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Immunological methods for the quantitation of the industrially important bioleaching microorganisms

Métodos inmunológicos para la cuantificación de los microorganismos biolixiviantes de importancia industrial

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To monitor the levels of *Thiobacillus ferrooxidans* in bioleaching operations, we have developed a specific and very sensitive dot-immunobinding assay. Polyclonal antisera against whole *T. ferrooxidans* cells was used, and the bacteria-antibody reaction was visualized by employing either ^{125}I-labeled or peroxidase-conjugated protein A or ^{125}I-labeled or peroxidase-conjugated goat anti-rabbit immunoglobulin G. A minimum of 10^3 cells per dot could be easily detected. Therefore, the method allows the sensitive, and specific simultaneous processing of numerous samples in a short time.

The bioleaching of minerals consists in the dissolution of ores by the action of bacteria. *Thiobacillus ferrooxidans* is the predominant species amongst a number of other chemolithotrophic and heterotrophic microorganisms usually found in bioleaching operations (Harrison Jr., 1984; Hutchins *et al.*, 1986; and Lundgren and Silver, 1980). Therefore, to asses the relative participation of each kind of microorganism in an industrial operation, specific methods for the detection and enumeration of the different bioleaching bacteria are required.

Methods currently employed involve determinations of most probable numbers or growth in solid media of iron-oxidizer microorganisms (Harrison Jr., 1984; Hutchins *et al.*, 1986 and Lundgren and Silver, 1980). However, more specific immunological methods have recently been developed. Apel *et al.* (1976), Baker and Mills (1982) and Gates and Pham (1979) have reported the use of fluorescent antibodies for the determination of pyrite-oxidizing microorganisms in acid mine drainage waters. More recently, we described the use of specific antisera against whole *T. ferrooxidans* cells (Jerez *et al.*, 1986). These antisera did not react with *Leptospirillum ferrooxidans,* one of the most common iron-oxidizers accompanying *T. ferrooxidans* in mining environments. Furthermore, the antisera were useful not only to specifically detect *T. ferrooxidans* but also to distinguish two different *T. ferrooxidans* strains based on the analysis of the immunoprecipitated radioactively labeled components by SDS-PAGE (Jerez *et al.*, 1986).

Later, Muyzer *et al.* (1987) reported the use of similar antibodies for a combined staining technique employing immunofluorescence and DNA-fluorescence to assess the *T. ferrooxidans* abundance in coal samples containing pyrite. However, no data on the sensitivity of the method was reported.

Yates *et al.* (1986), have recently developed a procedure for the identification and enumeration of bioleaching organisms, based on the utilization of cloned *T. ferrooxidans* gene sequences as probes for determining both genetic homologies between strains and amount of DNA using either dot or Southern blot assays.

We recently described the use of an alternative and convenient dot immunoassay (DIMA) which is both specific and sensitive (Arredondo and Jerez, 1989). In this work we review this method and extend its application by using simplified colorimetric procedures, which avoid the

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use of radioactive materials. This makes our method of practical use in the industrial bioleaching operations.

MATERIALS AND METHODS

Microorganisms and growth conditions. The *Thiobacillus ferrooxidans* strain employed was R2 (Rodríguez *et al.*, 1986), which was purified as single colonies by growing it in ferrous iron-containing agar as previously described by Jerez *et al.* (1986) and Arredondo *et al.* (1988). The microorganisms were then routinely grown in modified liquid 9K medium as published by MacKintosh (1978) and Tuovinen and Kelly (1973) at 30° with rotatory shaking.

Immunological procedures. Whole cells of *T. ferrooxidans* strain R2 were used as immunogens to prepare the antisera. They were titrated and checked by immunodiffusion as described by Jerez *et al.* (1986) and Arredondo *et al.* (1988).

Protein A (Sigma Chemical Co.) was labeled with ^{125}I to a specific activity of 1.7×10^7 cpm/ug as described by Arredondo and Jerez (1989) and goat IgG anti-rabbit immunoglobulin, affinity purified and labeled with the same isotope (specific activity = 2×10^7 cpm/ug), was a kind gift of A. Ferreira. Alkaline phosphatase-protein A and peroxidase-conjugated immunoglobulin were obtained from Bio-Rad laboratories.

For the DIMA assay, cells of *T. ferrooxidans* were either applied directly from the cultures to nitrocellulose membranes (Bio-Rad Trans-Blot transfer medium) or after prior concentration to a smaller volume by centrifugation at 12,000 x g for 10 min. This last procedure was used when the samples were manually applied (usually in a volume of 5 ul). Some of the samples were also applied to the membrane by using the Bio-Rad Bio-Dot apparatus according to the manufacturer's instructions. The number of *T. ferrooxidans* cells applied was determined by direct counting under the microscope.

