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Interactions of Thiophenes and Acidophilic, Thermophilic Bacteria

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ABSTRACT

The growth and oxygen consumption of a variety of thermophilic, acidophilic bacteria in the presence of thiophene-2-carboxylate (T2C) and dibenzothiophene (DBT) have been determined. T2C was extremely toxic to the acidophiles in comparison with neutrophiles, but appeared to be degraded by a heterotrophic *Sulfolobus*-like thermophile. DBT proved to be unstable at high temperatures, even in the absence of bacteria, and was not a substrate for the thermophiles.

Index Entries: *Sulfolobus solfataricus;* thermophilic acidophiles; dibenzothiophene; thiophene-2-carboxylate.

INTRODUCTION

There have been numerous laboratory studies of the removal of sulfur from coal by microbial activity (*see 1* for review). The major inorganic sulfur fraction in many coals is found in pyrite, which can be efficiently removed by acidophilic, mineral-oxidizing bacteria. The organic sulfur fraction, however, comprises various forms, including thiols, sulfides, and thiophenes, which, in contrast to pyrite, are distributed in the coal matrix and therefore more difficult to remove (*2,3*). Most studies of organic sulfur removal have concentrated on the use of heterotrophic, neutrophilic bacteria with dibenzothiophene (DBT) frequently chosen as a model compound for assessing the bacterial activity. The application of acidophilic

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bacteria to oxidize pyrite and of neutrophiles to degrade organosulfur compounds would require a two-stage process. However, both the oxidation of pyrite in coal (*4,5*) and the degradation of DBT (*6*) by *Sulfolobus acidocaldarius* have been described, which implies considerable nutritional versatility of this thermophilic archaebacterium. However, the lack of sulfur oxidation by some culture collection strains of *S. acidocaldarius* and *Sulfolobus solfataricus* (*7*), and the phenotypic variation of other cultures of these extreme thermophiles (*8*) perhaps raise the question of whether certain characteristics assigned to some named cultures are valid in all cases for pure strains.

In this article, the activity of a heterotrophic extreme thermophile that resembles *Sulfolobus* is compared with that of acidophilic archaebacteria which can oxidize sulfur as well as utilize organic substrates. DBT and thiophene-2-carboxylate (T2C) were used to assess the capacity of the bacteria for degrading recalcitrant organosulfur compounds. The interactions of these compounds with moderately thermophilic, acidophilic, heterotrophic bacteria and with mineral sulfide-oxidizing moderate thermophiles were also assessed with reference to some comparable experiments with the neutrophilic mesophiles *Pseudomonas putida* and a *Rhodococcus* species.

METHODS

Organisms

Sulfolobus solfataricus DSM 1616 as obtained from the German Culture Collection in 1982 proved to be unable to oxidize sulfur (*7*). Heterotrophic growth of the culture was obtained with yeast extract, but it appeared that this growth was that of a heterotroph that resembled *Sulfolobus* in morphology rather than the growth of a clone of *S. solfataricus* which had lost the capacity for chemolithotrophic growth (Norris, unpublished work). It is this heterotrophic strain that was used in this work and is, pending further characterization, still referred to as *S. solfataricus* in this article. The iron-, sulfur-, and pyrite-oxidizing *Sulfolobus brierleyi* (since renamed *Acidianus brierleyi*), and the sulfur-oxidizing *Sulfolobus* B6-2 were kindly provided by J. A. Brierley and W. Zillig, respectively.

The acidophilic, moderately thermophilic, gram-positive strains ALV and TH3 were isolated from coal spoil and copper leach dump samples, respectively, and are both capable of pyrite oxidation as well as heterotrophic growth on yeast extract (*9*). The obligately heterotrophic and moderately thermophilic strains K2 and N were isolated from acid coal spoil in Warwickshire, UK, and from a hot spring in Yellowstone National Park, USA, respectively (Norris, unpublished work); these strains have not been fully characterized, but resemble acid-tolerant isolates of *Bacillus coagulans*. *Rhodococcus* TTD-1, capable of thiophene-2-carboxylate degradation (*10*), and *Pseudomonas putida* (NCIB 11767) were also used.

Growth Media and Conditions

All bacteria were grown from 5% (v/v) inocula using serial culturing in a medium containing (m*M*) $(NH_4)_2SO_4$ (1.5), $MgSO_4 \cdot 7H_2O$ (1.6), K_2HPO_4 (0.6), KCl (1.3), and $FeSO_4 \cdot 7H_2O$ (0.03). pH was adjusted with H_2SO_4 to 2.5 or 3 for growth of the acidophiles, whereas *Ps. putida* and *Rhodococcus* TTD-1 were grown at pH 7. Yeast extract (usually 0.05% w/v) and supplements of T2C and DBT were added as indicated in the Results.

