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Salinity Requirements of a Marine *Thiobacillus intermedius*

David W. Smith and Susan F. Finazzo

School of Life and Health Sciences, University of Delaware, Newark, Delaware 19711, USA

Abstract. *Thiobacillus intermedius* was isolated from a salt marsh sediment with an interstitial water salinity of $30^0/_{00}$. This bacterium was cultured in a chemostat for 9 months. The optimum salinity for CO_2 fixation by this *Thiobacillus* was $10^0/_{00}$, much less than the salinity of its natural environment. Respiration of cultures increased at high salinities and the pathway of thiosulfate oxidation was altered so that polythionates accumulated rapidly. One ecological conclusion from these results is that in nature this bacterium probably grows at its maximum possible rate only rarely.

Key words: *Thiobacillus* – Salinity – Marsh – Thiosulfate – Sulfur cycle – CO_2 fixation – Respiration

There has been much work on the question of what distinguishes marine bacteria from bacteria of other environments (MacLeod 1965, 1968; Pratt 1974). As expected, the salt relations of bacteria from marine environments have received careful scrutiny. Most of these organisms display an absolute requirement for Na^+ for growth, although the magnitude of this requirement can be altered to some extent by the presence of K^+ (MacLeod 1968; Pratt and Tedder 1974). These monovalent cations protect against cell lysis by increasing intracellular osmotic pressure and by stabilizing the cell membrane (Buckmire and MacLeod 1965; Pratt and Tedder 1974). The optimum salinity for growth of marine isolates has been examined much less often. In the few cases examined it has been found that this optimum is clearly below the salinity of the environment from which the organism was isolated (MacLeod 1965; Tilton et al. 1967; Murphy et al. 1971; Pratt and Tedder 1974; Reichelt and Baumann 1974).

The current project was undertaken following qualitative observations on the rate of acid production in enrichment cultures of sulfur-oxidizing bacteria in marsh sediment samples. It was found that acid production and cell yield were greatest in media with salinities equal to one half that of sea water (B. A. Bradley and D. W. Smith, Abstr. Ann. Meet. ASM 1976, I102, p. 128). A similar result has been obtained with thiobacilli from Australia (J. Bauld, personal communication). Detailed investigation of this phenomenon was simplified by the fact that the *Thiobacillus* isolate is capable of growth as a lithotrophic autotroph; that is, it can obtain energy from the oxidation of inorganic compounds while reducing carbon dioxide as its sole carbon source (see Rittenberg 1969, 1972 for terminology). Therefore the carbon and energy metabolism of this isolate could be examined virtually independently of each other.

Offprint requests to: D. W. Smith

Materials and Methods

Organism Isolation and Growth. Marsh sediment was collected from a zone of short-form *Spartina alterniflora* in the Canary Creek marsh in Lewes, Delaware (see Dicker and Smith 1980 for description of marsh characteristics). The salinity of the interstitial water of the sediment sample was $30^0/_{00}$ as measured with a refractometer. Sulfur-oxidizing bacteria were enriched in a thiosulfate-limited chemostat inoculated with marsh sediment. The dilution rate of the chemostat was $0.10\,h^{-1}$. Thiosulfate limitation was demonstrated by the inability to detect thiosulfate in the culture vessel or overflow after a steady state was attained. The growth medium was that described by Smith and Rittenberg (1974), containing 3 g thiosulfate per liter and adjusted to $30^0/_{00}$ with NaCl. Culture pH was continuously monitored and held at 6.8 by adding 2 M Na_2CO_3 using an Impulsomat (Metrohm Ltd., Herisau, Switzerland). After approximately ten volume changes in the chemostat, a species of *Thiobacillus* predominated. This organism was isolated by repeated subculture on the same medium solidified with agar (1.5% w/v). This pure culture was inoculated into a thiosulfate-limited chemostat to establish the culture used for all physiological experiments. The dilution rate of the chemostat was $0.10\,h^{-1}$. The isolate was capable of growth from 0 to $40^0/_{00}$ added NaCl, although very slowly at the extremes.

