Autotrophic Growth of *Thiobacillus acidophilus* in the Presence of a Surface-Active Agent, Tween 80

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Received for publication 2 August 1979

Cellular protein, pH, dissolved oxygen concentration, and static surface tension were measured during growth of *Thiobacillus acidophilus* on elemental sulfur in the absence and presence of up to 5,000 mg of Tween 80 per liter. The decrease in pH and the increase in sulfate production were observed to be less accurate measurements of growth when compared with the increase in cellular protein. The doubling time of the bacterium decreased approximately 50% with the addition of 500 mg of Tween 80 per liter. The bacteria did not appear to synthesize any wetting agents as demonstrated by the constant surface tension of the medium during growth. Morphological alterations in the presence of Tween 80 were also observed.

Bacterial oxidation of metal sulfides has been reported (6, 13) to be significantly augmented with the addition of surface-active agents. Surface-active agents stimulate growth (8) by reducing surface tension, thus facilitating contact between the organisms and their insoluble substrate. This is particularly true for the oxidation of inorganic compounds. Torma et al. (31), however, reported that the leaching rate was dependent on the concentration of wetting agent added, as higher concentrations significantly decreased the rate of oxidation.

As oxidation of elemental sulfur by thiobacilli is believed to occur at the cell envelope (16), direct contact between the substrate and the bacteria must occur. To accomplish this, the thiobacilli decrease the surface tension in the growth medium by producing phospholipid wetting agents (1, 12, 23). Recently, the growth of *Thiobacillus acidophilus* on elemental sulfur (19) was investigated, and the generation time was found to be about 2 days, which was two- to fourfold the doubling times of other sulfur-oxidizing thiobacilli (24, 33). As a result, we examined the influence of Tween 80 and the effect of decreased surface tension on the growth of this organism.

Growth of thiobacilli has in the past been monitored by using increase in acidity (3, 23), production of sulfate (5, 12), utilization of substrate (14), and total bacterial counts (10, 33). These methods were used rather than the increase in cellular protein because of the severe interference by elemental sulfur on the Folin reaction (15). A recent modification of this method (19) has enabled accurate determination of cellular protein, which we believe to be a better indicator of growth than the previous methods.

(A portion of this investigation was presented at the 28th annual meeting of the Canadian Society of Microbiologists, Montreal, Quebec, 11 to 15 June 1978.)

MATERIALS AND METHODS

Heterotrophically grown *T. acidophilus* was adapted to autotrophic growth on elemental sulfur as previously described (11). Routinely, the bacteria were grown on a medium containing 5 g of tyndallized sulfur, 350 ml of 9K mineral salts solution (25), and 150 ml of distilled water in low-profile Fernbach flasks. The initial pH of the medium was adjusted to 3.5 with concentrated sulfuric acid (12 N). Sterilization was accomplished by autoclaving the medium at 121°C for 20 min. The elemental sulfur and 0.5 ml of a 10% (wt/vol) suspension of inoculum were added after cooling to ambient temperature. The flasks were then incubated at 25 ± 1.0°C with agitation at 200 cycles/min on a gyratory shaker housed in a humidity-controlled growth chamber.

Various parameters of growth were monitored during the growth of the bacterium in the absence and presence of up to 5,000 mg of Tween 80 per liter, which was added to the medium from a stock solution before sterilization. Dissolved oxygen concentration was measured by an azide modification of the iodometric method of Winkeler (20) at 25 ± 1°C. Surface tension was determined by the capillary rise method (18) at 25 ± 1°C, using the equation:

\[ \gamma = \frac{1}{2} (h + r/3) \text{ rpg} \]

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where $\gamma$ = surface tension (dynes per centimeter), $h$ = height of the ascended liquid (centimeters), $r$ = radius of the capillary tube (centimeters), $\rho$ = density of the liquid (grams per cubic centimeter), and $g$ = acceleration due to gravity (centimeters per second$^2$).

Care was taken to ensure that the capillary tubes were clean by washing them in hot nitric acid (15 N) and rinsing them copiously with glass-distilled water and acetone, followed by complete drying. Each capillary tube was standardized, using various liquids of known surface tension. Cellular protein was determined by the method of Proteau and Silver (19), sulfate was determined by the method of Glenn and Quastel (9), and the pH was determined with an expanded-scale pH meter. The generation times were calculated from the exponential growth phase and are defined as the number of hours required for the cellular protein to double, using the expression:

$$k = \frac{\ln 2}{(t_b - t_i)}$$

where $(t_b - t_i)$ is the generation time.

Differential staining procedures for intracellular lipid inclusions (poly-$\beta$-hydroxybutyrate) were performed on bacterial cells according to the methods of Burdon, Hotchkiss, and Albert as described by Norris and Swain (17).

Photomicrographs were taken with a Zeiss phase-contrast photomicroscope, model 1719.

All chemicals used were of the highest purity commercially available. Sublimed sulfur and Tween 80 were purchased from Fisher Scientific Co. Ltd, Montreal.

