Versatile Medium for the Enumeration of Sulfate-Reducing Bacteria

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ABSTRACT

POSTGATE, JOHN R. (University of Illinois, Urbana). Versatile medium for the enumeration of sulfate-reducing bacteria. Appl. Microbiol. **11**:265–267. 1963.—A lactate-yeast extract-sulfate medium, making use of both thioglycolate and ascorbate to poise the E_h gave valid colony counts of sulfate-reducing bacteria with both pure cultures and natural samples.

The estimation of numbers of sulfate-reducing bacteria in natural environments has been discussed (Anderson, 1958; Postgate, 1959*a*, *b*), and deficiencies in available procedures were pointed out. The cysteine media recommended by Grossman and Postgate (1953) for pure cultures of *Desulfovibrio desulfuricans* are inconvenient when used with natural samples, since cysteine-decomposing bacteria can cause blackening of colonies or media and so give false positive results; hence, colony counts are rarely practicable, and the cysteine procedure is restricted to most probable number (MPN) determinations using fluid media (Drummond and Postgate, 1955). Media poised with ascorbic acid as sole reducing agent are not always reliable; media poised with thioglycolate risk inhibitory effects (Grossman and Postgate, 1953; Postgate, 1959*a*). A procedure using a reduced iron nail (Abd-el-Malek and Rizk, 1958, 1960) is less tedious than Drummond and Postgate's (1955) modification of the cysteine procedure but is also restricted to MPN determinations. Allred, Mills, and Fisher (1954) described a medium using both ascorbate and thioglycolate, the latter at a concentration below that known to inhibit growth, but they published no data on its quantitative validity. Their medium, designed for use in connection with the oil industry, included 1% NaCl, which would be expected to give falsely low counts with some sulfate-reducing bacteria from fresh-water environments (Littlewood and Postgate, 1957). This report describes tests of a modification of their medium.

MATERIALS AND METHODS

Strains of sulfate-reducing bacteria were obtained from the National Collection of Industrial Bacteria; the species and collection numbers are given in Table 1. Mesophilic bacteria were grown at 30 C in the lactate-yeast extract of medium C of Butlin, Adams, and Thomas (1949) under either N_2 or H_2 plus 1% (v/v) CO_2; for marine strains, 2.5% (w/v) NaCl was added to the culture medium; in certain instances, for reasons unconnected with the present work, the sodium lactate and sulfate concentrations were augmented to 54 and 27 μmoles/ml, respectively; Na_2S or sodium thioglycolate (10^{-3} M) was added to poise the E_h of the parent culture. The thermophilic strain was grown at 55 C under N_2. Dilutions were made by serial 1:10, $1:10^2$, or $1:10^4$ dilutions as appropriate into the growth medium prepared without lactate and yeast extract. Portions of appropriate dilutions (0.2 or 0.3 ml for colony counts, 0.5 ml for MPN measurements) were distributed among replicate (three or five) tubes of the media being tested. Agar media were in tubes (15 by 1 cm) and were sealed, when mixed and set, with a plug of uninoculated agar.

Three types of counting medium were tested. Medium 1 was the sodium lactate-yeast extract medium C of Butlin et al. (1949) supplemented with 0.075% cysteine and 0.05% ferrous sulfate just before use; for pure cultures, it was set with 1.5% agar (Difco; *see* Grossman and Postgate, 1953). Medium 2 was that of Allred et al. (1954) autoclaved immediately before use following their instructions; this was an agar medium based on calcium lactate and yeast extract and included 0.01% ascorbate and 0.01% thioglycolate plus 1% NaCl. Medium 3 was medium C of Butlin et al. (1949) supplemented with 0.01% ascorbate, 0.01% thioglycolate, 0.05% ferrous sulfate, 1.5% agar, and, for salt-water organisms, 2.5% NaCl. It was sterilized by autoclaving and, like medium 2, used at once. Media 2 and 3 probably differ essentially only in that the former regularly contained 1% NaCl; other differences in the chemicals used in their published recipes are probably secondary. The ingredients of medium C were more readily available to the author, hence most of the comparative work reported here was done with media 1 and 3.

Colony counts usually involved 10 to 60 black colonies per tube; MPN values were read from tables (Ministry of Health, 1939) after the second, cysteine-free, subculture of Drummond and Postgate (1955).

