Deoxyribonucleic Acid Base Composition and Taxonomy of Thiobacilli and some Nitrifying Bacteria

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SUMMARY

The DNA base composition of 14 authentic strains of the genus *Thiobacillus*, determined by caesium chloride density gradient centrifugation, was found to vary from 51 to 68 mole % guanine + cytosine (G + C). The mole % G + C values for the various species are as follows: *T. thiooxidans* and *T. concretivorus* 51–52, *T. neopolitanus* and *T. ferrooxidans* 56–57, and *T. thioparus*, *T. thiocyanoxidans*, *T. denitrificans*, *T. novellus* and *T. trautweinii* 62–68. Another group of chemoautotrophic bacteria, the nitrifiers *Nitrosomonas europaea* and *Nitrobacter agilis*, were found to have a G + C content of 52 and 65 %, respectively. The results are compared with other types of taxonomic studies made with these and related bacteria.

INTRODUCTION

Thiobacilli are Gram-negative, non-sporulating, rod-shaped bacteria, usually found in soils and in marine and fresh water (Vishniac & Santer, 1957; Trudinger, 1967*a*). It might be assumed from their common function in oxidizing certain inorganic sulphur-containing compounds and in utilizing carbon dioxide as sole carbon source, that species within the genus *Thiobacillus* are closely related genetically. These physiological criteria may not however be sufficient in themselves to support this conclusion, since many different types of micro-organisms fix carbon dioxide, e.g. the nitrifying bacteria (Alexander, 1961), and some heterotrophic organisms readily oxidize sulphur compounds (Trudinger, 1967*b*).

The taxonomy of thiobacilli has recently been studied by Hutchinson, Johnstone & White (1965, 1966, 1967). They used a multivariate analysis (Beers & Lockhart, 1962; Sneath, 1957) to demonstrate that the genus consists of well-defined species and an unusual absence of intermediate forms. This method, however, has the disadvantage that it does not analyse directly the relationships between all the species of the genus *Thiobacillus*. This is because some of these species are aerobic, others anaerobic, some acidophilic, whereas others prefer more neutral pH conditions. Moreover, although this numerical analysis was based upon 100 or so phenotypic properties, it accounts for only a small proportion of the bacterial genome. Another way of examining the interrelationships between a group of micro-organisms is to compare the over-all base composition of their DNA complement. It has been applied with success to the pseudomonads (Mandel, 1966) and other bacteria. A list of the DNA base compositions of some bacteria was given by Hill (1966). For bacterial species to be closely related, it is necessary that their DNA should have a similar over-all base composition.

This method has the advantage over multivariate analysis in that a comparison of the thiobacilli can be made directly on the basis of the whole genome. We have extracted DNA from various species of thiobacilli and from some nitrifying bacteria, and examined the over-all base composition of each by CsCl density gradient centrifugation.

METHODS

Organisms. Strains of *Thiobacillus thiooxidans*, *T. concretivorus*, *T. novellus*, *T. trautweinii* and *T. ferrooxidans* were supplied by the National Collection of Industrial Bacteria (NCIB), Torrey Research Station, Aberdeen, Scotland (see Table 1). Strains 1 P and 2 P, originally from C. P. Parker, Melbourne, Australia, were procured from Dr R. Swaby, C.S.I.R.O., Division of Soils, Adelaide, South Australia. Samples of *T. neopolitanus* and *T. denitrificans* were obtained from Dr P. A. Trudinger (Baas-Becking Laboratories, Canberra). Dr I. M. H. Aleem (Research Institute for Advanced Studies, Baltimore, Maryland, U.S.A.), supplied strains of *Ferrobacillus ferrooxidans* (hereafter referred to as *T. ferrooxidans*; for discussion of the nomenclature see Hutchinson *et al.* 1966) and *Nitrobacter agilis*. The *Nitrosomonas europaea* strain used was obtained from Dr Jane Meiklejohn (Rothamsted Experimental Station, England).

