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Direct and Rapid Analysis of the Adhesion of Bacteria to Solid Surfaces: Interaction of Fluorescently Labeled *Rhodococcus* Strain GIN-1 (NCIMB 40340) Cells with Titanium-Rich Particles

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A fluorimetric assay which enables direct and accurate analysis of the adhesion of bacteria to solid particles was developed. The assay is based on labeling of the bacteria with fluorescamine, which reacts with primary amino groups on the cell surface to yield a yellow fluorescence that is easily detectable by both fluorescence microscopy and spectrofluorimetry. As an example, fluorescent labeling of *Rhodococcus* strain GIN-1 (NCIMB 40340) cells enabled the detection and quantitative determination of their adsorption to TiO_2 and coal fly ash particles. Exposure of the cells to 10% acetone during the labeling reaction affected neither their viability nor their ability to adhere to these particles. Only a small fraction (~2%) of the total cell protein was labeled by fluorescamine upon staining of intact bacterial cells, which may indicate preferential labeling of certain proteins. Specificity studies carried out with the fluorescence assay confirmed previous findings that *Rhodococcus* strain GIN-1 cells possess high affinities for TiO_2, ZnO, and coal fly ash and low affinities for other metal oxides. In principle, the newly developed fluorimetric assay may be used for determination of cell adhesion to any solid matrix by either microscopic examination or epifluorescence measurements. In the present work, the adhesion of several other microorganisms to TiO_2 particles was tested as well, but their ability to adhere to these particles was significantly lower than that of *Rhodococcus* strain GIN-1 cells.

Bacteria often tend to adhere to insoluble particles contained in their habitats, usually via specific components located on the bacterial surface (3). The matrices to which they adhere may be either biotic, e.g., tissue components or plant surfaces, or abiotic (clays, soils, etc.) (13). In either case, analysis of the adhesion of bacteria to these matrices by common optical methods such as turbidometry (light scattering) and microscopy is hindered by the fact that the solid particles usually form turbid suspensions that do not transmit light. Traditionally, the extent of adhesion is determined by measuring the amount of free bacteria in the supernatant before and after the adsorption reaction (2). Although these assays are commonly used, their results may be subject to error when (i) complete separation of free and adsorbed bacteria is not achieved or (ii) cellular aggregates or clumps suspended in solution interfere with the assay. Recently, more sophisticated methods have been developed by using radioactively labeled bacteria (4, 5), fluorescent PCR-enriched oligonucleotide probes (1), ethidium bromide staining (15), or fluorescein-labeled specific antibodies (8) to analyze bacterial adsorption onto solid particles or biofilms.

We recently described the isolation of *Rhodococcus* strain GIN-1 (NCIMB 40340) from the marine environment of a bacterium that adheres strongly to TiO_2 particles (11). The unique adhesive properties of the bacterium were exploited for biomagnetic separation of titanium-rich fractions from coal fly ash (CFA) generated in coal-operated electrical power plants (10, 11).

Examination of the adsorption of *Rhodococcus* strain GIN-1 cells to insoluble particles of metal oxides required the development of appropriate tools to enable qualitative and quantitative analysis of adhesion. As *Rhodococcus* strain GIN-1 cells tend to cluster, both microscopic and viable cell counting methods were highly inaccurate. Similarly, turbidometry measurements were hampered by the high turbidity of the oxide suspension. An accurate cell protein assay which was developed to determine the degree of cell adhesion to TiO_2 particles (9) required destruction of the cells by alkaline treatment at 100°C and could not be used for direct analysis of bacterial adsorption. The goal of the present study was to develop a sensitive and direct fluorimetric assay in which mild fluorescent labeling of the cell surface would not interfere with its adhesion to metal oxides.

Here we describe the fluorescent labeling of *Rhodococcus* strain GIN-1 cells with fluorescamine (12, 14) and the development of an assay to monitor their adhesion to titanium-containing particles, both (i) qualitatively by microscopic visualization and (ii) quantitatively by spectrofluorimetric measurements. We chose to label the bacteria with fluorescamine because this compound was found to react rapidly and efficiently with primary amines on the cell surface without interfering with its adhesive properties.