The organism was identified as *Thiobacillus intermedius*. Cells were small ($0.5 \times 1.0\,\mu m$), Gram negative, motile by polar flagellation, sensitive to pH values below 5 (slow growth down to pH 2), capable of producing tetrathionate in thiosulfate medium, capable of growth on FeS and S°, and capable of vigorous heterotrophic growth on complex organic medium (ZoBell marine agar, Difco). The organism was transferred between inorganic and organic media with no loss of ability to grow on either type.

$^{14}CO_2$ Incorporation. Culture fluid was removed from the chemostat and centrifuged at 4°C for 45 min at $15{,}000 \times g$. Sedimented cells were resuspended in sterile growth medium containing no added NaCl, a procedure which did not affect cell viability. The volume used for resuspension was equal to that removed from the chemostat, so that final cell density was the same as in the chemostat culture. Replicate 25 ml portions of this suspension were distributed to 125 ml flasks. Different solute concentrations were created by adding the requisite amount of sterile, solid solute to the suspensions in the flasks. This procedure insured maximum uniformity in cell distribution among flasks and also insured that all cultures in a given experiment had the same amount of time for adaptation to their new solute conditions before the addition of radioisotope. After 15 min preincubation, 1 ml of $NaH^{14}CO_3$ (New England Nuclear) (approximately 100,000 cpm) was added to each flask. Flasks were closed with cotton and incubated at room temperature (25°C) without shaking. Preliminary experiments showed that stationary incubations were necessary to prevent loss of $^{14}CO_2$. Duplicate samples

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(2 ml) were removed after 30, 60, and 90 min, and the cells were collected on 0.22 µm membrane filters (Millipore Corp.) by vacuum filtration of the suspension. Filters were washed with 5 ml of "pH 4 water" ($\approx 10^{-4}$ M H_2SO_4) to remove residual $H^{14}CO_3^-$, dried, and placed in vials containing 10 ml Formula 963 (New England Nuclear) for scintillation counting.

Respiration Measurements. Cells were harvested from the chemostat culture and suspended in medium lacking electron donor (thiosulfate), and solute concentrations were adjusted as described above. Respiration was measured for 100 min following conventional Warburg manometry procedures (Umbreit et al. 1972) at 25° C with air as the atmosphere in the reaction vessels.

Sulfur Chemistry Experiments. Cell suspensions were prepared in complete growth medium and adjusted to different NaCl concentrations as above. Replicate 100 ml suspensions were incubated in 250 ml flasks with shaking at 25° C. Duplicate 5 ml samples were removed from each flask at each sampling time (80, 130, 165, 225, 505 min), and duplicate assays were made of each sulfur species from each sample. Each sample was divided into two portions: 1 ml was mixed with 5 ml CS_2 for elemental sulfur extraction, and the remainder was immediately filtered through 0.22 µm membrane filters to remove cells. The filtrates were refrigerated at 4° C until assays could be performed, always within 4 h. Preliminary experiments showed no changes in any of the sulfur species during refrigeration.

Chemical Assays. Thiosulfate was measured iodometrically by reaction of the sample with known amount of I_2 and titration of the residual I_2 (Szekeres 1974).

For elemental sulfur determination, the CS_2 extract was evaporated to dryness at room temperature in a hood, the residue was redissolved in petroleum ether and cyanolytic assay of sulfur was performed following the Fliermans and Brock (1973) modification of the Bartlett and Skoog (1954) procedure. Sulfate was measured turbidimetrically as the barium precipitate (APHA 1971). Polythionates were measured by the procedure of Starkey (1934) in which alkaline hydrolysis converted polythionates to sulfate and thiosulfate which were assayed as above. Protein was measured with the procedure of Lowry et al. (1951) using bovine serum albumin (Sigma) as the standard.

Results

$^{14}CO_2$ Fixation. The ability of *Thiobacillus* cells to incorporate $^{14}CO_2$ into cell material was measured in media of varying NaCl concentration (Fig. 1). Fixation ability was strongly related to NaCl concentration, with maximum activity occurring at 10 ‰, i.e. one third strength sea water. Cells exposed to a salinity of 100 ‰ for 2 h retained the 10 ‰ optimum, although the incorporation was only 55 % of that in the unstressed cultures (Fig. 1). This reduction may reflect either cell death or physiological damage from the exposure to the high salinity.

