Isolation of Thermophilic Mutants of *Bacillus subtilis* and *Bacillus pumilus* and Transformation of the Thermophilic Trait to Mesophilic Strains

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Thermophilic mutants were isolated from mesophilic *Bacillus subtilis* and *Bacillus pumilus* by plating large numbers of cells and incubating them for several days at a temperature about 10 °C above the upper growth temperature limit for the parent mesophiles. Under these conditions we found thermophilic mutant strains that were able to grow at temperatures between 50 °C and 70 °C at a frequency of less than 10⁻¹⁰. The persistence of auxotrophic and antibiotic resistance markers in the thermophilic mutants confirmed their mesophilic origin. Transformation of genetic markers between thermophilic mutants and mesophilic parents was demonstrated at frequencies of 10⁻³ to 10⁻² for single markers and about 10⁻⁷ for two unlinked markers. With the same procedure we were able to transfer the thermophilic trait from the mutant strains of *Bacillus* to the mesophilic parental strains at a frequency of about 10⁻⁷, suggesting that the thermophilic trait is a phenotypic consequence of mutations in two unlinked genes.

INTRODUCTION

Every organism has its own optimal temperature for growth, being either a mesophile, a thermophile or a psychrophile. The factors that dictate the optimum growth temperature have not been established. Identification of genes controlling growth temperatures of these different classes of organisms by genetic means, e.g. transformation, should provide information that will improve our understanding of the mechanisms that determine the range of temperatures over which different organisms can grow. McDonald & Matney (1963) claimed the transformation of a characteristic which permitted a strain of *Bacillus subtilis* to grow at temperatures as high as 55 °C to other *B. subtilis* strains whose upper temperature limit for growth was 50 °C. Furthermore, Friedman & Mojica (1978) and Lindsay & Creaser (1975) both claimed transformation of the thermophilic trait from thermophilic '*Bacillus caldolyticus*' or *Bacillus stearothermophilus* to mesophilic *B. subtilis*. These observations have not been substantiated. The major difficulty in characterizing the thermophilic trait has been a lack of genetic evidence, because the organisms used for these studies were not genetically characterized.

In this communication we report the isolation of thermophilic mutants from two genetically marked mesophilic species (*B. subtilis* and *Bacillus pumilus*) and transformation of the thermophilic trait to mesophilic strains. Differences in their gene expression and behaviour over a range of temperatures are discussed.

METHODS

Bacteria. The *B. subtilis* strains used were 168 DB-1 from Dr Roy Doi, University of Texas, Arlington, Tx., USA; 1A248 *trpC2 rpoB500*, resistant to 50 µg rifampicin ml⁻¹, and 1E4(pLS13), resistant to 50 µg lincomycin ml⁻¹, from the Bacillus Genetic Stock Center, Ohio State University, USA; and 168 BR151 *lys-3 trpC2*, requiring lysine and tryptophan. The *B. pumilus* strains used were Bp203 Arg⁻, requiring arginine, and wild-type BpB1 from Dr M. Bramucci, Hahnemann University. In addition, Dr F. C. Welder, Pennsylvania State University, College Park, Pa., USA, supplied us with the thermophile '*B. caldolyticus*' YT-P (Heinen & Heinen, 1972).

Media. A hard TBAB agar (3%, w/v) comprising tryptose blood agar base medium (Difco) plus additional 1.5% (w/v) granular agar (Difco) was used throughout for plating at elevated temperatures. L broth, consisting of 5 g yeast extract (Difco), 10 g tryptone (Difco) and 5 g NaCl per litre, supplemented with 0.02 M-sucrose, was used for liquid culture throughout this work. The minimal medium contained 7 g K_2HPO_4 , 2 g KH_2PO_4 , 1 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4 \cdot 7H_2O$ and 0.45 g trisodium citrate dihydrate per litre of distilled water. After autoclaving, 0.02 M-sucrose or 0.1% glucose was added. For minimal agar plates 3% (w/v) granular agar (Difco) was added to the above minimal medium.

Isolation of thermophilic mutants. Either *B. subtilis* or *B. pumilus* was grown in approximately 100 ml L broth to late exponential phase (more than 5×10^8 cells ml^{-1}). Bacteria were harvested by centrifugation at 7000 g, washed with 50 ml fresh L broth, centrifuged again and resuspended in 10 ml L broth. A small volume (0.1 to 0.3 ml) of the concentrated bacterial suspension was spread on TBAB plates. Because of the low frequency at which thermophilic mutants arose it was essential that the total number of bacteria exceeded 10^9 cells per plate and routinely 10–20 plates were inoculated at a time. The plates were incubated in a closed jar, to prevent excessive evaporation, for 10 or more days at 68 °C.

