Arch Microbiol (1989) 151:268–273

Archives of
Microbiology

Environmental control of metabolic fluxes in thermotolerant methylotrophic *Bacillus* strains

Anthony G. Brooke, Elizabeth M. Watling, Margaret M. Attwood, and David W. Tempest

Department of Microbiology, University of Sheffield, Sheffield, S10 2TN, UK

Abstract. Recently we have isolated a number of thermotolerant, spore-forming methylotrophic bacilli in pure culture. With a methanol-limited chemostat culture of strain Ts1, incremental increases in the incubation temperature from 45° C to 62.5° C revealed an optimum with respect to growth yield of 52.5° C, and a maximum of 62.5° C. Similar investigations revealed a pH optimum of 7.5 and a broad growth rate optimum with respect to growth yield. The organism displayed a low maintenance energy requirement and high growth yield (attained simultaneously with high growth rates) during growth on methanol. Under all conditions of methanol limitation, substrate was oxidized solely to biomass and CO_2 and carbon recoveries greater than 90% were manifest. Our data suggested that this resulted from an ability of the organism to precisely adjust its catabolic and anabolic pathways to suit prevailing growth conditions. These results are discussed in relation to previously reported data on thermophiles in both batch and chemostat culture.

Key words: Methylotrophy – Thermotolerance – Chemostat culture – Methanol limitation – Metabolic fluxes

A large number of microorganisms are able to grow on methanol, a compound of relatively low cost, which is attractive as a raw material for biotechnological processes (Anthony 1982; Crawford and Hanson 1984; van Verseveld and Duine 1987).

However, of the aerobic methylotrophic bacteria so far isolated, the vast majority have been Gram-negative mesophiles, which are very sensitive to the toxic effects of higher methanol concentrations (i.e. > 100 mM). Furthermore, amongst the methanol-utilizing bacteria using the ribulose monophosphate (RuMP) pathway of carbon assimilation, versatile facultative strains are rare, the only example reported to date being a Gram-positive actinomycete, *Nocardia* sp. 239 (Hazeu et al. 1983).

Since evidence has been published for the presence of methylotrophic *Bacillus* strains in nature (Colby and Zatman 1975; Akiba et al. 1970; Snedecor and Cooney 1974; Hitzman 1976), we set out specifically to isolate new representatives of these bacteria. Using specially developed techniques, we were able to isolate a number of thermotolerant, spore forming bacilli in pure culture (Clement et al. 1986; Brooke et al. 1987; Dijkhuizen et al. 1988). Our studies have demonstrated that three isolates which utilize the RuMP pathway of formaldehyde fixation (the most efficient pathway for the conversion of methanol into biomass), are able to tolerate methanol concentrations of up to 1.5 M, and that their molar growth yields on methanol are amongst the highest reported for methylotrophic bacteria (Anthony 1982).

Consequently, these novel methanol-utilizing thermotolerant bacilli may be of considerable industrial interest, both as synthesizers of thermostable enzymes, and also for the transformation of methanol to useful metabolites. However, in order to assess the biotechnological potential of these bacilli, a detailed knowledge of the regulation of metabolic fluxes from C_1 substrates needs to be acquired. This paper presents the first results of studies aimed at elucidating the extent of environmental control of metabolic fluxes in these organisms.

Materials and methods

Microorganisms

The three organisms used throughout this study, designated Ts1, Ts2 and Ts4 have recently been isolated in our laboratory from different environments. The methods employed for the enrichment, isolation and maintenance of pure cultures of these organisms have been described (Dijkhuizen et al. 1988).

Cultivation

The organisms were cultured, with 12 hourly subcultures on agar plates of the following composition (per litre): Bacteriological agar, 15 g; $(NH_4)_2SO_4$, 1.5 g; K_2HPO_4, 1.8 g; NaH_2PO_4, 0.35 g, $MgSO_4\ 7\,H_2O$, 0.2 g; trace elements (Vishniac and Santer 1957) 0.2 ml; yeast extract, casamino acids and peptone (0.5 g of each). Following sterilization by autoclaving at 121° C for 20 min, filter-sterilized methanol, 50 mM; and 1 ml of a vitamin mixture (mg · l^{-1}; biotin, 100; thiamine hydrochloride, 100; riboflavin, 100; pyridoxal

Offprint requests to: A. G. Brooke

phosphate, 100; pantothenate, 100; nicotinic acid amide, 100; folic acid, 100; p-amino benzoic acid, 20; vitamin B_{12}, 10; lipoic acid, 10) were added. For liquid batch culture medium, the agar was omitted.

