OXIDATIVE ACTIVITY OF PSYCHROPHILIC AND MESOPHILIC BACTERIA ON SATURATED FATTY ACIDS

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ABSTRACT

SULTZER, BARNET M. (Michigan State University, East Lansing). Oxidative activity of psychrophilic and mesophilic bacteria on saturated fatty acids. J. Bacteriol. **82**:492–497. 1961.—Comparisons were made of the capacity of representative psychrophilic pseudomonads and of the mesophilic *Serratia marcescens* and *Sarcina flava* to oxidize sodium salts of fatty acids at temperatures ranging from 7.5 to 40 C. Conventional manometric techniques were employed to determine respiration of whole cells and of cell-free extracts of the bacteria. Reaction velocity constants were measured with whole cell preparations on octanoate at temperatures between 7.5 and 40 C. Temperature characteristic (μ) values, obtained by an Arrhenius plot of these reaction velocity constants, revealed a dissimilarity in response of the different types of organisms to the influence of decreasing temperature. The psychrophiles exhibited significantly lower μ values while oxidizing octanoate at much higher rates throughout the same temperature range. Changes in the slopes of the Arrhenius plots at various temperatures emphasized the differences in μ values between the psychrophiles and mesophiles. Extracts of these organisms were tested for their cofactor requirements and all were found to require adenosine triphosphate and coenzyme A to activate the octanoate oxidase system. Thus, in this experimental system, differences between these types of bacteria in their oxidative response to a saturated fatty acid cannot be explained by the initial activating mechanism involved. The implications of these findings are discussed in terms of the growth and physiological activity of psychrophiles at low temperatures.

Bacteria capable of relatively rapid growth at temperatures near the freezing point of water represent a biological exception in nature, for they display all of the significant physiological activities at temperatures which ordinarily inhibit other poikilothermic microorganisms.

One approach to the problem of defining the possible reasons for this distinctive metabolic capability is a comparison of the kinetics of enzyme systems common to both mesophilic and psychrophilic forms. Hess (1934), Kiser (1944), and Greene and Jezeski (1954) reported kinetic studies of psychrophilic bacteria using the Q_{10} as a measure of growth rates and various biochemical functions. Comparative rate studies on the respiration of a psychrophilic pseudomonad and *Pseudomonas aeruginosa* were reported by Brown (1957). The effect of temperature on the growth and metabolism of psychrophilic and mesophilic bacteria has also been studied from a kinetic viewpoint by Ingraham (1958) and by Ingraham and Bailey (1959).

Since oxidative catabolism yields more energy per mole of substrate than other mechanisms available to the cell to supply energy for endergonic reactions, and since the organisms generally considered to be psychrophilic are highly aerobic, it appeared that the ability to grow and metabolize at low temperatures in a relatively vigorous manner may be directly associated with the oxidative capacities of an organism. One such capacity is reflected in the ability of a variety of aerobic bacteria to attack saturated fatty acids. With the hope that a study of rate reactions might reveal characteristics indicative of the functioning of organisms at low temperatures, the ability to oxidize saturated fatty acids has been employed to measure the response of representative psychrophiles and mesophiles to changes in

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temperature. An investigation was also undertaken to demonstrate any possible differences in the fatty acid oxidation mechanisms of these organisms on the basis of cofactor requirements.

MATERIALS AND METHODS

Selection of cultures. Cultures were chosen for study based upon their growth response on brain heart infusion agar (Difco) at various incubation temperatures. A laboratory stock culture of *Pseudomonas geniculata*, and isolates obtained from various foods spoiled at refrigerator temperatures and identified as species of *Pseudomonas*, were found to demonstrate abundant growth at 5 C in 72 hr and no growth at 37 C in more than 2 weeks. These cultures were considered to be representative psychrophiles. A strain of *Serratia marcescens* obtained from the laboratory collection demonstrated slight growth after 15 days at 5 C which did not proceed further, whereas growth was apparent at 40 C in less than 1 week. A strain of *Sarcina flava* showed no growth at 5 C and exhibited abundant growth after 1 week at 45 C. These cultures were chosen as representative mesophiles.