MATERIALS AND METHODS

Microbial strains. *Rhodococcus* strain GIN-1 (NCIMB 40340) was isolated in our laboratories and has been deposited as a patented strain in the National Collection of Marine Bacteria, Aberdeen, Scotland, United Kingdom. The other strains used in this study, *Acinetobacter calcoaceticus* A2, *Bacillus subtilis* 168, *Escherichia coli* K-12 CHS57, and *Pseudomonas aeroginosa* YS-7, were the same as those previously described (9).

Materials. TiO_2, PbO, Al_2O_3, SiO_2, GeO, and ZnO were purchased from Merck (Darmstadt, Germany). Fluorescamine (Fluram) was from Fluka (Buchs, Switzerland). CFA was obtained from the Israel Electric Company, Ltd., and prepared as previously described (9). Seawater was collected and filtered as previously described (9). Flat-bottom, black microtiter plates (96 wells) were purchased from Polyfiltronics (Rockland, Mass.).

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Cell disruption. *Rhodococcus* strain GIN-1 cells (10 to 50 mg (dry weight)/ml in 50 ml of 0.2 M NaCl) were subjected to 15 cycles of disruption in a French pressure cell (Aminco, Silver Spring, Md.) at 1,200 lb/in^2.

Fluorescent labeling of bacteria. *Rhodococcus* strain GIN-1 cells (10^9 to $5 \cdot 10^{10}$ cells per ml, corresponding to 0.4 to 20 mg of dry cell weight and 0.12 to 6 mg of cell protein) were suspended in 1 to 10 ml of fresh water, filtered seawater, or 0.2 M NaCl. The suspension was mixed with 0.2 volume of 1 M potassium borate buffer, pH 9.5. Fluorescamine (2 mg/ml of acetone, 0.12 volume) was added, and the solution was thoroughly mixed with a Vortex mixer for about 15 s. The labeled cells were then centrifuged at 12,000 $\times$ *g* for 10 min and resuspended in 0.2 M NaCl to yield the original bacterial concentration.

Viability of fluorescently labeled bacteria. The ability of the bacteria to continue growing after being exposed to the labeling reaction was tested as follows. A culture of intact cells (in 100 ml of growth medium [9]) was grown until it reached a turbidity of 0.1 U of optical density at 600 nm. The suspension was divided into two equal parts, and the bacteria were collected by centrifugation at 12,000 $\times$ *g* for 10 min in a Sorvall RC-5 rotor. One fraction served as a control. The other was resuspended in 10 ml of sterile 0.2 M potassium borate buffer, pH 9.5, and mixed with fluorescamine (2 mg in 1 ml of acetone). The labeled cells were then collected by centrifugation and washed with phosphate-buffered saline. Fresh medium (50 ml) was added to each of the fractions, and growth was resumed. The growth curves of the two subcultures were then compared.

In addition, a viability test was carried out by staining the labeled and nonlabeled bacteria with 5% methylene blue and evaluating the viability of the cells by microscopic examination.

Adsorption of labeled and nonlabeled bacteria onto TiO_2 particles. Labeled or nonlabeled bacteria (10^9 to $5 \cdot 10^{10}$ cells, corresponding to 0.12 to 6 mg of cell protein) suspended in 1 to 10 ml of 0.2 M NaCl were agitated for 15 min at room temperature with 0.5 g of TiO_2 particles which had been prewashed with 0.2 M NaCl. The suspension was then centrifuged for 4 min at 200 $\times$ *g* to obtain adequate separation of free and adsorbed cells. The precipitate, containing TiO_2-adsorbed cells, was resuspended in an equal volume of 0.2 M NaCl. To detect the fluorescence of labeled cells with a plate reader attachment to the Spectrofluorimeter (see below), the suspension was recentrifuged and the precipitate was resuspended in 0.2 M NaCl at a final concentration of 0.15 g of TiO_2 per ml and placed into the wells of a microtiter plate (0.2 ml per well) as described below.

Detection of adsorption of nonlabeled bacteria to TiO_2 particles by a protein assay. Bacterial cells, in suspension or adsorbed to TiO_2 particles, were boiled in 1 ml of 0.1 M NaOH for 20 min. Under these conditions, the cells were completely destroyed, as observed by microscopic examination. The solid material was then removed by centrifugation for 10 min at 12,000 $\times$ *g*. An aliquot of the supernatant (10 to 100 μl) was tested for protein content by the assay of Lowry et al. (6).