For growth in continuous culture a medium was employed with the following composition (per litre): $(NH_4)_2SO_4$, 2.0 g; K_2HPO_4, 1.0 g; NaH_2PO_4, 1.0 g, $MgSO_4 \cdot 7\,H_2O$, 0.2 g; trace elements (Vishniac and Santer 1957), 0.4 ml: The pH of this medium was adjusted to 5.0 with conc H_2SO_4 prior to sterilization by autoclaving in 20 l batches at 121° C for 40 min. After cooling, vitamin mixture (as above) 1 ml, methanol (filter sterilized as indicated in individual experiments) and antifoam (Dow Corning, RD Emulsion, BDH), 0.1 ml were added.

The organisms were grown routinely in chemostats (LH Fermentation Ltd., 500 Series) with either 2 l or 1 l growth vessels (1,250 and 975 ml working volumes respectively), with automatic temperature control. The pH was adjusted by automatic titration with 2 N NaOH. Dissolved oxygen levels were monitored with galvanic oxygen electrodes, and culture agitation speed adjusted, either automatically or manually, to maintain a value of approximately 40% saturation in the culture medium.

Preparation of inocula for continuous cultures

The isolates used throughout this study were extremely sensitive to the method of cultivation used for the preparation of inocula. The most reproducible approach involved aseptically transferring a small number of young (< 12 h old) colonies from a methanol agar plate into pre-warmed liquid medium (25 ml), followed by incubation at 52.5° C with shaking until the mid-exponential growth phase (t_d = 45–50 min). This inoculum was transferred to the culture vessel which initially did not contain any carbon source and fresh medium containing methanol was pumped into the vessel at the appropriate rate. This method minimized any sudden changes in the environmental conditions, which might stress the organism and trigger the onset of sporulation and subsequent lysis. Invariably this approach resulted in the establishment of stable cultures which were used to study the environmental control of metabolic fluxes, with particular emphasis placed upon defining optimum growth conditions under carbon-limitation.

Metabolic rates

The in situ rate of methanol consumption [expressed as mmol methanol $\cdot$ h^{-1} $\cdot$ (g dry wt. cells)$^{-1}$] was determined using the formula

$$q_{\text{methanol}} = \frac{fS - f'\bar{s}}{\bar{x} \cdot V} \tag{1}$$

where f is the medium flow, f' the output flow rate (i.e. medium plus titrant), S the input methanol concentration, $\bar{s}$ the residual methanol concentration, (usually immeasurably low with methanol-limited cultures), $\bar{x}$ the steady state bacterial equivalent dry weight concentration and V the culture volume.

Oxygen consumption rate and CO_2 production rate were determined from their concentrations measured in the effluent gas with a Servomax Model 570A paramagnetic oxygen analyser and a Model PA404 infra-red CO_2 analyzer (Servomax Ltd., Crowborough, Sussex). Metabolic rates [as mmol $\cdot$ h^{-1} (g dry wt. cells)$^{-1}$] were calculated

$$p_{O_2}\,(\text{or } CO_2) = \frac{\%\ \text{gas used/produced} \times \text{airflow}\,(h^{-1}) \times 41.3}{\bar{x} \cdot V} \tag{2}$$

where $\bar{x}$ is the steady-state bacterial dry wt. and V is the culture volume (ml). Dry air, at 20° C, was assumed to contain 20.9% oxygen and 1 mol of gas assumed to occupy 24.2 l at the monitoring temperature. The factor 41.3 arises from 1000 ÷ 24.2 mmol gas consumed or produced (g dry wt cells)$^{-1}$.

In addition the maximum potential rates of the cells to oxidize methanol, i.e. $Q^{\max}_{O_2\text{methanol}}$, were determined in vitro using a Clark-type oxygen electrode (Biological oxygen monitor, Model 53, Yellow Springs Instrument Co., Ohio, USA) according to Dijkhuizen and Harder (1975) and expressed as mmol $O_2 \cdot h^{-1} \cdot$ (g dry wt cells^{-1}). Aliquots (10 ml) of culture were withdrawn rapidly from the chemostat, centrifuged (5 min, 12,000 × g) and resuspended in 10 ml of air saturated culture medium minus methanol. After equilibration, methanol was added to a final concentration identical to S_R of the chemostat culture medium from which the cells were withdrawn.

Carbon balances were constructed from the rates of methanol consumption and CO_2 formation as follows

$$\text{Carbon balance}\,(\%) = \frac{[(41.7) \cdot D + q_{CO_2}] \cdot 100}{q_{\text{methanol}}} \tag{3}$$

In this equation, it is assumed that biomass contained 50%, w/w carbon (see Herbert 1976). The factor 41.7 arises from 1000 ÷ 24.0 mmol C $\cdot$ (g dry wt cells)$^{-1}$. Carbon conversion efficiencies (% input C into biomass) were determined from Eq. (3) above except that the q_{CO_2} factor was omitted from the equation.

