# The Effect of Culture Media on Antigenic Expression in Sulfate-Reducing Bacteria

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Sulfate-reducing bacteria constitute a group of morphologically different anaerobic bacteria that convert sulfate to hydrogen sulfide in dissimilatory reactions [4, 5, 12, 23, 26, 33, 45, 49]. The isolation and classification of SRB by conventional methods are very time consuming [20, 47]. Since growth is possible on many nutrients, enrichment and growth media based on only one carbon source might give rise to a biased and incomplete picture of the natural population being sampled [21, 32]. One way to overcome these problems would be to use immu-nofluorescence microscopy for the detection and enumeration of particular microorganisms [8, 11, 22, 35, 39, 46]. However, successful application of fluorescent antibodies can be affected by a range of factors, including specificity and cross reactivity [18, 22, 35, 46].

Serological work on SRB indicated cross-reactions between different *Desulfovibrio vulgaris* and *D. desul-furicans* strains [6, 35, 38, 43]; whereas in other studies few cross-reactions were found among different strains of *D. desulfuricans* and or among *D. desulfuricans*, *D. vulgaris*, and *D. salexigens* [1]. Immunofluorescence was found to be mainly strain specific with SRB [7, 20]. These studies suggested that whole cell and surface an- tigens of these organisms are different, at least for those organisms considered to be related at species level.

The objective of this study was, therefore, to investigate the effect of culture media on specificity of fluorescent antibodies prepared by using authentic SRB strains.

## Materials and Methods

**Organisms.** Cultures of *Desulfovibrio desulfuricans* subsp. *desulfuri-cans* (DSM No 1924), *Desulfovibrio africanus* (DSM No 2603), *Desulfovibrio gigas* (DSM No 1382), *Desulfotomaculum nigrificans* (DSM No 574), *Desulfotomaculum orientis* (DSM No 765), and *Desulfotomaculum guttoideum* (DSM No 4024) were obtain from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM).

**Preparation of antiserum.** Antigens used for antisera preparation were whole cells of *D. desulfuricans* subsp. *desulfuricans*, *D. gigas*, *D. orientis*, *D. guttoideum*, and *D. nigrificans* grown in IS medium [28]. Trisodium citrate (0.3 g/1000 ml) was added to the medium, and ferrous sulfate and iron (III) citrate were omitted to prevent the formation of iron sulfide precipitates as the result of $H_2S$ production by the bacteria. Cells were harvested by centrifugation (10,000 *g* for 20 min), washed in saline (8.5% wt/vol NaCl) and resuspended in saline. The suspensions were diluted to $10^9$ cells/ml [29] and boiled for 5 min. Two white New Zealand rabbits per bacterial strain were immunized. Before immunization, a serum control (10 ml) was taken from each rabbit. Antigens were administered by using the following schedule: days 1, 10, and 30, 1 ml of cell suspension in 1 ml incomplete Freund's adjuvant was injected intramuscularly. Boosters (1 ml of antigen) were given intravenously on day 37. Blood samples were collected at 7-day intervals [19] and left to clot overnight at 4°C. The serum was then collected by centrifugation at 3000 *g* for 20 min and stored at — 12°C. The agglutination titer of the different antiserum was >1024 (titers are reported as the reciprocal of the greatest dilution showing reaction).

**Preparation of fluorescent antibodies.** The immunoglobulins were precipitated from the prepared antisera by using polyethylene glycol 6000 (12% wt/vol) [10] and dissolved in phosphate-buffered saline (PBS). The protein concentration was determined by spectrophotometry [48].

The purified immunoglobulin fraction of the prepared antisera was conjugated with fluorescein isothiocyanate, isomer I (FITC) [10]. After conjugation, the unbound FITC was removed from the conjugate by gel filtration through Sephadex G-25 [10].