Fluorimetry. Fluorimetric measurements were done with an LS-50 Spectrofluorimeter (Perkin Elmer, Norwalk, Conn.). Two alternative measurements were made. (i) The suspension of free fluorescent cells was diluted with 0.2 M NaCl to a concentration of 10^7 to 10^8/ml and placed into a 1.0-ml cuvette. Fluorescence was measured at an excitation wavelength of 395 nm and a emission wavelength of 475 nm with the excitation and emission slits set at 10 nm. To avoid quenching, the absorbance of the suspension at 395 nm was always kept under 0.2 U. (ii) The suspension of particle-adsorbed fluorescent cells was placed into wells of an enzyme-linked immunoassay microtiter plate, and their fluorescence was analyzed with a plate reader attachment to the LS-50 Spectrofluorimeter. In this device, particles are excited by a light source placed at 180° from the detector. The device is optically and electronically connected to the fluorimeter by excitation and emission fiber optic cables and electric cables, respectively. To determine the fluorescence of oxide-adsorbed GIN-1 cells, 0.2-ml samples of cell-particle suspensions containing 0.03 g of TiO_2 were introduced into the wells of the microtiter plate and centrifuged at 200 $\times$ *g* for 4 min in a Sorvall RLC-4 centrifuge equipped with an adequate adapter. The supernatants were removed, and the precipitates were washed three times with 0.2 M NaCl. The fluorescence of the washed pellets was monitored at an excitation wavelength of 395 nm and an emission wavelength of 475 nm with the excitation and emission slits set at 10 nm. In parallel, the fluorescence of the supernatants containing mainly unbound cells was determined in 100-μl samples with the plate reader device. This method enabled concomitant analysis of up to 96 samples.

In the experiments described here, fluorescence of free cells in suspension was determined in a cuvette while that of particle-adsorbed cells was determined in a microtiter plate with a plate reader device. Fluorescence units are expressed accordingly.

Fluorescence microscopy. Adhesion of the labeled bacteria to TiO_2 and CFA was monitored with an Olympus BH-2 epifluorescence microscope equipped with UV excitation and emission filters.

Specificity studies of the adhesion of bacteria to metal oxides. To determine the specificity of the adhesion of *Rhodococcus* strain GIN-1 to various oxides, 10^{10} cells were agitated with 0.1 g of TiO_2, GeO, PbO, ZnO, Al_2O_3, Fe_2O_3, or CFA particles in 1 ml of 0.2 M NaCl for 1 h at room temperature. The particles had been prewashed with 0.2 M NaCl. The suspensions were then centrifuged for 4 min at 200 $\times$ *g* to separate free and adsorbed cells. The precipitates were resuspended in 0.2 M NaCl (0.66-ml final volume) and placed into the wells of a microtiter plate (divided into triplicates) as described above.

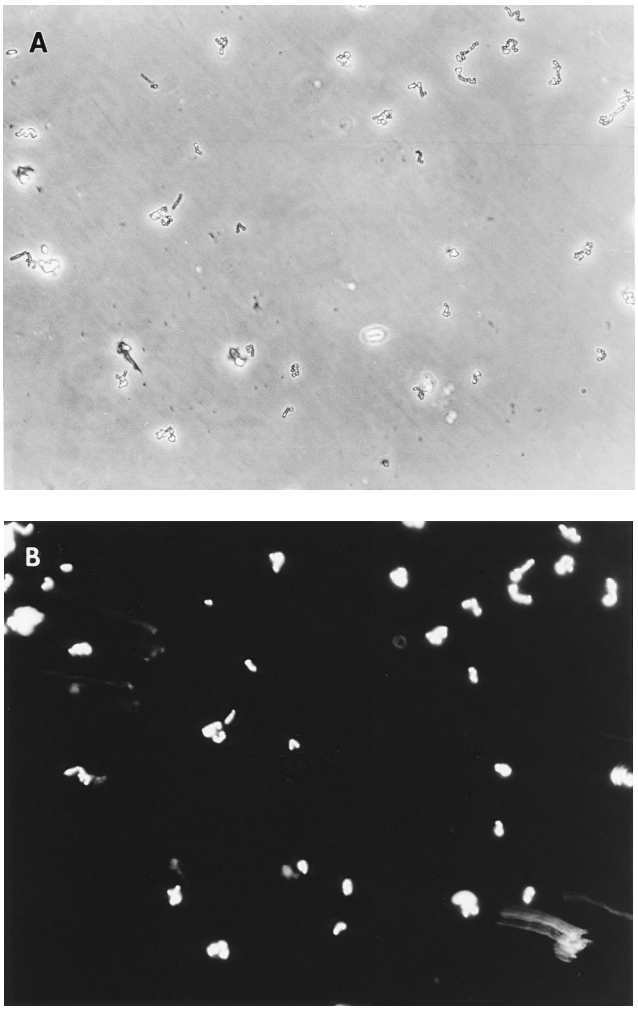

FIG. 1. Fluorescently labeled *Rhodococcus* strain GIN-1 cells. (A) Phase microscopy. (B) Fluorescence microscopy. Magnification, ×700.