Varying concentrations of KCl were used to determine the degree of specificity of the salt effect (Fig. 2). This experiment revealed the same pattern of optimal fixation ability at concentrations well below the in situ Na^+ levels. It is of interest to note that the optimum KCl concentration decreased from 20 to 10 ‰ as the incubation progressed. This pattern was reproduced in replicate experiments.

An artificial sea salts medium (final concentration, mM: NaCl, 335; $MgCl_2 \cdot 6H_2O$, 35; Na_2SO_4, 23; $CaCl_2 \cdot 2H_2O$, 12; $NaHCO_3$, 2) was tested in one series of $^{14}CO_2$ experiments. The results (Greenley unpublished observations) were the same as those obtained with just NaCl (30 ‰) addition.

To distinguish between ionic and osmotic effects on $^{14}CO_2$ incorporation, we measured the ability of our isolate to fix CO_2 at various concentrations of sucrose and glycerol. All concentrations of sucrose and glycerol lowered CO_2 incorporation (Fig. 3), but the effect of glycerol was much less severe than that seen with any of the other solutes. Concentrations of sucrose and glycerol are expressed as osmotic equivalent concentrations of NaCl (Scott 1957) to simplify comparison.

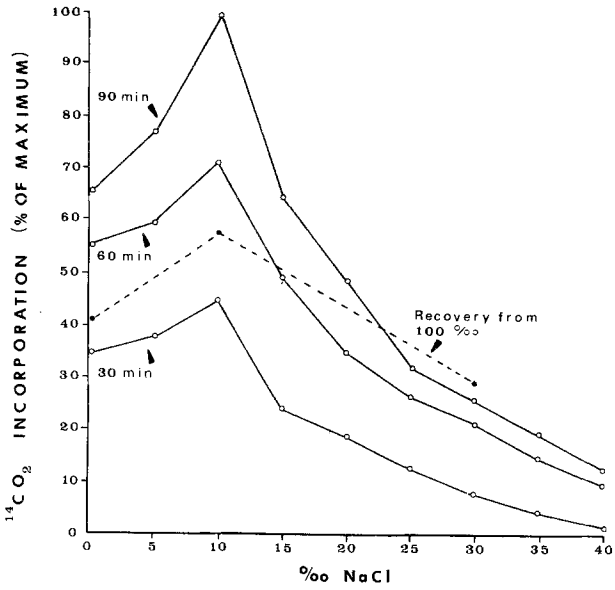

Fig. 1. $^{14}CO_2$ fixation by a marine *Thiobacillus intermedius* as a function of NaCl concentration. The three lines are cumulative incorporation at the indicated times. The dotted line is the result of a 90 min incubation of a suspension which had been exposed to 100 ‰ NaCl for 2 h. Each point is the average of duplicate samples which were within 5 % of each other. Maximum incorporation (100 %) was 2,540 cpm

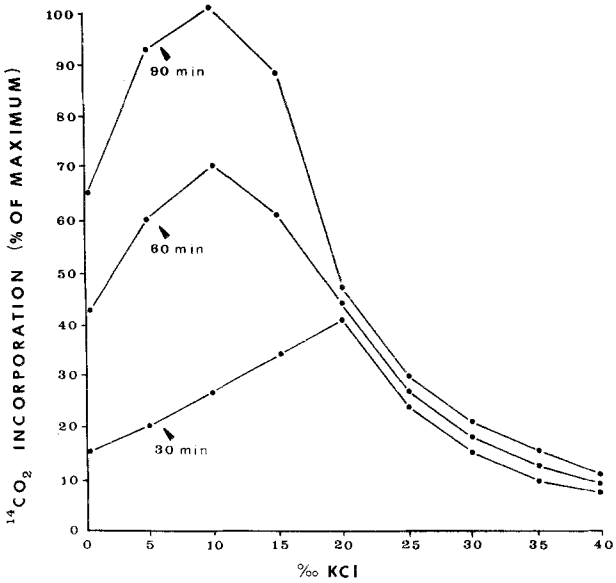

Fig. 2. $^{14}CO_2$ fixation by a marine *Thiobacillus intermedius* as a function of KCl concentration. Each point is the average of duplicate samples which were within 5 % of each other. Maximum incorporation (100 %) was 2,800 cpm