Other methods

For the determination of residual methanol concentrations, samples (10 ml) were rapidly withdrawn from the chemostat, the cells were removed by filtration (0.2 µm filters) and the methanol concentration in the cell-free medium determined using the gas chromatograph according to Brooke and Attwood (1983). Formaldehyde was assayed with the method of Nash (1953). Culture dry weights were measured by heating duplicate 40 ml aliquots of washed cells at 105° C to constant weight.

Protein concentrations in whole cells were measured using a modified version of the Folin-Ciocalteu method as described by Lowry et al. (1951). Bovine serum albumin was used as the standard. All chemicals were of analytical reagent grade, obtainable from commercial sources.

Results

Previously we have reported that these isolates grow on methanol-agar plated and in liquid batch cultures over the temperature range 35–60° C (Dijkhuizen et al. 1988). However, in order to further assess the effect of temperature upon the growth kinetics of these isolates, carbon (methanol)-limited continuous cultures (D = 0.2 h^{-1}; S_R = 50 mM methanol; pH 7.5) were studied with respect to metabolic

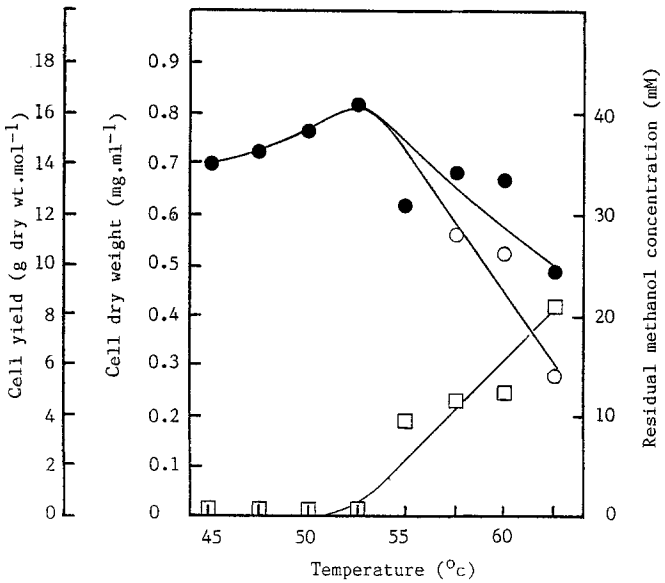

Fig. 1. Influence of cultivation temperature on the growth of *Bacillus* strain Ts1 in chemostat culture at a fixed dilution rate (0.2 h^{-1}), methanol concentration (50 mM) and pH value (7.5). *Symbols:* ○, cell dry weight; ●, cell yield; □, residual methanol concentration

Table 1. Influence of culture temperature on the kinetics of growth of *Bacillus* strain Ts1 in carbon-limited chemostat culture (D = 0.2 h^{-1}, S_R = 50 mM methanol, pH = 7.5)

Cultivation temperature (°C)	Rates: mmol · h^{-1} · (g dry wt · cells)$^{-1}$			
	$q_{methanol}$	q_{O_2}	q_{CO_2}	$Q^{max}_{O_2methanol}$
45	14.1	10.5	3.73	15.3
47.5	13.7	10.0	3.32	15.6
50	12.9	9.72	2.89	15.6
52.5	12.1	9.45	2.97	11.5
55	12.7	9.29	3.45	13.6
57.5	13.6	11.1	4.04	17.6
60	14.0	12.2	4.30	12.4
62.5	11.6	13.4	4.76	11.1

fluxes from methanol, over the temperature range 45–62.5° C. (Fig. 1, Table 1).

As the cultivation temperature was increased, the bacterial dry weight increased from 0.70 mg · ml^{-1} at 45° C to a maximum of 0.82 mg · ml^{-1} at 52.5° C, the optimum temperature (T.opt) with respect to biomass production. Thereafter the dry weight decreased markedly. Concomitant with the biomass increase an inverse pattern of CO_2 production (q_{CO_2}) was evident; that is, at temperatures above or below T.opt a larger proportion of the methanol carbon consumed by the cells ($q_{methanol}$) was oxidized to CO_2, and less was assimilated into biomass. These data are further supported by the carbon conversion efficiencies which vary between 59 and 69%, the highest value being at 52.5° C, (conditions under which 93.6% of the input carbon was recovered as biomass and CO_2, with a yield of 16.4 g · mol^{-1}). A comparison of the in situ rate of oxygen consumption (q_{O_2}) with the maximal ability of washed cells to oxidise methanol ($Q^{max}_{O_2methanol}$) measured in vitro, revealed that these two sets of data followed a closely similar trend [approximately 9.5–15.5 mmol O_2 · h^{-1} · (g cells)$^{-1}$], the $Q^{max}_{O_2methanol}$ invariably being 1.5-fold higher than the in situ q_{O_2} value. This result strongly suggests that, in response to temperature changes, these organisms are able, under carbon-limited growth conditions, to adjust their metabolism to suit the growth conditions. However, at temperatures of 55° C–60° C, residual methanol began to accumulate within the culture supernatant, indicating that the maximal rate of methanol oxidation (under these growth conditions) had been attained. Control experiments similar to those of Snedecor and Cooney (1974) have shown that methanol loss due to stripping within the temperature range is negligible. Ultimately the culture washed out at 62.5° C.