Survey of the oxidative activity on fatty acids. Twenty-four-hour cultures of the selected strains, previously transferred daily for 3 days, were harvested, the cells separated by centrifugation, washed three times with sterile distilled water, and the final suspension made in 0.05 M phosphate buffer at pH 7.4. All suspensions of cells were adjusted to a standardized turbidity at 600 mμ in the Bausch and Lomb Spectronic 20 spectrophotometer and aerated for 30 min at room temperature before use in the Warburg respirometer. Conventional manometric techniques were employed throughout. The acids tested as substrates included acetic, butyric, hexanoic, and octanoic as their sodium salts, and were used in a final concentration of 10 μmoles. The flasks were equilibrated for 15 min, and the uptake of oxygen was followed for a period of 3 hr at 30 C. All results reported represent values after subtracting the endogenous rates of oxidation.

Temperature characteristic (μ) studies with whole cell preparations. Since octanoate was found to be oxidized most rapidly by all of the cultures, it was selected as the substrate for determining reaction velocity constants at various temperatures. To eliminate the problem of the adaptive fatty acid oxidase systems of *S. marcescens* affecting reaction rates (Silliker and Rittenberg, 1951), and that of high endogenous rates of cells after growth on brain heart infusion agar, all of the test organisms were grown on the following medium: $(NH_4)_2SO_4$, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; K_2HPO_4, 8.0 g; KH_2PO_4, 2.0 g; yeast extract (Difco) 3.0 g; octanoic acid, 0.01 M; agar, 20.0 g; and distilled water, 1 liter. The medium was adjusted to pH 7.4 with NaOH before sterilization. To insure maximal cell crops, the psychrophilic cultures were grown at 20 C and the mesophiles were grown at 35 C on agar slants. The washed cells were made up in buffer to a concentration equivalent to a cell nitrogen content of 1 mg per ml as determined by the micro-Kjeldahl method.

Duplicate, and in some cases triplicate, reaction flasks were run at each temperature in the range of 7.5 to 40 C at 2.5 or 5.0 C intervals. Fresh cell suspensions, obtained from the original stock suspension and held at 1 C, were used at each temperature, and the flasks were equilibrated for 15 min.

Oxidation rates were followed for 60 min and endogenous rates were subtracted from the mean values of the replicate reaction flasks. $Q_{O_2}(N)$ values were determined from the slopes of the straight lines and the logarithms of these values plotted against the reciprocal of the absolute temperatures. The best straight line was drawn between the plotted points according to the least square method. From the slopes of these lines, μ values were determined according to the integrated Arrhenius equation

$$\log \frac{k_2}{k_1} = \frac{\mu}{2.3\,R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

where k_1 and k_2 are reaction velocity constants at absolute temperatures T_1 and T_2, R is the gas law constant, and μ is a constant designated as the temperature characteristic. Several runs were made for each organism over the cited temperature range.

Dried cell and cell-free extract preparations. Sufficient crops of cells of each culture were obtained by growth on agar plates (25 by 150 mm) of octanoate medium incubated at the temperatures appropriate for each strain. Twenty-four-hour cell-crop yields amounted to

about 1 g per liter of medium on a dry weight basis.

Acetone-dried cells were prepared according to the method of Colowick and Kaplan (1955) and stored at −15 C. The powders were tested for activity by preparing a suspension of 25 mg per ml of 0.05 M tris(hydroxymethyl)aminomethane buffer (tris) at pH 7.4. Sonic oscillation of the powders was accomplished at a concentration of 50 mg of powder per ml of tris buffer. The organisms differed in their susceptibility to such treatment, i.e., although the gram-negative cells were disrupted in 15 min, *S. flava* was quite refractory. Both fresh cells and powder took as long as 60 min of oscillation in a 250-w, 10-kc Raytheon sonic oscillator at a maximal obtainable output of 1.0 amp with the oscillator cup kept at less than 5 C by circulating ice water. The suspensions were then centrifuged in the cold at about 20,000 × *g* for 30 min. The clear, opalescent supernatant liquids were used immediately as the crude cell-free extracts in manometric studies.

