

THIOBACILLUS NOVELLUS

I. GROWTH ON ORGANIC AND INORGANIC MEDIA¹

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Thiobacillus novellus was first isolated and described by Starkey (1935) during the course of an investigation on thiosulfate-utilizing bacteria. Starkey was able to isolate this organism from several samples of soil and found that it grew very well on nutrient broth and could also grow on a thiosulfate-salts medium. Thus this species is the only member of the genus *Thiobacillus* which can grow both as an autotroph² and a heterotroph; all the other thiobacilli are obligate autotrophs. Since these first reports by Starkey, no further data on this organism have been published; Sijderius (1946), however, suggested that the bacterium isolated by Starkey may be similar to *Micrococcus denitrificans* Beijerinck.

The ability to grow under both autotrophic and heterotrophic conditions has been recognized in some hydrogenomonas species, some photosynthetic bacteria, and *Desulfovibrio desulfuricans* (Woods and Lascelles, 1954). Two alternate situations might account for the phenomenon of facultative autotrophy in a given species: (a) a mutation and selection phenomenon and (b) a situation in which all the cells are genetically equipotential. In the former case, a population of cells growing in one medium (e. g., heterotrophic) would contain a small per cent of cells capable of growth in the alternate environment (autotrophic); this small per cent of the population would be the only cells capable of growth on transfer to the autotrophic medium. At the present time, there is no evidence which allows us to distinguish between these alternate situations for any of the facultative autotrophs described. In the case of *T. novellus*, which, according to Starkey's description, requires 4 to 5 days for

growth in either medium, the conditions which permit mutation and selection would certainly appear to be present.

It seemed that methods were available to determine whether or not each cell of *T. novellus* was capable of growth on both heterotrophic and autotrophic media. Such information would be necessary for further studies on the biochemical basis of facultative autotrophy in this bacterium. This initial report presents evidence that each cell of a population of *T. novellus*, whether grown autotrophically or heterotrophically, is capable of growth in both environments. As a preliminary to further biochemical studies, the growth of *T. novellus* under defined conditions has also been studied.

MATERIALS AND METHODS

Professor Starkey kindly supplied us with a culture of *T. novellus* grown on nutrient agar and subcultures were made on the autotrophic medium described below. The bacterium has been transferred on this medium for more than a year with no decrease in its ability to grow autotrophically. Because of the question raised by Sijderius about the classification of *T. novellus*, a short description of the bacterium would be in order. The organism is a gram-negative rod, whether grown on organic or inorganic media, about 0.5 to 1 μ wide and 1 to 4 μ long. The rods are usually single but pairs attached end to end are observed. These observations are in agreement with Starkey's original report.

Autotrophic cultures were grown on an inorganic (thiosulfate) medium containing the following components per L of distilled water: Na₂S₂O₃·5H₂O, 8.0 g; KH₂PO₄, 4.0 g; K₂HPO₄, 4.0 g; NH₄Cl, 0.5 g; MgSO₄·7H₂O, 0.8 g; metal mix,³ 10 ml. The thiosulfate and the phosphates

³ Ethylenediaminetetraacetic acid, 50.0 g; ZnSO₄·7H₂O, 22.0 g; CaCl₂, 5.54 g; MnCl₂·4H₂O, 5.06 g; FeSO₄·7H₂O, 4.99 g; (NH₄)₆Mo₇O₂₄·4H₂O, 1.1 g; CuSO₄·5H₂O, 1.57 g; CoCl₂·6H₂O, 1.61 g; H₂O, 1 L. Adjusted to pH 6.0 with KOH.

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² Autotrophy refers to the ability to grow in an inorganic medium in which thiosulfate is the sole energy source and CO₂ the sole carbon source.

are each autoclaved separately and then added to the other components (Vishniac and Santer, 1957). To make up a solid medium, washed agar was added to the above components at a concentration of 20 g per L. The use of washed agar was discontinued when it was found that identical results were obtained using untreated agar.

