APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1996, p. 4243–4246 Vol. 62, No. 11
0099-2240/96/$04.00+0

Multiple Serotypes of the Moderate Thermophile *Thiobacillus caldus*, a Limitation of Immunological Assays for Biomining Microorganisms

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Received 9 April 1996/Accepted 5 September 1996

Phylogenetic and phenotypic analysis indicates that a moderately thermophilic isolate, C-SH12, from Australia belongs to the species *Thiobacillus caldus*. Antiserum generated against whole cells of *T. caldus* KU recognized protein antigens common to cell lysates of the three *T. caldus* strains KU, BC13, and C-SH12 but did not recognize whole cells of isolate C-SH12. Differences in the lipopolysaccharide (LPS) of strain C-SH12 and those of the other two *T. caldus* strains were found, and the anti-KU antiserum did not recognize the LPS from strain C-SH12. These data indicate that this *T. caldus* isolate belongs to a serotype different from that of strains KU and BC13.

The identification and especially the enumeration of bacteria in leaching systems are difficult tasks because of the presence of mineral particles. Also, it is difficult to obtain growth of leaching bacteria on defined solid media (6), although media which allow the growth of a variety of iron-oxidizing acidophiles have been developed recently (11). Even with this development, it is still difficult to distinguish bacteria by colony morphologies alone, and the incubation time for the development of colonies is usually on the order of 1 week. Therefore, other, quicker means of identification and enumeration of leaching bacteria have been developed.

One attempt in this direction has included the development of species-specific DNA probes for *Thiobacillus ferrooxidans* (18). Another means of identification and enumeration of leaching bacteria is through the use of immunochemistry techniques. Antibodies generated against whole cells of *T. ferrooxidans* were used in a study of the involvement of this species in the desulfurization of coal (14). Subsequent studies by another group showed that approximately 10^3 to 10^4 cells of *T. ferrooxidans* (2) and *Leptospirillum ferrooxidans* (10) could be rapidly counted in leaching systems by using antibodies. This same approach was taken with thermophilic acidophiles, including *Thiobacillus caldus* KU and BC13 and *Sulfolobus acidocaldarius* BC65 (1).

In leachates from a pilot-scale leaching plant in Sweden operating at 45°C, the sulfur-oxidizing *T. caldus* was found to constitute approximately 10% of the total bacteria present (1). Sulfur-oxidizing strains have been isolated from pilot-scale continuous leaching reactors in Australia which were operating at temperatures above 40°C, and the 16S rRNA gene of these strains was shown to be highly homologous to the 16S rRNA gene from *Thiobacillus* strain BC (3), now known as *T. caldus* BC13 (4). The data presented in this work show that one of the Australian isolates, strain C-SH12, is probably a third strain of *T. caldus* and that this strain belongs to a different serotype than that of the other two *T. caldus* strains, namely KU and BC13.

The three strains of bacteria used in this study were *T. caldus* KU (DSM 8584, ATCC 51756) and BC13 (ATCC 51577), from our laboratory stocks, and strain C-SH12, obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany, as DSM 9466. The bacteria were grown with 5 mM tetrathionate as the growth substrate as described previously (4) except that 0.02% (wt/vol) yeast extract was included in the growth medium (3). In one experiment, 0.5% (wt/vol) elemental sulfur replaced tetrathionate as the growth substrate.

Cell lysates for polyacrylamide gel electrophoresis (PAGE) analysis were prepared by suspending 5 mg (wet weight) of bacteria in 100 μl of sample buffer (0.625 M Tris-HCl, [pH 6.8], 2% [wt/vol] sodium dodecyl sulfate [SDS], 1% [vol/vol] β-mercaptoethanol, 10% [vol/vol] glycerol, and 0.001% [wt/vol] bromophenol blue). Samples for lipopolysaccharide (LPS) analysis were prepared as described above with the additional treatment of the lysates with proteinase K as previously described (8). LPS was separated in 15% (wt/vol) polyacrylamide gels without SDS, and this was followed by LPS-specific silver staining (8). After PAGE, the proteins and LPS were blotted onto nitrocellulose membranes with a semidry blotting apparatus (Bio-Rad). Western blot (immunoblot) analysis was performed as previously described (1).