Growth conditions. All the bacteria used here were grown in media listed below, either in flasks on a gyrotary shaker where the total volume was 1 l. or less, or in carboys up to 40 l. capacity. In the latter case sterile air was forced through the medium by using sintered glass aerators attached to a compressed air line. The culture media were either sterilized at 121° for 45 min. or, when more than 10 l. were used, the medium was filtered through a Millipore filter assembly (diam. 142 mm., pore size 0.22 μ , with prefilter). The larger carboys were sterilized by placing a specially constructed ultraviolet lamp inside them for several hours. The energy output of this lamp was 95 microwatts/cm.² at 1 m. distance, measured at 253.7 m μ .

A minimum of 10 % (v/v) inoculum was used to seed the cultures in all these experiments. All cultures were grown at 30° and the growth rate followed by counting the bacteria periodically in a haemocytometer. A generation time of not less than 14 hr was recorded for these chemoautotrophic bacteria.

Media. *Thiobacillus thioparus*, *T. novellus* and *T. trautweinii* were grown in the culture medium containing thiosulphate as described by Vishniac & Santer (1957) for *T. thioparus*; this medium was initially at pH 7. Growth of *T. thioparus* and *T. novellus* decreased the pH value of the medium, while that of *T. trautweinii* increased it. *Thiobacillus thiooxidans* and *T. concretivorus* were grown in a medium similar to that for *T. thioparus*, except that the initial pH value was made to 5.6 by substituting the following amounts of phosphate buffer (g./l. medium): K₂HPO₄, 0.5 and KH₂PO₄, 7.5.

The two strains of *Thiobacillus ferrooxidans* were grown in a medium containing (g.): (NH₄)₂SO₄, 3; KCl, 0.1; MgSO₄·7H₂O, 0.5; Ca(NO₃)₂, 0.01; KH₂PO₄, 0.2; in glass-distilled water, 700 ml. To this solution, which was autoclaved separately, was added 45 g. FeSO₄·7H₂O and 1 ml. 10 N-H₂SO₄ dispensed in 300 ml. distilled water. The medium was then at pH 2.7.

The medium for *Nitrosomonas europaea* contained (g./l. distilled water): (NH₄)₂SO₄, 4; KH₂PO₄, 0.5; and (mg./l.) MgSO₄·7H₂O, 50; CaCl₂·2H₂O, 4; chelated iron, 0.1

(Nicholas & Rao, 1964). Phenol red was added as internal indicator and the medium adjusted with 20% (w/v) K_2CO_3 solution to pH 8.

Nitrobacter agilis was grown in the following medium (g./l.): KNO_3 , 0.3; K_2HPO_4 , 0.175; $MgSO_4 \cdot 7H_2O$, 0.175; NaCl, 0.1; $KHCO_3$, 0.5; and chelated iron, 0.1. A sterile 10% (w/v) solution of KNO_3 was added automatically to maintain a value of 200 mg./l. during growth.

Isolation of DNA. About 1–4 g. wet weight of bacteria was collected from 10 to 40 l. of culture by continuous flow centrifugation in a Sorvall RC-2 refrigerated unit. The bacteria were stored at -17° . The procedure of Marmur (1961) for Gram-negative bacteria was used to prepare the DNA, except where otherwise stated in the results. The DNA preparations were characterized by u.v. absorption, and deoxy-pentose, pentose and organic phosphate determinations. The preparations were stored in 0.05 M-tris HCl (pH 8) at -17° .

Determination of DNA base composition. The base composition of the various samples was determined by CsCl density gradient centrifugation as follows: CsCl (1.128 g.) was dissolved in 0.86 ml. 0.01 M-tris HCl buffer (pH 8) containing approximately 5 $m\mu$ moles of the DNA to be tested and 5 $m\mu$ moles of a DNA from either calf thymus or *Escherichia coli* as a reference. *E. coli* DNA was prepared from *E. coli* B cells according to the method of Marmur (1961), and the DNA from calf thymus purified following the procedure of Kay, Simmons & Downce (1952). Optical grade CsCl was obtained from Stanley H. Cohen (Yonkers, New York). The refractive index of the solution containing the DNA samples was measured to check the final CsCl concentration. This solution was then used for density gradient centrifugation in a Spinco Model E analytical ultracentrifuge fitted with a titanium rotor. The u.v. photographs taken after 17 hr. at 44,770 rev./min. and 25° , were used to determine the density of the unknown DNA. For this purpose, tracings were made on a Beckman Analytical recording densitometer equipped with a film attachment. The buoyant densities of the reference DNA samples were taken as 1.710 g. cm^{-3} for *E. coli* DNA and 1.699 g. cm^{-3} for calf thymus DNA, and the base composition of the unknown sample calculated as described by Schildkraut, Marmur & Doty (1962), using the equation $\rho = 1.660 + 0.098 (GC)$, where ρ = buoyant density.

