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Effect of Growth Substrate on Thermal Death of Thermophilic Bacteria†

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The heat sensitivity of gram-negative, hydrocarbon-utilizing thermophilic bacteria was altered by a change in growth substrate. Thermophilic strains CC-6, BI-1, and LEH-1, grown with acetate or *n*-heptadecane as the carbon source, had a higher survival rate when incubated 5°C above their maximum growth temperature than cells of the same organism after growth on glucose or glycerol. There was a correlation between the growth substrate, heat resistance, and the ratios of cellular *n*-hexadecanoic acid/branched hexadecanoic acid and *n*-heptadecanoic acid/branched heptadecanoic acid. The bacterial cells that were more heat resistant had ratios of straight-chain/branched-chain fatty acids above 1.0, whereas the heat-sensitive cells had ratios below 0.6.

Many theories have been advanced to explain the ability of microorganisms to survive at a high temperature, and there has been considerable speculation as to the component(s) of the microbial cell that is critical in determining the maximum temperature at which the microorganism can survive (3, 9). Since thermal death is a first-order process, the loss of membrane integrity at a high temperature could be a limiting factor in establishing the upper limit for growth of some organisms (2).

Although there is little supportive evidence for explanations of thermophily (24), there is a direct relationship between cellular fatty acid composition and growth temperature. Studies with mesophilic bacteria indicate that there is an increase in the relative amount of unsaturation in the fatty acids of cells as the incubation temperature is lowered; conversely, increasing the growth temperature results in a decrease in unsaturated fatty acids (10, 13, 25).

The class of fatty acid present in greatest quantity in thermophilic bacteria is iso-branched (1, 20, 26). In the gram-positive organisms examined, lowering the incubation temperature for growth generally results in an increased proportion of unsaturated fatty acids, whereas a lowering of the incubation temperature for gram-negative thermophilic bacteria results in a larger proportion of small, branched-chain fatty acid in the cells (12, 23, 28).

The fatty acid composition of hydrocarbon-utilizing microorganisms is often dictated by the chain length of the hydrocarbon provided as the source of carbon and energy (4, 6-8, 14, 16). The isolation of *Thermomicrobium fosteri* (21) and other hydrocarbon-utilizing thermophilic bacteria makes it possible to study the effect of cellular fatty acid composition on the survival of thermophilic microbes at high temperatures. This report is concerned with the correlation between growth substrate, fatty acid composition, and survival of selected hydrocarbon-utilizing thermophilic bacteria at a temperature above that which will allow their growth.

MATERIALS AND METHODS

Microorganisms and cultural conditions. The organisms used in this study are designated strains LEH-1, CC-6, and BI-1. All are gram-negative, nonsporulating, obligately thermophilic bacteria that can utilize *n*-alkanes as the sole source of carbon and energy (G. J. Merkel, S. S. Stapleton, and J. J. Perry, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, I90, p. 126). The range of temperature for growth is 42 to 70°C, with an optimum at 60°C. Further characterization of these organisms will be the subject of a separate report.

The organisms were maintained at 60°C in L-salts basal medium (15) with 0.1% (vol/vol) *n*-heptadecane (ICN, New York, N.Y.) as the carbon source. Solid substrates were added to the basal medium at a concentration of 0.2% (wt/vol).

Measurement of thermal death. Survival of the organisms at 75°C, 5°C above the maximum temperature for growth, was measured by adding 1 ml of a stationary-phase culture grown at 60°C to 9 ml of diluent equilibrated to the temperature at which thermal death estimates were determined. The L-salts basal medium, physiological saline, and 0.07 M phosphate buffer were used as diluents to determine whether the suspending medium had a measurable effect on the pattern of thermal death. The microbial

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cell suspensions were incubated with continuous agitation in a circulating water bath. Samples were withdrawn periodically, and the number of viable cells was estimated by a plate count on a medium consisting of 0.5% tryptone (Difco Laboratories, Detroit, Mich.), 0.25% yeast extract (Difco), 0.1% glucose, and 1.5% agar (Difco). The number of colony-forming units on the plates was estimated after incubation for 20 h at 60°C in plastic bags sealed to prevent desiccation. There was less than 10% deviation in total colony count in duplicate experiments. The thermal death pattern was determined with cells obtained from at least five separate growth experiments for each substrate.

Fatty acid analysis. The fatty acid composition of the microbial cells was determined after growth to stationary phase at 60°C with glucose, glycerol, acetate, or *n*-heptadecane as the substrate. Fatty acids were extracted and transesterified directly from washed cell pellets by the BF_3-methanol-benzene procedure described by Dunlap and Perry (7, 8). The fatty acid profiles were compared with those obtained by method B of Moss et al. (18) to determine whether hydroxy and cyclopropane fatty acids were present.

