APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1994, p. 3470–3473 Vol. 60, No. 9
0099-2240/94/$04.00+0

An Immunological Assay for Detection and Enumeration of Thermophilic Biomining Microorganisms

ANA M. AMARO,[1] KEVIN B. HALLBERG,[2] E. BÖRJE LINDSTRÖM,[2] AND CARLOS A. JEREZ[1]*

Department of Applied Cell and Molecular Biology, University of Umeå, S-901 87 Umeå, Sweden,[2] and Departamento de Bioquímica, Facultad de Medicina, Universidad de Chile, Santiago, Chile[1]

Received 2 February 1994/Accepted 29 June 1994

A specific, fast, and sensitive nonradioactive immunobinding assay for the detection and enumeration of the moderate thermophile *Thiobacillus caldus* and the thermophilic archaeon *Sulfolobus acidocaldarius* was developed. It employs enhanced chemiluminescence or peroxidase-conjugated immunoglobulins in a dot or slot blotting system and is very convenient for monitoring thermophilic bioleaching microorganisms in effluents from industrial bioleaching processes.

A number of chemolithotrophic and heterotrophic microorganisms are normally present in bioleaching operations (5, 7). To study their ecological relationships and time evolution during the process, specific methods for the identification and enumeration of bioleaching microorganisms are required. Several approaches have been taken in this direction (14, 19). We have previously developed a sensitive, specific, and convenient dot immunobinding assay to enumerate *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* (1, 8), which are apparently the main bacteria involved in bioleaching systems (5, 17, 18).

In recent years, new thermophilic and moderate thermophilic microorganisms have been described and their role in bioleaching has been studied (2, 13, 15). The extreme thermophilic archaeon *Sulfolobus acidocaldarius* used in high-temperature leaching exhibits high oxidation rates (10, 12). Other moderate thermophilic microorganisms also offer a good potential for use in controlled industrial leaching (3, 15).

Although a plating method has been described for *S. acidocaldarius* (11), quick and sensitive methods for the identification of thermophiles and moderate thermophiles are not currently available. We therefore have developed a nonradioactive immunobinding assay for monitoring strains of the new moderate thermophile *Thiobacillus caldus* (4) and the thermophilic *S. acidocaldarius*. The method can be used by applying the samples to a nitrocellulose membrane in the form of dots or slots, and will be referred to as the dot immunobinding assay (DIMA) or slot immunobinding assay (SIMA), respectively. For detection of the bound immunoglobulins, we have employed both commercially available enhanced chemiluminescence (Amersham International) and peroxidase-conjugated antibodies (Bio-Rad Laboratories).

S. acidocaldarius BC65 was grown in liquid medium as described previously (11). *T. caldus* KU was isolated from a sample of moderate thermophiles kindly supplied by Paul Norris. The moderate thermophilic isolate BC13 was also provided by P. Norris. Both microorganisms were grown and maintained in autotrophic medium consisting of the basal salts (with grams liter^{-1} in parentheses) $(NH_4)_2SO_4$ (3.0), $Na_2SO_4 \cdot 10H_2O$ (3.2), KCl (0.1), K_2HPO_4 (0.05), $MgSO_4 \cdot 7H_2O$ (0.5), and $Ca(NO_3)_2$ (0.01) and the following trace elements (with milligrams liter^{-1} in parentheses): $FeCl_3 \cdot 6H_2O$ (11.0), $CuSO_4 \cdot 5H_2O$ (0.5), H_3BO_3 (2.0), $MnSO_4 \cdot H_2O$ (2.0), $Na_2MoO_4 \cdot 2H_2O$ (2.0), $CoCl_2 \cdot 6H_2O$ (0.6), and $ZnSO_4 \cdot 7H_2O$ (0.9). The basal salts were adjusted to pH 2.5 with H_2SO_4 and autoclaved before the filter-sterilized trace elements were added. The growth temperature was generally 45°C, and the growth medium was sparged with CO_2-enriched air (2%, vol/vol) (4). *T. ferrooxidans* ATCC 19859 and *T. thiooxidans* ATCC 19377 were grown as described previously (1, 8, 9), and *T. acidophilus* ATCC 27807 was grown as described by Pronk et al. (16).

