SULPHURETTED HYDROGEN PRODUCTION BY BACTERIA IN A LEAD MINE

By J. W. EDINGTON

From the Department of Bacteriology, Sheffield University

This investigation was initiated when twelve miners who were working in a certain part of the Mill Close Lead Mine complained of smarting of the eyes associated with marked congestion of the conjunctivae. They also suffered from a stinging sensation in the nose and throat. The particular area in which they were working differed from other parts of the mine in that there was a strong smell of sulphuretted hydrogen present. After recovery, and when the ventilation had been improved, the men were able to continue working in that area without experiencing further discomfort. The interesting part of the investigation was tracing the origin of the sulphuretted hydrogen. The odour proceeded from collections of mine water which the miners very appropriately called "stink water". In this there was a deposit of sulphur mixed with black sludge which also smelt strongly of H_2S. The sludge consisted of slightly gelatinous black masses mixed with a whitish felt work of a filamentous organism. On microscopical examination the filaments were seen to contain sulphur globules, and often to arise from a mass of black material. This black material consisted of sulphur and inorganic salts in a slimy matrix, the black colour being due to the presence of ferrous sulphide. On further examination these masses were found to be teeming with other bacteria, Gram-negative bacilli and spirilla, both of which were motile, the spirilla being actively so.

Thus altogether three organisms were present. The isolation of two of these in pure culture was exceedingly difficult and laborious, whilst that of the third, viz. the coliform bacillus, was easy.

Isolation of the coliform bacillus. The sludge was plated out on agar and MacConkey's medium, grown aerobically at 37° C. and single colonies picked off for further investigation. The organism was identified as *Bact. coli communior.*

Isolation of the Thiothrix nivea (*Rabenhorst*, 1865) *Winogradsky*, 1888. This was the filamentous organism which contained sulphur globules in its filaments. One end of each filament had a "slime cushion" which acted as an organ of attachment, whilst the other end showed constrictions which divided it into short segments, thus forming the so-called "conidia". The filaments were non-motile. The formation of the "slime cushions" accounts, in part at least, for the gelatinous consistence of the sludge. A modification of Keil's method (1912) was used to obtain pure cultures. Small pieces of the filamentous

material were washed thoroughly with 20–30 changes of the following sterile medium (Synthetic 1):

[1]$CaH_2(CO_3)_2$	3·4 g.	KCl 0·1 g.
$MgH_2(CO_3)_2$	2·7 g.	K_2S 0·1 g.
$CaSO_4$	3·1 g.	FeS 0·1 g.
$MgSO_4$	5·1 g.	CaS 0·1 g.
Na_2SO_4	2·1 g.	$(NH_4)_2SO_4$ trace
K_2SO_4	0·2 g.	Distilled water 1000 c.c.
$Ca_3(PO_4)_2$	0·2 g.	

[Another suitable medium could be obtained by filtering some of the "stink water" through Seitz E.K. filters. I am indebted to Mr F. L. Taylor, chemist to the Mill Close Mine, for the following analysis of the water:

NaCl	9·48	parts per	100,000
Na_2SO_4	4·82	,,	,,
$CaSO_4$	10·61	,,	,,
$CaCO_3$	14·21	,,	,,
$MgCO_3$	7·28	,,	,,
Al_2O_3	0·17	,,	,,
SiO_2	1·10	,,	,,
H_2S	0·56	,,	,,
$FeCO_3$	Trace		
*p*H	7·3		

This shows a marked increase in the $CaSO_4$ content, viz. 10·61, as compared with an average of 5·37 parts per 100,000 in the mine water from unaffected areas. H_2S is also of course an abnormal constituent.]

The washed filaments were then placed in test-tubes containing shallow layers of either of the above media. A gaseous mixture was prepared at ordinary room temperature (*c.* 16° C.) containing:

Oxygen at a pressure of 15·0 mm. Hg.