The fatty acid analysis was accomplished on a Varian Aerograph gas chromatograph (series 2400) equipped with a hydrogen flame ionization detector. The carrier gas was helium at a flow rate of 30 ml/min. The copper column (6 feet by ⅛ inch [ca. 183.08 by 0.32 cm]) was packed with 15% HiEFF-1BP (DEGS) on Gas-Chrom P (60–80 mesh). All determinations were isothermal at 170°C with the detector and injector ports at a temperature of 190°C. Fatty acids were identified and quantified (14), branching was located (19), and the presence of unsaturation was determined (7, 8), all by previously described methods.

RESULTS

Thermal death. The microbial cells grown with acetate or *n*-heptadecane as the substrate had a higher survival rate at 75°C than cells grown with glucose or glycerol (Fig. 1). The viability (percent survivors) of the cells after growth on *n*-heptadecane and exposure to 75°C for 1 h was 13, 10, and 6 for strains LEH-1, BI-1, and CC-6, respectively. When grown with acetate as the carbon source, the percent surviving 1 h at 75°C was 6 for LEH-1 and 1 for both BI-1 and CC-6. The cells grown on glucose had 0.002% survival after 1 h at 75°C in all three strains, and glycerol-grown cells had a viability of 0.3, 0.01, and 0.003% for LEH-1, BI-1, and CC-6, respectively. Incubation of equivalent cellular suspensions at 60°C for 1 h resulted in no detectable decrease in colony-forming units. The pattern of thermal death was not dependent on culture age, since there was no measurable difference between cultures harvested in early log, late log, or stationary phase.

The order of heat resistance in cell strains after growth on *n*-heptadecane or glycerol was LEH-1 > BI-1 > CC-6. The three strains had

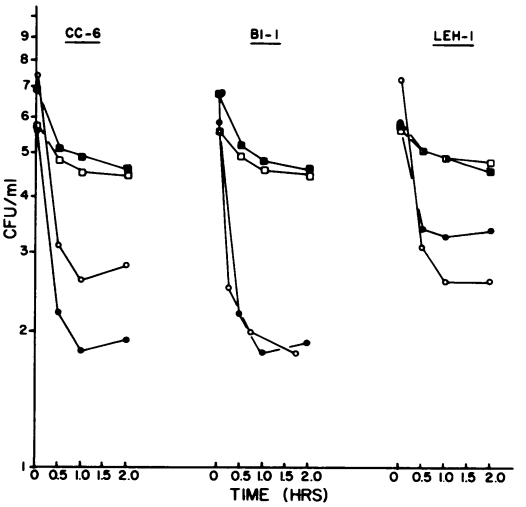

FIG. 1. *Thermal death curves for strains CC-6, BI-1, and LEH-1 at 75°C. The organisms were grown at 60°C to stationary phase with glucose* (○), *glycerol* (●), *n-heptadecane* (□), *or acetate* (■) *as the substrate. CFU, Colony-forming units.*

equivalent levels of heat resistance after growth on glucose or acetate. The thermal tolerance resulting from the growth substrate was *n*-heptadecane > acetate > glycerol > glucose.

The diluent for preparing the cell suspension used in the thermal death determinations had no measurable effect on survival. The same patterns of thermal death were observed with L-salts basal medium, 0.9% saline, and 0.07 M phosphate buffer. The effect of growth medium carryover in the preparation of all suspensions was negligible, since use of various concentrations of growth substrate as suspending medium resulted in thermal death patterns equal to those reported for phosphate buffer.

Various concentrations of calcium (as $CaCl_2$, up to 0.5 mg/ml) or magnesium (as $MgSO_4$, up to 4 mg/ml), added to the growth medium with glucose as substrate, did not affect viability of the resultant cells when they were subjected to a temperature of 75°C.

Fatty acid composition. Minor qualitative differences were observed in the cellular fatty acid composition of the three strains after growth on substrates rendering heat-resistant or -sensitive cells (Table 1). No unsaturated fatty acids were detected in any of the strains, and permanganate oxidation and chemical ionization spectrometry indicated that the majority of the branched-chain fatty acids were anteiso-branched.

The effect of incubation temperature on the fatty acid composition of the organisms was

TABLE 1. *Fatty acid profile of thermophilic bacteria (strains LEH-1, BI-1, and CC-6) after growth with various carbon sources*[a]

Fatty acid	LEH-1				BI-1[b]				CC-6[c]			
	Glu	Gly	Ac	C_{17}	Glu	Gly	Ac	C_{17}	Glu	Gly	Ac	C_{17}
C_{14Br}	1.0	2.2	0.5	0.3	1.2	2.3	0.5	0.5	1.0	3.1	0.6	0.4
C_{14}	0.8	2.3	2.3	0.2	0.2	1.6	1.8	0.4	1.0	1.6	2.0	0.3
C_{15Br}	9.3	15.6	25.7	10.5	8.1	16.7	26.2	12.8	12.1	17.9	20.8	17.1
C_{15}	4.0	6.7	6.2	25.5	1.5	4.9	6.7	17.6	5.2	5.8	6.6	18.0
C_{16Br}	40.5	38.1	17.6	6.2	38.8	46.1	17.7	8.1	40.3	43.2	19.3	10.9
C_{16}	13.2	22.2	19.5	2.3	11.7	12.7	20.4	4.9	12.6	10.8	22.2	3.0
C_{17Br}	17.6	7.6	19.5	7.5	15.0	11.2	19.8	9.7	13.0	12.9	18.8	14.3
C_{17}	6.1	3.1	4.5	43.1	5.3	2.9	4.1	37.7	5.2	3.1	4.5	32.9
C_{18Br}	4.0	1.1	0.8	0.7	8.5	0.5	0.5	ND[d]	1.4	0.8	1.2	ND
C_{18}	3.6	1.3	2.6	1.0	5.6	1.1	2.3	3.6	1.7	0.6	2.8	0.7

