Taxonomy of the Genus *Thiobacillus*: the Outcome of Numerical Taxonomy Applied to the Group as a Whole

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SUMMARY

Data from previous numerical analyses have been combined to produce an over-all classification of the genus *Thiobacillus*; 93 strains were examined using 38 tests and 106 character states. The results indicated the possible existence of a new species and demonstrated an unexpectedly close similarity between *T. neapolitanus* and *T. thio-oxidans*.

A number of diagnostic tests have been selected from those used for the numerical analysis. A brief description of the other important features of each species recognized is also given.

INTRODUCTION

The reasons for this investigation of the taxonomy of the thiobacilli and a description of the numerical methods used were given in an earlier paper (Hutchinson, Johnstone & White, 1965). Originally it had been intended to examine all the species using the same series of tests but this was not possible because of the differing physiological requirements of the various species. For this reason *Thiobacillus novellus*, *T. thioparus*, *T. neapolitanus* and '*T. trautweinii*' were tested with thiosulphate as the substrate at pH 6.6 (Hutchinson *et al.* 1965), whereas *T. thio-oxidans* and *T. ferro-oxidans* had to be tested in a similar medium at pH 5 (Hutchinson, Johnstone & White, 1966). The anaerobic species *T. denitrificans* and certain other strains were tested both aerobically and anaerobically at pH 6.6 and 7.0, respectively (Hutchinson, Johnstone & White, 1967). The data from these tests were analysed numerically and the results were highly significant for the groups of species under test. It was apparent, however, that an over-all indication of the interrelationships of the various species could only be obtained from a single numerical analysis which included all the species.

In an attempt to devise a test series which would be suitable for all the species, serious consideration was given to pH values in the range between 5.0 and 6.6, but no one value was satisfactory. The choice, therefore, was between a test series in which some of the species would not grow because of the initial pH value, and a numerical analysis in which the various groups of species were tested at, or near, their optimum pH value. The latter course was regarded as most appropriate although this involved the abandonment of strict Adansonian principles. To achieve further

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uniformity, thiosulphate was chosen as the basal substrate for these tests in that this substrate is utilized by all of the strains.

Numerical methods in taxonomy are designed to remove personal bias from the final result and the authors believe that the present study has been made as objectively as possible. In one instance, however, a separation between two species has been maintained despite the evidence from the numerical analysis.

METHODS

Organisms

From the 128 strains available 87 were selected for inclusion in the analysis together with six strains not described previously. The latter are listed in Table 1. The strains omitted were mainly from groups 3 and 4 and were in most cases duplicates of strains included in the analysis.

The results for all the tests based on thiosulphate were pooled, irrespective of the pH value at which the tests were made. Where necessary, new limits were drawn up for the quantitative tests and the data re-scored. Details of the tests are given in Table 2. In this final analysis 106 characters were scored for the 93 strains.

Table 1. *Strains not previously described and their sources of origin*

Source	Species	Code no.
R. L. Starkey, Rutgers University, New Jersey	<i>T. novellus</i> *	S 1
	<i>T. novellus</i> †	S 2
From lagoon system treating carbonization liquor	<i>T. neapolitanus</i>	6 G
	<i>T. neapolitanus</i>	7 G
Natural spring of sulphuretted water	<i>T. neapolitanus</i>	1 R
Laboratory scale plant treating gas liquor	<i>T. neapolitanus</i>	1 V

* Maintained on thiosulphate agar. † Maintained on nutrient agar.

Analysis of data

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

RESULTS

From the rearranged matrix of S values given in Fig. 1 it is apparent that the groups 0 to 7 which were found corresponded with those reported previously for the individual series of numerical analyses (Hutchinson *et al.* 1965, 1966, 1967). A new group was found which had been overlooked in the previous analyses and this has been designated group 1*a*. This new group does not correspond to any named species and is represented by only two isolates, 10G and 11G, from a lagoon system treating carbonization effluent.