Recently it has been reported that pH changes imposed upon continuous cultures of the thermophile *Bacillus stearothermophilus* result in profound metabolic changes (Pennock and Tempest, 1988). Thus, having established T.opt to be 52.5° C for isolate Ts1, we investigated the effect at T.opt, of cultural pH variations upon the metabolic fluxes during growth on methanol. This was achieved by first establishing a steady-state culture at pH 7.5, then varying the pH of the culture either above or below this value until wash-out occurred. This experiment revealed a pH-range for growth at D = 0.2 h^{-1} of 6.5 to 8.5 with washout occurring at 6.0 and 9.0. The optimum value with respect to biomass production was 7.5, at which point a yield of 16.3 g · mol^{-1}, and a carbon to biomass conversion efficiency of 69% was attained. Just as was observed previously with changes in temperature, when the pH was varied the $q_{methanol}$, q_{O_2} and q_{CO_2} values followed a similar pattern of variation i.e. the most efficient use of methanol and oxygen [12.1 and 9.45 mmol · h^{-1} · (g cells)$^{-1}$, respectively] and lowest rate of CO_2 production [2.97 mmol · h^{-1} · (g cells)$^{-1}$] were attained at the opt pH value. Carbon recoveries (average 94.5% – data not shown) again suggested that, throughout the range of pH values able to support growth, all the input C was either assimilated into biomass, dissimilated to produce CO_2 and energy or, at sub-optimal pH values remained unmetabolised in the culture filtrate. Interestingly, when the residual methanol concentrations are taken into account, the cells displayed a very broad pH optimum 7.0–8.5 with respect to yield. Only at pH 6.5 did this drop to approximately 50% of the optimal value of 16.2–16.5 g mol^{-1}. In addition a marked increase in the metabolic rate, i.e. a 2-fold in $q_{methanol}$, q_{O_2} and a 4-fold increase in q_{CO_2}, was observed.

It is generally accepted that one of the main advantages of thermophilic bacteria over their mesophilic counterparts is their potentially high growth rates, coupled with high turnover rates of substrates and products (see Zeikus 1979; Sonnleiter 1983). However, the precise growth rate of thermophilic bacteria in batch cultures can be difficult to determine due to very short exponential growth phases (see Kuhn et al. 1979, 1980). In these cases accurate results should be obtained by chemostat studies; Therefore, using the T.opt of 52.5° C and pH opt of 7.5, we investigated the effect of increasing the dilution rate of a methanol-limited chemostat culture of Ts1 (Fig. 2, Table 2). Progressive increases from D = 0.05 h^{-1} to the maximum dilution rate of 0.8 h^{-1} resulted in a linear increase in methanol and oxygen consumption rates with a concomitant increase in the CO_2 production rate. The optimal dilution rate range with respect to yield is 0.3 to 0.7 h^{-1}, however, at D = 0.5 h^{-1} methanol begins to accumulate in the culture supernatant, and washout occurs at D = 0.9 h^{-1} (Dc). Previously we have determined the

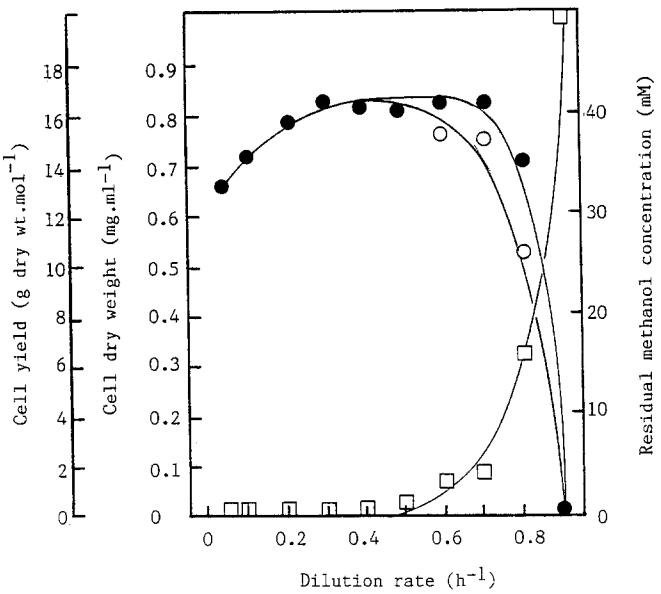

Fig. 2. Influence of dilution rate on the growth of *Bacillus* strain Ts1 in chemostat culture at a fixed temperature (52.5°C) pH (7.5) and methanol concentration (50 mM). *Symbols:* as in Fig. 1