The ability of other microorganisms to adhere to TiO_2 was examined by fluorescent labeling of cells of *A. calcoaceticus* A2, *B. subtilis* 168, *E. coli* K-12 CHS57, and *P. aeroginosa* YS-7 with fluorescamine under conditions that were the same as those used for labeling of *Rhodococcus* strain GIN-1, followed by incubation of the cells (10^{10}/ml) with 0.1 g of TiO_2 as described above. Cell adhesion was analyzed by a protein assay or by direct analysis of the fluorescence of the particle-adsorbed cells with a plate reader device (see above).

RESULTS

Exposure of *Rhodococcus* strain GIN-1 cells to fluorescamine resulted in their extensive labeling. Highly fluorescent cells, clearly visualized by fluorescence microscopy (Fig. 1), were obtained. Exposure to 10% acetone during the labeling reaction mixture and blocking of the free amino groups on the cell surface affected neither the viability nor the adhesive properties of the bacteria. Thus, the growth curves of intact and labeled cells were found to be identical. Furthermore, the labeled cells were positively stained with methylene blue to the same extent (>95%) as intact cells. As shown below (see Fig. 3), the labeled bacteria fully retained the ability to adhere to titanium-rich particles.

Since adhesion of *Rhodococcus* strain GIN-1 cells to TiO_2 requires the presence of salt (at least 0.1 N NaCl) and is usually tested in seawater (9), we compared the efficiency of the la-

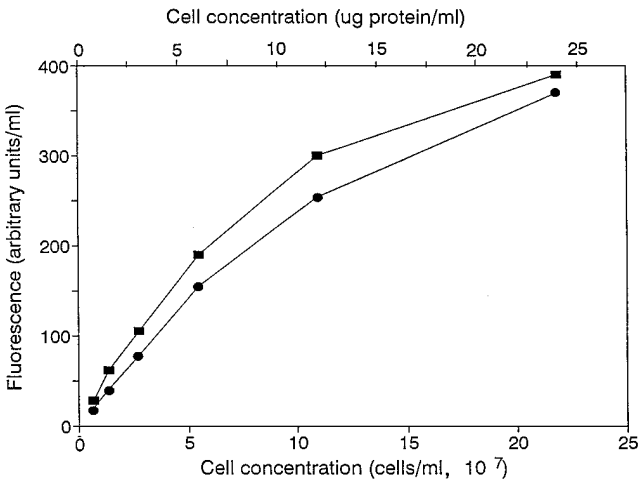

FIG. 2. Fluorescence labeling by fluorescamine of *Rhodococcus* strain GIN-1 cells as a function of the cell concentration in the reaction mixture. The extent of fluorescence after labeling in borate buffer (0.2 M, pH 9.5) (■) and in borate-buffered seawater (●) was measured spectrofluorimetrically by using the cuvette assay (see Materials and Methods for experimental details).

beling reaction in borate buffer and in borate-buffered seawater. Efficient labeling was achieved in both cases, even though the fluorescence intensity of cells labeled in the presence of seawater was about 20% lower, which may be attributable to the inhibitory effect of sodium ions on the fluorescamine reaction (12). As shown in Fig. 2, the fluorescence intensity of the cell suspension increased with the initial cell concentration in the reaction mixture. Typically, we obtained about 27,000 arbitrary fluorescence U/10^{10} cells when we recorded the fluorescence of cells in suspension.

It is pertinent to note that when the reaction of intact cells with fluorescamine was compared to that of homogenized cells, it was found that only a small fraction of the total cell protein (~2%) was labeled, indicating that some proteins of the intact cells are preferentially labeled. In a set of preliminary experiments, we indeed were able to isolate from the fluorescamine-stained bacteria a labeled subcellular protein fraction which is capable of specific adsorption to TiO_2. This protein, which is currently being purified and investigated by us, will be the subject of a separate report.