The essential data from the rearranged matrix of S values are listed in Table 3. This method, which has been used previously, permits the comparison of two adjacent groups with reference to the central strain of one of the groups. Thus, in the comparison of groups 3 and 4, strain P 2 was selected as the central strain, and the S values

Table 2. Key to tests used in the final analysis of the thiobacilli

Test	Concentration (%)	Comments	Characters
Sulphur	Excess	Sterilized by steaming on 3 successive days (Hutchinson <i>et al.</i> 1965)	2
Ammonium thiocyanate	0.02	Previously described (Hutchinson <i>et al.</i> 1965)	2
Hydrogen sulphide	.	Previously described (Hutchinson <i>et al.</i> 1965)	2
Ferrous iron	2	Growth determined visually	2
Thioacetamide	0.02	Titrated with iodine	4
Nutrient agar	.	Visible colonies	3
Citrate utilization	.	Growth with citrate as sole energy source	2
Thiosulphate liquid medium	1	Final pH attained	6
	1	Amount of thiosulphate used at 19°	3
	1	Amount of thiosulphate used at 29°	3
	1	Amount of thiosulphate used at 35°	3
	1	Rate of thiosulphate oxidation at 19°	3
	1	Rate of thiosulphate oxidation at 29°	3
	1	Rate of thiosulphate oxidation at 35°	3
	0.5	Amount of thiosulphate used	3
	2	Amount of thiosulphate used	3
	6	Amount of thiosulphate used	2
Anaerobic growth	.	Amount of thiosulphate used in S 8 liquid (Hutchinson <i>et al.</i> 1967)	3
	.	Gas production under anaerobic conditions	2
Thiosulphate agar	.	Sulphur deposition	2
	.	Irregular margin to the colony	2
Inhibitors			
Streptomycin	.	These tests were done on plates using 'Sentest' tablets (Evans Medical Ltd.)	2
Chloramphenicol	.		2
Bacitracin	.		2
Novobiocin	.		2
Ampicillin	.		2
Sulphonamide	.		2
Elemental sulphur	Excess	The results were scored as a percentage of the amount of thiosulphate oxidized relative to a parallel control series grown under the standard conditions with no inhibitor present	4
Nutrient broth	1		4
Mixed phosphate	4		4
Sodium chloride	5		2
Ammonium thiocyanate	0.02		3
Potassium nitrate	1		3
Calcium chloride	1		2
Ammonium chloride	2.5		3
Sodium glutamate	1		4
Phenol	0.0025		3
Phenol	0.01		3

of group 3 to this strain ranged from 74 to 89, with a mean value of 83. The highest S values of group 4 to strain P2 was 57, indicating a gap between the two groups of 17 units. If one assumes a normal distribution of S values about the central organism of each group of plus or minus three standard deviations, the separation of the groups is not as precise as that for the previous individual studies (Hutchinson *et al.* 1965, 1966, 1967). A certain degree of overlap has arisen largely as a result of the adaptation

of the scoring system to encompass the wide variety of strains. However, on the basis of the lowest S value of the group with the highest range of S values and the highest S value of the lower group, there is reasonable agreement between all the results of this and the previous analyses.

The overlap between groups 4 and 5 was rather unexpected because, as explained previously, these two groups cannot be tested at the same pH value. However, once this difference has been removed the two groups are of sufficient similarity to overlap each other on several important points; notably their tolerance of high salt concentrations and growth in 6 % (w/v) $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$.