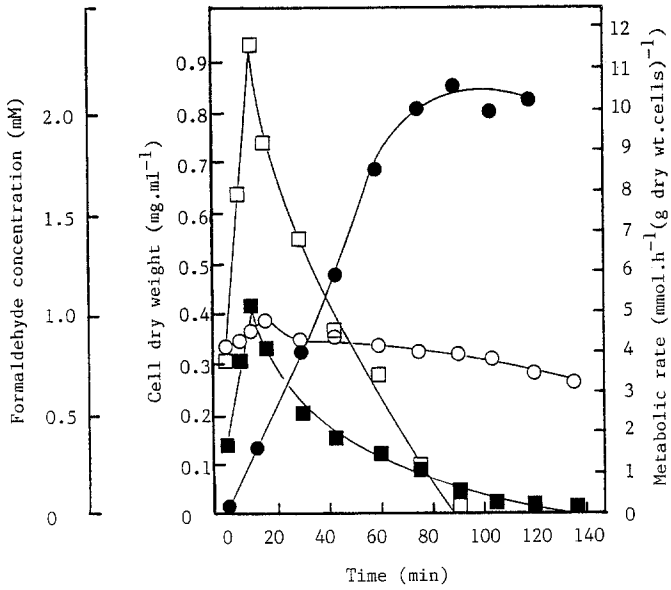

Fig. 3. Effect of pulse addition of methanol (25 mM) at time 0 on *Bacillus* strain Ts1 in chemostat culture under cultural conditions described in Fig. ■ with an initial substrate concentration of 25 mM methanol. *Symbols:* ○, cell dry weight; ● formaldehyde concentration in supernatant; q_{O_2}, □; ■, q_{CO_2}

Table 2. Influence of growth rate on the kinetics of growth of *Bacillus* strain Ts1 in carbon-limited chemostat culture (Temp = 52.5°C, pH = 7.5, S_R = 50 mM methanol

Dilution rate (h^{-1})	Rates: mmol · h^{-1} · (g dry wt · cells)$^{-1}$			
	$q_{methanol}$	q_{O_2}	q_{CO_2}	$q^{max}_{O_2 methanol}$
0.05	3.90	2.12	1.08	4.85
0.10	6.80	4.68	1.39	9.26
0.20	12.40	9.72	3.05	11.5
0.30	18.1	12.1	3.68	14.8
0.40	24.3	16.1	5.23	17.9
0.50	30.5	22.4	6.98	24.2
0.60	36.1	29.3	8.29	30.8
0.70	41.9	31.4	9.87	38.6
0.80	51.3	41.2	13.8	45.4

minimal doubling time (t_d) in batch cultures to be 45–50 min, (Dijkhuizen et al. 1988). The t_d determined from continuous cultures at μ_{max} under optimal cultural conditions is 46–52 min, which is in close agreement with the former figure. It has also been reported that a high maintenance energy requirement is a characteristic of thermophiles (Zeikus 1979; Ben-Basset and Zeikus 1981; Sonnleiter et al. 1982). If the methanol consumption rate ($q_{methanol}$) is plotted against D (data not shown), the resultant plot yields information regarding the maximum growth yield on methanol and the maintenance energy requirement (M). In the case of Ts1, linear regression analysis of the data contained in Table 2 gave a $Y^{max}_{methanol}$ of 17.0 g cells · mol methanol^{-1}, but more interestingly a relatively low maintenance energy requirement of 0.81 mmol methanol · h^{-1} · (g cells)$^{-1}$.

Methanol can be a toxic substrate for methylotrophic bacteria; indeed a mixed culture of 3 thermophilic methylotrophic bacilli was shown to be very sensitive to relatively low concentrations of methanol, the minimum inhibitory concentration was 25 mM methanol, and complete growth inhibition occurred with 37.5 mM methanol (Snedecor and Cooney 1974). Our isolates were able to grow in batch cultures with media containing up to 1.5 M methanol as sole carbon – and energy-source (Dijkuizen et al. 1988). With this in mind we investigated the metabolic response of Ts1 to increased methanol feed concentrations under optimal conditions for temperature and pH, at a dilution rate of 0.2 h^{-1}. Incremental increases in $S_{R_{methanol}}$ of 25 mM resulted in a linear increase in the culture biomass up to a level of 100 mM methanol, with a constant biomass yield of 16.0 ± 0.5 g cells · mol^{-1} across this range. Similarly, the in situ rates of methanol and O_2 consumption and CO_2 production remained virtually constant, whilst the optimal metabolic efficiency occurred when the S_R value was 75 mM methanol. Above 100 mM methanol, the cellular yield began to decrease and a larger proportion of the metabolised substrate appeared as CO_2. This suggested that as the steady-state substrate concentration increased, growth became energetically more expensive, a view which supported data from batch cultures in which t_d increased and $Y_{methanol}$ gradually decreased as the methanol concentration was increased (Brooke, A.G., Attwood, M. M., and Watling, E. M., personal communication). Growth at methanol concentrations above 125 mM was not performed, since oxygen-transfer rates within the culture became growth-limiting.