Stock solutions of T2C and DBT were prepared in distilled water and dimethylformamide (final concentration 0.1% v/v in media), respectively. T2C solutions were neutralized with NaOH before addition to medium used for growth of the nonacidophilic bacteria and, where indicated, DBT was added directly to the medium in powder form. Flasks (250 mL) containing 100 mL medium were shaken at 120 rpm at 30°C for growth of the mesophiles, 50°C for strains K2, N, ALV, and TH3, and 65°C for *A. brierleyi*, *Sulfolobus* B6-2, and *S. solfataricus*. A medium containing (m*M*) NH_4Cl (3.7), $MgCl_2 \cdot 6H_2O$ (1.9), K_2HPO_4 (0.6), and $FeSO_4 \cdot 7H_2O$ (0.03), and adjusted to pH 3 with HCl was used when DBT degradation was examined.

Assays

Growth of the bacteria was followed as culture optical density at 440 nm. The concentration of cell suspensions in oxygen uptake experiments was determined by the method of Lowry et al. (*11*) using bovine serum albumin as standard. The concentration of T2C was measured spectrophotometrically at 247.5 nm, which was the peak of its absorbance spectrum, with appropriate dilutions of samples and reference to a standard curve, which was linear up to 0.1 m*M* T2C. DBT concentrations were determined by gas chromatography using a Pye Unicam 4500 capillary gas chromatograph with detector and column temperatures of 250 and 210°C, respectively. Samples (entire culture volumes) were extracted with petroleum ether using phenanthrene (0.1 g/L) as an internal standard for the extraction efficiency. After evaporation of the petroleum ether, DBT and phenanthrene were redissolved in heptane and assayed with pentadecane (0.5 g/L) as an internal standard for the injection volume.

Oxygen Electrode Experiments

Cell suspensions were prepared by centrifuging late exponential phase cultures, two washes of cell pellets in growth medium minus substrate, and resuspension in the medium at room temperature prior to use. The screening of the various bacteria for substrate-induced oxygen uptake involved adding the potential substrates (0.1 mL) to a suspension of cells (1.9 mL) in a Clark oxygen electrode cell (Rank Bros., Cambridge, UK). The effect of the T2C concentration on oxygen uptake by *Sulfolobus solfataricus* was measured following addition of 0.1-mL vol of a concentrated cell suspension to 1.9 mL medium and substrate already equilibrated at 65°C in the electrode chamber.

Chemicals

Analar-grade inorganic salts and phenanthrene were obtained from Fisons (UK), yeast extract from Lab M, (Lancashire, UK), and dibenzothiophene, thiophene-2-carboxylate, heptane, petroleum ether, dimethylformamide, and pentadecane from Aldrich Chemical Co. (Dorset, UK).

RESULTS

Effects of Dibenzothiophene (DBT) and Thiophene-2-Carboxylate (T2C) on Growth

Growth of the acidophilic bacteria was generally prevented by lower concentrations of the organosulfur compounds in comparison with the neutrophilic bacteria. The higher toxicity of T2C probably reflected its greater solubility in comparison with that of DBT. *Pseudomonas putida* and *Rhodococcus* TTD-1 were not inhibited by 0.1 g DBT or T2C/L.

Moderate Thermophiles

With yeast extract (0.025% w/v) as the carbon source at pH 2 and 50°C, the obligately heterotrophic acidophiles (strains K2 and N) grew more rapidly than the bacteria which were also capable of chemolithotrophic growth (strains TH3 and ALV). The approximate doubling time of strains K2 and N was slightly less than 2 h compared to about 7 h for strains TH3 and ALV (these latter strains being capable of more rapid growth when oxidizing ferrous iron in the presence of yeast extract; data not shown). The optimum pH for growth of strains K2 and N was 3–4, whereas optimum heterotrophic growth of strains TH3 and ALV occurred at pH 2–2.5.

None of these bacteria appeared capable of growth with DBT or T2C as a sole carbon source, and all were sensitive to low concentrations of T2C when utilizing yeast extract. Growth of strains N, K2, TH3, and ALV on 0.05% (w/v) yeast extract occurred with only a slight reduction of yield in the presence of 1 mg T2C/L, but was prevented in the presence of 5 mg T2C/L (data not shown).

Growth of strains TH3 and ALV on yeast extract was slightly retarded in the presence of 0.1 g DBT/L, although the yield was unaffected. This concentration of DBT did not affect the growth of the obligately heterotrophic strains N and K2.