Table 3. *Comparison of S values to the central strains for adjacent groups*

A	B	S values of group A to central strain of group A				S values of group B to central strain of group A			
		1	2	3	4	1	2	3	4
Group 0 (1A is central strain)	Group 1a	89	78	6.1	68	55	52	*	49
Group 1 (a 1 is central strain)	Group 1a	97	87	7.6	78	55	54	*	52
Group 0 (1A is central strain)	Group 1	89	78	6.1	68	69	61	5.4	52
Group 1 (a 1 is central strain)	Group 2	97	87	7.6	78	64	60	2.8	58
Group 2 (8G is central strain)	Group 3	94	93	1.0	92	73	58	6.8	47
Group 3 (p 2 is central strain)	Group 4	89	83	5.0	74	57	50	3.0	43
Group 4 (1 I is central strain)	Group 5	97	85	5.7	74	81	74	4.6	66
Group 5 (2P is central strain)	Group 6	89	84	4.9	75	61	50	6.8	38
Group 6 (b7F is central strain)	Group 7	91	86	4.3	77	53	46	*	40

* Not applicable.

1 = Highest S value in the set.

2 = Mean S value in the set.

3 = One standard deviation of the S values for the set.

4 = Lowest S value in the set.

If the groups are drawn out spatially they form a network, not the succession of different types as the linear order of the rearranged S value matrix might suggest. An attempt to represent this in a two-dimensional plan is given in Fig. 2. This construction is based on the central strain of each group, i.e. the organism possessing the least standard deviation in its S values to the remainder of the strains of the group. These have been arranged relative to each other by way of 'best fits' using $\log_2 1/S$ (Rogers & Tanimoto, 1960) as the measure of distance. As the production of these diagrams involves the condensation of large amounts of data into two dimensions, it is considered that the method used here is sufficient to achieve a simple over-all picture of interrelationships. A more precise method for calculating the relative positions of the groups has been suggested by Rayner (1965).

The heterogeneity of the groups themselves is represented by the circle of radius

$2\sqrt{(\sum d^2/n)}$ (Gyllenberg, 1965), where d is the distance of the strains from the central strain of the group and n the number of strains within the group. Any overlap of these circles does not necessarily indicate that the two groups concerned merge into each other, the circles only indicate the diversity of the individual groups: in most cases the groups concerned are possibly in different planes. When drawn out in the two dimensional state, planes may be drawn which serve to segregate the species according to certain characteristics which were highly correlated with other tests in the analyses, e.g. autotrophy/heterotrophy and final pH attained in culture; tests which would be termed 'key features' of conventional systematics.

Fig. 2. Plan of the relationships of the groups of thiobacilli showing planes which separate the groups according to specific characters. Relative distance of the groups is based on the relationship $\log_e 1/S$ for the centrotypic of each group. The area of the circles is proportional to the range of S values within the group according to the expression: radius = $2\sqrt{(\sum d^2/n)}$, where d is the distance in terms of difference in S value between each strain and the centrotypic of the group and n is the number of strains within the group.

For the reasons given, it would be unwise to regard this total analysis as representing a true picture of the relationships of the various groups of thiobacilli. Therefore greater attention should be paid to the individual analyses where the strains have been compared directly with others under the same condition of growth. This is not so much a failing of Adansonian principles but is specific to the groups of organisms

involved, for there can be few genera of bacteria in which the species cannot be tested under the same physiological conditions so that direct comparisons may be made between them.

The results of this work indicate that the classification of Parker & Temple (1957) is basically correct, though certain of the diagnostic criteria have been found to be invalid. Our assessment of the status of the various species is given in Table 4.