When the fluorescent bacteria were mixed with TiO_2, adherent bacteria were clearly visible on top of the oxide particles (Fig. 3B). As shown in the control experiment, the oxide particles by themselves possessed no fluorescence under these conditions (Fig. 3A). Quantitative analysis of the adhesion of the cells to the particles required separation of free and adherent cells, which was achieved by differential centrifugation, in which the particle-adsorbed cells sedimented while unbound cells remained in solution. Since the bacterium-oxide suspensions were too turbid to be analyzed directly in the fluorimeter cuvette, a plate reader device attached to the Spectrofluorimeter was used. The particle-adsorbed, labeled cells were sedimented in wells of a microtiter plate, which was then inserted into the device. The fluorescence emitted by the upper surface of the particle layer was collected at 180° from the exciting light with minimal interference by the turbid suspension. Since the light is emitted from a relatively thin layer and energy is lost in the fiber optic cables connecting the device with the main body of the fluorimeter, the fluorescence detected was much lower than in solution measurements. Typically, we obtained 300 fluorescence U/10^{10} TiO_2-adsorbed cells (the sensitivity of the spectrofluorimeter under these conditions was 1 fluorescence U).

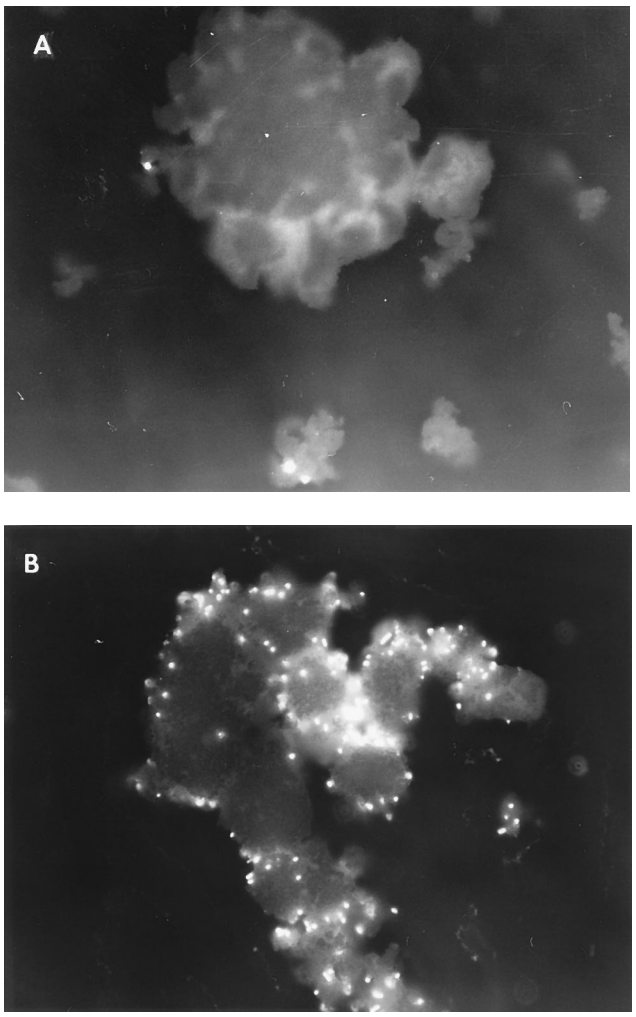

FIG. 3. Adsorption of fluorescently labeled *Rhodococcus* strain GIN-1 cells to TiO_2 particles observed by epifluorescence microscopy. (A) TiO_2 particles with no bacteria. (B) Fluorescent cells adsorbed to a TiO_2 particle. Magnification, ×280.

As shown in Fig. 4, cell adhesion to TiO_2 particles increased with the amount of cells in the incubation mixture. Since the adhesion of labeled GIN-1 cells, measured directly by fluorimetry, was similar to that of nonlabeled cells measured by the cell protein assay (9), it was concluded that the labeled cells fully retained the ability to adhere to TiO_2 particles. The linear dependence of the fluorescence on cell input indicated that although fluorescence was collected only from the upper surface of the sedimented cells, no saturation of the fluorescence signal was observed, even at high loads of cells on the TiO_2 particles.