In initial experiments, nutrient broth was used as the medium for heterotrophic growth. Subsequently, a defined organic medium was prepared containing the following components per L of distilled water: sodium glutamate, 5.0 g; dibasic ammonium citrate, 5.0 g; K_2HPO_4 , 8.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeCl_2$, 0.05 g; and $MnCl_2$, 0.05 g. The final pH of this medium is 6.8. In the text of this paper, the components of this medium minus the glutamate are referred to as the citrate minimal medium.

Bacteria were grown at 30 C. For resting cell experiments, the bacteria were centrifuged, washed twice in cold distilled water, and resuspended in 0.16 M tris(hydroxymethyl)amino-methane buffer, pH 7.2. The optical density of cell suspensions was measured in a Klett-Sumner colorimeter using the 540 m μ filter.

RESULTS

In the process of developing a defined medium for *T. novellus*, various carbon compounds were tested for their ability to support heterotrophic growth. Growth experiments were carried out in Nephelco-culture tubes (Belloco Glass Company, Vineland, New Jersey). The inoculum was grown on an autotrophic medium, centrifuged, resuspended in water, and inoculated into the various media. Data on the growth on several media are presented in table 1. It was observed during the course of these experiments that a particular subculture of the strain originally obtained from Starkey ("slow growers") gained the ability to grow more rapidly on both organic and inorganic media. (This subculture will be called "fast grower.") This is apparent from the data in table 1 and in a later experiment (figure 3). No explanation can be offered at this time for the behavior of the fast growers; however, no difference other than rate of growth has been observed between the fast growers and the slow growers.

Glutamic and aspartic acids were the only two amino acids which supported considerable heterotrophic growth and it is obvious that glutamic acid is a superior carbon source to aspartic acid. Glucose, sucrose, lactose, acetate, malate, and

TABLE 1
Comparative growth characteristics of "fast" and "slow growing" strains of *Thiobacillus novellus* on various media

Medium	Fast Growers			Slow Growers		
	Incubation time in hr:			Incubation time in hr:		
	24	48	72	24	72	120
	<i>Klett units</i>			<i>Klett units</i>		
Thiosulfate medium	3	13	31	—	—	2
Citrate minimal . . .	0	7	270	2	6	9
Citrate minimal + aspartate*	6	15	260	0	6	8
Citrate minimal + glutamate*	42	280	380	2	21	53

* Aspartate and glutamate added in a concentration of 5 g per L.

succinate can not serve as the sole carbon source. Citrate does support some growth of *T. novellus* after a 24- to 48-hr lag period, however its primary role is to prevent the precipitation of the metals contained in the medium.

The following experiment was designed to determine whether each cell of *T. novellus* is capable of growth under both autotrophic and heterotrophic conditions. Autotrophically and heterotrophically grown cells were collected after 24 or 48 hr growth and washed twice with 5 ml of sterile cold distilled water. Serial dilutions of each population were prepared and equal aliquots of each dilution were plated on thiosulfate and nutrient agar or thiosulfate and glutamate-citrate agar. The results of these experiments are shown in tables 2 and 3 and indicate that essentially equal numbers of colonies appeared on both media. In the course of various growth experiments, several thousand cells have been plated on both heterotrophic and autotrophic media; in no case was there any significant discrepancy between the counts on the two media. From these data it is quite clear that each cell in an autotrophic or heterotrophic population is capable of growth when placed on either a heterotrophic or an autotrophic medium.

Corroborative evidence for the above conclusion was obtained from replica plating (Lederberg and Lederberg, 1952) experiments. *T. novellus* was grown on glutamate-citrate agar and on thiosulfate agar until the colonies were just