Table 4. *List of species and redundant names of the thiobacilli*

Species	Original description	Comments
<i>Thiobacillus novellus</i>	Starkey (1934, 1935 <i>a, b</i>)	Good species
<i>T. denitrificans</i>	Beijerinck (1904 <i>a, b</i>)	Good species
<i>T. thioparus</i>	Starkey (1935 <i>a, b</i>)	Type species
<i>T. neapolitanus</i>	Parker & Prisk (1953)	Good species
<i>T. thio-oxidans</i>	Waksman & Joffe (1921, 1922)	Good species
<i>T. ferro-oxidans</i>	Temple & Colmer (1951)	Good species
<i>T. intermedius</i>	London (1963)	Good species
Group 1 <i>a</i>	Hutchinson <i>et al.</i> (this work)	Doubtful species
<i>Ferrobacillus ferro-oxidans</i>	Leathen & Braley (1954)	Redundant, new name <i>T. ferro-oxidans</i>
<i>F. sulfo-oxidans</i>	Kinsel (1960)	Redundant, new name <i>T. ferro-oxidans</i>
<i>T. thiocyanoxidans</i>	Happold <i>et al.</i> (1954)	Redundant, new name <i>T. thioparus</i>
<i>T. concretivorus</i>	Parker (1945)	Not examined in the present study
<i>T. coproliticus</i>	Lipman & McLees (1940)	Not examined in the present study
<i>T. thermophilica</i>	Egorova & Deryugina (1963)	Probably different genus
Strain 1 <i>D</i>	Hutchinson & White (1964)	Probably different genus
<i>T. trautweinii</i>	Trautwein (1921)	Many heterotrophic strains are of this type and should not be regarded as thiobacilli

Procedure for the identification of an unknown strain of Thiobacillus

To achieve an unambiguous identification, the following procedure should be strictly adhered to, paying particular attention to the time factor.

The culture should first be inoculated into both S5 and S6 media to determine the appropriate pH value for the tests. Experience has shown that it is necessary to passage iron oxidizing strains suspected of being thiobacilli, through a sulphur medium before testing them in thiosulphate.

Test media should be inoculated with approximately 0.02 ml. of a 3-day-old culture under the following standard conditions which should be adhered to throughout the test series. These conditions consist of growing the strains at 28° in 10 ml. quantities of the appropriate medium contained in 50 ml. conical flasks in static culture to ensure an adequate but uniform, degree of aeration. Growth is determined by the amount of thiosulphate oxidized and the pH value after 28 days.

The deposition of sulphur intercolonially is determined by microscopical examination of the culture on thiosulphate agar over a period of up to 28 days. Sulphur appears, by transmitted light, as a black opaque material which may be of a crystalline or globular form. Both of these forms have proved to be sulphur by X-ray analysis. Certain strains of group 0 produce a white intercolonial deposit which appears as a 'brown' amorphous deposit on microscopical examination by transmitted light. This material has been identified as sodium sulphate by X-ray analysis.

Table 5. *Useful diagnostic tests for the thiobacilli*

Species ... Group ...	A 0	B 1a	C 1	D 2	E 3	F 4	G 5	H 6	I 7
Medium									
S6/S5	> 6.6	6.5-5.0	6.6-5.0	6.6-5.0	6.6-3.5	3.5-2.8	< 2.0	< 2.0	> 2.0- < 2.8
S6/S5	< 90 %	< 50 %	< 30 %	< 90 %	> 90 %	> 90 %	> 90 %	> 90 %	> 90 %
Criterion									
Final pH	> 6.6	6.5-5.0	6.6-5.0	6.6-5.0	6.6-3.5	3.5-2.8	< 2.0	< 2.0	> 2.0- < 2.8
% thiosulphate oxidation	< 10 %	< 10 %	< 10 %	< 10 %	< 10 %	> 10 %	> 10 %	< 10 %	< 10 %
% thiosulphate	+/-	+	+	+	+	-	-	+	+
Inhibition	+	+	+	+	+	-	+/-	+	+
Final pH	ng	6.1-6.25	ng	5.15	4.6-4.1	3.2-2.8	< 2.0	< 2.0	> 2.0- < 2.8
Sulphur	+	+	-	+	+/-	-	-	-	-
S8	+	+	-	+	+	-	-	-	-
Anaerobic conditions	-	-	-	+	+	-	-	-	-
S7	-	-	-	+	+	-	-	-	-
Thiocyanate oxidation	-	-	-	+	+	-	-	-	-
pH change	-	-	-	-	+/-	-	+	-	-
Iron oxidation	-	-	-	-	-	-	-	+	-
Growth	+	+	+	-	-	-	-	-	+
Koser's citrate	+	+	-	-	-	-	-	-	+
Thiosulphate agar	-	-	+	+	+	+	+	-	+

KEY TO SPECIES. A = 'trautweinii' types; B = group 1a; C = *Thiobacillus novellus*; D = *T. denitrificans*; E = *T. thioparus*; F = *T. neapolitanus*; G = *T. thiooxidans*; H = *T. ferro-oxidans*; I = *T. intermedius*; ng = no growth.