Respiration. The endogenous respiration of chemostat-grown cells had its maximum value, 0.001 µl O_2 consumed/min × mg

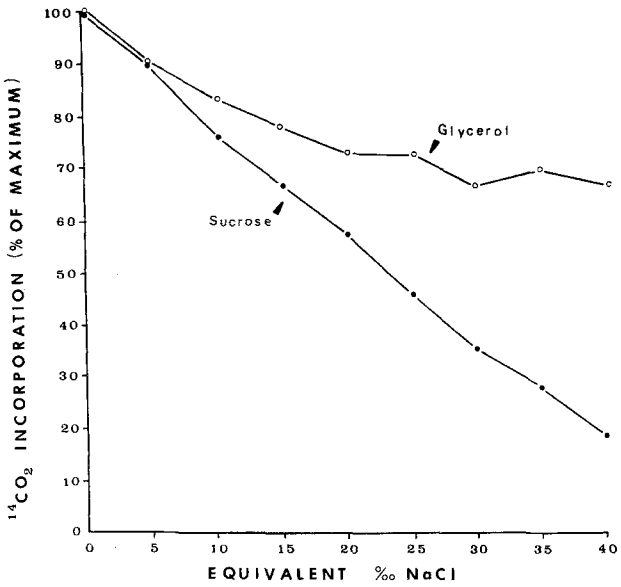

Fig. 3. $^{14}CO_2$ fixation by a marine *Thiobacillus intermedius* as a function of sucrose and glycerol concentration expressed as equivalent NaCl concentrations. The curves are cumulative incorporation after 90 min. Each point is the average of duplicate samples which were within 5% of each other. Maximum incorporation (100%) was 2,150 cpm

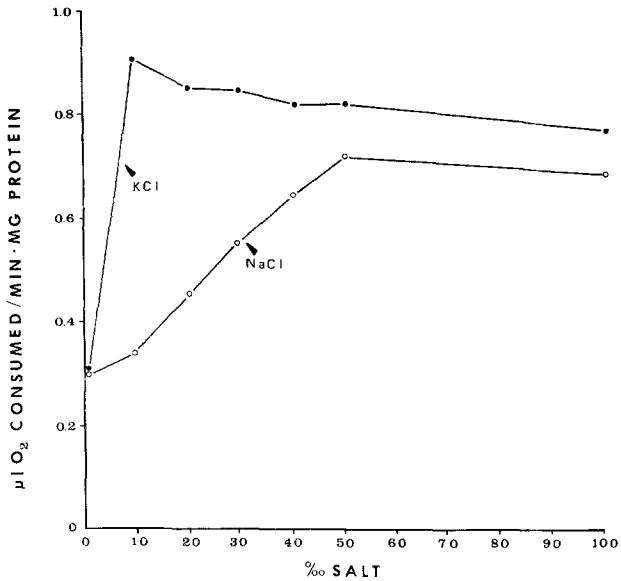

Fig. 4. Thiosulfate respiration by a marine *Thiobacillus intermedius* as a function of NaCl and KCl concentration. Points are the averages of triplicate measurements which were within 10% of each other

protein, at $50\,^0/_{00}$ NaCl. This value is nearly 100 times less than that reported for batch cultures of a terrestrial *Thiobacillus* (Vogler 1942). It may be that the energy-limited chemostat culture had no excess energy available to synthesize organic storage polymers whose oxidation can be a significant contribution to the rather complicated phenomenon of endogenous respiration (Dawes 1976).

Thiosulfate respiration was measured in media of varying NaCl and KCl concentrations (Fig. 4). Oxygen consumption was stimulated by both salts, reaching maximum levels at $10\,^0/_{00}$ (KCl) or $50\,^0/_{00}$ (NaCl) with no appreciable decline, even at the highest concentration tested ($100\,^0/_{00}$). There was no sharp maximum at $10\,^0/_{00}$ as was observed for CO_2 fixation.