DNA extraction and preparation of competent recipients for transformation. DNA was extracted by the alkaline procedure of Saito & Miura (1963). Bacteria were grown in 100 ml L broth with shaking, centrifuged and resuspended in 10 ml 0.15 M-NaCl/0.1 M-EDTA, pH 8. Lysozyme (6 mg; Sigma) was added and the cells were incubated at 37 °C for 30 min. The lysozyme-treated cells were mixed with 10 ml Tris/SDS (0.1 M-Tris/HCl, 1% SDS, 0.1 M-NaCl, pH 9), quickly frozen in liquid nitrogen and then thawed in a 60 °C water bath. The freeze-thaw step was repeated and the lysed cells added to an equal volume of distilled phenol saturated with Tris/SDS. The mixture was slowly stirred at 4 °C for 30 min, centrifuged at 2000 g for 30 min and the aqueous phase collected. DNA was precipitated with 2 vols ethanol and isolated by spooling on a glass rod. DNA was resuspended in a small volume (about 0.5 ml) of $10 \times$ sodium saline citrate (SSC) ($1 \times$ SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7.0), then 2 to 4 ml $1 \times$ SSC was added and the DNA was stored at 4 °C over a drop of chloroform.

For transformation we used the following procedure, which is adapted from the method of Bott & Wilson (1967). Bacteria from a single colony were picked off TBAB plates, grown overnight with shaking in L broth, diluted one to 100 in fresh L broth and grown for about 3 h to reach late exponential phase. The cultures were washed twice with, and resuspended in, fresh L broth. With *B. pumilus* species competence was dependent on the addition of $CaCl_2$ to a final concentration of 0.03 M (cf. Mandel & Higa, 1970). For transformation, bacteria were incubated for 1 h at 37 °C with a high concentration of DNA ($\geq 10 \mu g\ ml^{-1}$) and 0.2 ml samples were plated on TBAB or minimal agar. When selection was made for thermophily, the plates were incubated at 60 °C or 68 °C in a closed jar for 48 h; prototrophic transformants were selected on minimal agar plates that were incubated at 37 °C for 48 h. As a control, DNAase (1 mg ml^{-1}) was added to a DNA sample before it was incubated with the competent recipient cells.

RESULTS

Growth temperature ranges for mesophilic B. subtilis

Many micro-organisms can grow over a wide range of temperatures. It has been reported that *B. subtilis* can grow at temperatures between 5 °C and 55 °C (Gibson & Gordon, 1974). However, the upper temperature limit for growth of *B. subtilis* mesophiles was found to be dependent on the kind of medium used. On minimal agar the highest temperature permitting growth of *B. subtilis* was 50 °C. However, addition of 0.1% Casamino acids to the minimal agar allowed slow growth at a temperature of 56 °C. TBAB supported growth up to an even higher temperature (60 °C). When *B. subtilis* was cultured in L broth with shaking, growth was supported up to 56 °C. Thus it appears that the products of some nutritional biosynthetic genes are temperature-sensitive, although they have yet to be identified.

Isolation and characterization of thermophilic mutants of B. subtilis

From the results described above, a temperature of 68 °C, which is about 10 °C above the upper limit for growth of *B. subtilis* on a rich agar medium, was chosen for selection of thermophilic mutants. Using TBAB plates we first isolated thermophilic mutants from *B. subtilis* 168 strains DB-1 and BR151 at a frequency of less than 10^{-10} (Table 1). All of the thermophilic *B. subtilis* mutants that were isolated were designated T/r and grew at temperatures between 50 °C and 70 °C, but were no longer able to grow at temperatures below 50 °C. However, the thermophilic mutants of *B. subtilis* BR151 retained their requirements for lysine and tryptophan at 50 °C (Table 1). These amino acid requirements could not be demonstrated above 55 °C because the organisms grow only on rich nutrient media under these conditions.

Nevertheless, the demonstration that the auxotrophic requirements of the parent strain were retained unequivocally confirmed that these thermophilic mutants were derivatives of *B. subtilis* strains.