The identification and description of the species of the genus Thiobacillus

In view of the extended period for which the diagnostic tests must be run, the normal dichotomous key has been discarded in favour of the diagnostic table suggested by Cowan & Steel (1961). The relevant test media and diagnostic criteria are listed in Table 5.

The most important of these criteria is the final pH attained in the S6 or S5 medium and the majority of the other tests should be regarded as confirmatory. Data on the individual species which are not easily tabulated are included in the following brief descriptions.

Table 6. *The effect of the initial pH of the medium and its effect on the utilization of thiosulphate by certain facultative autotrophic strains*

Group...	0					I
Strain...	d 1		P 4		b 2	
Initial pH of thiosulphate medium	Thio-sulphate used (%)	Final pH	Thio-sulphate used (%)	Final pH	Thio-sulphate used (%)	Final pH
5.05	0	5.05	0	5.05	0	5.05
6.5	42	7.65	44	7.7	10	5.5
7.6	17	8.2	20	8.55	19	5.15

Results were read after 28 days incubation at 28°.

Group 0 (*T. trautweinii*). The members of this group are facultatively autotrophic and oxidize thiosulphate to tetrathionate with a corresponding increase of the pH value. As reported previously (Hutchinson *et al.* 1965), this group is very heterogeneous and subsequent electron micrographs have shown that one of the two authentic strains of '*T. trautweinii*' is peritrichously flagellated whereas the genus *Thiobacillus* is defined as consisting of Gram-negative, rod-shaped cells; non-motile or motile, by means of a single polar flagellum (Parker & Temple, 1957). For these reasons it is proposed to exclude these organisms from the genus *Thiobacillus* even though certain of them may oxidize greater amounts of thiosulphate than other undoubted thiobacilli, as shown in Table 6. They have been included in this study for completeness because representatives of this group are commonly encountered in nature (Trudinger, 1967).

Group 1 (*T. novellus*). These organisms are facultative autotrophs which oxidize thiosulphate with the production of a small amount of acid; the reaction of S6 medium may fall below that of the control but never below pH 5.0. Thiosulphate utilization is small (usually less than 20 %) though this is probably a reflexion of the limited acid tolerance of these strains (Table 6). The addition of greater amounts of buffer does not significantly increase the amount of thiosulphate used, because these strains are very sensitive to increased salt concentrations. They do not tolerate the inhibitory compounds used in the test scheme.

Heterotrophically, these organisms are very limited in their reactions; they are oxidase-positive, catalase-negative, unable to utilize citrate as sole source of carbon, do not reduce nitrate, do not hydrolyse arginine, tributyrin, tallow or starch, acid is not formed in glucose or lactose, acetylmethylcarbinol and indole are not produced,

gelatin is not liquefied and there is no change in litmus milk. Contrary to the findings of Starkey (1935*a*), growth on a potato plug has not been observed and no pigments were formed on any media. The only readily discernable heterotrophic property for these strains is their growth in proteinaceous media, which is characterized by the formation of streamer like growths extending from the surface of the medium to the bottom of the vessel. This form of growth was described by Starkey (1935*b*).

The original strain isolated by Starkey is widely available. An organism (1 L) was isolated in the course of the present study which resembled the authentic strains.