Polyclonal antibodies against whole cells of *T. caldus* KU and *S. acidocaldarius* were generated as described before (1, 8). For the DIMA and SIMA, the different types of cells were applied to nitrocellulose membranes (Trans-Blot transfer medium; Bio-Rad Laboratories) by using a slot or dot blot apparatus (Bio-Rad Laboratories) according to the instructions of the manufacturer. The number of cells applied was determined by direct counting under a microscope. The samples were then fixed by heating the membrane under an infrared lamp for 10 min. The membranes were then blocked with skim milk as previously described (1) and incubated in the diluted primary antiserum (1:3,000 or 1:5,000 in Tris-buffered saline–1% bovine serum albumin) according to the instructions given for the ECL Western blotting (immunoblotting) analysis system (Amersham International), except that five washings in the presence of 0.5% Tween 20 for 10 min each were done. With this modification, a much lower background was obtained. The membranes were exposed to X-ray film for various times (usually between 10 and 60 s). The development of the colorimetric immunological reaction by using a peroxidase-conjugated immunoglobulin was done as described previously (1, 8). For quantification, the autoradiograms or color-developed membranes were scanned with a Scan Jet Plus device (Hewlett-Packard) and the images were processed with a GelPerfect image analysis program (developed by S. Bozzo, Universidad de Chile).

To determine the nature of the antigens recognized by the antisera prepared against thermophiles and moderate thermophiles, the different bacterial cells were lysed in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. After the total lysates were subjected to SDS-PAGE, the cell components were transferred to a nitrocellulose membrane and immunoblotted as shown in Fig. 1. In general, the products reacting with the antibodies appeared to be proteins, since they did not show the typical ladder-like pattern characteristic of the lipopolysaccharide (LPS) present

* Corresponding author. Mailing address: Departamento de Bioquímica, Facultad de Medicina, Universidad de Chile, Casilla 70086, Santiago 7, Chile. Phone: (56 2) 735 6384. Fax: (56 2) 735 5580.

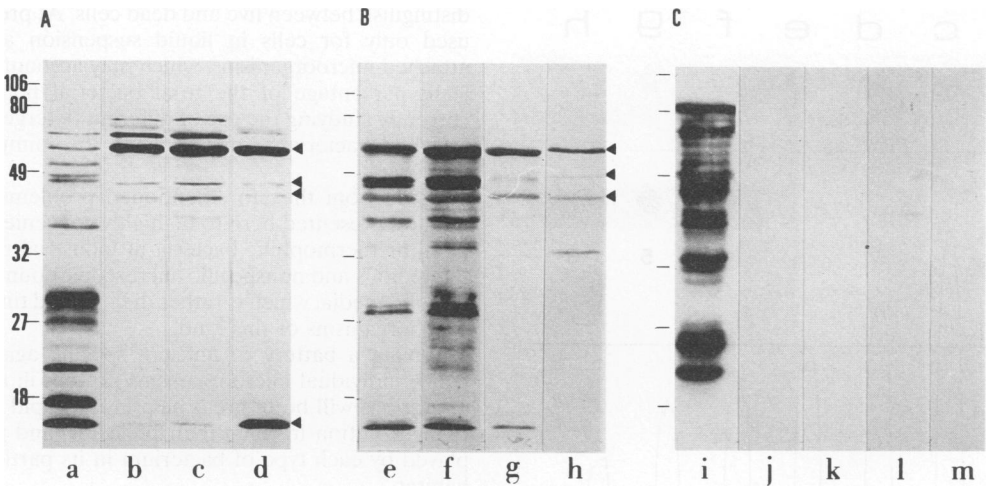

FIG. 1. Detection of the main antigenic components in different microorganisms by Western blotting. Twenty-five micrograms (wet weight) of *T. ferrooxidans* (lanes a, h, and m), *T. caldus* KU (lanes b, e, and j), isolate BC13 (lanes c, f, and k), *T. thiooxidans* (lanes d, g, and l) or *S. acidocaldarius* (lane i) was separated by SDS–12% PAGE, and the products were transferred to a nitrocellulose membrane. The membrane was reacted with antiserum against *T. ferrooxidans* (1:3,000) (A), *T. caldus* KU (1:5,000) (B), or *S. acidocaldarius* (1:5,000) (C) and was developed by the chemiluminescence assay. Arrowheads, protein bands apparently common to the different microorganisms. Molecular weights (in thousands) of prestained standards are indicated on the left.