RESULTS AND DISCUSSION

DNA isolation

Since most of the chemoautotrophic bacteria investigated are Gram-negative, they were easily lysed by detergent as described by Marmur (1961). There were, however, two exceptions, *Thiobacillus concretivorus* and *T. ferrooxidans*.

Thiobacillus concretivorus, strain 1P. Unlike strains 2P and 9514, strain 1P did not lyse with sodium lauryl sulphate. To prepare a DNA sample from this strain, it was necessary to break the bacteria with aqueous phenol mixtures. Bacteria (2 g. wet wt) were suspended in 20 ml. 0.05 M-tris HCl (pH 8) containing 2% (w/v) sodium lauryl sulphate, then shaken with 20 ml. phenol saturated with aqueous M-tris HCl (pH 8). After centrifugation at 6000 g for 10 min., the upper aqueous layer was collected and the DNA precipitated from it with 2 vol. of ethanol. The method of Marmur (1961) was then followed to achieve further purification. Despite the different extraction technique used, the base composition of the DNA prepared from strain *T. concretivorus* 1P was the same as that for strains 2P and 9514 (see Table 1).

Thiobacillus ferrooxidans. In these experiments, *T. ferrooxidans* organisms were not lysed by detergent alone, lysozyme followed by detergent, grinding with glass powder (500-mesh), or even by shaking with aqueous phenol mixtures at 20° or 60°. A DNA preparation was obtained by suspending 0.6 g. wet wt bacteria in a mixture containing 2.5 ml. of 0.1 M-tris HCl (pH 8) and 2.5 ml. phenol previously saturated with aqueous M-tris HCl (pH 8). This suspension was given ultrasonic treatment for 1 min. at 0° with an M.S.E. model 60 W. ultrasonic disintegrator (20 kcyc./sec.). After centrifugation at 6000 g for 10 min. the upper aqueous layer was removed and the DNA precipitated with 2 vol. of ethanol. The purification was then continued as described by Marmur (1961). This method, however, did not always yield DNA. It is possible that small amounts of ferric oxide and perhaps other compounds of iron in the bacteria

Table 1. *DNA base compositions of Thiobacillus species*

Species	Strain	Buoyant density (g. cm. ⁻³)	Base composition (mole % G+C)
<i>T. trautweinii</i>	NCIB 9549	1.725	66
<i>T. novellus</i>	NCIB 9113	1.727	68
	NCIB 8093	1.725	66
<i>T. denitrificans</i>	Baas-Becking (Trudinger)	1.723	64
<i>T. thioparus</i>	NCIB 8349	1.725	66
	NCIB 8370	1.721	62
<i>T. thiocyanoxidans</i>	NCIB 5177	1.722	63
<i>T. neopolitanus</i>	Baas-Becking (Trudinger)	1.715	56
<i>T. ferrooxidans</i>	(Aleem)	1.716	57
<i>T. thiooxidans</i>	NCIB 9112	1.711	52
	NCIB 8085	1.711	52
<i>T. concretivorus</i>	NCIB 9514	1.710	51
	1P	1.710	51
	2P	1.711	52

complexed with some or all of the DNA and RNA, rendering them insoluble during cell breakage. Ferric ions are known to precipitate DNA (Hammerstein, 1924). Although differential centrifugation removed some of the free ferric compounds from the whole bacteria, this did not improve the extraction of the nucleic acids, presumably because of the iron compounds within the cells (Dugan & Lundgren, 1965). Addition of Na-EDTA during lysis was without effect. After several attempts, DNA was isolated from Aleem's strain of *T. ferrooxidans* but as yet none has been obtained from strain NCIB 9490. The DNA preparation from Aleem's strain gave a band much broader than usual in the CsCl density gradient, indicating a relatively low molecular weight. This was probably a result of shearing forces operating during the ultrasonic treatment.