Group 1a. Although only two isolates of this group have been examined, the characters studied would suggest that they represent a valid group in that they are facultatively autotrophic and produce a slight acidity (pH 6.0) in S6 medium. Moreover, the amount of thiosulphate oxidized is greater than that of *T. novellus* under standard conditions. These strains differ from *T. novellus* in their ability to grow anaerobically in thiosulphate media, but fail to produce visible evidence of denitrification. In nitrate peptone broth, however, nitrogen gas is formed. Citrate is utilized as the sole source of carbon. The numerical analysis has shown that these strains may represent a new species, but it is thought that more strains should be examined before a name is given. Similar strains were described by Tjulpanova-Mossevitich (1930) and Sijderius (1946).

Group 2 (T. denitrificans). These are autotrophic strains which are able to oxidize sulphur, thiosulphate and thiocyanate under anaerobic conditions in the presence of nitrate. This latter anion is reduced to nitrogen gas which is clearly visible when these organisms are grown in completely filled and stoppered bottles. Good growth is obtained on solid media containing thiosulphate and nitrate when incubated in a McIntosh & Fildes jar and large amounts of elementary sulphur are deposited intercolonially.

Organisms of this group are also capable of aerobic growth but under these conditions the amount of thiosulphate used is considerably reduced. This is a reflexion on the unfavourable pH attained (pH 5.0). This does not occur under anaerobic conditions as the fall in pH is moderated by the products of the nitrate reduction. (Baalsrud & Baalsrud, 1954). This species resembles *T. thioparus* in its sensitivity to inhibitory compounds.

Strains of this species appear to have been isolated at intervals since the original description by Beijerinck (1904*a, b*), but no authentic strains of *T. denitrificans* could be located. The strains of this group isolated during the course of this work have been deposited with the National Collection of Industrial Bacteria, accession numbers 9546, 9547 and 9548.

Group 3 (T. thioparus). As with group 2, these organisms oxidize thiocyanate, thiosulphate and sulphur under aerobic conditions. The final pH value with the two latter substrates is always below 5.0 but never less than pH 3.5. Although essentially aerobic, they possess a limited ability to grow anaerobically in that colonies are produced on thiosulphate-nitrate agar in a McIntosh & Fildes jar; unlike *T. denitrificans*, they die out with prolonged anaerobic cultivation. In liquid anaerobic culture, gas production has never been observed, nor appreciable amounts of thiosulphate or thiocyanate used. These strains are inhibited by many of the compounds listed in Table 2, and in some cases the final pH is greater than 7.0, indicating that the oxidation of thiosulphate has not proceeded beyond the tetrathionate stage.

Thiobacillus thioparus is regarded as the type species of this genus. Many authentic strains of this species are available and it is readily isolated from a wide variety of sources. All the named strains of *T. thiocyanoxidans* examined were grouped with *T. thioparus*.

Group 4 (*T. neapolitanus*). This species differs from *T. thioparus* in that it is unable to oxidize thiocyanate, or grow anaerobically. Thiosulphate and sulphur are oxidized with the production of acid which becomes inhibitory when the pH falls to 2.8. In 1 % thiosulphate liquid medium the amount of sulphur deposited varies from strain to strain. Strains of *T. neapolitanus* are very resistant to inhibitors and in general are not affected by 6 % thiosulphate, 4 % phosphate, 5 % sodium chloride, 1 % sodium glutamate or 100 p.p.m. phenol.

They have been isolated from many sources, and some strains of this species have been incorrectly described as *T. thioparus*.

Group 5 (*T. thio-oxidans*). This species oxidizes sulphur, hydrogen sulphide and thiosulphate with the production of pH values below 2.0. As Kempner (1966) has shown, when grown in a medium containing sulphur pH values below 1.0 may be recorded and viable bacteria recovered. Initiation of growth in thiosulphate media only occurs in the pH range 4.0 to 5.7 but in sulphur-containing media this pH range extends from 3.0 to 6.4.