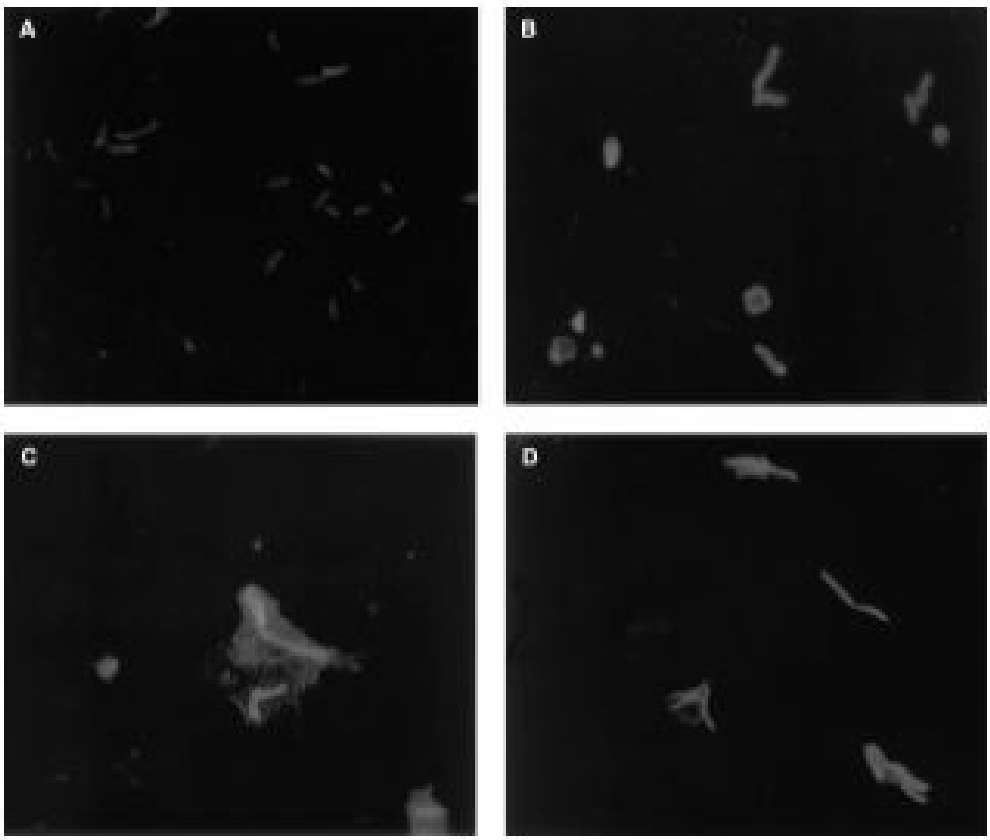


Fig. 1. Specific reactions between the fluorescent-antibody conjugates and homologous cells of (A) *Desulfovibrio desulfuricans*, (B) *Desulfotomaculum orientis*, (C) *Desulfotomaculum guttoideum*, and (D) *Desulfovibrio gigas*.

**Direct fluorescent antibody (FA)-stains.** Air-dried smears of the SRB strains used for antiserum preparation were used as antigens in FA-stains. The different FITC-antibody-conjugates were double diluted to 1/2048 and used in FA stains with the homologous antigen smear by placing a drop of a conjugate on a smear and incubating the slide in a humidity chamber in the dark for 30 min. The slides were rinsed in PBS and mounted in sodium carbonate-buffered glycerol for optimum fluorescence [36] and examined for fluorescence by using a Zeiss epi-fluorescent UV microscope fitted with a HBO-200 mercury vapor lamp. The highest dilutions of the various FITC-antibody conjugates at which fluorescence could be observed were used to test for cross-reactions between the different FITC-antibody conjugates and the following antigens: (a) homologous SRB-cells cultivated in IS-medium [28]; (b) homologous SRB-cells cultivated in synthetic medium [34] with lactate as carbon source; (c) heterogeneous SRB-cells cultivated in IS-medium [28].

**Sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE).** Cells of *D. gigas* and *D. nigrificans* were cultured in IS-medium [28] without an iron source (tryptone, 10 g; sodium sulfate, 0.5 g; sodium (III) citrate, 0.3 g; lactic acid, 6 ml of a 60% solution; $MgSO_4$. $7H_2O$, 2 g; ascorbic acid, 0.75 g; dist. $H_2O$, 1000 ml; pH 7.6). *D. desulfuricans*, *D. orientis* and *D. africanus* cells were cultured in both IS-medium and modified synthetic medium [34] without iron. Cells were harvested by centrifugation (10,000 *g* for 20 min), washed in saline, and resuspended in saline. For the extraction of membrane proteins, the pH of the bacterial suspensions was lowered to 1.5 by adding 10 M HCl in order to degrade extracellular polysaccharides. The cells were then washed three times in saline and the membrane proteins extracted according to the method of De Maagd *et al*. [15]. The membrane protein pellets were dissolved in 5% mercaptoethanol, 4.6% SDS, and 2% Tris-HCl (pH 6.8).

