Taxonomy of Anaerobic Thiobacilli

By M. HUTCHINSON, K. I. JOHNSTONE AND D. WHITE

*The Houldsworth School of Applied Science and the Department of
Bacteriology, The University, Leeds*

(Accepted for publication 22 October 1966)

SUMMARY

The taxonomic position of *Thiobacillus denitrificans* was investigated by using six newly isolated strains which were compared with other species of this genus, under aerobic and anaerobic growth conditions. The validity of *T. denitrificans* is shown. *Thiobacillus intermedius* and *T. thermophilica*, two species recently described by other authors, were also investigated.

INTRODUCTION

The species *Thiobacillus denitrificans* was first described by Beijerinck (1904*a, b*) and said to be capable of autotrophic growth; aerobically in the presence of thiosulphate or thiocyanate and anaerobically in the presence of thiosulphate and nitrate. In previous studies of the genus *Thiobacillus* (Hutchinson, Johnstone & White, 1965, 1966) many strains were isolated from a wide variety of sources, but strains corresponding to *T. denitrificans* were not found. As aerobic enrichment cultures had been used in these experiments the present paper describes attempts to isolate *T. denitrificans* using anaerobic enrichment cultures and isolation techniques. Twenty sources were examined and strains which produced gas from nitrate-containing media isolated in pure culture. These organisms proved to be facultative anaerobes but unlike the strains described by Baalsrud & Baalsrud (1954) and Woolley, Jones & Happold (1962) they did not lose their ability to grow anaerobically after cultivation for a period of 6 months. During this period the strains were subcultured at 21-day intervals.

As with the acidophilic species previously studied (Hutchinson *et al.* 1966) there was considerable difficulty in deciding the test conditions to be used when comparing these strains with the other species. There appeared to be no logical method for selecting either aerobic or anaerobic test conditions and therefore these organisms were compared with closely related species in two separate series of tests under aerobic and anaerobic conditions.

During the course of this work two rather unusual species of the thiobacilli were described by other authors. *Thiobacillus intermedius* (London, 1963) was an acidophilic facultative heterotroph which decreased the pH value of thiosulphate media to below pH 2.8; a culture of the original strain together with a similar organism isolated by us have been included in the aerobic series of tests. The other species, *T. thermophilica* (Egorova & Deryugina, 1963), is a spore-forming autotrophic thermophil; our examination of this organism was limited to confirming the original description.

METHODS

Organisms. As no authentic strains of *Thiobacillus denitrificans* were available to us and no isolates corresponding to the original description had been found by us in previous work (Hutchinson *et al.* 1965, 1966), twenty sources were examined specifically for anaerobic thiobacilli. Enrichment cultures of the various samples were set up in the S 6 thiosulphate medium (Hutchinson *et al.* 1965) with the addition of NaHCO_3 0.5 g./l. as carbon source and KNO_3 5.0 g./l. as oxidant (medium S 8). These cultures were contained in completely filled and stoppered bottles. When gas formation was observed in the cultures two-thirds of the culture medium was replaced aseptically with fresh medium. This accelerated the rate at which the gas accumulated and was

Table 1. *Sources which yielded nitrogen-forming enrichment cultures on anaerobic incubation*

Source	pH value of source	Isolates
Lagoon system, carbonization effluent	—	8g, 9g, 10g, 11g
Lagoon system, domestic sewage	7.0	1s, 2s, 3s, 4s
Anaerobic digestion tank (1), domestic sewage	6.8	2t, 2t', 3t, 4t
Anaerobic digestion tank (2), domestic sewage	6.4	1u, 2u
Biological filter, carbonization effluent	7.1	1w*
Fertile soil	6.7	—†
Activated sludge plant, carbonization effluent	7.1	—†

* Could not be obtained in pure culture.

† No nitrogen-forming strains could be isolated.