Direct analysis of the adhesion of cells to solid particles by using labeled cells was also used to determine binding specificity, as in tests carried out previously with the cell protein assay (9). Fluorescent *Rhodococcus* strain GIN-1 cells were incubated with various metal oxides or with CFA under the same conditions as described for TiO_2. As shown in Fig. 5, the affinity of *Rhodococcus* strain GIN-1 cells was highest for TiO_2 and slightly lower for ZnO. The affinities for the other oxides

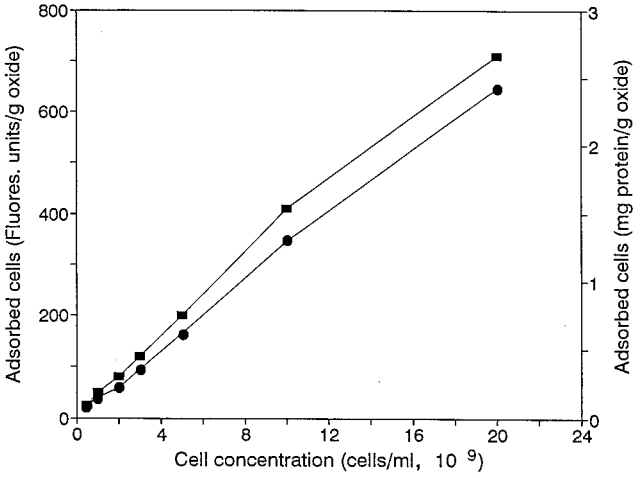

FIG. 4. Adsorption of *Rhodococcus* strain GIN-1 cells to TiO_2 particles measured by fluorescence labeling (■) and by a cell protein assay (●) as a function of the cell concentration in the incubation mixture. Fluorescence was measured with a plate reader device. Fluorescence units are expressed accordingly (see Materials and Methods for experimental details).

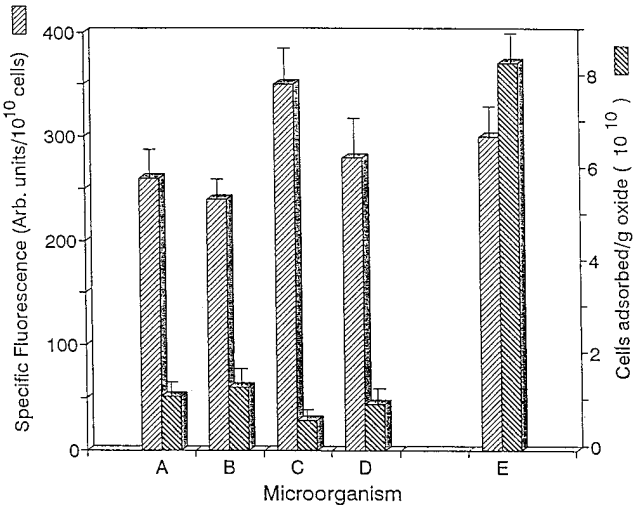

FIG. 6. Specific fluorescence of several bacterial strains labeled by reaction with fluorescamine and adsorption of the labeled bacteria to TiO_2 particles. A, *A. calcoaceticus* A2; B, *B. subtilis* 168; C, *E. coli* K-12 CHS57; D, *P. aeroginosa* YS-7; E, *Rhodococcus* strain GIN-1 (NCIMB 40340). The experiment was done in triplicate. The error bars represent standard deviations. Arb., arbitrary.

tested were much lower, in agreement with previous observations obtained with the cell protein assay (9).

To demonstrate the applicability of the direct fluorescent binding assay to other microorganisms, several other bacterial strains were labeled with fluorescamine and their adhesion to TiO_2 was tested with the fluorescence assay. Cells of *A. calcoaceticus* A2, *B. subtilis* 168, *E. coli* K-12 CHS57, *P. aeroginosa* YS-7, and *Rhodococcus* strain GIN-1 (NCIMB 40340) were labeled by reaction with fluorescamine. Efficient labeling, yielding specific fluorescence values of 240 to 350 fluorescence $U/10^{10}$ cells, was achieved for all of the strains tested. However, the degree of adhesion of these bacteria to TiO_2 particles varied; *Rhodococcus* strain GIN-1 cells showed maximal adhesion to these particles, while the adsorption of the other bacteria tested was much lower. As above, the results of this test were in good agreement with those obtained by the cell protein assay (Fig. 6).