The sequence of the 16S rRNA gene from strain C-SH12 was obtained from the EMBL database (accession number, X72851) and was found to be 99.8% identical over 1,354 bases with the gene from the *T. caldus* isolate KU. The 16S rRNA gene sequence from strain C-SH12 was aligned with the sequences of the other thiobacilli obtained from the ribosomal database project (13) by using CLUSTAL V (7). This alignment was used to construct a phylogenetic tree by the neighbor-joining method (16) provided in the PHYLIP software, version 3.5c, obtained from Joseph Felsenstein, University of Washington.

The phylogenetic tree (Fig. 1) shows that strain C-SH12 is highly related to the *T. caldus* strains KU and BC13. In addition to showing the relatedness indicated by the 16s rRNA analysis, the three strains of bacteria share many common phenotypic characteristics. All three acidophilic strains have a higher optimal growth temperature, 45 to 50°C, than do the other acidophilic thiobacilli (3, 4). In addition, the three strains use reduced inorganic sulfur compounds, but not ferrous iron or metal sulfides, as growth substrates (3, 4). Taken together, these data indicate that strain C-SH12 is another strain of *T. caldus.*

We have recently described an immunological method, designated the slot immunobinding assay, to detect the moderately thermophilic *T. caldus* strains KU and BC13 (1). Whether the antiserum generated against strain KU, which recognizes

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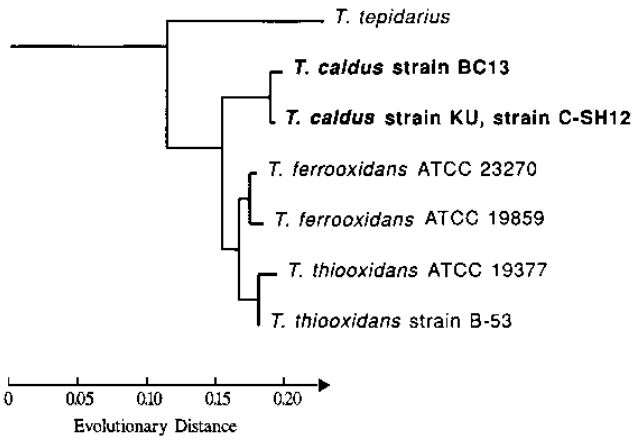

FIG. 1. Phylogenetic tree based on 16S rRNA sequences and rooted with *Escherichia coli* as the outgroup (not shown). The three strains used in this study are indicated in boldface print.

whole cells of both *T. caldus* KU and BC13, would recognize the Australian isolate C-SH12 was also of interest. The antiserum generated against whole cells of *T. caldus* KU recognized equally well whole cells of strain KU grown on tetrathionate and those grown on sulfur (Fig. 2). In contrast, the antiserum recognized whole cells of *T. caldus* C-SH12 only slightly, irrespective of the growth substrate, as evidenced by the faint signals in the slots to which these bacteria were applied (Fig. 2).

Since the phylogenetic and phenotypic data indicated that the three strains belong to the same species and yet the anti-KU antiserum only slightly recognized strain C-SH12, we further characterized the nature of the immunological reactions of the antiserum with the three isolates. Cell lysates of the three strains of bacteria were analyzed by SDS-PAGE. Coomassie staining of the separated proteins showed that all three strains exhibited almost identical protein profiles (Fig. 3A), further indicating the relatedness of C-SH12 to *T. caldus* KU and BC13.

After separation of cell lysates by SDS-PAGE and blotting onto a nitrocellulose filter, the filter was incubated with the anti-KU antiserum (Fig. 3B). Several proteins with identical molecular weights from each of the three strains reacted with the antiserum. In the previous study using the same anti-KU antiserum (1), several cross-reacting proteins from lysates of *T. ferrooxidans* and *Thiobacillus thiooxidans* were identified, but

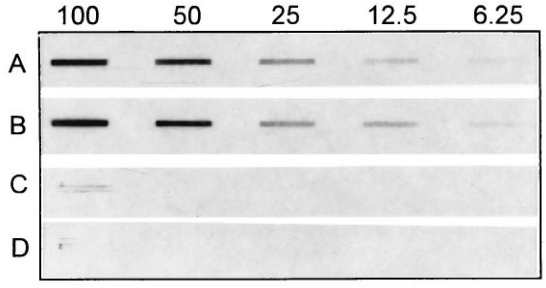

FIG. 2. Slot immunobinding assay of *T. caldus* KU grown on sulfur (row A) and tetrathionate (row B) and C-SH12 grown on sulfur (row C) and tetrathionate (row D). The numbers above the slots indicate the number of bacteria (10^4) which were applied to the nitrocellulose filters. The anti-KU antiserum used was diluted 1:5,000.