Table 2. *Aerobic strains*

Collection or source	Species	Code number
NCIB 8370	<i>Thiobacillus thioparus</i>	b5
P. A. Trudinger	<i>T. neapolitanus</i>	c1
F. C. Happold	<i>T. thioparus</i>	h1
		h2
		h3
D. P. Kelly	<i>T. neapolitanus</i>	k1
J. London	<i>T. intermedius</i>	l1
Mrs M. Townshend	<i>T. neapolitanus</i>	m1
Miss E. S. Pankhurst	<i>T. thioparus</i>	p1
T. G. Tomlinson	<i>T. thioparus</i>	t1
W. Vishniac	<i>T. neapolitanus</i>	v1
New isolate	<i>T. thioparus</i>	1b
New isolate	<i>T. intermedius</i>	2r

accompanied by the deposition of elementary sulphur. At this stage the cultures were plated on S 8 medium agar and incubated at 28° in a McIntosh & Fildes jar. All the different types of colonies seen were purified by three consecutive single-colony isolations, and all the isolates except 2t' were maintained under anaerobic conditions before testing. Gas formation was observed in only six of the twenty enrichment cultures examined. As may be seen from Table 1, successful isolations (14) were limited to four sources. De Kruyff, van der Walt & Schwartz (1957) and Woolley *et al.* (1962) reported similarities between *Thiobacillus denitrificans* and *T. thioparus*; therefore representative strains of the latter species were included in the tests. The authentic

strain of *T. intermedius*, very kindly supplied by Dr J. London, and a very similar strain isolated in our laboratories were also included in the aerobic series of tests. These organisms and their origins are listed in Table 2.

Tests. All isolates were examined under aerobic and anaerobic conditions according to the standard test scheme used previously. The anaerobic tests were made in the anaerobic medium (S 8) in completely filled and stoppered bottles.

Some of the tests did not differentiate between the organisms under study and were therefore omitted from the analysis. Of the 30 tests investigated anaerobically only 18 were finally utilized and these yielded 44 character states. In the aerobic series only 24 out of 40 tests were used giving 62 character states.

All measurements and analyses were made as in previous studies (Hutchinson *et al.* 1965, 1966). The two sets of data were analysed independently though certain tests were common to both series of scorings. The tests used are summarized in Table 3.

Table 3. *Tests*

Test compound or variable	Concentration (%)	Comments	Characters	
			Aerobic	Anaerobic
Sulphur (excess)		*	3	2
Ammonium thiocyanate	0.02	*	2	2
Hydrogen sulphide		*	2	—
Thioacetamide	0.02	*	3	—
Nutrient medium		*	3	—
19°		Rate of thiosulphate oxidation	2	—
29°		*	2	—
35°		*	2	—
29°		Amount of thiosulphate oxidation	4	2
pH 8.25		Amount of thiosulphate oxidation	—	2
S6 or S8 medium agar		Sulphur deposition	2	3
S8 medium agar		Growth in McIntosh & Fildes jar	2	—
S8 medium		Gas production	—	2
		Formation of nitrite	—	3
		Deposition of sulphur	—	3
S6 medium		Final pH value	4	—
Bacitracin		*	2	—
Novobiocin		*	2	2
Streptomycin		*	—	2
Chloramphenicol		*	—	2
Mixed phosphate	4	*	3	—
Sodium chloride	5	*	2	—
Potassium nitrate	1	*	3	3
Ammonium chloride	2.5	*	2	3
Ammonium thiocyanate	0.02	*	2	2
Phenol	0.01	*	—	3
	0.02	*	4	3
Sodium glutamate	1	*	3	2
Nutrient broth	1	*	3	3
Sulphur	Excess	*	3	—
Calcium chloride	1	*	2	—

* Previously described (Hutchinson *et al.* 1965).

Chemical analysis. The amount of thiosulphate utilized was determined by titration with 0.01 N-iodine, and gas formation (presumed to be nitrogen) by visual observation.

Analysis of data. The results were scored according to the method of Beers & Lockhart (1962) and the S values calculated by the method of Sneath (1957). The order of the rearranged matrices was obtained by inspection.

RESULTS

The results of the numerical analyses for the aerobic and anaerobic test series are shown in Figs. 1 and 2, in which the strains have been arranged in order of similarity. The strains may be segregated into four groups reported previously (Hutchinson *et al.* 1965): 0 (the trautwein types), 3 (*Thiobacillus thioparus*) and 4 (*T. neapolitanus*) together with a newly formed group containing the anaerobic denitrifying thiobacilli which has been termed group 2.