Transformation of the thermophilic trait (T/r) from the mutant thermophilic B. subtilis to mesophilic B. subtilis strains

Prototrophic markers (*lys*⁺ or *trp*⁺) were transferred from thermophilic *B. subtilis* DB-1 T/r to the mesophilic recipient *B. subtilis* BR151 by transformation at a frequency of 10^{-3} to 10^{-2} (Table 2), which is similar to the frequency observed for the transfer of prototrophic markers between two mesophilic strains (cross 2, Table 2). This efficient transformation also supports the idea that these *B. subtilis* thermophilic mutants are derived from mesophilic *B. subtilis* strains.

We were also able to transfer the thermophilic trait from *B. subtilis* thermophiles to *B. subtilis* mesophiles. In a cross between thermophilic strain DB-1 T/r and mesophilic BR151, transfer of both the thermophilic trait and prototrophic markers was scored (cross 1, Table 2). The transformation frequency of the thermophilic trait was about 10^{-7} while the transformation frequency of prototrophic markers was 10^{-3} to 10^{-2} for a single marker and about 10^{-7} for the cotransfer of two unlinked prototrophic markers (*lys*⁺ and *trp*⁺) (see Table 2). Similar data were obtained with five *B. subtilis* T/r mutant isolates. Since the transformation frequency of the thermophilic trait is similar to that found for transformation of two unlinked genes, it can be suggested that mutations in two unlinked genes are sufficient to cause thermophily in *B. subtilis*.

When we attempted to transform *B. subtilis* BR151 with DNA isolated from an existing thermophilic species, '*B. caldolyticus*' YT-P, however, the frequency of transformation between these two strains was about 400- to 1000-fold lower than the frequency observed between the *B. subtilis* thermophilic mutants and their parental strains. The low frequency of transformation between '*B. caldolyticus*' and *B. subtilis* may be explained by the differences in their base compositions: the mol % GC of '*B. caldolyticus*' is in the region 62 to 65 (Sharp, 1982) while that of *B. subtilis* is 42 to 48 (Normore, 1976).

Viability and stability of thermophilic mutants

All the thermophilic mutants and transformants that were isolated were found to be unstable. The thermophilic *B. subtilis* strains grew within a rather confined temperature range of 50 °C to 70 °C. The mutants were unable to survive above 71 °C or below 49 °C. When thermophilic strains were incubated at 47 °C the c.f.u. remained about the same for approximately 2 h, but dropped more than 1000-fold during the next 6 h. Incubation at 72 °C caused a decrease in c.f.u. by 10-fold after 1 h and about 10^5 -fold after 6 h. However, the thermophilic *B. subtilis* strains remained viable during storage at -50 °C for at least 1 year. The basis of the instability of the thermophilic mutants remains to be studied.

Expression of antibiotic resistance markers in thermophilic B. subtilis transformants

Thermophilic transformants of mesophilic *B. subtilis* carrying antibiotic resistance markers were tested for antibiotic sensitivity at the upper and lower thermophilic temperature range. Rifampicin resistance was expressed in T/r transformants of *B. subtilis* 1A248 at both 50 °C and 68 °C, showing that the mesophilic parental gene is present and expressed when bacteria are incubated over the entire thermophilic temperature range. The thermophilic (T/r) transformants of *B. subtilis* 1E4 containing the Lm^r plasmid (pLS13), however, expressed lincomycin resistance at the lower temperature range (50 °C–55 °C) but not at the higher temperature range (65 °C–68 °C). Clones isolated from T/r transformants grown at 65 °C were no longer able to express lincomycin resistance at 50 °C. This observation suggests that the elevated temperature results in the curing of the Lm^r plasmid (pLS13) from 1E4 transformants.

Thermophilic mutants of B. pumilus

Studies on the production of thermophilic mutants and transformation of the thermophilic trait were extended to another *Bacillus* species, namely *B. pumilus*. Unlike *B. subtilis*, nutritional enrichment does not expand the upper temperature range for growth of *B. pumilus* above 50 °C.