The second method involved stirring the powder at a concentration of 50 mg per ml of tris buffer at pH 7.4 at 5 C for 4 hr. The suspension was centrifuged as described and the supernatant liquid used for the manometric studies. Determinations of the protein content of cell-free extracts were made by means of turbidity measurements of trichloroacetic acid precipitates at 600 mμ, compared to standard solutions of purified albumin.

Several cofactors known to be necessary for enzyme activation in animal fatty acid oxidase systems were used to determine the requirements, if any, of these bacterial extracts. These included coenzyme A (CoA), Mg^{++}, adenosine triphosphate (ATP), cytochrome *c*, diphosphopyridine nucleotide (DPN), flavin adenine dinucleotide (FAD), and yeast concentrate. All cofactor reagents were obtained from the Sigma Chemical Company.

RESULTS

The activity of the selected psychrophiles and mesophiles on short chain fatty acids is shown in Table 1. Except for acetate, increased oxidation generally paralleled increased chain length, and the psychrophilic organisms appeared to oxidize almost all of the acids tested at greater rates than *S. marcescens* and *S. flava*. The failure of *S. marcescens* to oxidize butyrate to any extent was also found to be the case with another pseudomonad and a psychrophilic isolate of *Achromobacter*. Silliker and Rittenberg (1951) previously reported that dried cell preparations of *S. marcescens* likewise fail to oxidize butyrate; apparently this rules out a permeability barrier as an explanation. In view of the deficiency in several genera, this particular anomaly seems to be significant, and any theory of fatty acid oxidase mechanisms in aerobic bacteria should take it into account.

TABLE 1. *Activity of psychrophilic and mesophilic bacteria on saturated fatty acids*

Organism	Microliters of oxygen uptake in 120 min*			
	Acetate	Butyrate	Hexanoate	Octanoate
Pseudomonas geniculata	241	47	132	166
Pseudomonas sp. 1	155	34	170	197
Pseudomonas sp. 2	129	−16	126	89
Pseudomonas sp. 3	60	16	59	86
Pseudomonas sp. 4	36	60	74	123
Pseudomonas sp. 5	34	45	64	85
Pseudomonas sp. 6	183	22	94	129
Achromobacter sp.	145	5	111	123
Alcaligenes sp.	184	67	180	283
Serratia marcescens	33	0	54	58
Sarcina flava	45	23	74	46

* Reaction mixture contained: 1.0 ml of cell suspension, 1.0 ml of 0.05 M phosphate buffer at pH 7.4, 1.0 ml of substrate as the sodium salt at 10 μmoles, 0.2 ml of 20% KOH in center well, incubation at 30 C in air atmosphere, values corrected for endogenous rates.

The plots of the $Q_{O_2}(N)$ values obtained at each temperature in the presence of sodium octanoate reveal the influence of temperature changes on the measured reaction rates for each organism tested (Fig. 1). Both of the psychrophilic pseudomonads chosen, compared to either mesophile, were found to oxidize octanoate at greater rates throughout the temperature range of 7.5 to 35 C. The maximal rates achieved by all of the organisms occurred at nearly the same temperature, 35 to 37.5 C. Therefore, no correlation exists between the maximal growth temperatures of these organisms and the maximal rates of oxidative activity with octanoate. In fact, under the experimental conditions employed, the enzyme system in vivo functioned at temperatures somewhat higher than the maximal

growth temperature for the psychrophilic pseudomonads, whereas the converse was true with the mesophilic cultures.