1	1U	00	—	—	—	—	—												
2	2T	89	00	—	—	—	—												
3	9G	89	00	00	—	—	—												
4	8G	72	83	83	00	—	—												
5	2T	71	71	71	82	00	—												
6	2s	71	65	65	82	87	00												
7	1B	39	39	39	28	18	12	00	—	—	—	—	—	—	—	—	—	—	—
8	3T	44	44	44	39	29	24	83	00	—	—	—	—	—	—	—	—	—	—
9	p1	28	39	39	44	24	35	67	72	00	—	—	—	—	—	—	—	—	—
10	h1	33	28	28	44	29	35	72	72	78	00	—	—	—	—	—	—	—	—
11	b5	20	13	13	27	27	29	67	60	80	87	00	—	—	—	—	—	—	—
12	1s	22	17	17	17	12	18	78	72	67	72	87	00	—	—	—	—	—	—
13	4T	28	22	22	28	29	29	72	78	67	72	80	83	00	—	—	—	—	—
14	h3	17	8	8	8	9	9	67	58	50	58	75	83	92	00	—	—	—	—
15	11G	33	28	28	28	24	24	56	50	39	50	53	72	56	50	00	—	—	—
16	4s	28	28	28	28	18	18	56	50	39	44	33	44	39	42	67	00	—	—
17	m1	9	9	9	9	10	9	45	36	36	36	37	55	36	40	82	91	00	—
18	cl	12	12	12	12	14	12	50	37	37	37	37	50	37	40	87	00	00	00
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18

group 2 (rows 1-6)
group 3 (rows 7-14)
group 0 (rows 15-16)
group 4 (rows 17-18)

Fig. 1. Re-arranged matrix of S values—anaerobic organisms tested anaerobically.

1	4s	00	—	—	—														
2	11G	54	00	—	—														
3	10G	54	96	00	—														
4	2s	50	50	50	00	—	—												
5	8G	46	50	46	92	00	—												
6	9G	42	46	42	83	92	00												
7	2T	42	46	42	83	92	00	00											
8	h2	42	50	46	67	71	79	79	00	—	—	—							
9	t1	37	50	46	67	75	75	75	87	00	—	—							
10	h1	33	37	33	54	62	71	71	83	87	00	—							
11	b5	33	37	33	54	58	67	67	87	83	92	00							
12	2R	42	50	46	54	54	62	62	71	62	62	67	00	—	—				
13	11	37	62	58	50	50	50	50	62	62	58	54	71	00					
14	v1	21	25	21	21	25	33	33	50	37	42	46	42	46	00	—			
15	k1	21	25	21	21	25	33	33	50	37	42	46	42	46	00	00			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15			

group 0 (rows 1-3)
group 2 (rows 4-7)
group 3 (rows 8-11)
group 7 (rows 12-13)
group 4 (rows 14-15)

Fig. 2. Re-arranged matrix of S values—anaerobic organisms tested aerobically.

Although similarities exist between group 2 and group 3 especially in their ability to oxidize thiocyanate, the differentiation between these two groups is shown in Table 4. These data indicate that there is no overlap between the highest observed S values for one group and the lowest S value for the other. Moreover, with the exception of case 4 in Table 4, these two groups are distinct even if one adopts the criteria of a probable distribution of S values, plus or minus three standard deviations about the mean for the median organism of either of the two groups. This differentiation was also supported by certain cultural characteristics. These were:

- (1) Only members of group 2 were able to oxidize thiocyanate anaerobically.
- (2) Only members of group 2 were capable of active denitrification in a nitrate-containing liquid medium under anaerobic conditions. Although certain other strains, notably group 3 and group 0, grew under these conditions to a limited extent, they did not possess the ability to denitrify actively producing visible amounts of nitrogen gas.
- (3) Members of group 2 differed from those of group 3 in that the final pH values attained during aerobic cultivation were never below pH 5.0 with sulphur or thio-sulphate as substrate. When the substrate was thiosulphate the amount oxidized was limited by the acidity resulting from the oxidation. For example in medium S 6, 18 % of the thiosulphate was removed by these organisms in 8 days when the pH value had fallen to 5.45 and this was unchanged after incubation for a further 20 days. Similarly, cultures inoculated into media containing 0.5, 1, 2 % (w/v) thiosulphate, oxidized in 28 days 31 %, 15 % and 6 %, of the initial thiosulphate, the corresponding final pH values were 5.35, 5.4 and 5.6. All members of group 3 under aerobic conditions oxidized all the thiosulphate in medium S 6 with a final pH value between 3.5 and 4.0.