Carbon-limited chemostat cultures of microorganisms have been shown to be able to instantaneously accelerate their rate of carbon consumption when pulsed with an excess of carbon (Teixeira de Mattos et al. 1984; Crabbendam et al. 1985; Pennock and Tempest 1988), and in some cases this led to a transient-phase of growth in which extensive dissociation of catabolism from anabolism occurred (Pennock and Tempest 1988). The metabolic response of Ts1 growing in chemostat culture (T = 52.5°C, pH 7.5, D = 0.2 h^{-1}, $S_{R_{methanol}}$ 25 mM) to a 25 mM methanol pulse is shown in Fig. 3. Immediately following the pulse, the q_{O_2} and q_{CO_2} increased dramatically (2.5-fold) and attained maximum values after 10 min of 11.57 and 5.21 mmol · h^{-1}

(g cells)$^{-1}$ respectively. Over the same time period the culture biomass increased only 1.12-fold and more importantly, formaldehyde began to accumulate within the culture supernatant. After 90 min the formaldehyde concentration in the medium attained the maximum value of 2.2 mM. From these data it is clear that, at least transiently, catabolism can be dissociated from anabolism. However, the toxic effects exerted by elevated levels of formaldehyde within the culture-filtrate resulted in cell death.

The data described in detail above was obtained with Ts1 but the effect of temperature, pH, growth rate and substrate concentration was also investigated with two other isolates, Ts2 and Ts4, and similar metabolic responses were seen.

Discussion

Based upon the definitions used to categorise thermophilic bacteria (see Zeikus, 1979; Sonnleiter, 1983; Sundaram, 1986), the T.opt together with the temperature range of our isolates suggests that they do not fall within the true thermophile category. However, these isolates represent the first pure cultures of true thermotolerant methylotrophs studied, and as such open up a new area of microbial metabolism/potential hitherto uninvestigated. At present we may only speculate as to the factors which determine the limits of the temperature range for growth, however, conformational changes in one or more essential enzymes leading to loss of catalytic activity, or modification of an important regulatory characteristic could prevent growth below T.min (Ljungdahl 1979) whereas membrane instability, protein instability and possible loss of ribosome function (Sundaram 1986) together with methanol volatility may determine T_{max}. These are questions that will be addressed in future work.

With respect to pH, all thermophilic bacteria so far described fall into two major categories (i) thermoacidophiles with pH optima in the range 1.5–4.0 and (ii) thermophilic neutrophiles with pH optima in the range 5.8–8.0 (Sonnleitner 1983; Sundaram 1986). However, it has been noted that a disregard for the dependence of pH on temperature may lead to considerable errors between medium pH when prepared, and medium pH at the growth temperature (Sonnleitner 1983). The control systems for pH used in our studies automatically compensated for these discrepancies and revealed a broad pH optimum with respect to yield of 7.0 to 8.5 thereby placing our organism in the thermotolerant neutrophile class. Only at the pH 6.5 did the yield drop with an accompanying increased metabolic rate; this suggested that at the lower extreme of the pH range, the maintenance of cytoplasmic pH was energetically more expensive than at the optimal pH (see Pennock and Tempest 1988). Why a similar response did not occur at high pH extremes remains to be elucidated.

Sundaram (1986) has highlighted not only the problem of accurate and reproducible growth rate determinations in batch cultures of a variety of thermophilic bacteria, which is due possibly to reduced oxygen supply at high temperatures with increasing cell densities, but also the relatively little data obtained from chemostat studies. In our isolates t_d determined from μ_{max} in continuous cultures corresponds exactly to t_d determined from batch cultures with low methanol concentrations (i.e. conditions ensuring oxygen sufficiency) (Dijkhuizen et al. 1988).

