-1}$ eventually eliminated heterotrophs from even this culture. Serial dilution into this latter medium ensured that pure *T. ferrooxidans* cultures were obtained. Purity of cultures, checked by plating onto mannitol-TSB agar, has been maintained subsequently through at least ten subcultures in 9K medium. Continued subculturing of mixed cultures NCIB 8455 and 10435 in L+ medium has failed to eliminate heterotrophic bacteria.

Pure cultures of *T. ferrooxidans* obtained from NCIB 8455 and NCIB 10435 have been returned to the NCIMB, where they have been given new accession numbers. Purified *T. ferrooxidans* from NCIB 8455 is NCIB 11820 and purified *T. ferrooxidans* from NCIB 10435 is NCIB 11821.

Characteristics of the acidophilic heterotrophic contaminant bacteria

All heterotrophic isolates were Gram-negative, non-sporulating motile rods which were acidophilic but incapable of growth on either ferrous iron or elemental sulphur. Each formed colonies which could be distinguished by their colour and size on mannitol-TSB plates. Heterotroph 10/A outnumbered 10/B in both 9K and L+ media by about 20 to 1. All three heterotrophic isolates (10/A, 10/B and 84/A) were inhibited by 0.1% sodium acetate, but grew in medium containing 0.5% glucose (pH 3.5).

Isolates 84/A, 10/A and 10/B have been returned to the NCIMB and have been given accession numbers 11822, 11745 and 11746, respectively.

DISCUSSION

We have confirmed that cultures of *T. ferrooxidans* are likely to be contaminated with heterotrophic bacteria, and that these may display a remarkable tenacity in remaining associated with the autotroph. *Thiobacillus ferrooxidans* can readily adapt to high concentrations of copper ions in its growth medium (Tuovinen *et al.*, 1971) and this can be used as a basis for purifying contaminated cultures, since the heterotrophs may display much lower tolerance and thus be selectively eliminated from liquid cultures. The method is relatively simple and rapid, and it removes the need for repeated subculturing (Norris & Kelly, 1982). Serial dilution by itself is less satisfactory as numbers of contaminants may approach those of the autotroph (e.g. as with *T. acidophilus*). Finally, single colony isolation of *T. ferrooxidans* on agar containing ferrous iron may prove unsatisfactory since *T. ferrooxidans* is difficult to grow on solid media (Manning, 1975).

Tests for the presence of heterotrophic bacteria showed that the NCIMB type culture of *T. ferrooxidans* (NCIB 8455) was contaminated whilst its ATCC equivalent (ATCC 23270) was not. This result was not anticipated because the ATCC type strain was originally derived from its NCIMB equivalent (ATCC Catalogue of Strains, 1974). The medium used by the ATCC for propagating *T. ferrooxidans* contains $4 \text{ g Fe}^{2+} \text{ l}^{-1}$ (ATCC Catalogue of Strains, 1974), whilst NCIMB strains are freeze-dried from a medium containing $200 \text{ mg Fe}^{2+} \text{ l}^{-1}$ (A. R. MacKenzie, NCIMB, personal communication). Since heterotrophic contaminants 84/B and 84/C are readily eliminated from mixed cultures and 84/A shows gradual loss of viability in media containing high iron concentrations (such as 9K medium) it is likely that an originally impure ATCC culture has lost its heterotrophic population, whilst the corresponding NCIMB culture has remained contaminated.

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