Table 4. *Highest and lowest S values to the centrotypes of group 2 and group 3*

	Group 2			Group 3		
	High	Mean	Low	High	Mean	Low
A. Anaerobic tests						
(i) 8G (centrotype of group 2)	83	80	72	44	29	9
(ii) 1s (centrotype of group 3)	22	17	12	87	77	67
B. Aerobic tests						
(iii) 8G (centrotype of group 2)	100	92	83	75	66	58
(iv) h2 (centrotype of group 3)	79	74	67	87	86	83

Table 5. *The sensitivity of groups 2 and 3 to certain antibiotics when tested under aerobic and anerobic conditions*

	Novobiocin		Streptomycin		Bacitracin	
	a	b	a	b	a	b
Group 2	R	R	S	R	S	S
Group 3	S	R	S	S	S	R

a, aerobic conditions; b, anaerobic conditions. R = resistant; S = sensitive.

- (4) Differences exist between these two groups with respect to their antibiotic sensitivity patterns when examined by using Sentest sensitivity discs (Evans Medical

Ltd., Speke, Liverpool) on plate cultures incubated aerobically or anaerobically. The sensitivity patterns of both groups are given in Table 5. Moreover, the sensitivity pattern for these strains differed according to the conditions in a manner comparable to the findings of Kogut, Lightbown & Isaacson (1965) who found that *Escherichia coli* under anaerobic conditions was not inhibited by streptomycin because these conditions interfered with the uptake of this compound. A similar phenomenon may be operative here for group 3 organisms with respect to bacitracin and novobiocin.

The examination of *Thiobacillus intermedius* confirmed that this organism in addition to producing an acidity pH of 2.8 in thiosulphate or sulphur media also grew heterotrophically in common organic media. The isolate 2R was 71 % similar to the authentic culture and should probably be regarded as another strain of the same species.

Thiobacillus thermophilica, very kindly supplied by Dr A. A. Egorova, was not tested with the other strains because of its abnormal temperature requirement; growth did not occur at 37°, but at 57° small colonies were visible after 18 hr which increased to 5 mm. diam. in 10 days. Similar results were obtained in liquid culture and growth only occurred in the presence of either thiosulphate or sulphide. The amount of thiosulphate oxidized was small, only 8 % of a 0.5 % (w/v) thiosulphate medium, in 28 days. This was accompanied, however, by a slight fall in the pH value of the medium and a positive test for sulphate, indicating that some of the thiosulphate had been completely oxidized.

No growth could be obtained in any of the common organic media nor in the basal mineral salts medium without thiosulphate.

The vegetative cells were Gram-negative and rather larger than the other thiobacilli, 2–3 μ by 0.6–0.8 μ . Many terminal spores were observed and the chain formation as described by Egorova & Deryugina (1963) was very characteristic.

DISCUSSION

As may be seen in Fig. 1, the denitrifying group 2 appears distinct from the established species, *Thiobacillus thioparus* (group 3). There is considerable intragroup variation in the similarity values of these two groups which may be the result of the comparatively small selection of tests used in this instance. This is supported to some extent by the results for 2T and 2T' which only resemble each other at 71 % level; these are the same strain but differed in that 2T' was grown aerobically prior to testing.