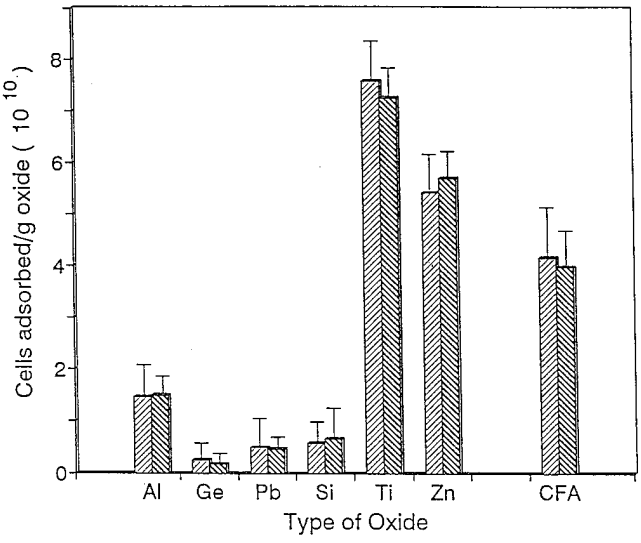

FIG. 5. Binding specificity of *Rhodococcus* strain GIN-1 cells for various metal oxides measured by fluorescence labeling and by a protein assay. The experiment was done in triplicate. The error bars represent standard deviations.

DISCUSSION

Direct examination of the adhesion of bacteria to solid particles is usually complicated, as both viable counting and optical methods have very limited applicability in the quantitative determination of particle-bound bacteria. Instead, adhesion of cells to such particles is usually determined by monitoring the disappearance of bacteria from the supernatant. Although convenient, this method is not very accurate, especially when the percentage of adsorbed bacteria is low or when the cells tend to form aggregates that coprecipitate with the solid particles. Fluorescent labeling of bacteria provides a rapid and sensitive way to monitor their adhesion on solid particles, as exemplified by the adhesion of fluorescamine-labeled *Rhodococcus* strain GIN-1 cells to particles of TiO_2 and CFA.

Fluorescamine was chosen for labeling because it reacts readily with free amino groups of the bacterial cells, yielding a yellow fluorescence easily detectable by both fluorescence microscopy and spectrofluorimetry. Since this reagent undergoes rapid hydrolysis in aqueous solutions (12), a relatively high concentration of fluorescamine in the reaction mixture was required to achieve efficient labeling of the cells. It was expected that the short half-life of the reagent under such conditions (less than 1 s) would allow preferential labeling of the outer surface of the bacteria, as the reagent is not likely to be internalized into the cells within this short period. Since the hydrolysis products of fluorescamine do not emit fluorescence, very low levels of background fluorescence were observed.

Reaction of the cell surface with fluorescamine caused no apparent damage to cellular functions. The cells continued to grow normally and stained positively by methylene blue. Moreover, no interference with the adhesion of the labeled cells to TiO_2 was observed. Thus, the results of the cell adhesion tests obtained with the fluorimetric assay were in good agreement with those obtained with the assay based on total cell protein measurements.

Fluorescent labeling of *Rhodococcus* strain GIN-1 cells was

used for quantitative evaluation of cell adhesion to titanium-rich particles by spectrofluorimetry, as well as visualization of adhesion by fluorescence microscopy. To overcome turbidity problems, a plate reader device attached to a Spectrofluorimeter was used in which the fluorescence emitted by the sedimented, particle-adsorbed bacteria was determined.

In principle, the method described here may be applied to the study of the adhesion of bacteria and other microorganisms to any solid particles or surfaces by using epifluorescence measurements with Spectrofluorimeters equipped with fiber optic technology. The only requirement is that the interaction with the fluorescent dye should not interfere with cell adhesion. Since adsorption of bacteria to solid surfaces is often based on hydrophobic interactions (7), as was also shown for the adhesion of *Rhodococcus* strain GIN-1 to TiO_2 (9), it is anticipated that blocking of the positively charged amino groups on the cell surface by reaction with fluorescamine should not interfere with cell adhesion. We have already labeled several microorganisms by reaction with fluorescamine and measured their adhesion to TiO_2 particles. The successful application of this method to study of the adhesion of *Rhodococcus* strain GIN-1 cells on other metal oxides and on CFA particles as described here points to its potentially wider applicability in the investigation of cellular adhesion to solid particles.

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