The molar growth yield on methanol (after taking into account the maintenance energy requirement) of 17.0 g cells^{-1} (mol methanol)$^{-1}$ measured under optimal growth conditions is relatively high when compared with figures reported for other methylotrophic bacteria (Anthony, 1982). Furthermore, at growth rates close to μ_{max}, this yield drops by only 7%, thereby affording a very desirable culture system (from a biotechnological view point) in which maximum growth rates and almost maximal growth yields are attainable simultaneously. It has been suggested that a high maintenance energy requirement is a characteristic property of (extreme) thermophiles (Zeikus, 1979; Ben-Basset and Zeikus 1981). For example $M_{glucose}$ and M_2 values for *Bacillus caldotenax* are reported to be 10-fold higher than the values obtained for mesophiles (Pirt 1965; Mainzer and Hempfling 1976; Neijssel and Tempest 1976). Conversely the values for *Termus thermophilus*, an extreme thermophile, were even lower than the values for mesophiles (McKay et al 1982). The maintenance energy value for Bacillus Ts1 is low when compared with mesophilic methanol utilizers (see Anthony 1982). However, since the uncoupling of growth from energy production is one factor that increases the value of maintenance energy requirement, and the data suggest that our isolates precisely adjust their catabolic and anabolic processes to suit prevailing carbon-limited growth conditions, this may contribute to a reduction in the maintenance energy requirement and account for the relatively low value measured.

Some thermophiles have been found to decelerate and even stop growth in the presence of relatively high substrate concentrations resulting in extremely low yield coefficients. It has been suggested that this may result from medium pH fluctuations, depletion of unknown growth factor requirements or simply from an inherent survival property of non-sporulating thermophiles (Sonnleiter 1983). Our sporulating strains were able, over a range of conditions, to completely metabolise all the supplied substrate in chemostat cultures with relatively little drop in yield, and only when oxygen becomes a limiting factor, or under extreme growth conditions, did incomplete substrate oxidation occur. Invariably under carbon-limited growth conditions heterotrophic aerobes metabolize the substrate solely to biomass and CO_2. Such a situation can be seen in our isolates when generally more than 90% of the input carbon source was recovered as biomass or CO_2. It has been reported in the literature, that excretion of metabolites by such cultures may occur if the carbon-limitation is transiently relieved. This is due to the fact that organisms in chemostat culture modulate the synthesis of their anabolic machinery to match the imposed overall rate of the cell synthesis (Kjeldgaard and Kurland 1963), whereas synthesis of their catabolic enzymes is not similarly adjusted (O'Brien et al. 1980). We have shown that with isolate Ts1, at least after a pulse of methanol and for a transient period of time, anabolism can be dissociated from catabolism. This resulted in the excretion of the intermediary metabolite formaldehyde. Unfortunately, such is the toxicity of this compounds to cells (Walker 1964; Attwood and Quayle 1984) that the accumulation of formaldehyde caused this culture to die. This raises the question as to how these novel strains respond to steady-state cultural conditions in which the rate of anabolism is severely constrained by the availability of anabolic substrate, whilst the availability of catabolic substrate remains in excess. Such studies are currently in progress.

Acknowledegements. AGB and EMW were financially supported by the SERC Biotechnology Directorate.

References

Akiba T, Ueyamas H, Yamauchi Y, Seki M, Fukimbara T (1970) Utilization potency of lower alcohols by soil bacteria. J Ferm Technol. 48:323–328

Anthony C (1982) The biochemistry of methylotrophs. Academic Press, London

Attwood MM, Quayle JR (1984) Formaldehyde as a central intermediary metabolite of methylotrophic metabolism. In: Crawford RL, Hanson RS (eds) Proceedings 4th Int. Symp. Microbial. Growth on C_1 compounds. Minneapolis. Am Soc Microbiol Washington, DC. pp 315–323

Ben-Basset A, Zeikus JG (1981) *Thermobacteroides acetoethylicus* gen. nov. and spec. nov., a new chemoorganotrophic anaerobic, thermophilic bacterium. Arch Microbiol 128:365–370

Brooke AG, Attwood MM (1983) Regulation of enzyme synthesis during the growth of *Hyphomicrobium* X on mixtures of methylamine and ethanol. J Gen Microbiol 129:2399–2404

Brooke AG, Watling EM, Attwood MM, Tempest DW (1987) Regulation of methanol metabolism in thermotolerant *Bacilli.* In: Abstracts 3rd Int. Symp Microb Physiol for Biotechnological Innovation. London

Clement W, Oosterwijk RJ van, Veldkamp J, Dijkhuizen L (1986) Isolation and characterisation of methanol-utilizing thermotolerant bacteria. In Verseveld HW van, Duine JA (eds) Abstracts 5th Int. Symp. Microbial. Growth on C_1 compounds. Free University Press, Amsterdam Haren, The Netherlands

Colby J, Zatman LJ (1975) Enzymological aspects of the pathways for trimethylamine oxidation and C_1 assimilation in obligate methylotrophs and restricted facultative methylotrophs. Biochem J 148:513–520

Crabbendam PM, Neijssel OM, Tempest DW (1985) Metabolic and energetic aspects of the growth of *Clostridium butyricum* on glucose in chemostat culture. Arch Microbiol 142:375–382