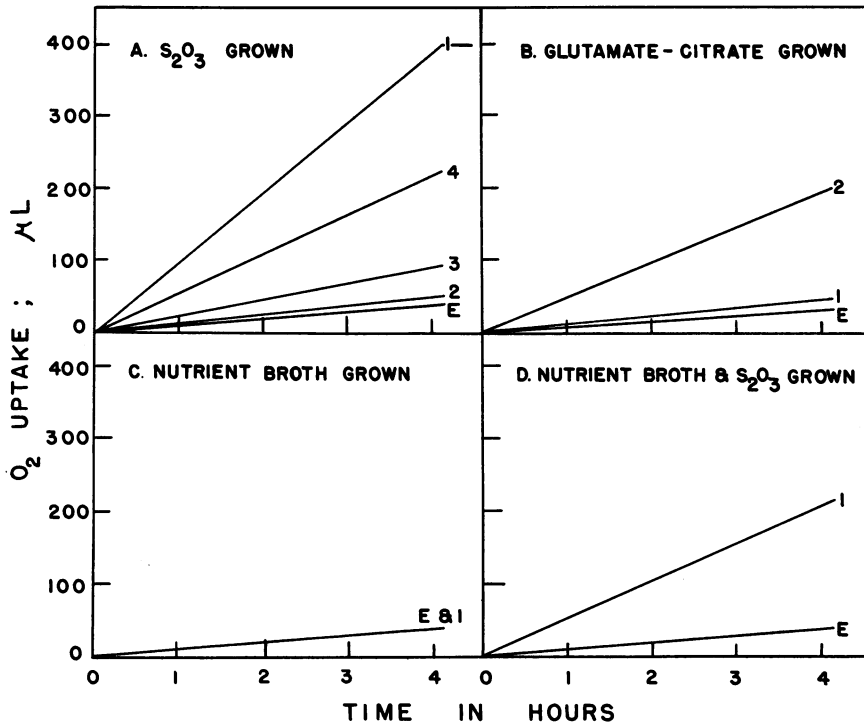


Figure 1. Substrate oxidation by organic and inorganic grown *Thiobacillus novellus*. Curves numbered 1, S₂O₃ as substrate; 2, glutamate as substrate; 3, nutrient broth as substrate; 4, tetrathionate as substrate; E, value for endogenous flask. Flask no. 1 in experiment A contained 9 μmoles of S₂O₃; no. 2, 1 mg of glutamate; no. 3, 1 mg nutrient broth; no. 4, 10 μmoles of tetrathionate. Each flask contained 0.1 ml of 0.3 M phosphate buffer, pH 7.2, 1 ml cells adjusted to a Klett reading of 400 suspended in 0.16 M tris(hydroxymethyl)aminomethane buffer, pH 7.2; water to make total volume 2 ml; 0.2 ml 5 N KOH in center well. In experiments B, C, and D there were 100 μmoles S₂O₃ in each flask; all other components are the same as in experiment A.

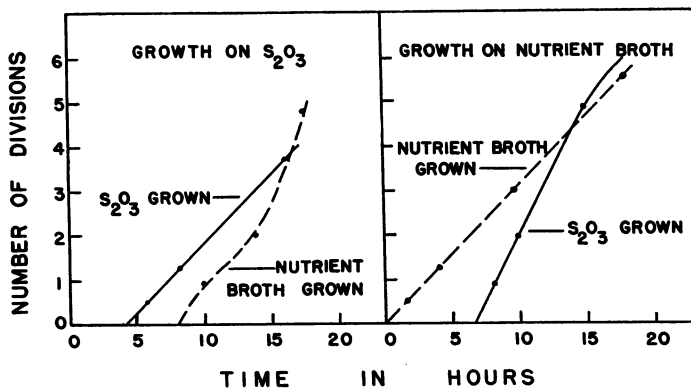


Figure 2. Growth rates of thiosulfate and nutrient broth grown *Thiobacillus novellus* ("slow growers") on thiosulfate and nutrient broth media. Bacterial counts were made by serial dilutions and plating of each dilution on both inorganic and organic agar.

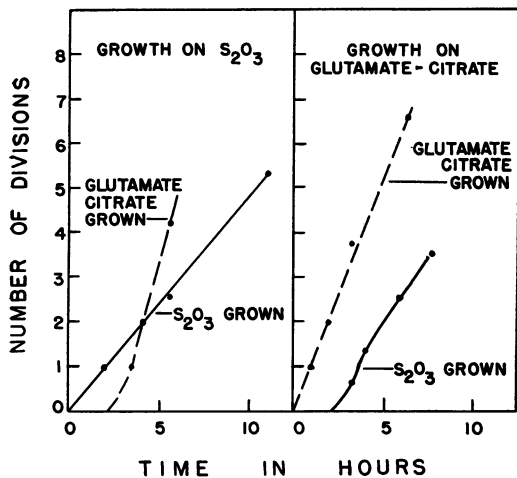


Figure 3. Growth rates of thiosulfate and glutamate-citrate grown *Thiobacillus novellus* ("fast growers") on thiosulfate and glutamate-citrate medium. Bacterial counts were made by serial dilutions and plating of each dilution on inorganic and organic agar.

visible; there were approximately 100 colonies per plate. Each of these plates was replicated onto glutamate-citrate and thiosulfate agar. Identical numbers of colonies in identical positions appeared on both replicas.