in gram-negative bacteria (6). This was also clear in the case of *T. ferrooxidans* (Fig. 1A). In the case of *S. acidocaldarius*, no LPS-like products should be expected, and therefore, all the bands observed probably corresponded to proteins. Figure 1C shows that the antiserum against *S. acidocaldarius* was very specific for the archaeon used, giving no reaction with any of the moderate thermophilic or mesophilic bacterial species tested. Figures 1A and B also show the existence of some cross-reaction between the lysates of the different microorganisms. For example, the bands labeled with arrowheads appear to be common to both *T. ferrooxidans* and *T. caldus* KU. Figures 1A and B also show that *T. caldus* KU and isolate BC13 gave essentially the same band patterns when the antiserum against *T. ferrooxidans* or *T. caldus* KU was used. This result was expected, since the moderate thermophilic isolate BC13 is also a strain of *T. caldus* (4).

It will be important to evaluate the antisera prepared against other thermophilic and moderate thermophilic microorganisms previously described (15). If some cross-reaction was to be found with some of these bacteria, it could be removed by preadsorption of the antisera with the microorganism giving the undesired reaction. Nevertheless, with similar types of antisera against *T. ferrooxidans* and *L. ferrooxidans*, we found very low, if any, cross-reaction with other microorganisms normally found in bioleaching habitats (1, 8).

To study the sensitivity of the method for the enumeration of different microorganisms in liquid suspension, we applied decreasing numbers of cells to the nitrocellulose membrane as shown in Fig. 2A. With this method we could detect as few as 10^3 bacteria in the slot. However, it is possible to detect a smaller number of microorganisms by increasing the time of exposure of the X-ray film during the chemiluminescent reaction with the secondary antibody. The specificity of the antibodies prepared against *S. acidocaldarius* was confirmed in this assay, since as shown in the SIMA of Fig. 2B, the antiserum against this archeon did not cross-react with any of the bacterial species tested. The antiserum against *T. caldus* KU was also highly specific against whole cells (Fig. 2B). The

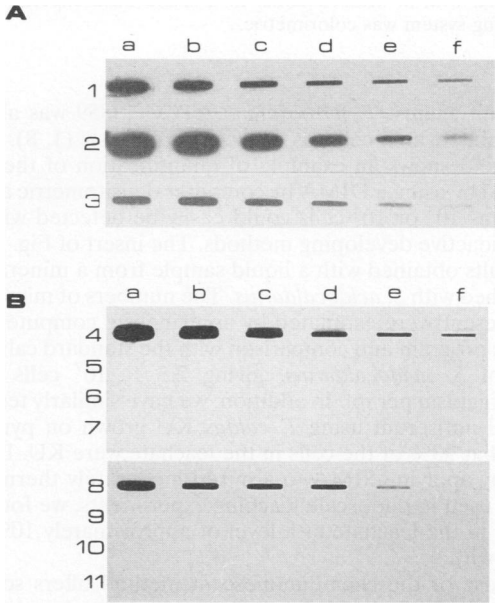

FIG. 2. Sensitivity and specificity of SIMA. (A) *T. ferrooxidans* (row 1), *T. caldus* KU (row 2), and *S. acidocaldarius* (row 3) were applied to nitrocellulose membranes, and the primary antisera employed were against the respective bacteria. (B) *T. caldus* (row 4), *T. thiooxidans* (rows 5 and 9), *T. ferrooxidans* (rows 6 and 10), *T. acidophilus* (rows 7 and 11), and *S. acidocaldarius* (row 8) were applied to nitrocellulose membranes. The primary antisera employed were that against *T. caldus* (rows 4 to 7) and that against *S. acidocaldarius* (rows 8 to 11). Antiserum dilutions and the developing method were as described for Fig. 1. The numbers of cells applied to the membranes were 1×10^5 (lane a), 5×10^4 (lane b), 2.5×10^4 (lane c), 1.25×10^4 (lane d), 6×10^3 (lane e), and 3×10^3 (lane f).