Carbon dioxide at a pressure of 25·0 mm. Hg.

Hydrogen sulphide at a pressure of 1·0 mm. Hg.

Nitrogen: sufficient to bring the gaseous mixture to the existing atmospheric pressure.

Some of this was bubbled through the medium in the culture tubes by means of a sterile Pasteur pipette. The culture tubes were then placed in a jar and means were taken to keep a steady stream of the above gaseous mixture flowing slowly through the jar. The incubation temperature was 30° C. After about a fortnight fresh filaments were detected, and on these the "slime cushions" were easily seen. If the H_2S is eliminated from the gaseous mixture the sulphur globules slowly disappear from the filaments (Winogradsky, 1887).

[1] As the calcium and magnesium bicarbonates are unstable the equivalents of calcium and magnesium carbonates were used and converted into the bicarbonates after sterilization by bubbling sterile CO_2 aseptically through the medium.

Pure cultures of the *Thiothrix* were not easy to obtain, as it was exceedingly difficult to get rid of the *Bact. coli communior*. It was only by repeating the above procedure with subcultures on many occasions that a pure culture could be procured.

Isolation of Vibrio desulfuricans (*Beijerinck*, 1895). Synonyms: *Bacterium sulfureum* (Hölschewnikoff, 1889); *Bacterium hydrosulfureum ponticum* (Zelinsky, 1893); *Spirillum desulfuricans*; *Microspira desulfuricans*. This was very troublesome, as the colonies of the *Vibrio* developed almost without exception in the *Bact. coli* colonies. The *Vibrio* is a strict anaerobe, but this was no help in its isolation, as the *Bact. coli* also grows anaerobically.

A pure culture was eventually obtained by employing a modification of a method recommended by Van Delden (1904). The following medium (Synthetic 2) was employed:

Dipotassium hydrogen phosphate	0·5 g.
Sodium lactate	5·0 g.
Asparagine	1·0 g.
Magnesium sulphate	1·0 g.
Sodium sulphite	0·5 g.
Sodium carbonate	1·0 g.
Ferrous ammonium sulphate	Trace
Tap water	1000·0 c.c.

(Filtered "stink water" could also be used.)

This was used as a fluid culture medium for the propagation of pure cultures. For the isolation of the *Vibrio* solid media were used by incorporating either 2 % agar or 10 % gelatin in the above. Plates were made with the agar medium and inoculated with the sludge. They were incubated anaerobically at 30° C. until black colonies appeared.

The *Bact. coli* colonies appeared slightly black, whilst those of the *Vibrio* were intensely so, the colonies of the latter appearing in the *Bact. coli* colonies. In no case was it possible at this stage to pick off separate colonies of the *Vibrio*.

A mixed colony was chosen which contained a relatively large number of *Vibrios*. This was determined by either (1) making a film from a portion of the mixed colony and staining it by Gram's stain; the relative number of *Vibrios* could then be ascertained; or (2) making a wet film of the colony and examining it for motility. Both organisms are motile, but the *Vibrio* is very much the more active of the two.

A suitable mixed colony having been obtained it was emulsified in some sterile salt solution (0·85 % NaCl), and varying amounts of the emulsion were inoculated into shake gelatin tubes of the above medium. Incubation was carried out at 22° C. until intensely black colonies developed. These were obtained separate from those of the *Bact. coli* only after repeating the above procedure many times and depending on luck more than on good guidance for a happy result.

When the colonies were eventually separate, the tube was cut across opposite a suitable colony, and with aseptic precautions this was removed from the culture medium and placed in a tube of the fluid medium and incubated anaerobically at 30° C.

It was found advantageous, if the position of the colony permitted, to remove it surrounded with a portion of the gelatin medium. If the colony is only touched with the inoculating wire the *Vibrios* are naturally freely exposed to oxygen with the result that, being very susceptible to that gas, they are liable to be killed during the process of transfer.