It has been shown that each *T. novellus* cell is capable of reproducing on organic and inorganic media. These experiments, however, do not tell us whether an autotrophically grown cell can immediately metabolize the components of the organic medium or whether organically grown cells can immediately metabolize thiosulfate; that is, are the cells constitutive for both capabilities or must they adapt to the new environment? In order to answer this question, resting cell and growth experiments were carried out.

T. novellus was grown on the following media: nutrient broth, nutrient broth plus 8 g of thiosulfate per L, thiosulfate medium, and glutamate-citrate medium. The cells were harvested, washed, and incubated with various substrates. The oxygen consumption data for these cells are shown in figure 1. These data show that cells grown on thiosulfate medium or in a heterotrophic medium containing thiosulfate are capable of immediately oxidizing thiosulfate and also tetrathionate (curves 1 and 4). Cells grown on glutamate-citrate medium or nutrient broth are incapable of metabolizing thiosulfate at a rate significantly

TABLE 2

Comparative colony counts of autotrophically grown *Thiobacillus novellus* on inorganic and organic media

Expt No.	Thiosulfate Grown Cells			
	Plated on:	No. of colonies		
		Dilutions:		
		10 ⁻³	10 ⁻⁴	10 ⁻⁵
1	S ₂ O ₃ agar.....	—	450	40
	Nutrient agar.....	—	550	44
2	S ₂ O ₃ agar.....	310	46	4
	Glutamate-citrate agar..	440	60	5

TABLE 3

Comparative colony counts of heterotrophically grown *Thiobacillus novellus* on inorganic and organic media

Nutrient Broth Grown Cells				
Plated on:	No. of Colonies			
	Dilutions:			
	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	
S ₂ O ₃ agar.....	800	73	5	
Nutrient agar.....	786	90	9	
Glutamate-Citrate Grown Cells				
S ₂ O ₃ agar.....	150	12	0	
Glutamate-citrate agar.....	139	18	1	

above the endogenous rate. Cells grown on thiosulfate medium are incapable of oxidizing nutrient broth or glutamate for a period of at least 4 hr.

Studies on the growth characteristics of cells grown on one medium and transferred to another further suggest that a definite period of adaptation is needed before growth can commence. Cells were grown in thiosulfate medium for 48 hr, washed, and inoculated into heterotrophic (nutrient broth or glutamate-citrate medium) and thiosulfate media. Similarly, a heterotrophically grown inoculum was transferred to thiosulfate and the original heterotrophic medium. Growth was followed by plating and counting. Each dilution was plated on both

organic and inorganic agar. These platings gave essentially similar numbers of colonies, corroborating the evidence presented in tables 2 and 3. The results of the growth experiments are given in figures 2 and 3.

It is evident that cells exhibit a longer lag period when inoculated into a medium different from that in which they have been grown than when reinoculated into the same medium. This is true for both the fast and the slow growing *T. novellus*, although the fast growers show a marked decrease in lag period and generation time. For instance, when thiosulfate grown cells are inoculated into thiosulfate, the fast growers show no noticeable lag period and have a generation time of about 2 hr, whereas the slow growers have a lag period of about 4 hr and a generation time of about 3 hr.

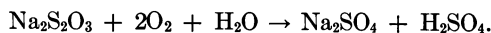
These growth experiments suggested that the adaptation observed under conditions of growth might also be observed with heavy resting cell suspensions in the Warburg apparatus. In a few cases, adaptation has indeed been obtained with heavy suspensions of autotrophically grown cells incubated with glutamate for more than 8 hr. Under these conditions, some growth of the cells did occur, but it was not enough to account for the marked increase in the rate of oxygen consumption. The results with resting cells, however, have not been consistently reproducible.