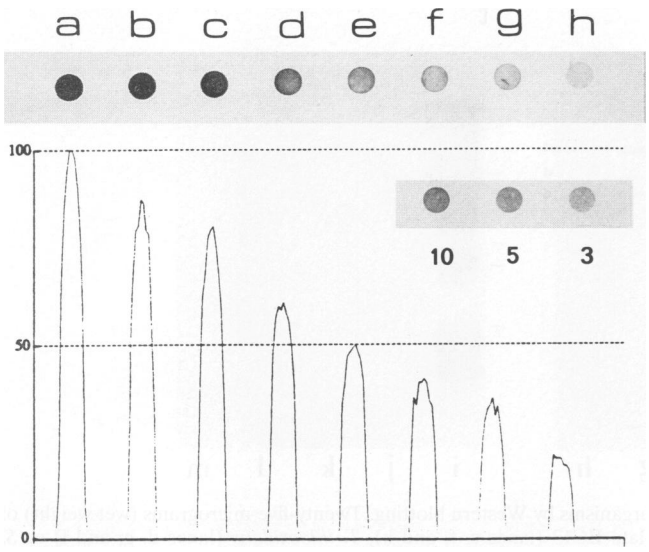

FIG. 3. DIMA of *S. acidocaldarius* and quantitation by computer image analysis. The following numbers of *S. acidocaldarius* organisms were applied to a nitrocellulose membrane: 1.5×10^6 (a), 5×10^5 (b), 2.5×10^5 (c), 1.5×10^5 (d), 5×10^4 (e), 2.5×10^4 (f), 1.5×10^4 (g), and 5×10^3 (h). The primary antiserum employed was that against *S. acidocaldarius*. The densitometric tracings of the dots were obtained by computer image analysis. The *y* axis represents the percent relative intensity of the dot, and the *x* axis reflects the relative positions of the dots. The insert shows the dots obtained with the indicated volumes (in microliters) of a liquid sample obtained from pure pyrite (1%, wt/vol) bioleached with *S. acidocaldarius* in a shake flask experiment. The developing system was colorimetric.

antiserum against *T. ferrooxidans* ATCC 19859 was also specific against whole cells, as reported previously (1, 8).

Figure 3 shows an example of quantification of the results obtained by using a DIMA by computer densitometric analysis. As few as 10^3 or 10^4 cells could easily be detected with both nonradioactive developing methods. The insert of Fig. 3 shows the results obtained with a liquid sample from a mineral being bioleached with *S. acidocaldarius*. The numbers of microorganisms present were estimated by applying the computer image analysis program and comparison with the standard calibration curve of *S. acidocaldarius*, giving 2.5×10^7 cells of this microorganism per ml. In addition, we have similarly tested the anti-KU antiserum using *T. caldus* KU grown on pyrite and found that 90% of the cells in the leachate were KU. Furthermore, by applying SIMA to a mixed moderately thermophilic culture used in pilot scale leaching experiments, we found KU present in the leachate at a level of approximately 10% (data not shown).

The use of the chemiluminescent method offers some advantages compared with the colorimetric procedure of immunodetection. Not only is it very fast (only a few seconds of exposure to X-ray film), but the X-ray films can be stored permanently without loss of intensity due to light exposure, as usually happens with color reagents. In addition, these films not only offer a higher intensity of reaction (providing better sensitivity) but allow a better photographic record and computer-assisted image analysis.

Although the current SIMA and DIMA methods for the enumeration of thermophilic and moderately thermophilic species are fast, specific, and sensitive and provide identification and enumeration results simultaneously, they cannot distinguish between live and dead cells. At present, they can be used only for cells in liquid suspension and not for ore-attached microorganisms, which may account for a very significant percentage of the total bacterial population. We are currently studying the use of different detergents to release the attached bacteria before applying the immunological detection.

Aside from the aforementioned problems, we believe the method presented here to be highly convenient and specific to monitor thermophilic bacteria in industrial reactors, avoiding the tedious and nonspecific microscopic counts and the plating on solid media, which is rather difficult and time-consuming for microorganisms of this kind.

Having a battery of antisera specific against most of the major individual microorganisms present in many bioleaching operations will be of great help in the rapid determination of their evolution in time, their behavior, and possibly, the role played by each type of bacterium in its particular bioleaching habitat.

This research was supported by grants from SAREC, Stockholm, Sweden, to both laboratories, and an UNIDO grant to C.A.J.

We thank R. Amils for a critical reading of the manuscript.