RESULTS AND DISCUSSION

The increase in acidity (Fig. 1) due to the formation of sulfate is the most common method of determining growth of acidophilic thiobacilli when elemental sulfur is used as the substrate (12, 27, 30). The growth of *T. acidophilus* caused the pH of the medium to decrease from an initial value of 3.5 to approximately 1.0 after 300 h of growth on elemental sulfur. This decline in pH corresponds to an increase in sulfate production by *T. acidophilus*, as is the case with *T. ferrooxidans* grown on elemental sulfur (24). Although the effect of dissolved oxygen concentration on bacterial growth has not been intensively studied, it is apparent that an increase in bacterial numbers causes a decrease in the dissolved oxygen concentration. For the thiobacilli, this can be explained on the basis of the amount of oxygen required for the oxidation of elemental sulfur not only for the oxygenation to sulfite (28) but also as a terminal electron acceptor for the oxidation to sulfate (4). The time required to reduce the overall dissolved oxygen concentration decreases with increased concentration of surfactant. Increased bacterial growth in the presence of higher concentrations of surfactant seems to be a plausible explanation for the more rapid decrease in the dissolved oxygen concentration. The surface tension of the medium remains constant at a level of 70 dyn/cm and may be the reason for the slow growth of *T. acidophilus* on elemental sulfur.

Addition of increasing concentrations of Tween 80 to the growth medium of *T. acidophilus* results in different initial rates of protein production. A cellular protein doubling time of 42.5 ± 4.6 h was obtained in the control. This is in agreement with data previously published by Proteau and Silver (19). The doubling times for *T. acidophilus* and morphological alterations in the presence of various concentrations of Tween 80 are indicated in Table 1. During the first 5

**Table 1. Growth of *T. acidophilus* in the presence of elemental sulfur and Tween 80**

<table>
<thead>
<tr>
<th>Tween 80 concn (mg/liter)</th>
<th>Sulfate produced after 300 h (µmol/ml)</th>
<th>Doubling time (h)</th>
<th>Bacterial size (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Length</td>
</tr>
<tr>
<td>0</td>
<td>230 ± 10.6</td>
<td>42.5 ± 4.6</td>
<td>1.01 ± 0.1</td>
</tr>
<tr>
<td>1</td>
<td>242 ± 4.3</td>
<td>42.4 ± 4.3</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>5</td>
<td>416 ± 3.9</td>
<td>41.6 ± 3.9</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>10</td>
<td>408 ± 3.8</td>
<td>40.8 ± 3.6</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>25</td>
<td>383 ± 2.9</td>
<td>38.3 ± 2.9</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>50</td>
<td>252 ± 2.4</td>
<td>25.2 ± 2.4</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>100</td>
<td>246 ± 12.3</td>
<td>24.6 ± 1.8</td>
<td>1.65 ± 0.1</td>
</tr>
<tr>
<td>200</td>
<td>264 ± 3.3</td>
<td>26.4 ± 3.3</td>
<td>2.7 ± 0.13</td>
</tr>
<tr>
<td>500</td>
<td>385 ± 2.6</td>
<td>38.5 ± 2.6</td>
<td>1.65 ± 0.1</td>
</tr>
<tr>
<td>1,000</td>
<td>224.6 ± 11.7</td>
<td>224.6 ± 11.7</td>
<td>1.65 ± 0.1</td>
</tr>
<tr>
<td>5,000</td>
<td>224.6 ± 11.7</td>
<td>224.6 ± 11.7</td>
<td>2.7 ± 0.13</td>
</tr>
</tbody>
</table>
days of growth, the presence of surfactant caused little change in morphological structure. Increasing concentrations of surfactant resulted in elongation of the bacteria. Little change was observed from 7 to 10 days between the control and the addition of 100 mg of surfactant per liter; however, a marked lengthening of the bacterium from 1.0 to 1.65 μm was apparent with 500 mg of Tween 80 per liter. The addition of 1,000 and 5,000 mg of Tween 80 per liter resulted in the bacteria being more elongated and thinner (1.9 by 0.4 μm and 2.7 by 0.28 μm, respectively) than in the absence of surfactant (1.0 by 0.5 μm). In addition, a great deal of cell debris was observed. The overall effect of surfactant addition was an increase not only in cell length but also in the size of the refractile inclusions (possibly poly-β-hydroxybutyrate). These changes may be due to an increase in bacterial growth processes, the incorporation of Tween 80 into the bacteria, or the decrease in the surface tension which has a profound effect on the ability of the bacterium to maintain its normal shape. Similar morphological and physiological alterations have been observed with *T. ferrooxidans* with increased aeration (26).