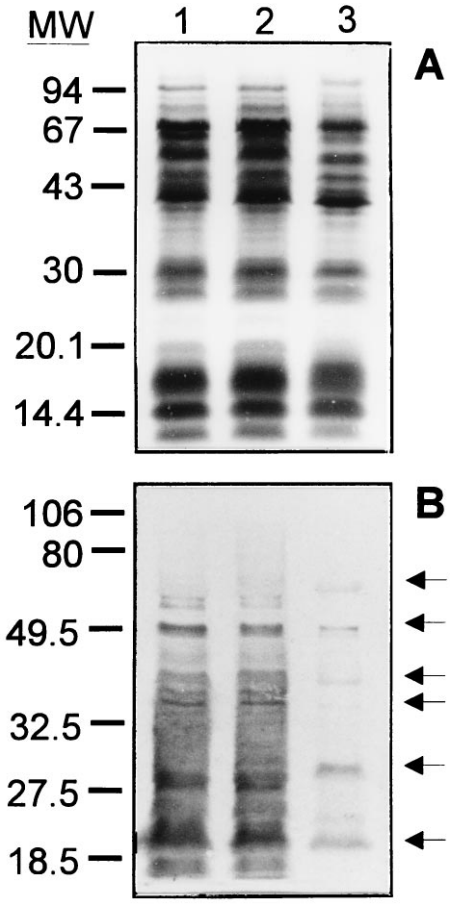

FIG. 3. SDS-PAGE of *T. caldus* cell lysates separated on SDS–12% (wt/vol) PAGE gels. The proteins were visualized by staining with Coomassie blue (A) and Western blotting of a duplicate gel using the anti-KU antiserum diluted 1:5,000 (B). Protein antigens common to the three strains are indicated by arrows. Lane 1, strain KU; lane 2, strain BC13; and lane 3, strain C-SH12. The positions of molecular weight markers (MW) are given in thousands.

the size and the number of proteins as well as the strength of the reaction differed from those of the cross-reacting proteins found in C-SH12. Taken together, the SDS-PAGE data and Western blot data indicate that the anti-KU antiserum recognizes common protein antigens from all three of the *T. caldus* strains used in this study but does not recognize whole cells of strain C-SH12.

A common antigenic component of gram-negative bacteria is the LPS. We therefore analyzed the LPSs of the three moderate thermophiles and determined the reaction of each LPS with the anti-KU antiserum. As shown by the LPS-specific silver staining of the cell lysates separated by PAGE, all three *T. caldus* strains have LPSs (Fig. 4A). The pattern of banding of the LPSs was visualized more easily after treatment of the lysates with proteinase K. Each of the three strains had a ladder-like banding pattern indicative of a smooth-type LPS. Some minor differences in the banding pattern of the LPS of strain C-SH12 and the other two *T. caldus* strains, which had similar LPS patterns (compare lanes 2, 4, and 6 of Fig. 4A), were noted. These differences were more pronounced at higher molecular weights.

The antigenic relatedness of the LPSs from the three strains was determined by using anti-KU antiserum. The anti-KU antiserum recognized the LPSs of both *T. caldus* KU and *T.*