Thiobacillus thio-oxidans shares with group 4 a resistance to high salt concentrations which are inhibitory to most other species of thiobacilli. Tolerance to intense acidities is the only reliable character which serves to differentiate these two groups. On solid media this intense acid formation by *T. thio-oxidans* produces a characteristic fogging of the agar around the colonies.

Strains of this species are easily isolated from acidic environments, particularly those associated with mining operations. The only named culture of *T. concretivorus* which could be included in the analysis was classified in group 5 and no new isolate corresponding to this species was found.

Group 6 (*T. ferro-oxidans*). In sulphur- and thiosulphate-containing media this species resembles *T. thio-oxidans* with respect to final pH value but, unlike the latter species, ferrous iron may also be used as the sole energy source. It also differs in that hydrogen sulphide is not metabolized by *T. ferro-oxidans* and growth is inhibited by the high concentrations of salts and organic compounds used in this study.

A further diagnostic criterion is their colony form on thiosulphate agar on which characteristic colonies are produced with a flat profile, irregular shape and a crystal-line intercolonial deposit. This material gives them the frosty appearance described by Colmer, Temple & Hinkle (1950); Colmer (1962).

Strains of this species have been isolated from acid mine waters, a natural ferruginous water and domestic sewage. All the named strains of the iron-oxidizing species *T. ferro-oxidans*, *Ferrobacillus ferro-oxidans* and *F. sulfo-oxidans*, were placed in one group by the numerical analysis. Because all of these species oxidize sulphur, thiosulphate and ferrous iron, it is considered that certain of these names are redundant. A possible explanation of the reported differences in the substrate utilization by these species was reported previously (Hutchinson *et al.* 1966).

Group 7 (*T. intermedius*). Only two strains of this group have been examined; the original isolate of London (1963) and a similar strain isolated during this study. They both possess the unusual combination of characters of facultative heterotrophy and

acid tolerance. In sulphur or thiosulphate media they produce final pH values in the range 2.0 to 2.8.

In thiosulphate media these strains are inhibited by the various inorganic compounds used in the test scheme but, in general, the organic inhibitors have no effect.

Heterotrophically, these organisms grow to a limited extent in nutrient broth or nutrient agar and this is marginally improved by the addition of yeast extract. Citrate is utilized as the sole source of carbon.

Thiobacillus thermophilica (Egorova & Deryugina, 1963) was also examined and it was confirmed that this organism is strictly a thermophilic autotroph, though its exceptional morphology in being a spore former probably singles it out as belonging to a different genus. Similarly, the evidence for strain 1 D, a heterotrophic isolate which possesses the ability to grow autotrophically on thiocyanate but not on thiosulphate, is against its inclusion in the genus *Thiobacillus* (Hutchinson & White, 1964).

DISCUSSION

The apparent degree of success achieved by the application of numerical analysis in this genus may be attributed to the occurrence of species which form discrete groups with no intermediate forms. In certain other analyses the species, and even genera, were not discontinuous and in these cases numerical analysis was of limited applicability (Graham, 1964; Kreig & Lockhart, 1966). Although numerical taxonomy gives rise to groupings it does not in any way identify them. This may be done by the inclusion of authentic cultures in the analysis or by the comparison of the original test results with descriptions from the literature. Both methods have been used to name the groups found in this analysis and in general, most of the groups correspond to the species recognized by Parker & Temple (1957).

A recent adjunct to systematics utilizes DNA base composition in that related bacterial species should have a similar over-all base composition. This concept was applied by Jackson, Moriarty & Nicholas (1968) to test the homogeneity of certain autotrophs, notably authentic cultures of thiobacilli. Their findings are almost in complete agreement with the present multivariate analysis. However, although it is possible for two species to have the same over-all base composition, the base sequences can be entirely different resulting in completely different phenotypic expression. This is well illustrated in the work of Jackson *et al.* (1968), who found G+C base compositions of 56 % and 57 % (± 1 %) for *Thiobacillus neapolitanus* and *T. ferro-oxidans* which are very different physiologically and biochemically. It is considered that DNA base ratios may not in themselves be diagnostic at the species level.