The samples were then fixed by heating the membrane under an infrared lamp for 10 min. The membranes were then blocked as previously described by Arredondo and Jerez (1989). Briefly, they were treated with 5% powdered skim milk (Nestle) in buffer A (50 mM Tris-HCl, pH 8, 0.15 M NaCl) by shaking for 10 min at room temperature. Membranes were then incubated in normal serum or antiserum diluted in buffer A (1: 100 or 1: 1,000) for 60 min and washed 3 times, 10 min each, with 5% milk solution in buffer A, at 50 rpm. Then, 1.5 uCi of protein A or goat anti-rabbit immunoglobulin G, both labeled with ^{125}I, were added in the presence of 5% milk in buffer A and shaken for 60 min. The radiolabeled protein was removed and the membranes were washed three times in buffer A with 5% milk as described above. Finally, the nitrocellulose sheets were washed twice with buffer A, shaking for 30 min each time.

The membranes were then dried and autoradiographed (Arredondo and Jerez, 1989). The colorimetric procedures were essentially the same, except that the enzyme-conjugated components were used and the development of color was according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Fig. 1 summarizes the methods employed. As we and others have previously described, the main antigens on the surface of *Thiobacillus ferrooxidans* correspond to lipopolysaccharides (LPS) or a few outer membrane proteins (Arredondo and Jerez, 1989 and Arredondo *et al.*, 1988). Therefore, the rabbit antibodies obtained against whole *T. ferrooxidans* cells would attach to these components on the surface of the bacteria (Fig. 1a). Once the antibodies are attached to the bacteria, protein A or a second goat-antirabbit antibody radioactively labeled or conjugated with an enzyme are added. These components will attach to the first bound antibody in a specific way (Fig. 1b). To perform these reactions, the bacterial cells are previously fixed to a nitrocellulose membrane as a support (Fig. 1c). The antigen-antibody reaction is then visualized by autoradiography or by adding the enzyme substrate which originates a colored product.

An example of the application of the method, developed with ^{125}I-labeled protein A is shown in Fig. 2. We can see that there is a clear intensity of response which correlates well with the number of *T. ferrooxidans* cells applied. This is specially evident when the autoradiogram is analyzed by densitometry and the relative absorbances are plotted against cell numbers. As we previously showed (Jerez *et al.*, 1986; Arredondo and Jerez, 1989 and Arredondo *et al.*, 1988), this antiserum is specific for several strains of *Thiobacillus ferrooxidans*. It did not react against other microorganisms which are normally present in the bioleaching operations, such as *Leptospirillum ferrooxidans, Thiobacillus thiooxidans, Thiobacillus versutus, Thiobacillus intermedius, Thiobacillus neapoli-*

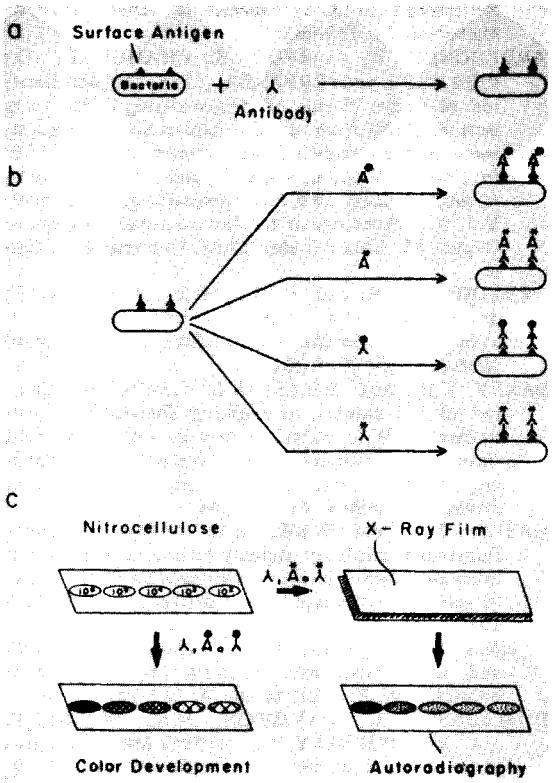

Fig. 1: Dot-Immunobinding assay (DIMA). a) *T. ferrooxidans* cells react with antibodies against its surface components. b) Once this reaction has taken place, a second reaction is performed in the presence of protein A conjugated with peroxidase (A•), ^{125}I-labeled protein A (A*), peroxidase-conjugated goat antirabbit immunoglobulin (Y) or ^{125}I-labeled antirabbit immunoglobulin (Y*). c) The different reactions are developed either by autoradiography or colored product formation by enzymatic reaction. The intensity of the response is directly related to the number of cells applied to the nitrocellulose membrane used as support.

