Some Metabolic Differences Between *Thiobacillus thioparus*, *T. denitrificans* and *T. thiocyanoxidans*

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SUMMARY

Differences in behaviour of *Thiobacillus thioparus*, *T. denitrificans* and *T. thiocyanoxidans* were found in growth experiments carried out in thiosulphate or thiocyanate containing media and using aerobic or anaerobic conditions. These differences were: *T. denitrificans* and *T. thioparus* grew anaerobically in thiosulphate + nitrate and thiocyanate + nitrate media; *T. thiocyanoxidans* did not. During growth of *T. thioparus* in thiocyanate + nitrate medium, nitrite accumulated but thiocyanate was not completely oxidized; no nitrite accumulated during growth of *T. denitrificans* under similar conditions. After aerobic, serial subculture in thiosulphate medium *T. denitrificans* no longer grew anaerobically in thiosulphate + nitrate or thiocyanate + nitrate media whereas *T. thioparus* did. *T. thioparus* produced copious growth on thiosulphate agar slopes under aerobic conditions; *T. denitrificans* proved difficult to culture under such conditions, i.e. there was a failure to guarantee growth on each occasion, and where growth occurred this was poor. *T. denitrificans* did not produce the mucoid slime associated with *T. thioparus* cultures. *T. thioparus* accumulated and subsequently used up tetrathionate during aerobic growth in thiosulphate medium; *T. denitrificans* did not.

INTRODUCTION

De Kruyff, Van der Walt & Schwartz (1957) claimed, as a result of growth experiments, that under anaerobic conditions in thiosulphate + nitrate or in thiocyanate + nitrate media *Thiobacillus denitrificans* reduced nitrate to nitrogen but that *T. thioparus* and *T. thiocyanoxidans* reduced nitrate only to nitrite. They suggested, moreover, that the two latter organisms were metabolically identical. Happold, Jones & Pratt (1958) agreed that *T. thioparus* and *T. thiocyanoxidans* grew similarly at the expense of thiocyanate under aerobic conditions. Pratt (1958) showed that *T. thioparus* cultures accumulated polythionates at an earlier stage of growth than was the case with *T. thiocyanoxidans* cultures, grown aerobically in thiosulphate medium. Jones & Happold (1961), in confirming Pratt's results, showed that oxidation of the polythionates was potassium ion dependent. We have consequently attempted to define further the similarities and dissimilarities between these organisms. Vishniac & Santer (1957) stated that *T. denitrificans* 'differed from *T. thioparus* only in ability to grow under anaerobic conditions with nitrate as terminal respiratory electron acceptor, and that *T. denitrificans* could grow aerobically in absence of nitrate in thiosulphate medium but rapidly lost denitrifying ability on aerobic subculture, thus becoming indistinguishable from *T. thioparus*'. The experiments reported in the present paper were designed to resolve the apparent

difference in the views of the above authors, namely, that de Kruyff *et al.* (1957) could grow *T. thioparus* anaerobically but that Vishniac & Santer (1957) could not, and to obtain information which might be used in differentiating between the three species of *Thiobacillus* used here.

METHODS

Organisms. The initial culture of *Thiobacillus denitrificans* was obtained from Dr K. S. Baalsrud, and was subcultured weekly in screw-capped bottles completely filled with the liquid thiosulphate + nitrate medium of Baalsrud & Baalsrud (1954).

The culture of *Thiobacillus thiocyanoxidans* used was developed from a single cell isolated from a crude culture which oxidized thiocyanate.

The initial culture of *Thiobacillus thioparus* was obtained from the National Collection of Industrial Bacteria (NCIB 8370) described as 'Starkey's original non-motile strain'. From this culture a single colony on a thiosulphate agar plate was picked, grown and used for subculture.

The *T. thiocyanoxidans* and *T. thioparus* cultures were subcultured weekly on thiosulphate agar slopes.

Media. For anaerobic cultures the thiosulphate + nitrate medium of Baalsrud & Baalsrud (1954) and thiocyanate + nitrate medium of de Kruyff *et al.* (1957) were used. For aerobic cultures the thiosulphate medium described by Jones & Happold (1961) was used; thiosulphate agar was prepared according to these authors.