DISCUSSION

It has been demonstrated that each cell of a *T. novellus* population, whether grown on an inorganic or an organic medium, is capable of growth in both kinds of environments. Platings on the two kinds of media, as well as replica plating experiments, have shown this. In fact, in a period of over a year, during which time populations of several thousand cells have been plated in organic and inorganic media, no significant discrepancy in counts on the two kinds of media has ever appeared. Consequently, each cell is capable of metabolizing in two ways: as a chemolithotroph, like the other thiobacilli, and as a chemoorganotroph.

As a chemolithotroph, *T. novellus* can oxidize thiosulfate to sulfate and can couple this oxidation to some energy yielding reactions. The ability to oxidize thiosulfate to sulfate is shown by the data contained in figure 1. In incubation flask number 1 there were 9 μ moles of thiosulfate present; the complete oxidation of these 9 μ moles

would require the consumption of 18 μ moles of oxygen or 403 μ L. Close to 400 μ L were actually consumed, a figure which is in good agreement with the theoretical value. Thus the over-all stoichiometry for thiosulfate oxidation to sulfate by *T. novellus* is the same as that given for *Thiobacillus thioparus* (Vishniac, 1952):



T. novellus can also lead the more conventional existence typical of heterotrophic forms, deriving its energy and carbon from an organic compound. However, heterotrophic growth seems to be supported by only a few organic substrates. Glucose, sucrose, and lactose are not utilized for growth; neither are succinate, malate, or acetate; glutamic acid appears to be the best substrate.

Although *T. novellus* is genetically capable of living as both an autotroph and a heterotroph, it is evident that inorganic and organic grown cells differ from each other in their metabolic capabilities. In the case of heterotrophically grown cells, either resting cell suspensions or cells under growing conditions can immediately metabolize the organic substrate. Consider, for example, the data in figure 3. When glutamate-citrate grown cells are placed in thiosulfate medium, there is no increase in the number of viable cells for 2 hr; after this lag period the cells begin to divide and within 90 min they are capable of growing as rapidly on the thiosulfate medium as cells previously grown on this same medium. The same situation holds for thiosulfate grown cells when they are placed in glutamate-citrate medium. Again there is about a 2 hr lag period followed by a rapid increase in the number of bacteria; the growth rate of these cells very nearly parallels the growth rate of cells previously grown on glutamate-citrate medium.

Both resting cell and growth experiments have demonstrated that autotrophically and heterotrophically grown cells differ from each other. In growth experiments, a finite amount of time must elapse before a cell can grow on the alternate medium. In resting cell experiments, the cells are incapable of metabolizing the alternate substrate for some time but occasionally acquire the ability to do so. This situation appears to be similar to the phenomenon which has been called adaptation (Gale and Davies, 1953) or induction (Cohn 1957). Although it is not known what specific enzymes or other factors are involved in this adaptation, it seems possible that there is both a

gain and a loss of several enzymes during the change in both directions. Evidence which supports the idea that more than one enzymatic activity is acquired is the data on tetrathionate oxidation (figure 1). Thiosulfate grown *T. novellus* can oxidize both thiosulfate and tetrathionate, whereas heterotrophically grown cells oxidize neither. Tetrathionate has been proposed as an intermediate in the oxidation of thiosulfate to sulfate (Vishniac, 1952). This appears to be a case of "sequential induction" (Stanier, 1954) since growth on one substrate (thiosulfate) adapts the cell to a metabolic intermediate (tetrathionate).

The adaptation to the heterotrophic state seems to be limited to a few substrates. This may indicate a relatively recent acquisition of heterotrophic capabilities or may represent some remaining capabilities of an organism evolving in the direction of obligate autotrophy.

SUMMARY

Each cell of *Thiobacillus novellus* is capable of growth on both heterotrophic and autotrophic media. Consequently it can grow on an inorganic medium with thiosulfate as the sole energy source and carbon dioxide as the sole carbon source; it can also grow on either nutrient broth or a chemically defined glutamate-citrate-salts medium. Glutamate, of various carbon compounds tested, appears to be the best substrate supporting growth. Growth on the autotrophic medium by heterotrophically grown cells and on the organic

medium by inorganic grown cells occurs in an adaptive manner.

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