The *Vibrios* in pure culture are very actively motile and measure $2{\cdot}0–5{\cdot}0 \times 0{\cdot}6–1{\cdot}0\mu$. They are Gram-negative but counterstain badly with weak carbol fuchsin. To get a good preparation it is necessary to counterstain with strong carbol fuchsin for at least 1 min.

Each *Vibrio* has a single terminal flagellum measuring about 2–3 times the length of the organism. They can be stained by silver impregnation methods, e.g. that recommended by Pownall (1935) gave good results.

These *Vibrios* produce a large amount of H_2S when actively growing in a suitable environment.

Discussion

Two reactions were being carried on in the "stink water and sludge".

The *Vibrio* was producing H_2S by the reduction of sulphates. What probably happens is that the *Vibrio* dehydrogenates some organic substrate present in the sludge, and with the hydrogen so obtained hydrogenates the sulphates (Kluyver & Donker, 1925).

Regarding the organic substrate Baars (1930) has shown that a great variety of different organic substances can act as such in reactions of this kind.

In the other reaction the *Thiothrix* was oxidizing the H_2S to form sulphur and sulphuric acid, the latter being converted by the carbonates present into sulphates.

The action of the *Bact. coli communior* was probably to remove some inhibitory agent possibly by producing a suitable anaerobic environment for the *Vibrios*. Cultures of the *Vibrio* grow quite well in the absence of *Bact. coli*, therefore no essential accessory food factor seems to be involved.

No stimulation of the growth of the *Vibrios* was observed when the following fluids were added in varying amounts to tubes of synthetic medium (2) inoculated with that organism and incubated anaerobically at 30° C.:

(1) Heat-killed (60° C. for ½ hr.) broth cultures of the *Bact. coli communior* grown at 37° C. for 2 days.

(2) Sterile Seitz filtrates of broth cultures of the *Bact. coli communior* grown at 37° C. for 2 days.

(3) Heat-killed (60° C. for ½ hr.) synthetic medium (2) cultures of the *Bact. coli communior* grown at 37° C. for 2 days.

(4) Sterile Seitz filtrates of synthetic medium (2) cultures of the *Bact. coli communior* grown at 37° C. for 2 days.

ACKNOWLEDGEMENT. I have great pleasure in expressing my thanks to Mr L. B. Williams, Manager of the Mill Close Mine, for permission to publish these results, for sending me various samples of the mine water, and also for an account of the symptoms of the men who were exposed to the sulphuretted hydrogen.

REFERENCES

BAARS, J. K. (1930). Over sulfaatraductie door bacteriën. Dissert., Delft.

BEIJERINCK, M. W. (1895). *Zbl. Bakt.* Abt. 2, **1**, 1, 48 and 104.

VAN DELDEN, A. (1904). *Zbl. Bakt.* Abt. 2, **11**, 81 and 113.

HÖLSCHEWNIKOFF (1889). *Fortschr. Med.* **7**, 201.

KEIL, F. (1912). *Beitr. Biol. Pfl.* **11**, 335.

KLUYVER, A. J. & DONKER, H. J. L. (1925). *Verh. Akad. Wet. Amst.* **28**, 297 and 605.

POWNALL, M. (1935). *Brit. J. exp. Path.* **16**, 157.

RABENHORST, L. (1865). *Flora Europaea Algarum.* Leipzig.

WINOGRADSKY, S. (1887). *Bot. Ztg*, **45**, 489.

—— (1888). *Beiträge zur Morphologie und Physiologie der Bacterien.* Heft 1. *Zur Morphologie und Physiologie der Schwefel Bacterien Leipzig* (A. Felix), p. 39.

ZELINSKY, N. D. (1893). *Proc. Russ. phys. chem. Soc.* **25**, 298. Referred to in *Microorganisms in Water*, by P. and G. Frankland, p. 458, 1894. London: Longmans, Green and Co.

(*MS. received for publication* 16. VI. 1938.—Ed.)