RESULTS AND DISCUSSION

Counts of pure populations using the three media are recorded in Table 1. With the species *D. desulfuricans*, microscopic total counts were not performed, since Gross-

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TABLE 1. *Viable counts of sulfate-reducing bacteria in three media*

Strain	NCIB no.	Habitat	Medium* 1	Medium* 2	Medium* 3
Desulfovibrio desulfuricans					
Hildenborough	8303	Fresh water	7.3×10^8	5.3×10^8	—
Wandle	8305	Fresh water	8.7×10^7	2.3×10^7	7.1×10^7
Wandle	8305	Fresh water	1.3×10^8	4.3×10^7	1.2×10^8
Beckton	8319	Fresh water	1.6×10^9	1.6×10^9	—
Walvis Bay	8347	Salt water	8×10^7	—	9.8×10^7
El Agheila Z	8380	Salt water	2.2×10^8	—	2.9×10^8
D. orientis					
Singapore	8382	Fresh water	1.3×10^7	—	2×10^6
Clostridium nigrificans					
Teddington Garden	8351	Fresh water (55 C)	3.5×10^6	—	2.3×10^6
Unclassified					
Coleman's (1960) organism	8452	Fresh water	1.1×10^7	—	1.7×10^7

* Pure cultures of the strains indicated were counted in lactate-yeast extract-sulfate agar media poised with either cysteine (1) or a mixture of thioglycolate and ascorbate (2, 3). Medium 2 had 1% NaCl; media 1 and 3 had salt concentrations matched to that of the sample. Results expressed as viable count per ml.

man and Postgate (1953) had shown that the cysteine medium (medium 1) gave 30 to 70% recoveries of examples of this species. These recoveries were probably nearer to 100% than those authors realized, since the systematic errors of microscopic counting pointed out by Norris and Powell (1961) were not then appreciated. The inhibitory effect of medium 2 with fresh-water strains, predicted from the experiments of Littlewood and Postgate (1957), was clear with the Wandle strain. Medium 3 gave similar recoveries to cysteine medium with all strains of *D. desulfuricans*. With the mesophilic sporeformer, Coleman's (1960) organism, medium 3 was also as effective as medium 1; with *D. orientis* it was less effective. With the thermophilic *Clostridium nigrificans*, medium 3 was marginally less effective than medium 1. However, Bufton (1959) showed that no existing medium counts *C. nigrificans* quantitatively; the count of 3.5×10^6 viable organism/ml in medium 1 represented 3.7% of the total microscopic count. The highest recoveries in the experiments quoted, involving Coleman's organism and *D. orientis*, were 13.6 and 8.9% of the total microscopic counts, respectively, indicating that the difficulties in the quantitative estimation of the thermophiles reported by Bufton (1959) apply also to the sporulating mesophiles.

Table 2 shows determinations using samples of natural origin; the pond water gave a low count in medium 2, indicating that the inhibitory effect of NaCl may also be encountered with natural samples; in all instances, medium 3 gave counts insignificantly different from the MPNs obtained with medium 1. Black and white colonies were readily distinguishable, even when white were in the majority.

Organisms that form sulfide from thioglycolate might introduce error when media poised with this compound are used with samples of natural origin but, though they exist (Fuchs and Bonde, 1957), they appear to be sufficiently rare to be disregarded unless there is special reason to expect them; enrichment cultures for thioglycolate-decomposing bacteria from polluted water and sewage failed to yield them (*see* Anderson, 1958). Medium 3 thus appears to be as reliable as the cysteine medium for determining numbers of sulfate-reducing bacteria in most circumstances, provided the salt concentration is appropriate to the sample being examined; it has the advantage of permitting colony counts even with natural samples. The absolute validity of counts so obtained is subject to restrictions mentioned by Grossman and Postgate (1953), Postgate (1959*b*), and Bufton (1959).

TABLE 2. *Viable counts for sulfate-reducing bacteria in natural samples*

Origin	Medium* 1	Medium* 2	Medium* 3
Stream water	6	4	6.5
Enrichment from gas holder water	5×10^6	2.3×10^5	4.9×10^6
Sewage sludge yielding CH_4	7×10^4	6.7×10^4	6.1×10^4
Pond water	2.2×10^2	4	2.5×10^2
Water from gravel pit	About 10^3	—	7×10^2
Soil from Llanelly, S. Wales	3.4×10^2†	—	3.3×10^2†

* The media in Table 1 were tested, except that medium 1 was liquid and used for MPN determinations (see text). The soil sample from Llanelly was treated by Pochon's (1955) procedure. Results expressed as viable count per ml.

† Viable count per g of soil.

A recipe and instructions for the use of medium 3 follows: KH_2PO_4, 0.5 g; NH_4Cl, 1 g; Na_2SO_4, 1 g; $CaCl_2 \cdot 6H_2O$, 1 g; $MgSO_4 \cdot 7H_2O$, 2 g; Na lactate, 3.5 g; yeast extract, 1 g; ascorbic acid, 0.1 g; thioglycolic acid, 0.1 g; $FeSO_4 \cdot 7H_2O$, 0.5 g; agar, 15 g/liter; distilled water; NaCl as appropriate; and NaOH to pH 7.6. Autoclave for 15 min at 15 lb/in^2, hold at 40 to 44 C, and add aseptically to

tubes containing not more than a 15% volume of sample being tested. Mix, allow to set, and seal with a 1.5-cm plug of agar to prevent access of air to the inoculated portion; incubate in air until the number of black colonies (sulfate-reducing bacteria) shows no further increase (3 to 16 days, depending on species and environment).

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