DNA base composition of Thiobacillus species

The buoyant densities of CsCl solutions and the corresponding base compositions of DNA preparations from the *Thiobacillus* species are listed in Table 1. In general, the value for buoyant density is reliable to ± 0.001 g. cm.⁻³, and so the probable error in base composition as determined by this method is $\pm 1\%$ G+C (Schildkraut, Marmur & Doty, 1962).

The DNA base composition for all the members of the genus *Thiobacillus* tested were in the range 51–68 mole % G + C. Further subdivision into three groups can be made, based on this parameter. Thus one group with 62–68 % G + C included *T. trautweinii* (the only facultative autotroph in the genus), *T. novellus*, *T. denitrificans*, *T. thioparus* and *T. thiooxyanoxidans*. These organisms thus have G + C content similar to some of the pseudomonads (Hill, 1966), in particular *Pseudomonas aeruginosa* (64–68 %) and *Pseudomonas fluorescens* (60–64.5 %). Since these heterotrophs also oxidize certain inorganic sulphur compounds (Trudinger, 1967*b*), it would be of interest to study further their relationship to the genus *Thiobacillus*.

Another group which included *Thiobacillus thiooxydans* and *T. concretivorus* showed a base composition of 51–52 % G + C, and was therefore clearly separated from the rest of the genus. *Thiobacillus neopolitanus* and *T. ferrooxydans* were distinct from both these groups since the base composition was intermediate (56–57 % G + C). This does not necessarily mean that these two bacteria are closely related to each other, but it does suggest that they are not closely allied to the other members of the genus.

The DNA preparation from *Thiobacillus thiooxyanoxidans* NCIB 5177, by CsCl density gradient centrifugation, gave a second minor band. This band made up to 10–20 % of the total DNA present, and had a buoyant density of 1.718 g. cm.⁻³, and a G + C content of 59 %. This observation illustrates an advantage of the buoyant density method over other techniques for base composition determination, since it shows that all the preparations investigated in the present work with the exception of *T. thiooxyanoxidans* NCIB 5177, had only one species of DNA present with respect to over-all base composition. We have no proof at present that this minor band is not derived from a second or contaminating organism in the *T. thiooxyanoxidans* culture. This minor band, like the major component, is shifted by 0.013 g./cm.⁻³ to a higher buoyant density in CsCl solutions following heat denaturation, and so is double stranded. It may be of interest that *Pseudomonas stutzeri*, another organism capable of oxidising inorganic sulphur compounds, was found by Mandel (1966) to have a minor 'satellite' DNA component.

Brief reports of the DNA base composition of two *Thiobacillus* species have previously been recorded. Marmur, Falkow & Mandel (1963) quote a value of 68–70 mole % G + C for *T. thioparus* (method unspecified); this is reasonably close to the figure reported here. Bohacek, Kocur & Martinec (1965) give a value of 58–59 % G + C for *T. novellus* which is different from our measurements made on two authentic strains.

Comparison with a multivariate analysis of Thiobacillus species

Hutchinson *et al.* (1965, 1966, 1967) were able to recognize six groups within the genus *Thiobacillus* by means of a multivariate analysis. The order of similarity (S values) of these groups, as indicated by this numerical analysis, is compared in Table 2 with data from the over-all DNA base composition. Where S values can be compared directly (groups 0–4), the order tends to follow that of the base composition.

To make this comparison, *T. concretivorus* is included in group 5 with *T. thiooxydans*, whereas *T. thiooxyanoxidans* is placed in group 3 with *T. thioparus*. Hutchinson *et al.* (1965) stated that there was not sufficient difference in S values between *T. thiooxyanoxidans* and *T. thioparus*, or between *T. concretivorus* and *T. thiooxydans*, to justify subdividing either group 3 or group 5. The results for DNA base composition

reported here tend to support this view (see Table 1). Another point of agreement between the two investigations is the unequivocal demonstration of the difference between *T. thioparus* and *T. neopolitanus* (Table 2). Some doubt had been expressed earlier about this (see discussion by Hutchinson *et al.* 1965).