REFERENCES

1. **Arredondo, R., and C. A. Jerez.** 1989. A specific dot-immunobinding assay for detection and enumeration of *Thiobacillus ferrooxidans*. Appl. Environ. Microbiol. **55:**2025–2029.
2. **Brierley, J. A., and C. L. Brierley.** 1986. Microbial mining using thermophilic microorganisms, p. 279–305. *In* T. D. Brock (ed.), Thermophiles: general, molecular and applied microbiology. John Wiley & Sons, Inc., New York.
3. **Budden, J. R., and P. A. Spencer.** 1993. Tolerance to temperature and water quality for bacterial oxidation: the benefits of BacTech's moderately thermophilic culture. FEMS Microbiol. Rev. **11:**191–196.
4. **Hallberg, K. B., and E. B. Lindström.** Characterization of *Thiobacillus caldus* sp. nov., a moderately thermophilic acidophile. Microbiology (United Kingdom), in press.
5. **Harrison, A. P., Jr.** 1984. The acidophilic *Thiobacilli* and other acidophilic bacteria that share their habitat. Annu. Rev. Microbiol. **38:**265–292.
6. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154:**269–277.
7. **Hutchins, S. R., M. S. Davidson, J. A. Brierley, and C. L. Brierley.** 1986. Microorganisms in reclamation of metals. Annu. Rev. Microbiol. **40:**311–336.
8. **Jerez, C. A., and R. Arredondo.** 1991. A sensitive immunological method to enumerate *Leptospirillum ferrooxidans* in the presence of *Thiobacillus ferrooxidans*. FEMS Microbiol. Lett. **78:**99–102.
9. **Jerez, C. A., I. Peirano, D. Chamorro, and G. Campos.** 1986. Immunological and electrophoretic differentiation of *Thiobacillus ferrooxidans* strains, p. 443–456. *In* R. W. Lawrence, R. M. R. Branion, and H. G. Ebner (ed.), Fundamental and applied biohydrometallurgy. Proceedings of the Sixth International Symposium on Biohydrometallurgy, Vancouver, British Columbia, Canada. Elsevier/North-Holland Publishing Co., Amsterdam.
10. **Lindström, E. B., E. Gunneriusson, and O. H. Tuovinen.** 1992. Bacterial oxidation of refractory sulfide ores for gold recovery. Crit. Rev. Biotechnol. **12:**133–155.
11. **Lindström, E. B., and H. M. Sehlin.** 1989. High efficiency of plating of the thermophilic sulfur-dependent archaebacterium *Sulfolobus acidocaldarius*. Appl. Environ. Microbiol. **55:**3020–3021.
12. **Lindström, E. B., S. Wold, N. Kettaneh-Wold, and S. Sääf.** 1993. Optimization of pyrite bioleaching using *Sulfolobus acidocaldarius*. Appl. Microbiol. Biotechnol. **38:**702–707.
13. **Marsh, R. M., and P. R. Norris.** 1983. The isolation of some thermophilic, autotrophic, iron-oxidizing bacteria. FEMS Microbiol. Lett. **17:**311–315.

14. **Muyzer, G., A. C. de Bruyn, D. J. M. Schmedding, P. Bos, P. Westbroek, and G. J. Kuenen.** 1987. A combined immunofluorescence-DNA-fluorescence staining technique for enumeration of *Thiobacillus ferrooxidans* in a population of acidophilic bacteria. Appl. Environ. Microbiol. **53:**660–664.
15. **Norris, P. R.** 1990. Acidophilic bacteria and their activity in mineral sulfide oxidation, p. 3–27. *In* H. L. Ehrlich and C. L. Brierley (ed.), Microbial mineral recovery. McGraw-Hill, Inc., New York.
16. **Pronk, J. T., P. J. W. Meesters, J. R. van Dijken, P. Bos, and J. G. Kuenen.** 1990. Heterotrophic growth of *Thiobacillus acidophilus* in batch and chemostatic cultures. Arch. Microbiol. **153:**392–398.
17. **Sand, W., T. Gehrke, R. Hallmann, K. Rohde, B. Sobotke, and S. Wentzien.** 1993. In-situ bioleaching of metal sulfides: the importance of *Leptospirillum ferrooxidans*, p. 15–27. *In* A. E. Torma, J. E. Wey, and V. I. Lakshmanan (ed.), Biohydrometallurgical technologies, vol. I. Bioleaching processes. Minerals, Metals & Material Society, Warrendale, Pa.
18. **Sand, W., K. Rohde, B. Sobotke, and C. Zenneck.** 1992. Evaluation of *Leptospirillum ferrooxidans* for leaching. Appl. Environ. Microbiol. **58:**85–92.
19. **Yates, J. R., and D. S. Holmes.** 1986. Molecular probes for the identification and quantitation of microorganisms found in mines and mine tailings. Biotechnol. Bioeng. Symp. **16:**301–309.