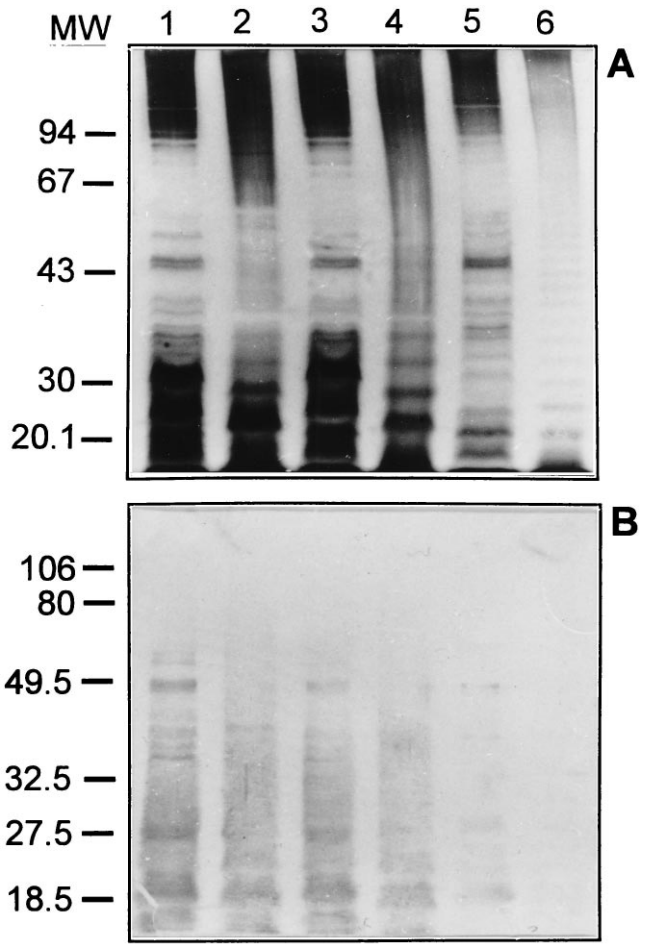

FIG. 4. PAGE analysis of *T. caldus* LPS followed by silver staining (A) and Western blotting of a duplicate gel (B) of cell lysates from strain KU (lane 1), strain BC13 (lane 3), and strain C-SH12 (lane 5) or proteinase K-treated cell lysates from strain KU (lane 2), strain BC13 (lane 4), and strain C-SH12 (lane 6). The positions of molecular weight markers (MW) are given in thousands.

caldus BC13 (Fig. 4B). In contrast, the antiserum only slightly recognized the LPS from *T. caldus* C-SH12. Most of the recognition of the *T. caldus* C-SH12 lysate was due to proteins, as the signals disappeared when the lysate was treated by proteinase K. The anti-KU antiserum slightly recognized the low-molecular-weight region of the LPS from *T. caldus* C-SH12, which could account for the slight recognition of whole cells of this isolate by the anti-KU antiserum. The low-molecular-weight substance of *T. caldus* C-SH12 recognized by the anti-KU antiserum is probably the core lipid A-oligosaccharide region of the LPS to which the repeating O-specific antigen subunits are attached (9). It is the O-specific antigen which is the most antigenic part of LPS and is used in determining the different serotypes of bacteria (15). The lack of recognition by the antiserum is most likely due to the fact that the LPS of strain C-SH12 differs from that of the other two *T. caldus* strains, and thus C-SH12 can be considered to belong to a serotype different from that of strains KU and BC13.

The finding that the three strains of *T. caldus* examined to date belong to two different serotypes is significant. Examination of the LPSs from iron-oxidizing bacteria presumptively identified as *T. ferrooxidans* has revealed at least six different LPS structures (17). The six different LPS structures were identified by differences in appearance of the LPS following PAGE analysis. This LPS variation could be explained by the fact that *T. ferrooxidans* is represented by several different DNA homology groups (5), indicating that bacteria commonly identified as belonging to this species could actually belong to other, undefined species.

In another study, it has been found that *T. ferrooxidans* ATCC 23270, ATCC 19859, and DSM 583 belong to the same serotype while four other *T. ferrooxidans* strains each belong to their own serotype (12). The three strains in the one serotype are members of one DNA homology group, as defined by Harrison (5), while it is not known to which group the other *T. ferrooxidans* strains used in the study belong. As in the study presented here, the LPSs of the bacteria were the major antigens recognized by the antisera used in whole-cell immunoassays (12). The occurrence of LPSs with different antigenic characteristics among different strains of the same species indicates that acidophiles can belong to different serotypes as do other gram-negative bacteria.

With the presence of different serotypes of biomining microorganisms such as *T. ferrooxidans* or *T. caldus*, caution must be used when interpreting results from immunoassays of such organisms. Even though such assays could be suitable for use in the commercial leaching environment, it must be noted that the antisera used could fail to recognize all bacteria present in the leaching environment, leading to the underestimation of bacterial numbers. However, this potential drawback to the use of immunoassays can be avoided by the selection of an antiserum which recognizes a common antigen, such as a protein which is shared among bacteria of the same species or even among bacteria of other species. Also, monitoring one or a couple of organisms which have a large influence on the bioleaching reaction could allow a simple and immediate means to determine the health of the microorganisms, which could be predictive of the entire bioleaching process.

We thank Siv Sääf for her excellent technical assistance.

This research was supported by grants from Närings- och Teknikutvecklingsverket (NUTEK), Boliden Mineral AB, SAREC, and the Carl Tryggers Foundation. K.B.H. received additional support from the J. C. Kempe Foundation, Umeå, Sweden.

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