Crawford RL, Hanson RS (eds) (1984) Proceedings of the 4th International Symposium. Microbial growth on C_1 compounds. Minneapolis, Am Soc Microbiol Washington DC

Dijkhuizen L, Harder W (1975) Substrate inhibition in *Pseudomonas oxalaticus* OX1: a kinetic study of growth inhibition by oxalate and formate using extended cultures. Antonie van Leeuwenhock 7. Microbiol Serol 41:135–146

Dijkhuizen L, Arfman N, Attwood MM, Brooke AG, Harder W, Watling EM (1988) Isolation and initial characterisation of thermotolerant methylotrophic *Bacillus* strains. FEMS Microbiol Lett 52:209–214

Hazeu W, Bruyn JC de, Dijken JP van (1983) *Norcardia sp. 239* a facultative methanol utilizer with the ribulose monophosphate pathway of formaldeyde fixation. Arch Microbiol 135:205–210

Herbert D (1976) Stoichiometric aspects of microbial growth. In: Dean ACR, Ellwood DC, Evans CGT, Melling J (eds) Continuous culture 6 Ellis Horwood Ltd., Chichester, pp 1–30

Hitzman DO (1976) United States Patent No. 3.981.774

Kjeldgaard NO, Kurland CG (1963) The distribution of soluble and ribosomal RNA as a function of growth rate. J Mol Biol 6:341–348

Kuhn HJ, Cometta S, Fiechter A (1980) Effects of growth temperature on maximal specific growth rate, yield, maintenance and death rate in glucose-limited continuous culture of the thermophilic *Bacillus caldotenax*. Eur J Appl Microbiol Biotechn 10:303–315

Kuhn HJ, Friederich U, Fiechter A (1979) Defined minimal medium for a thermophilic *Bacillus* sp developed by a chemostat pulse and shift technique. Eur J Appl Microbiol Biotechnol 6:341–349

Ljungdahl LG (1979) Physiology of thermophilic bacteria. Adv Microb Physiol 19:150–331

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Mainzer SE, Hempfling WP (1976) Effects of growth temperature on yield and maintenance during glucose-limited continuous culture of *Escherichia coli.* J Bacteriol 126:251–256

McKay A, Quilter J, Jones CW (1982) Energy conservation in the extreme thermophile *Thermus thermophilus* HB8. Arch Microbiol 131:43–50

Nash T (1953) The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. Biochem J 55:416–421

Neijssel OM, Tempest DW (1976) Bioenergetic aspects of aerobic growth of *Klebsiella aerogenes* NCTC 418 in carbon-limited and carbon-sufficient chemostat culture. Arch Microbiol 107:215–221

O'Brien RW, Neijssel OM, Tempest DW (1980) Glucose phosphoenolpyruvate phosphotransferase activity and glucose uptake rate of *Klebsiella aerogenes* growing in chemostat culture J Gen Microbiol 116:305–314

Pennock J, Tempest DW (1988) Metabolic and energetic aspects of the growth of *Bacillus stearothermophilus* in glucose-limited and glucose-sufficient chemostat culture. Arch Microbiol (in press)

Pirt SJ (1965) The maintenance energy of bacteria in growing cultures. Proc R Soc London Ser B. 163:224–231

Snedecor B, Cooney CL (1974) Thermophilic mixed culture of bacteria using methanol for growth. Appl Microbiol 27:1112–1117

Sonnleiter B (1983) Biotechnology of thermophilic bacteria – Growth, products, and application. Adv Biochem Bioeng 28: 69–138

Sonnleiter B, Cometta S, Fiechter A (1982) Growth kinetics of *Thermus thermophilus.* Eur J Appl Microbiol Biotechnol 15:75–82

Sundaram TK (1986) Physiology and growth of thermophilic bacteria. In: Brock TD (ed) Thermophiles, general molecular and applied microbiology. John Wiley & Sons, New York, USA

Teixeira de Mattos MJ, Streekstra H, Tempest DW (1984) Metabolic uncoupling of substrate level phosphorylation in anaerobic glucose-limited chemostat cultures of *Klebsiella aerogenes* NCTC 418. Arch Microbiol 139:260–264

Van Verseveld HW, Duine JA (eds) (1987) Proceedings of the 5th International Symposium, Microbial growth on C_1 compounds. Free University Press, Amsterdam Haren, The Netherlands

Vishniac W, Santer M (1957) The Thiobacilli Bact Rev 21:95–213

Walker JF (1964) Formaldehyde, 3rd ed. Reinhold Publishing Corp. New York

Zeikus JG (1979) Thermophilic bacteria; ecology, physiology and technology. Enzyme Microb Technol 1:243–252

Received July 25, 1988/Accepted November 9, 1988