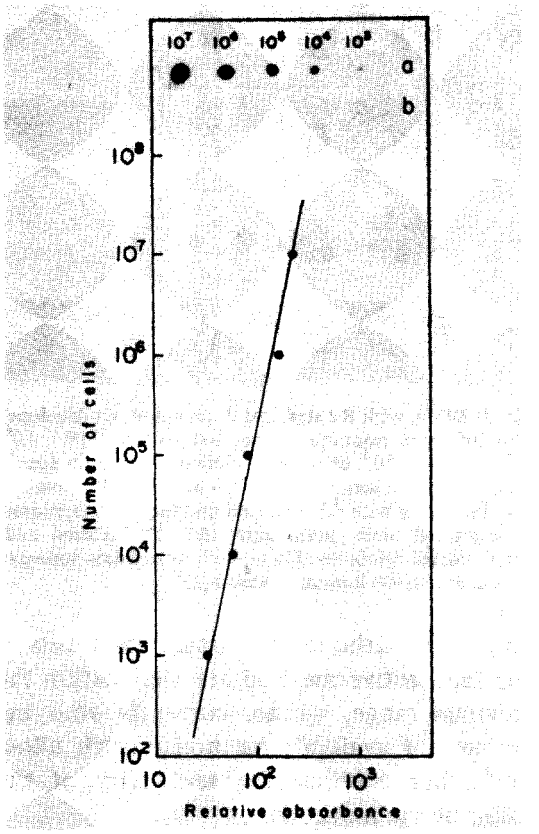

Fig. 2: Dot immunoblot with R2 strain of *T. ferrooxidans*. The indicated amounts of cells were applied. Antiserum (1: 1,000) against R2 strain of *T. ferrooxidans* (a) or preimmune serum (b) was used. The reaction was developed with ^{125}I-labeled protein A. The autoradiography shown was quantitated by densitometry. The relative absorbance of the X-ray film was measured at 550 nm.

tanus and *Thiobacillus novellus* (Arredondo and Jerez, 1989).

The radioactive method allows to determine directly as few as 10^3 cells. However, it may be possible to detect a lower number of microorganisms depending on the specific activity of the radioactively labeled iodinated protein employed to develop the assay and the time of exposure of the X-ray film during autoradiography. Therefore, it is much more sensitive than previous immunological methods, some of which require 10^7 cells or 10^8 cells per gram of sample (Apel *et al.*, 1976 and Muyzer *et al.*, 1987).

The use of short-lived radioisotopes is a limitation of our method, specially if it is to be applied directly in industrial bioleaching operations. For this reason, we explored the use of enzyme-conjugated proteins to replace the radioactively labeled reagents. Fig. 3 shows the results obtained when protein A conjugated with peroxidase is used (Fig. 3B) and when a goat antirabbit antibody conjugated with peroxidase is used (Fig. 3D). It is evident that under these conditions there is a good response which depends on the number of cells. When compared with the radioactively labeled reagents, (Fig. 3 A, C), there was a similar degree of sensitivity, since as few as 10^3 cells could be directly determined in each case. The range of *T. ferrooxidans* titers found in acid mine drainages generally varies between 10^4 and 10^6 cells per ml.

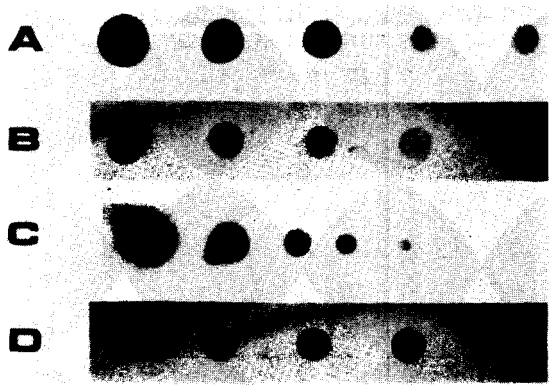

Fig. 3: DIMA with R2 strain of *T. ferrooxidans* developed with different reactions. From left to right, 10^7, 10^6, 10^5, 10^4 and 10^3 cells were applied. Antiserum against R2 strain at a dilution of 1: 1,000 was used. The reaction was developed with ^{125}I-labeled protein A (A), protein A-conjugated with peroxidase (B), ^{125}I-labeled anti-rabbit immunoglobulin (C) or with peroxidase-conjugated goat antirabbit immunoglobulin (D).

Therefore, although the detection limits of our radioactive method are well within the previous range, we are currently studying the use of available methods which allow to further enhance the sensitivity of the colorimetric methods.

In summary, the screening methods we have developed for the determination of *T. ferrooxidans* in liquid suspension, do not require isolation of bacteria and provide identification and enumeration results simultaneously. They are much faster, specific and perhaps more accurate than the traditional methods such as plate counting, which in the case of *T. ferrooxidans* takes between 7 and 10 days (Harrison, Jr., 1984; and Jerez *et al.*, 1986). However, the DIMA does not distinguish between live and dead cells. Therefore, some viability measurements could also be used to estimate the proportion of dead cells in the samples.

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