Extreme Thermophiles

The growth on yeast extract of the obligately heterotrophic *Sulfolobus solfataricus* at 68°C with a doubling time of about 10 h was slightly more than twice as rapid as the growth of *Acidianus brierleyi* and *Sulfolobus* B6-2

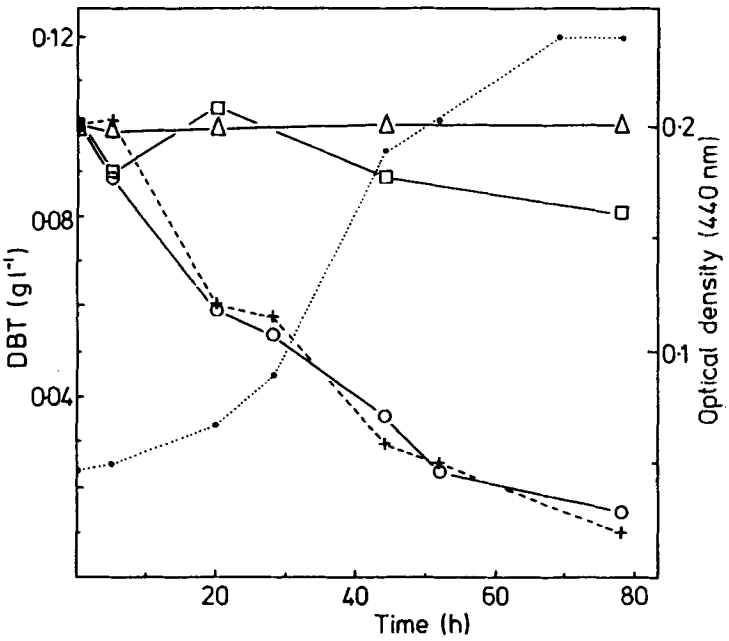

Fig. 1. The concentration of DBT (initially 0.1 g/L) in the medium during incubation without bacteria at 30°C (△), 50°C (□), and 68°C (○), and during the growth of *S. solfataricus* (culture optical density,•) at 68°C (+).

under the same conditions, these latter bacteria being capable of more rapid growth chemolithotrophically (data not shown). As with the moderate thermophiles, these archaebacterial acidophiles did not grow with the organosulfur compounds as sole carbon sources. The growth of *S. solfataricus* on yeast extract was less sensitive to T2C, with inhibition between 10–50 mg T2C/L, than that of the moderate thermophiles. The exponential growth phase of *S. solfataricus* utilizing yeast extract was shortened when 0.1 g DBT/L was in the medium, and the growth of *A. brierleyi* was more severely inhibited.

Degradation of DBT and T2C

DBT and T2C showed contrasting stabilities at high temperature. In the absence of bacteria, some loss of DBT (added in powder form) from an initial 0.1 g/L was seen at 50°C, and complete loss was approached during incubation at 68°C for 80 h (Fig. 1). The growth of *S. solfataricus* in the medium initially containing 0.1 g DBT/L (and only a trace sulfate ion concentration; *see* Methods) was substantial by the time about half of the DBT would have been lost abiotically, but did not increase the rate of its disappearance (Fig. 1). In the absence of bacteria, the initial concentration of T2C in solution (0.1 g/L) was maintained at 68°C for 80 h (data not shown). However, a simple examination of any degradation by *S. solfatar-*

icus of the T2C at readily measurable concentrations of this compound in the growth medium was precluded by interference from yeast extract with the spectrophotometric assay for the T2C and by the T2C toxicity to this strain. The interaction of the bacteria and T2C was assessed through the measurement of respiration using an oxygen electrode and resting cell suspensions.

Effect of T2C on Oxygen Uptake

The addition of pyruvate (1 m*M*) to washed cell suspensions of *Pseudomonas putida* at 30°C resulted in oxygen consumption of 400 nmol/min/mg protein. The rate of oxygen uptake increased from 4 (endogenous respiration) to 10 nmol/min/mg protein on addition of T2C (0.1 g/L, 0.78 m*M*), but this concentration appeared inhibitory in that subsequent addition of pyruvate stimulated oxygen consumption at about half the rate seen with pyruvate in the absence of T2C.

Moderate Thermophiles

The heterotrophic metabolism of the chemolithotrophic acidophiles has been little studied, since it has not been an area of special interest with these mineral-leaching bacteria. However, succinate was found to be a suitable substrate for establishing the potential for exogenous respiration of carbon compounds by cell suspensions of all the acidophiles prior to the addition of the organosulfur compounds.