For the purpose of numerical analysis, several new differential tests have been investigated and certain of these have proved to be diagnostic. The diagnostic tests are listed in Table 5. Of all the tests, the most conclusive are those which depend upon the utilization of an unusual substrate such as thiocyanate or ferrous iron. The differentiation based on final pH attained is also important, provided a strict experimental procedure is adopted which allows growth to proceed to completion without any physical or chemical constraints being applied. Inhibitory conditions tend to produce an incomplete metabolism of thiosulphate to tetrathionate with a corresponding rise in pH, $4\text{Na}_2\text{S}_2\text{O}_3 + 2\text{H}_2\text{O} + \text{O}_2 = 2\text{Na}_2\text{S}_4\text{O}_6 + 4\text{NaOH}$. Vishniac & Trudinger (1962) have described a similar phenomenon with respect to the availability of oxygen.

Several of the diagnostic tests which have been suggested previously for this genus were found to be unsatisfactory. All the strains examined produced polythionates under our test conditions with the possible exception of *Thiobacillus novellus*. With this organism the amount of thiosulphate metabolized was so small that the detection of polythionates was outside the experimental error of the analytical method. Although these results differ from those of Parker & Temple (1957) similar findings have been reported by Pankhurst (1964).

It has been suggested (Parker & Prisk, 1953) that the form of the nitrogen source might be used as a diagnostic test, but our experiments suggest that it is difficult to obtain an absence of growth in nitrogen free media used for control purposes despite all reasonable precautions to exclude possible sources of nitrogen contamination.

Baalsrud (1954) suggested that the thiobacilli may represent a spectrum of types in that he could arrange all of his isolates in a series according to the final pH attained in culture. In this study considerable attention has been given both to the pH value at which growth may be initiated and also the final value after 28 days. The latter closely paralleled the findings of Baalsrud (1954). Our work would indicate, however, that the spectrum is not continuous as there has been no evidence of interchange of strains of one group with another and the lower limits of the final pH values are extremely stable. This property has proved the most fundamental and reliable diagnostic feature of these organisms and is in every case correlated with other characteristics.

Bunker (1936) was of the opinion that a spectrum of types merging from the autotrophic thiobacilli to common heterotrophs may be possible. The number of thiobacilli which possess heterotrophic abilities is increasing with the discovery of new species such as *T. intermedius*, and possibly the group 1*a* and strain 1*D* in the present study. This is further supported by reports in the literature of an iron-oxidizing strain which has been reported to grow on glucose (Remsen & Lundgren, 1963) and similarly for a strain of *T. thio-oxidans* (Borichewski & Umbreit, 1964, 1966). This suggests that there may be a series of these organisms, each of which could conceivably represent a heterotrophic analogue of the unknown obligate autotrophs.

No difficulty was experienced in isolating a sufficient number of strains of most species with the exception of *T. novellus*, *T. intermedius* and the new group 1*a*. This may have occurred because they may be comparatively rare in nature, or alternatively special techniques are required for their isolation. Such species, however, do give rise to difficulties in numerical analysis because the possible extent of the group cannot be judged from only one or two strains. These considerations suggest that the absence of intermediate forms between the groups in this study may simply be a function of the number of isolates examined, and increasing this by a suitable factor may have led to the recognition of intermediate forms.

As interspecies change had been encountered previously in this genus (Johnstone, Townshend & White, 1961) all the stock cultures were carefully observed at frequent intervals for a repetition of this phenomenon. However, there were only four instances in which unusual strains were found associated with the stock cultures which, in view of the maintenance of over 100 cultures for a period of three years, could possibly represent contamination. No unusual strains were encountered when a number of representative isolates were regularly subcultured either on solid media, liquid then solid media, or solely in liquid over a period of six months.

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