Sulfur Metabolism. The concentrations of appropriate sulfur compounds were determined as a function of NaCl concentration and incubation time in complete growth medium. Table 1 presents the results from the initial and final sampling times. Samples removed at three intermediate times were also analyzed, and the results were consistent with the overall trend. The major findings were as follows. Initial effects: 1) most rapid thiosulfate consumption at high salinity; 2) most rapid pH decline at high salinity; 3) most rapid elemental sulfur production at low salinity; and 4) most rapid polythionate production at high salinity. Final effects: 1) total thiosulfate consumption more extensive at low salinity; 2) extensive elemental sulfur production at all salinities, with a peak of $30\,^0/_{00}$; 3) total polythionate accumulation greatest at low salinity; and 4) extensive sulfate production at all salinities, with a peak at $20\,^0/_{00}$.

Discussion

The metabolic activities of a microorganism may be conceptually separated into the processes by which the organism obtains carbon (for cellular synthesis) and energy (i.e. generates ATP). It is just this distinction which was employed by van Niel (1946) as the basis for nutritional/physiological classification of bacteria. A key advantage to examining an organism which is growing as a lithotrophic autotroph as we did here is that to a large extent these two aspects of metabolism can be analyzed independently. Although simplified, this approach has many advantages and will be used as a framework for analyzing the present results.

Carbon Metabolism. The fixation of $^{14}CO_2$ by our autotrophically grown *Thiobacillus* was clearly affected by the salinity of the medium (Figs. 1 and 2), with the optimum

Table 1. Changes in sulfur species and pH as a function of incubation of *Thiobacillus intermedius* in a complete growth medium[a]

Salinity ($^0/_{00}$)	Thiosulfate		Polythionates[b]		Sulfate		Sulfur		pH	
	80 min	505 min	80 min	505 min	80 min	505 min	80 min	505 min	80 min	505 min
0	41	24	0	27	0	8	0.25	1.0	7.1	6.5
10	41	23	1.8	24.2	0	10.9	0.25	2.0	6.8	5.6
20	40	24	2.9	22.4	0	11.2	0.15	2.5	6.5	5.3
30	39	23	4.9	24.6	0	10.2	0.1	2.7	6.4	6.0
50	37	26	8.6	22.7	0	6.8	0	1.9	6.3	5.3
100	35	29	13.0	18	0	5.9	0	1.8	6.2	5.4

[a] All concentrations are μmol/ml. Initial thiosulfate concentration was 41 μmol/ml

[b] Polythionate values are total μmol of S atoms in polythionates/ml. Various polythionate species were not quantified individually

salinity being much lower than that of the environment from which the culture was isolated. This finding was corroborated by two observations. First, the optimum NaCl concentration remained unchanged over 9 months incubation at $30^0/_{00}$ NaCl in the laboratory. Second, the identical salinity optimum was found for an isolate of *Thiomicrospira pelophila* (Kuenen and Veldkamp 1972) from the same marsh location (R. B. Ketcham and D. W. Smith, Abstr. Ann. Meet. ASM 1977, N65, p. 239). This optimum was retained by *T. intermedius* even after cells were exposed to high ($100^0/_{00}$) salinity, although the rate of CO_2 fixation was lower, reflecting either physiological damage or cell death by the high salt level (dotted line on Fig. 1). The stability of this optimum is especially interesting in its lack of relation to the salinity regime of the bacterium's environment. The salinity of the in situ marsh sediment varies only slightly from $30^0/_{00}$ during the year, with the exception of occasional (less than 5% of the time) periods during the summer when interstitial salinity can be as high as $80^0/_{00}$ (Smith, unpublished observations).

Ions other than Na^+ have been shown to be significant in the physiology of marine bacteria. The Na^+ requirement is often replaceable by K^+ as it was for our *Thiobacillus* (Fig. 2). The initial (30 min) optimum concentration of K^+ was $20^0/_{00}$, but it shifted to $10^0/_{00}$ after 60 min incubation. This shift was reproducible and may reflect a relatively slow equilibration of this bacterium with external K^+ as compared to Na^+. The effect of K^+ was examined in a separate experiment (data not shown) with a small amount ($6^0/_{00}$) of added NaCl since most marine bacteria, including thiobacilli, have obligate requirements for Na^+ (MacLeod 1965; Pratt 1974). The two experiments gave identical results; when both cations were present, their effects were additive.