Table 1. Isolation of thermophilic mutants from mesophilic *Bacillus* strains

Mesophilic parental strain	Total no. of T/r mutants isolated	Characteristics of T/r mutants		
		Auxotrophic markers expressed	Frequency of occurrence	Temp. range for growth (°C)
<i>B. subtilis</i> DB-1	9	NA	0.3×10^{-10}	50-70
<i>B. subtilis</i> BR151	5	Lys ⁻ Trp ⁻ at 50 °C	0.6×10^{-10}	50-70
<i>B. pumilus</i> BpB1	4	NA	0.3×10^{-10}	50-70

NA, Not applicable (no auxotrophic markers carried by these strains).

Table 2. Transformation of the thermophilic trait from mutant thermophilic *Bacillus* strains to mesophilic strains

Cross	Donor	Recipient	Transformation frequency				
			Arg ⁺	Trp ⁺	Lys ⁺	Lys ⁺ Trp ⁺ *	T/r
1 (Thermophile × mesophile)	<i>B. subtilis</i> DB-1 T/r	<i>B. subtilis</i> BR151 <i>lys-3 trpC2</i>	—	5×10^{-3}	9×10^{-3}	4×10^{-7}	2×10^{-7}
2 (Mesophile × mesophile)	<i>B. subtilis</i> DB-1	<i>B. subtilis</i> BR151 <i>lys-3 trpC2</i>	—	7×10^{-3}	6×10^{-3}	2×10^{-7}	NA
3 (Thermophile × mesophile)	<i>B. pumilus</i> BpB1 T/r	<i>B. pumilus</i> Bp203 <i>Arg⁻</i>	3×10^{-3}	—	—	—	8×10^{-8}
4 (Mesophile × mesophile)	<i>B. pumilus</i> BpB1	<i>B. pumilus</i> Bp203 <i>Arg⁻</i>	7×10^{-3}	—	—	—	NA
5 (Thermophile × mesophile)†	' <i>B. caldolyticus</i> '	<i>B. subtilis</i> BR151 <i>lys-3 trpC2</i>	—	2×10^{-5}	1×10^{-5}	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$

NA, Not applicable (transformation of the thermophilic trait is not possible).

* Transformation of two unlinked genes.

† Cross between two different species.

We isolated thermophilic mutants of *B. pumilus* at a frequency of less than 10^{-10} (Table 1). These thermophilic mutants grew at temperatures above 50 °C and required rich medium at the higher thermophilic temperature range (55 °C–70 °C). Transformation of the thermophilic trait to mesophilic *B. pumilus*, made competent by incubation in the presence of CaCl_2 , occurred at a frequency of about 10^{-7} , while transfer of a single prototrophic marker between these mesophilic and thermophilic *B. pumilus* strains occurred at a frequency of about 10^{-3} to 10^{-2} (Table 2). These data are similar to those obtained with *B. subtilis* and support the hypothesis that mutations in two genes are involved in the acquisition of thermophily.

DISCUSSION

The upper temperature range for growth of both mesophiles and thermophiles of *B. subtilis* and thermophiles of *B. pumilus* was increased by growing on rich media; this is consistent with the findings of Baker *et al.* (1953). Thus it appears that the products of some nutritional genes are temperature-sensitive. It has not, therefore, been possible to demonstrate the presence of auxotrophic markers in strains growing at the upper part of their thermophilic temperature range. Moreover, Gray & Jackson (1973) reported that with mesophilic mutants obtained from the psychrophile, *Micrococcus cryophilus*, the phenotype associated with some of the parental markers cannot be demonstrated at mesophilic temperatures because of nutrient requirements.

The genetics of thermophilic growth is a controversial subject. Johnson (1979) suggested that difficulties of studies of thermophiles is mainly due to the lack of established methods for isolating thermophilic mutants and the unavailability of previously isolated thermophilic mutants for genetic studies on the thermophilic trait. Confirmation of some of the previous reports, for example transformation of the thermophilic trait to mesophiles (Friedman & Mojica, 1978; Lindsay & Creaser, 1975), has been hampered by the unavailability of thermophilic derivative strains. The present paper explains that such thermophilic strains may be easily lost due to their instability. However, we found that viability was maintained for a long period of time during storage of the thermophilic mutants at –50 °C. With this knowledge we have now been able to establish a method that permits genetically marked strains of both *B. subtilis* and *B. pumilus* to mutate to form thermophilic strains which grow at temperatures from 50 °C to 70 °C. Our hypothesis that mutations in two genes are responsible for the thermophilic nature of our mutants is consistent with the low frequencies of mutation to thermophilic growth and the observed frequency of transformation of the thermophilic trait to mesophiles.

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