[a] Recorded as percentage of the total fatty acids present. The substrate was added at 0.1% for *n*-heptadecane (C_{17}) and 0.2% for acetate (Ac), glucose (Glu), and glycerol (Gly). The cells were grown to stationary phase at 60°C.

[b] Strain BI-1 had 2.3% C_{19} after growth on glucose.

[c] Strain CC-6 had 3.5% C_{13Br} after growth on glucose.

[d] ND, None detected.

examined in strain LEH-1. An increase from 45 to 60°C resulted in slight increases in the relative amount of straight-chain saturated fatty acid, with a concomitant decrease in the level of branched-chain fatty acid. This effect was evident when LEH-1 was grown with *n*-heptadecane as substrate and was not observed after growth on other substrates.

Correlations were found between the growth substrate, heat resistance, and the ratio of *n*-hexadecanoate to branched (Br) hexadecanoate or *n*-heptadecanoate to branched heptadecanoate (Table 2). Growth of the organisms on substrates that yielded more heat-sensitive cells had a ratio below 0.6. In cells grown on substrates that resulted in less sensitivity to heat, however, the ratio of C_{16}:C_{16Br} or C_{17}:C_{17Br} was above 1.0. The strains had equivalent ratios of normal to branched-chain acids when grown on glucose or acetate, whereas, on glycerol or *n*-heptadecane, the ratio increased in the order of heat resistance for each strain (LEH-1 > BI-1 > CC-6).

TABLE 2. *Ratio of saturated to branched fatty acids in the thermophilic strains after growth on various substrates*

Strain	Growth substrate[a]			
	Glu	Gly	Ac	C_{17}
LEH-1				
C_{16}/C_{16Br}	0.3	0.6	1.1	0.4
C_{17}/C_{17Br}	0.3	0.4	0.2	5.7
BI-1				
C_{16}/C_{16Br}	0.3	0.3	1.1	0.6
C_{17}/C_{17Br}	0.3	0.3	0.2	3.9
CC-6				
C_{16}/C_{16Br}	0.3	0.2	1.1	0.3
C_{17}/C_{17Br}	0.4	0.2	0.2	2.3

[a] Abbreviations as in Table 1.

DISCUSSION

The thermal death patterns of LEH-1, CC-6, and BI-1 differed, and each organism had a varying degree of thermal sensitivity or resistance. This relative resistance can be correlated with the ratio of straight-chain/branched-chain fatty acids in the cells. The higher the ratio, the greater the heat resistance. The presence of more straight-chain saturated fatty acid would be associated with increased survival, since increasing the concentration of saturation in a heterogeneous mixture of fatty acids would broaden the lipid phase transition temperature. The higher melting point of straight-chain fatty acids as compared with branched-chain fatty acids (5) could result in a cell membrane that would retain integrity over a wider temperature range.

Although alterations in fatty acid composition of mesophilic and thermophilic bacteria have been studied as a function of incubation temperature, little attention has been devoted to the structural and functional alterations that differences in fatty acid composition might induce. It has been reported that the maximum growth temperature for thermophilic bacteria is dependent on the growth substrate (11, 22). It may be that this maximum growth temperature is determined as an effect of that substrate on the membrane fatty acid composition.

Metal ions have been found to stabilize certain thermophilic bacteria at high temperatures and

thus to increase survival rate (17). In light of our experimental results with varying calcium and magnesium concentrations, this is insufficient to explain the increased resistance of these thermophilic microorganisms when they are grown on substrates that produce an increasedly heat-resistant cell.

It has been suggested that inactivation of key enzymes or genes in cells is a factor that determines the maximum temperature of growth for an organism (27). This may hold true for extreme temperatures; however, since the maximum growth temperature for these thermophilic bacteria is near 70°C regardless of the growth substrate, it would be necessary to assume that the organism possessed key enzymes or genes that are inactivated over a narrow (5-degree) temperature range. The results of investigations conducted on enzymes from thermophilic bacteria suggest that this would be a limited range for such extensive inactivation to occur. It would be probable that alterations in fatty acid composition, resulting from growth of the organism on substrates that yield more heat-sensitive cells, would result in a loss of membrane integrity when the organism is shifted to a high temperature.

We are currently examining in detail the inner- and outer-membrane lipid composition of thermophilic bacteria grown on substrates that result in more heat-resistant or heat-sensitive cells. These studies should give further indications of the involvement of the cellular membrane in determining maximum temperatures for growth.

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