SDS-PAGE was performed by the method of Laemmli [27], modified according to Kiredjan *et al*. [25]. Electrophoresis was carried out by using an HSI vertical slab gel unit SE-600 series (Hoefer Scientific Instruments, San Francisco) at a constant current of 15 mA and 25 mA per stacking and separation gel respectively, at 10°C. Membrane protein gels were stained with Coomassie Brilliant Blue according to the method of Jackman [24] and destained according to Anderson and Anderson [3]. Gels were scanned on a Hoefer GS 300 Transmittance/Reflectance Scanning Densitometer (Hoefer Scientific instruments). Numerical analysis, based on the correlation coefficient (r), which was determined by using the unweighed average linkage cluster analysis, was done using the Gel Compar program version 1.3 supplied by Helix C.V., Belgium.

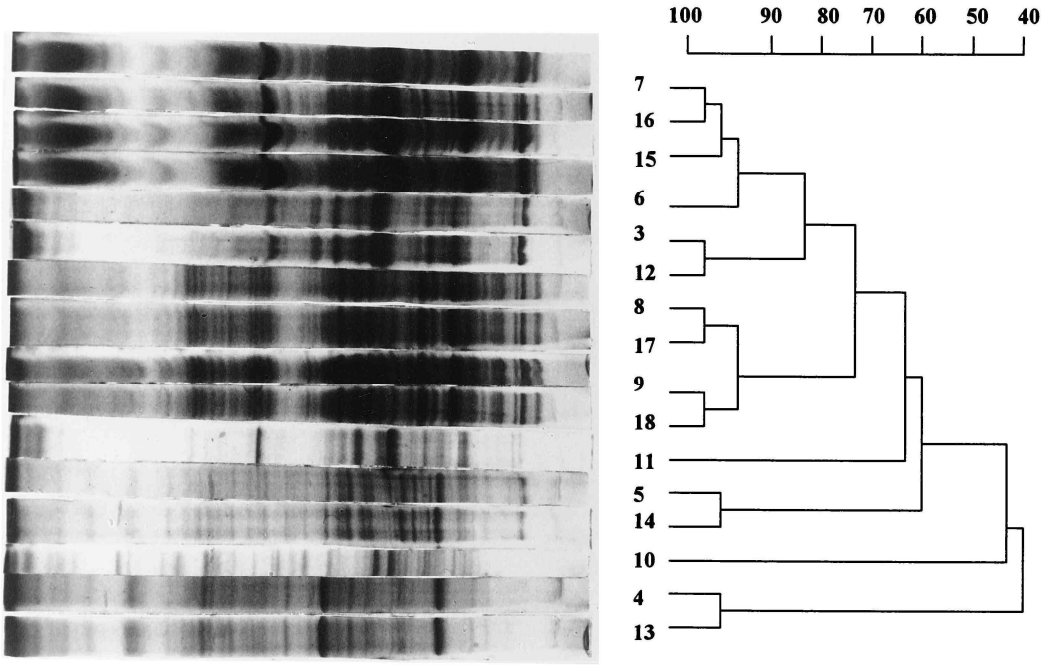


Fig. 2. Dendrogram of the relationships of different SRB-strains cultivated in IS-medium [22] and synthetic medium [28] based on r values, as calculated by the unweighted average pair group method with SDS-PAGE of total membrane proteins. Lanes 3, 12 represent the protein profile of *D. gigas*; 4, 13, *D. orientis*[a]; 5, 14, *D. orientis*; 6, 15, *D. desulfuricans*[a]; 8, 17, *D. africanus*[a]; 9, 18, *D. africanus*; and lane 11, *D. nigrificans*. All the cells were cultured in IS-medium except for the strains indicated with[a], which were cultured in synthetic medium. The profile of the total soluble proteins of *Psychrobacter* was used as a standard (lane 10).