All solutes have osmotic activity, but the monovalent cations have other effects as well in marine bacteria, such as protein stabilization and transport (Buckmire and MacLeod 1965; Pratt and Tedder 1974; Niven and MacLeod 1978, 1980). In order to understand the role of osmotic pressure more directly, we examined non-ionic solutes (glycerol and sucrose) for their effect on $^{14}CO_2$ fixation (Fig. 3). It is clear that, at any concentration, sucrose or glycerol depresses fixation, with sucrose being more inhibitory, possibly as the result of permeability differences (Buckmire and MacLeod 1970). An alternative explanation is that the organic solutes have metabolic effects on the mixotrophic *Thiobacillus*. However, this *Thiobacillus* does not metabolize either of these compounds (Bugher and Smith unpublished results). It is interesting to note that glycerol is a significant osmoregulatory compound synthesized intracellularly in high salinity environments by other microorganisms, for example the halophilic alga *Dunaliella* (Ben-Amotz and Avron 1973). We conclude that the salinity effects we observed results from a specific salt requirement, either Na^+ or K^+, and that the osmotic aspects are much less important.

Energy Metabolism. Two aspects of energy metabolism were examined: respiration and chemistry of sulfur metabolism in the culture. These two differ in that respiration is a coupled, whole-cell phenomenon requiring among other things an intact cell membrane for sustained activity. The changes in sulfur species on the other hand may be analyzed more directly in terms of individual chemical reactions and possibly specific enzyme activities. However, for lithotrophically growing *Thiobacillus* both are reflections of the same basic process: the aerobic oxidation of reduced sulfur compounds for the presumed purpose of generating useful energy (ATP) for the cell.

As summarized by Roy and Trudinger (1970), two fundamentally different pathways for the oxidation of thiosulfate to sulfate have been proposed: 1) cleavage of thiosulfate to produce elemental sulfur as an intermediate (Peck and Fisher 1962; Charles and Suzuki 1966); 2) combination of two thiosulfate molecules to form tetrathionate ($S_4O_6^=$) and other polythionates (Jones and Happold 1961; London and Rittenberg 1964). It is clear from Table 1 that salinity variations affect the pattern of metabolic sulfur changes brought about by the *Thiobacillus* isolate. These patterns may reflect pathway shifts within the organism, but great care must be used in deducing pathways from the accumulation of intermediates, especially in light of the instability of most aqueous sulfur species (Roy and Trudinger 1970).

The respiration and sulfur chemistry experiments are consistent in indicating that the initial metabolic response of the *Thiobacillus* isolate was greatest at high salinities (Fig. 4, Table 1). Prolonged incubation altered the sulfur chemistry pattern (Table 1), especially in accumulation of polythionates and sulfate. These two aspects of energy metabolism in *Thiobacillus* are clearly affected by salinity variations. However, the response is quite different from that seen for carbon metabolism.

Our isolate, identified as *Thiobacillus intermedius*, is capable of growth as a lithotrophic autotroph, a mixotroph, or an organotrophic heterotroph. If this *Thiobacillus* grows predominantly as a lithotrophic autotroph in nature, then our data show it will produce new cell material at a rate far below its potential (Figs. 1 and 2) even though it is rapidly respiring reduced sulfur compounds (Fig. 4). However, we did not examine the effect of salinity on the metabolic activities of this organism while growing as an organotrophic heterotroph or a mixotroph. Since the sediments in this marsh are up to 40% organic matter (Dicker and Smith 1980), it is conceivable that the total effect of salinity on the physiology of this bacterium may be different from the conclusions derived here.

Even accepting from our data that growth of this *Thiobacillus* does not occur at its maximum rate in situ very often, one should not conclude that it is not well adapted to its environment. It is necessary to keep in mind that a given environment is a complex of a large number of factors. An organism's fitness and survival depend not on its response to any one of these features, but to the environment as a whole, including biotic factors such as competition and predation. It is therefore reasonable to suggest that there are factors in addition to salinity important to growth and survival of our *Thiobacillus* which we have yet to examine.

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