Considering the obligate heterotrophs, more attention was given to strain K2, since it was found that a washed cell suspension of this strain was much more active than one of strain N, even though these bacteria appeared to be at least superficially similar from their growth characteristics and their morphology. The rate of respiration of strain K2 at 45°C was increased from 42 (endogenous respiration) to 219, 112, 122, and 169 nmol O_2 consumed/min/mg protein following the addition of succinate, acetate, glucose (all 1 m*M*), and pyruvate (0.1 m*M*), respectively. The rate of respiration following the addition of T2C (0.78 m*M*) was 12.7 nmol O_2 consumed/min/mg protein and, thus, reduced to less than the endogenous rate of the cells to which no substrate had been added. The prior exposure of strains K2 and N to subinhibitory levels of T2C during growth on yeast extract did not induce oxidation of T2C by the cells.

The oxygen uptake rate by strain TH3 at 45°C was 30.5 nmol O_2 consumed/min/mg protein in the absence of added substrate and remained at this rate following addition of T2C (to 0.78 m*M*). The addition of succinate (1 m*M*) to the cells after the addition of T2C increased the rate to 84 nmol/min/mg protein, which illustrated some residual inhibition by the T2C when compared with the 296 nmol O_2 consumed/min/mg protein when succinate (1 m*M*) alone was added. With strain ALV, the rate of oxygen consumption at 45°C was reduced from 14.7 in the absence of

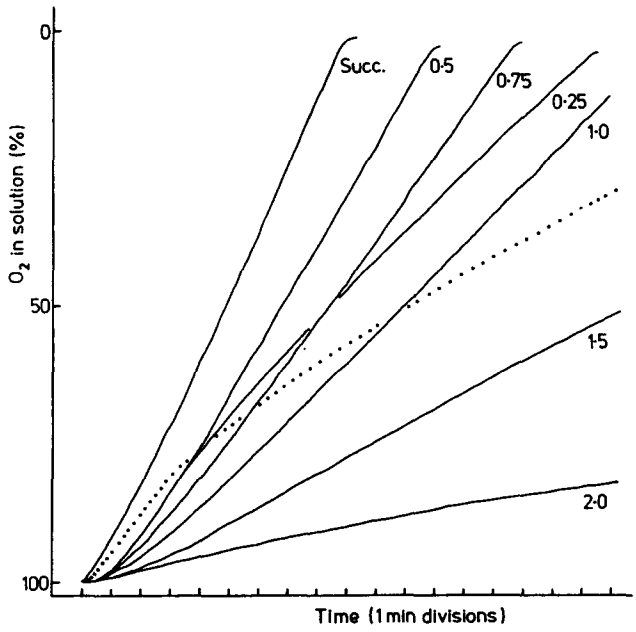

Fig. 2. Superimposed oxygen electrode traces showing oxygen consumption at 65°C following addition of *S. solfataricus* (0.32 mg protein/mL) to medium without substrate (dotted line) and to medium containing succinate (1 m*M*) or the indicated concentrations (m*M*) of T2C.

added substrate to 3.6 nmol/min/mg protein following T2C addition (to 0.78 m*M*), but increased to 38 nmol min/mg protein by the addition of succinate (1 m*M*) alone.

Extreme Thermophiles

The sulfur-oxidizing *Acidianus brierleyi* and *Sulfolobus* B6-2, which were grown in a medium containing yeast extract (0.05% w/v) and T2C (10 mg/L), were unable to oxidize T2C (0.5 m*M*). At 65°C, their rate of oxygen consumption (endogenous respiration) increased 2- to 2.5-fold on addition of succinate (1 m*M*). In contrast, oxygen uptake by *Sulfolobus solfataricus* at 65°C increased from 3- to 4.5-fold on the addition of succinate (1 m*M*), and T2C was also oxidized. When *S. solfataricus* was grown in the presence of T2C (10 mg/L), the cell pellets after centrifugation were darker (light brown), and T2C oxidation rates were almost doubled in comparison with cells not previously exposed to T2C. Both sets of cells showed similar specific rates of succinate oxidation. The oxygen electrode traces are shown to illustrate the effect of the T2C concentration on its oxidation by *S. solfataricus*, which had been grown on yeast extract (0.05% w/v) in the presence of T2C (Fig. 2). A steady rate of oxygen uptake could not be obtained for the lower concentrations of T2C (0.1 m*M*, trace not