## Results and Discussion

The antisera prepared against different SRB-species cross-reacted only with the cells of homologous SRB-species cultivated in the same medium (IS-medium) that were used during the preparation of antigens for antisera production. No cross-reactions were observed between the antisera and nonhomologous species.

Smith [44] also found FAs prepared against *D. salexigens*, *D. desulfuricans*, *D. vulgaris*, and *D. nigrificans* to be mainly strain specific. Polyvalent cocktails comprised of antisera prepared against various strains of *D. desulfuricans*, *D. gigas*, *D. salexigens*, *D. vulgaris*, *Desulfobacter postgatei*, and *D. nigrificans* were successfully used to detect SRB in nature by using the ELISA technique [7, 20]. The antisera prepared by Bobowski and Nedwell [7] were prepared against cell extracts and not whole cells. These authors all used Postgate's media [37] for the preparation of antigens for antisera production. We chose IS-medium because of the high yield of SRB cells obtained with this medium in industrial water systems [14]. Postgate medium differs from IS-medium in that Postgate medium contains yeast extract and IS medium, tryptone. There is no sulfite present in Postgate medium. Iron was omitted from IS-medium, whereas iron sulfide precipitates were removed from the SRB-cells after culturing of SRB cells in Postgate medium for antisera preparation.

Various studies indicated that FA staining reactions were highly specific [9, 17, 18, 39, 40, 41]. According to Aketagawa *et al.* [2], *D. vulgaris* and *D. desulfuricans* are heterogeneous on the basis of antigenic diversities of its cell-surface antigens. When culturing the cells of SRB under different conditions (synthetic medium), no cross-reaction was observed between the antisera prepared and its homologous SRB-species. This places severe limitations on the possible use of FA-SRB identification in environmental samples. It would, therefore, possibly be better to use SRB strains isolated from the environment for FA production. Although the antigens obtained when preparing antisera by injecting boiled whole cells could be from any portion of the cell, the FA-staining technique identifies only intact cells that express an appropriate antigen on its surface. Different results could, therefore, have been obtained with the ELISA technique. Species cross-reactivity was observed with hydrogenases, adenosine 5'-phosphosulfate reductase, sulfite reductase, and cytochromes with the ELISA technique and can possibly be used for the detection of SRB species in environmental samples [2, 31, 42, 50].

The SDS-PAGE of membrane protein profiles of different SRB-species showed prominent differences among the bacteria (Fig. 2). The three species of the genus, *Desulfovibrio*, i.e., *D. desulfuricans, D. gigas,* and *D. africanus,* clustered

together at r = 0.73. *Desulfotomaculum nigrificans* clustered at r = 0.63 with the genus *Desulfovibrio.* Although *D. orientis* and *D. nigrificans* belong to the same genus, they clustered at only r = 0.6. Differences could also be observed in the same SRB-species cultivated in IS-medium and synthetic medium. The appearance of new protein bands, the disappearance of bands, and a difference in the amount of the expression of certain proteins was observed. *D. orientis* cultivated in synthetic medium clustered at less than r = 0.5 with the rest of the organisms.

When SRB cells were cultivated in synthetic medium, different membrane proteins were expressed when compared with those from cells cultivated in IS-medium, a more nutritious medium (Figs. 1, 2). Iron did not influence the expression of the proteins, since cells cultured in both IS-medium and synthetic medium were starved for iron. The cultivation of cells in different media had an influence on *D. orientis. D. orientis* cultured in synthetic medium fell outside the group (r = 0.6) formed by the SRB strains and clustered with the other SRB strains and *D. orientis* cultured in IS-medium with r less than 0.5. *D. orientis* cultured in IS-medium clustered with *D. nigrificans* at r = 0.6. When a study of the cell envelope proteins in SRB was performed by Norqvist and Roffey [30], they also concluded that *D. orientis* was unique. A relationship between DNA relat-edness and level of similarity of 16S rRNA was defined and indicated that many pairs of *Desulfovibrio* species shared less than 10% sequence homology [16]. The results from our study are similar to those of Davies *et al.* [50], who indicated that the outer-membrane protein profiles in SDS-PAGE of *Pasteurella haemolytica* demonstrated significant differences in the synthesis of certain *P. haemolytica* outer-membrane proteins under various growth conditions [13]. The above indicate that caution should be exercised when using FA for ecological studies.

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