Baalsrud & Baalsrud (1954) and Woolley *et al.* (1962) have expressed the view that with prolonged aerobic cultivation, *Thiobacillus denitrificans* reverts to a *T. thioparus* type. However, the instance of 2T' quoted above would suggest that this organism resembles more closely the members of group 2 than those of group 3. Further evidence for this differentiation is shown in the physiological characters of denitrification in anaerobic culture, the ability to oxidize thiocyanate anaerobically and in aerobic culture a final reaction not below pH 5.0. The differentiation of groups 2 and 3 under aerobic conditions of cultivation (Fig. 2) is also evident. These data would strongly suggest that *T. denitrificans* is a valid species and distinct from the other named thiobacilli.

From the account of London (1963) and our experimental data there is little doubt that *Thiobacillus intermedius* is a member of the genus *Thiobacillus* and is probably sufficiently different from *T. thio-oxidans* to warrant species rank.

The problem of the classification of *T. thermophilica* is rather more complex for, although it appears to be an obligate autotroph which utilizes reduced sulphur compounds, the morphology of this organism is very different from that of the other thiobacilli. Although it cannot by definition be a member of the Pseudomonadales we suggest that it should be retained within the genus *Thiobacillus* until more evidence of its relationship is forthcoming. To some extent this situation is similar to that of the anaerobic sulphur reducing genus *Desulfovibrio* which included both monotrichous non-sporulating and peritrichous sporulating bacteria. In this group the sporulating organisms have been separated from the non-sporulating types and placed in a different genus (Campbell & Postgate, 1965). However, no indication was given of whether the two genera were to be separated by the removal of the new genus *Desulfotomaculum* from the Pseudomonadales.

A diagnostic table for the identification of all the species of the thiobacilli will be given in a later paper.

The authors wish to thank Professor A. L. Roberts for his continued support of this programme and the British Coke Research Association for financial assistance. We are grateful to the research workers who supplied the cultures given in Table 2.

REFERENCES

- BAALSRUD, K. & BAALSRUD, K. S. (1954). Studies on *Thiobacillus denitrificans*. *Arch. Mikrobiol.* **20**, 34.
- BEERS, R. J. & LOCKHART, W. R. (1962). Experimental methods in computer taxonomy. *J. gen. Microbiol.* **28**, 633.
- BEIJERINCK, M. W. (1904*a*). Phénomènes de réduction produits par les microbes. *Archs néerl. Sci. Ser.* **2**, 9, 131.
- BEIJERINCK, M. W. (1904*b*). Über die Bakterien, welche sich im Dunkelm mit Kohlensäure als Kohlenstoffquelle ernähren können. *Zentbl. Bakt.* (2 Abt.), **11**, 593.
- CAMPBELL, L. L. & POSTGATE, J. R. (1965). Classification of the spore-forming sulphate-reducing bacteria. *Bact. Rev.* **29**, 359.
- DE KRUYFF, C. D., VAN DER WALT, J. P. & SCHWARTZ, H. M. (1957). The utilization of thiocyanate and nitrate by Thiobacilli. *Leeuwenhoek med. Tijdschr* **23**, 305.
- EGOROVA, A. A. & DERYUGINA, A. P. (1963). The spore-forming thermophilic thiobacterium, *Thiobacillus thermophilica* Imschenetskii. *Mikrobiologiya*, **32**, 439.
- HUTCHINSON, M., JOHNSTONE, K. I. & WHITE, D. (1965). The taxonomy of certain thiobacilli. *J. gen. Microbiol.* **41**, 357.
- HUTCHINSON, M., JOHNSTONE, K. I. & WHITE, D. (1966). Taxonomy of the acidophilic thiobacilli. *J. gen. Microbiol.* **44**, 373.
- KOGUT, M., LIGHTBOWN, J. W. & ISAACSON, P. (1965). Streptomycin action and anaerobiosis. *J. gen. Microbiol.* **39**, 155.
- LONDON, J. (1963). *Thiobacillus intermedius* nov.sp.: a novel type of facultative autotroph. *Arch. Mikrobiol.* **46**, 329.
- SNEATH, P. H. A. (1957). The application of computers to taxonomy. *J. gen. Microbiol.* **17**, 201.
- WOOLLEY, D., JONES, G. L. & HAPPOLD, F. C. (1962). Some metabolic differences between *Thiobacillus thio-parus*, *T. denitrificans* and *T. thiocyanoxidans*. *J. gen. Microbiol.* **29**, 311.