Estimations. Thiosulphate was estimated by titration with iodine according to Vogel (1951); polythionate by titration with iodine after treatment with KOH according to Starkey (1934*b*); thiocyanate spectrophotometrically by a modification of the method described by Bowler (1944); nitrite spectrophotometrically by the method of Rider & Mellon (1946). To estimate nitrate in presence of nitrate advantage was taken of the observation of Skerman, Lack & Millis (1951) that nitrate and nitrite yielded the same colour when determined spectrophotometrically by the brucine method of Noll (1945), and that with mixtures of nitrate and nitrite the colour densities were additive. Thus it was possible to estimate the nitrate concentration in a mixture of nitrate + nitrite when the concentration of nitrite had been determined by the method of Rider & Mellon (1946). None of the other ions of the concentrations found in the media used affected any of these estimations.

Chromatography. The methods of Jones & Happold (1961) for detecting polythionates were used. Potassium pentathionate prepared by the method of Goehring & Feldmann (1948) was used as an additional marker.

RESULTS

Anaerobic cultures

Thiobacillus denitrificans and *T. thioparus* grew anaerobically in both nitrate-containing media; *T. thiocyanoxidans* did not. These results contrast with those of de Kruyff *et al.* (1957) who reported growth of *T. thiocyanoxidans* anaerobically in both nitrate-containing media, and with the statement of Vishniac & Santer (1957) that '*T. thioparus* was a strictly aerobic bacterium'. Figures 1*a* and 1*b* show the thiocyanate, nitrate and nitrite concentrations measured during growth of *T. denitrificans* and *T. thioparus* in screw-capped bottles completely filled with thio-

cyanate + nitrate medium. With *T. thioparus* nitrite accumulated and thiocyanate was not completely used up. These facts may be associated, for the rate of thiocyanate utilization decreased when the nitrite concentration reached 150 $\mu\text{g./ml.}$ and ceased when the nitrite concentration was 200 $\mu\text{g./ml.}$ The decrease in rate and subsequent cessation of thiocyanate utilization by *Thiobacillus thioparus* in thiocyanate + nitrate medium may have been related to nitrite concentration; inhibitory concentrations of nitrite for growth of thiobacilli have been shown as follows: 1×10^{-2} M- NO_2 and 1.4×10^{-2} M- NO_2 for anaerobic growth of *T. denitrificans* in thiosulphate + nitrate and thiocyanate + nitrate media respectively (Baalsrud & Baalsrud, 1954; Woolley, 1961); 2.1×10^{-2} M- NO_2 for aerobic growth of *T. thioparus*

Fig. 1a. Utilization of thiocyanate $\circ$, and nitrate ∇ and production of nitrite $\bullet$ by a culture of *Thiobacillus denitrificans*.

Fig. 1b. Utilization of thiocyanate $\circ$, and nitrate ∇ and production of nitrite $\bullet$ by a culture of *Thiobacillus thioparus*.

in thiosulphate medium (Starkey, 1934a); 1.4×10^{-3} M- NO_2 for aerobic growth of *T. thiocyanoxidans* in thiocyanate medium (Youatt, 1953). No quantitative comparisons were made of the anaerobic growth of *T. denitrificans* and *T. thioparus* in thiosulphate + nitrate medium because of difficulties associated with the estimations of thiosulphate and nitrite in the presence of each other.

Aerobic cultures

After aerobic, serial subculture in the thiosulphate medium of Jones & Happold (1961), *T. denitrificans* no longer grew anaerobically when inoculated into thiosulphate + nitrate or thiocyanate + nitrate media, whereas *T. thioparus* which before such subculture has been maintained aerobically, grew immediately under anaerobic conditions in nitrate-containing media.

Whereas *Thiobacillus thioparus* needed 24–48 hr. to produce copious growth on thiosulphate agar slopes under aerobic conditions, *T. denitrificans* proved extremely

difficult to grow on thiosulphate agar under aerobic or anaerobic conditions; minute white colonies took between nine and seventeen days to appear under these conditions. *T. denitrificans* did not produce the mucoid slime associated with *T. thioparus* cultures; cultures of the latter organism took appreciably longer to filter because they were mucoid.

Figures 2a and 2b show pH values and thiosulphate and polythionate concentrations in 50 ml. cultures of the two organisms grown in 250 ml. conical flasks containing thiosulphate medium (Jones & Happold, 1961). Differences between the organisms

Fig. 2a. Utilization of thiosulphate by *Thiobacillus denitrificans* $\circ$ and by *T. thioparus* $\bullet$; production of polythionate by *Thiobacillus denitrificans* ∇ and by *T. thioparus* $\blacktriangledown$.