Figure 2 illustrates the relationship between the synthesis of cellular protein with respect to time and the presence of varying concentrations of wetting agent added to the growth medium. The synthesis of cellular protein is stimulated by increasing concentrations of surfactant to the growth medium. Hydration of elemental sulfur is promoted by Tween 80, which reduces the energy required by the bacterium to overcome the dynamic surface tension between the substrate and the liquid medium, thus facilitating contact between bacterium and substrate. The energy saved may be utilized for increased metabolic activity (21, 32, 34) or protein synthesis. Although *T. acidophilus* was unable to grow solely in the presence of Tween 80, the organism may be able to incorporate the Tween 80 into cell lipids and consequently save on their biosynthesis. An energy source (elemental sulfur) may be required for growth in the presence of Tween 80, as mixotrophic growth of the bacteria on pyruvate in the presence of elemental sulfur has been observed (unpublished observations). The cellular protein doubling time is significantly reduced with the addition of 100 mg of Tween 80 per liter, and it is at this concentration of surfactant that the most substantial quantity of cellular protein is produced. At 500 mg of Tween 80 per liter, the cellular protein doubling time is about half that of the control, and late logarithmic phase is reached after approximately 200 h. The final cellular protein concentration is slightly lower than that obtained with the addition of 100 mg of Tween 80 per liter. At concentrations of 1,000 and 5,000 mg/liter, the cellular protein doubles at a substantially faster rate than the control; however, lysis of the cells occurs at approximately 200 h. The final protein concentrations obtained before lysis differ slightly with respect to the control.

The pH, monitored during growth, varied only slightly in the absence and presence of varying concentrations of Tween 80. In the absence of surfactant a final pH of 1.0 was obtained, whereas in the presence of 5,000 mg of Tween 80 per liter, the pH decreased to 1.25. This apparent buffering effect may be a result of lysis of the bacteria with the concomitant release of cell contents; however, control acidity experiments with Tween 80 and growth medium illustrated that the surfactant acted as a buffer at high concentration.

The pH decrease is in close agreement with the increase in sulfate concentration (Fig. 1) for all of the concentrations of Tween 80 (0 to 5,000
mg/liter) used in this investigation. This similarity indicates that the rate of sulfur oxidation is similar in all of the experimental situations. It is important to note that the addition of Tween 80 does not increase the rate of sulfur oxidation but facilitates contact by wetting the insoluble substrate. The amount of protein synthesized, however, does vary with surfactant concentration. Because of this phenomenon, we question the use of pH or sulfate concentration, or both, as reliable indicators of growth for acidophilic thiobacilli when grown in the presence of surfactants since they measure metabolic activity and not bacterial growth. We have observed that the increase in cellular protein is a much more accurate indicator of growth and consequently would recommend its use.

The surface tension must be overcome by the bacteria before oxidation of elemental sulfur can occur (12, 23). Figure 3 illustrates the surface tensions determined during the growth of *T. acidophilus* in the absence and presence of various concentrations of surfactant. The surface tension in the control is relatively stable; however, the addition of Tween 80 immediately reduces the overall surface tension. Addition of 100 mg of Tween 80 per liter results in an initial surface tension of 46 dyn/cm; however, the surface tension continues to decrease for approximately 100 h, after which it increases to a final value of 57 dyn/cm. This increase in surface tension may be due to the adsorption of surfactant by the bacteria (2). A correlation between surface tension and the rate of synthesis of cellular protein is noted; as the surface tension increases, the rate of synthesis of cellular protein decreases. The addition of higher concentrations of surfactant results in a further decrease in surface tension; in the presence of 5,000 mg/liter, the initial surface tension is 38 dyn/cm. During growth, the bacteria break up as a result of the further decline in surface tension. Surface tension also influences the concentration of dissolved oxygen at saturation in aqueous systems (7, 30). Alexander and Soltys (2) reported that a surface tension of 40 to 50 dyn/cm is optimal for bacterial growth. More recently, Takakuwa et al. (29) have illustrated that decreased availability of oxygen inhibits attachment of *T. thiooxidans* to elemental sulfur. This inability to attach to the substrate would result in a decrease in cell numbers.

It has been reported that *T. thiooxidans* and *T. ferrooxidans* are able to produce phospholipid wetting agents (1, 12, 23) which facilitate oxidation of elemental sulfur by decreasing the surface tension between the sulfur particle and the liquid medium. This enables the bacterium to come in direct contact with the sulfur particle for oxidation (22, 27).

Because of these observations we conclude that dissolved oxygen concentration and surface tension directly influence growth of *T. acidophilus* and its capacity to oxidize elemental sulfur (12, 23).

ACKNOWLEDGMENTS

We thank G. A. Proteau for advice and encouragement and R. Bojanowski for use of photographic facilities.

This investigation was supported by a grant from the National Research Council of Canada (NRCC-A6054).

LITERATURE CITED


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**Fig. 3.** Changes in static surface tension during growth on elemental sulfur in the absence or presence of Tween 80: (C) 0 mg/liter; (□) 100 mg/liter; (△) 500 mg/liter; (◇) 1,000 mg/liter; (○) 5,000 mg/liter.
AUTOTROPHIC GROWTH OF T. ACIDOPHILUS