Table 2. *Comparison of multivariate analysis and over-all DNA base composition*

Species	Group no. (multi- variate analysis)	Range of S values*	Mean S value*	DNA base composition (mole % G + C)
<i>T. trautweinii</i>	0	94-63	71	66
<i>T. novellus</i>	1	49-46	47	66-68
<i>T. denitrificans</i>	2	—	—	64
<i>T. thioparus</i> †	3	45-24	35	62-66
<i>T. neopolitanus</i>	4	37-18	27	56
<i>T. ferrooxidans</i>	6	—	—	57
<i>T. thiooxidans</i> ‡	5	—	—	51-52

* The S values listed are taken from Table 4 of Hutchinson *et al.* (1965). They refer to S values of the various strains, all with respect to the 3F strain of *T. trautweinii*. This particular strain was chosen since it represents an extreme case, and so enables all the species to be arranged in an order of similarity. *T. denitrificans* is listed between *T. novellus* and *T. thioparus* by reason of the results of aerobic tests made by Hutchinson *et al.* (1967).

† Includes *T. thiocyanoxidans*.

‡ Includes *T. concretivorus*.

Table 3. *DNA base composition of Nitrobacter agilis and Nitrosomonas europaea*

Bacterium	Energy yielding reaction	Buoyant density (g. cm. ⁻³)	DNA base composition (mole % G + C)
<i>N. agilis</i>	$\text{NO}_2^- \rightarrow \text{NO}_3^-$	1.724	65
<i>N. europaea</i>	$\text{NH}_4^+ \rightarrow \text{NO}_2^-$	1.711	52

Starkey (1935) and Baalsrud (1954) suggested that *Thiobacillus trautweinii* be excluded from the genus *Thiobacillus*. The results of Hutchinson *et al.* (1965) support this view, although they state that their investigations were not designed to test this point specifically. Although it has a G + C content within the range found for other species classified in the genus, the present DNA base composition studies carried out on this organism do not allow a positive decision to be made on this point.

As mentioned in the Introduction, a DNA base composition study enables a comparison to be made between all members of the genus, regardless of cultural conditions. Thus, as well as substantiating the suggestion of Hutchinson *et al.* (1965) that *Thiobacillus thiooxidans* and *T. ferrooxidans* are clearly separable, the present work goes further and establishes that *T. thiooxidans* (including *T. concretivorus*) are not closely related to the rest of the genus.

DNA base composition of the nitrifying bacteria

Although *Nitrosomonas europaea* and *Nitrobacter agilis* are both nitrifying bacteria (Alexander, 1961), they are quite distinct from each other since they utilize different inorganic nitrogen compounds, and have a distinctive cell ultrastructure (Murray,

1963). This is also confirmed by their widely different DNA base composition (Table 3). The value obtained here for *N. europaea* is similar to that quoted by Marmur *et al.* (1963) for a '*Nitrosomonas* sp.' (54–56 % G+C), and by Anderson, Pramer & Davis (1965) who found 50–51 % G+C for *N. europaea*. The DNA samples from the nitrifying bacteria have a G+C content within the range found for the other group of chemoautotrophs, *Thiobacillus* spp. Because it is possible to have the same over-all base composition with entirely different base sequences (and thus with different genetic 'messages'), this method does not permit us to decide whether or not these chemoautotrophs are closely related genetically.

CONCLUSIONS

DNA base composition studies of species of the genus *Thiobacillus* have confirmed some of the similarities and differences previously indicated between the species in a numerical analysis (Hutchinson *et al.* 1965, 1966, 1967). The present work suggests a possible relationship between some members of the genus (*T. trautweinii*, *T. novellus*, *T. denitrificans* and *T. thioparus*) and certain pseudomonads, e.g. *Pseudomonas aeruginosa* and *P. fluorescens*, which oxidize sulphur compounds. Although the base compositions of the chemoautotrophic nitrifying bacteria examined are in the range obtained with *Thiobacillus* species the method does not permit any conclusions about the relationship between them.

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