Fig. 2b. Acid production during culture of *Thiobacillus denitrificans* $\circ$ and *T. thioparus* $\bullet$.

Table 1. Utilization of thiosulphate and production of polythionate by *Thiobacillus thioparus* and *T. denitrificans*

Time (days)	Medium a				Medium b			
	<i>T. thioparus</i>		<i>T. denitrificans</i>		<i>T. thioparus</i>		<i>T. denitrificans</i>	
	S ₂ O ₃	P	S ₂ O ₃	P	S ₂ O ₃	P	S ₂ O ₃	P
0	187.5	2.5	190	3.0	93.5	1.5	91.5	0.5
1	186.5	0.5	192.5	2.3	88.5	3.0	91	0.5
2	180	3.5	189.5	6.0	83	—	83	2.5
3	176	6.0	182.5	1.5	76	7.0	72	2.0
4	169.5	9.5	175.5	2.5	70.5	8.0	66	0.5
5	164	13.5	171.5	1.5	59.5	15	59.5	1.0
7	150	26.0	156.5	1.5	40	24.5	44.5	0
9	133.5	33.0	143	4.0	24.5	30.5	32.5	0
11	114.5	41.0	130	1.0	5.5	39	18.5	0
14	76.0	59.0	108	17.5	0	21.5	16	0
15	—	—	—	—	0	20	—	—
21	0	30.0	49.5	36.5	0	19	13.5	0.5

Medium a: thiosulphate (S₂O₃) of Jones & Happold (1961).

Medium b: thiosulphate of Baalsrud & Baalsrud (1954), omitting nitrate.

P = polythionate.

Concentrations expressed as μ equiv. I₂/5 ml. culture.

can be seen (Fig. 2) in respect of polythionate production and rate of acid production. Table 1 shows differences in the amounts of titratable polythionate produced by the two organisms during growth of 500 ml. cultures in 1 l. flasks. The media used were: *a* that described by Jones & Happold (1961); *b* that described by Baalsrud & Baalsrud (1954) but without nitrate. During the latter experiment concentrates of culture filtrates were chromatographed on paper to detect polythionates; Table 2 shows some differences between the two organisms with respect to the polythionates which accumulated.

Table 2. *Chromatographic detection of polythionates in culture filtrate concentrates from cultures of Thiobacillus denitrificans and T. thioparus*

Time (days)	Medium	Organism	Polythionates		
			S ₃ O ₆	S ₄ O ₆	S ₅ O ₆
14	<i>a</i> , S ₂ O ₃ (Jones & Happold, 1961)	<i>T. thioparus</i>	+	—	+
		<i>T. denitrificans</i>	+	—	+
	<i>b</i> , S ₂ O ₃ : no NO ₃ (Baalsrud & Baalsrud, 1954)	<i>T. thioparus</i>	+	—	+
		<i>T. denitrificans</i>	—	—	—
21	<i>a</i>	<i>T. thioparus</i>	+	+	?
		<i>T. denitrificans</i>	+	+	+
	<i>b</i>	<i>T. thioparus</i>	?	+	+
		<i>T. denitrificans</i>	—	?	—

DISCUSSION

The strains of *Thiobacillus denitrificans* and *T. thioparus* used here were capable of anaerobic growth in the presence of nitrate at the expense of thiosulphate or thiocyanate. In contrast to the report of de Kruffy *et al.* (1957), our *T. thiocyanoxidans* did not grow anaerobically in thiosulphate + nitrate or thiocyanate + nitrate media. In contrast to the statements of Vishniac & Santer (1957) our cultures of *T. denitrificans* and *T. thioparus* were easily distinguished when grown aerobically (as indicated above). Of the differences described, we attach most weight to the loss of capacity for anaerobic growth by *T. denitrificans* in thiosulphate + nitrate or thiocyanate + nitrate media after aerobic subculture, and to the production of slime by the non-motile strain of *T. thioparus*. The differences observed with respect to rate of acid production and polythionate production may have been of degree only for, as Jones & Happold (1961) showed, the type of polythionate which accumulated in cultures of *T. thioparus* was altered by changing the sodium:potassium ion ratio of the growth medium. Furthermore, we found manometrically (Woolley, 1961) that the oxidation of potassium tetrathionate by washed whole cell suspensions of *T. denitrificans* proceeded at different rates according to the sodium:potassium ion ratio of the buffers used to suspend the organisms.

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