Besides the differences in over-all activity, the μ values for the oxidation of octanoate emphasize other points of dissimilarity as is shown in Table 2 and Fig. 1. The various plots of the reaction velocity constants did not obey the Arrhenius equation over the entire temperature range studied. The change in slope is to be noted with each psychrophile at about 15 C, whereas with the mesophilic species the change in slope occurs at 20 and 25 C. These specific differences are further emphasized by the lower μ values displayed by the psychrophile over the range of 7.5 to 37.5 C.

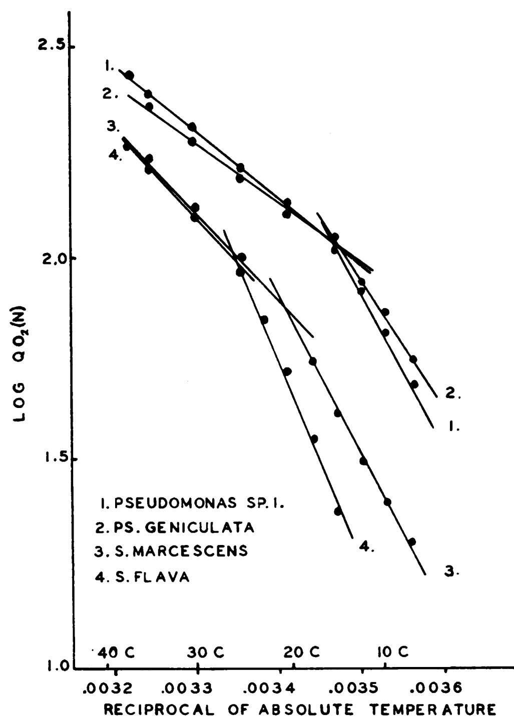

FIG. 1. *Temperature characteristic* (μ) *plot of the oxidation of sodium octanoate by psychrophilic and mesophilic bacteria.*

The acetone powder preparations were found to be sufficiently active for determining cofactor requirements and the cell-free extracts prepared from these powders responded in a similar manner. Representative results of the activity of psychrophilic and mesophilic extracts on sodium

TABLE 2. *Temperature characteristic* (μ) *values for the oxidation of sodium octanoate*

Organism	μ	Temp range
	cal/mole	*C*
Pseudomonas sp. 1	7630	15–37.5
	16,030	7.5–15
Pseudomonas geniculata	6870	15–35
	14,660	7.5–15
Sarcina flava	9160	25–37.5
	22,900	15–25
Serratia marcescens	9160	20–35
	18,320	7.5–20

TABLE 3. *Activity of extracts of psychrophilic and mesophilic (bacteria) on sodium octanoate*

Organism	Substrate concn	Cofactors added	Oxygen uptake in microliters* at time (min): 30	60	90	120	150	180
	moles							
Pseudomonas sp. 1	2	None	0	0	0			
	2	ATP	1.0	0	0			
	2	CoA, ATP	3.7	6.5	9.8	13.5	19.8	21.5
	2	CoA	2.3	6.1	5.7			
	5	CoA, ATP	16.7	20.0	39.8	37.5	46.9	52.2
Serratia marcescens	2	None	0	0	0			
	2	CoA	1.0	0	0			
	2	ATP	2.7	6.3	5.4			
	2	CoA, ATP	17.3	20.4	21.5			

* Reaction mixture contained: 1.0 ml *Pseudomonas* sp. 1 acetone powder (25 mg/ml) or 1.0 ml of the *S. marcescens* extract (7 mg protein/ml), 2 μmoles of $MgCl_2$, 0.12 mg CoA, 0.1 μmole reduced glutathione, 1 μmole ATP, 5 μmoles phosphate in 0.05 M tris buffer at pH 7.4, and substrate as indicated. Incubation at 30 C, air atmosphere with 0.2 ml 20% KOH in center well. The total volume of the reaction mixture was 3.2 ml. All data are corrected for endogenous respiration in the absence of substrate.

octanoate are presented in Table 3. With each organism, both CoA and ATP were required. The addition of cytochrome *c*, DPN, yeast concentrate, or FAD had no stimulatory effect over levels achieved with CoA and ATP. As shown, the activity amounted to about ½ μmole of oxygen per μmole of substrate. The results obtained with several preparations of *S. flava* were the same, but significant residual amounts of CoA were present in *P. geniculata* extracts since either ATP alone or CoA and ATP stimulated activity to about the same extent. It must also be noted that the activity of the *S. marcescens* preparations was variable, i.e., of six different batches, three demonstrated activity when appropriately supplemented, whereas the remaining preparations were inactive.