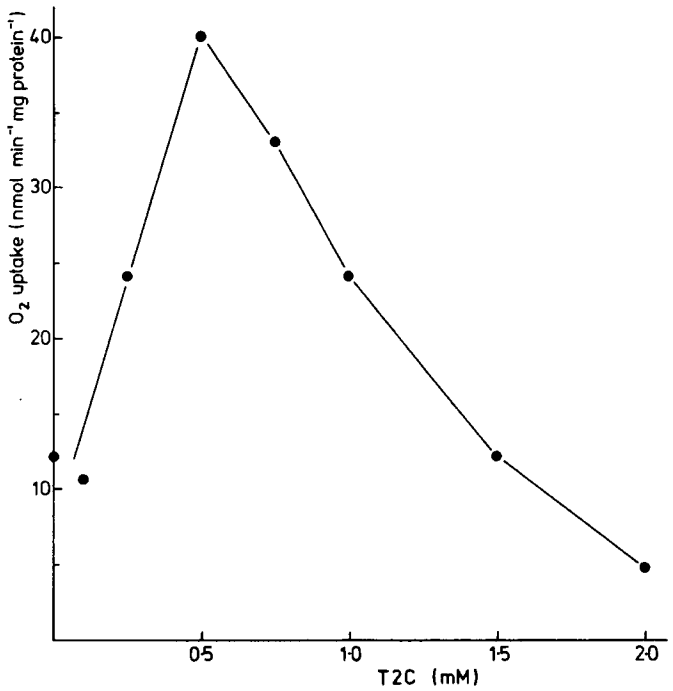

Fig. 3. The rate of oxygen consumption by *S. solfataricus* at different T2C concentrations (from Fig. 2, *see text*).

shown, and 0.25 m*M*, Fig. 2). The initial rates possibly reflected some of the oxygen uptake that would have occurred in the absence of any additions (Fig. 2), and the subsequent uptake rate (particularly with 0.1 m*M* T2C) steadily declined towards the endogenous rate. Steady rates of oxygen consumption were established with between 0.25–1 m*M* T2C, whereas higher concentrations restricted the consumption to less than that observed in the absence of added substrates. The influence of the T2C concentration has been summarized (Fig. 3) by selecting the maximum oxygen uptake rates obtained with 0.5–1 m*M* T2C, the endogenous rate about 10–15 min after adding the cells to the electrode chamber (avoiding the rapid, initial rate of oxygen uptake), and the rates at the higher T2C concentrations also after about 15 min, which in these cases was before maximum inhibition was reached (*see* Fig. 2). *S. solfataricus* also oxidized thiophene-3-carboxylate (T3C), although three times more slowly than T2C (data not shown), but did not oxidize DBT.

DISCUSSION

Two major problems were encountered in trying to assess the capacity of the thermophilic bacteria to degrade thiophenes. First, the acidophiles were easily inhibited by T2C, the moderate thermophiles in particular

being inhibited at concentrations probably several hundred-fold lower than those inhibiting the neutrophiles. The high toxicity of T2C to the acidophiles could arise through the mechanism proposed to explain the sensitivity of acidophilic *Thiobacillus ferrooxidans* to organic acids (*12*). If there is uptake of uncharged T2C from the acid medium as a result of the large transmembrane pH difference, it could be followed by dissociation of its carboxyl group at the nearly neutral internal pH and lead to acidification of the cytoplasm. Alternatively, accumulation could be followed by a specific inhibition reaction.

The second problem was the instability of DBT at high temperature in the absence of bacteria. On the basis of a lack of sulfate solubilization, DBT was reported to be stable under similar conditions in an earlier work that showed DBT degradation by *Sulfolobus acidocaldarius* (*6*). Bacterial DBT oxidation was not seen in the present study, but the oxidation of T2C by *S. solfataricus* confirms that some acidophilic archaebacteria can degrade thiophenes. The pyrite-oxidizing *Acidianus brierleyi* and sulfur-oxidizing *Sulfolobus* B6-2 did not oxidize T2C, however, and whether a single strain might efficiently degrade pyrite and thiophenes requires further study with reference to the many types of *Sulfolobus*-like bacteria that have now been isolated. The application of separate pyrite- and organosulfur compound-degrading strains in a mixed culture in a single-stage coal desulfurization process might be feasible if more active acidophilic examples of the latter strains could be identified. The complete mineralization of DBT has already been obtained with enrichment cultures of mesophiles from soils and sediments at neutral pH (*13*) and with a pure culture of a species of *Brevibacterium* (*14*). The oxidation of T2C by *S. solfataricus* was slower than that seen with some species of *Vibrio* (*15*), but the activity shown by the thermophilic archaebacterium is sufficient for further study of the degradation pathway and eventually a comparison with that in the eubacteria where the pathway also remains to be resolved (*10,12,15,16*).

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