DISCUSSION

To compare the oxidative capacities of psychrophiles and mesophiles, one must consider both the absolute reaction rates at any one temperature and the effect of varying the temperature on the change in such rates. If there were no inherent differences to a decrease in temperature, then regardless of the reaction velocity constants, the μ values would be similar. Conversely, μ values of psychrophiles and mesophiles would correspondingly differ whether reaction velocity constants were higher or lower at any specific temperature. The results obtained in this work appear to support the latter proposition. The psychrophilic pseudomonads oxidized octanoate at higher rates and their μ values were significantly lower than the mesophiles throughout the cited temperature range. In this regard, Brown (1957) found that a psychrophilic pseudomonad exhibited a greater oxidative activity on glucose than that demonstrated by a strain of *P. aerguinosa* over a range of 0 to 40 C. Ingraham and Bailey (1959) also found the psychrophilic *Pseudomonas perolen* to show greater rates of glucose oxidation than the mesophilic *Escherichia coli* or *P. aeruginosa*. However, quite interestingly, these workers described a psychrophilic pseudomonad that demonstrated lower rates of glucose oxidation than these same mesophiles, but nevertheless exhibited significantly lower Q_{10} values. Thus, the evidence to date suggests a working hypothesis that the oxidative activity of psychrophilic bacteria is less sensitive than mesophilic bacteria to a decrease in temperature. Whether there exists a causal relationship between such a response and the over-all physiological activity and growth of psychrophiles at low temperatures awaits the results of more direct experimentation.

The change in the linearity of the Arrhenius plots at various temperatures with each of the organisms used in these studies may be interpreted from at least two standpoints. According to Johnson, Eyring, and Polissar (1954), if an equilibrium exists between the native and denatured forms of an enzyme, a shift in this equilibrium toward the denatured state is subject to temperature increases and may be observed at temperatures well below the optimum. If so, changes in μ values at different temperatures observed with the two types of organisms used here may be a function of the differential sensitivity of the corresponding enzyme systems to a rise in temperature. On the other hand, Sizer (1943) claims the postulate of a single rate-limiting step governing the over-all rate of a catenary series of reactions is consistent with the fact that so many enzyme processes conform to the Arrhenius relation over a significant biokinetic temperature range. This suggests the concept of rate-limiting reactions in the fatty acid oxidase system of the organisms studied as being differentially sensitive to temperature. However, factors other than enzymatic degradative steps may themselves become rate limiting, e.g., the accumulation of intermediates due to a block in the catabolic mechanism, or differences in cell membrane permeability. The nature of the reaction rates with cell-free systems was precluded in this work, and a study in vitro is necessary if rate-limiting reactions are to be defined in terms of specific enzymes.

Although the whole cell studies revealed certain differences in the response of psychrophiles and mesophiles to octanoate, the results of the acetone powder and cell-free extract work emphasized basic similarities. The requirements for CoA and ATP appeared to be consistent with each organism, suggesting the degradation of this acid in aerobic bacteria by means of CoA derivatives. These results support the work of Ivler, Wolfe, and Rittenberg (1955) and Murray and Dawes (1956) with *Pseudomonas fluorescens* and *Sarcina lutea*, respectively. Furthermore, assuming the mechanism of β-oxidation to be functioning, the quantitative data and the failure of DPN to

stimulate oxidation with each organism indicate the acetone powder and cell-free extraction techniques apparently resulted in the isolation of the thiokinase and acyl dehydrogenase enzymes.

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