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INVESTIGATION OF THE MOLECULAR MECHANISM
OF THERMAL TOLERANCE IN BACILLUS SUBTILIS

Final Report

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ABSTRACT

We have studied Bacillus subtilis, Bacillus pumilus and Bacillus caldolyticus to ascertain the molecular mechanism of the ability to grow at high temperatures. B. subtilis wild type strains grow in minimal salts medium at a mesophilic temperature (37°C) but not at a thermophilic temperature (56°C). However, they do grow at 56°C in complex rich media. Analysis of their nutritional requirements revealed that these bacteria require pyridoxine or aspartic acid and threonine to grow at 56°C indicating that they are temperature sensitive mutants. Furthermore, mutants of B. subtilis which are able to grow on minimal salts media at 56°C can be readily isolated. Therefore, it appears that the lack of growth of the wild type strain in minimal salts media at 56°C is due to the instability of an anabolic enzyme.

In contrast to B. subtilis, B. pumilus has never been observed to grow above 50°C. However, our studies show that mutants of B. pumilus and B. subtilis which grow at 68°C can be easily isolated. The isolation of such mutants strongly supports the idea that one gene can determine the ability to grow at extreme temperatures.

INTRODUCTION

We have sought to determine if reports of transformation of a "thermophilic" gene in bacilli can be confirmed. McDonald and Matney (3) found that a gene linked to streptomycin resistance determined the ability of certain strains of Bacillus subtilis to grow at 55°C. Subsequently, Lindsay and Creaser (2) reported that transformation of a gene(s) linked to streptomycin enabled B. subtilis to grow at 70°C. A similar finding was reported by Friedman and Mojica (1), however, these transformants were found to be unstable. Our findings pertaining to these experiments are presented in the present report.

METHODS

To confirm the experiments conducted by Lindsay and Creaser (2) and Friedman and Mojica (1), requests for the organisms used in their experiments were made in July, 1980. Unfortunately, we have not heard from them yet. In order to begin the research project without further delay, the experiments were conducted with B. subtilis DB-1 (168 wt) (kindly supplied by Dr. Roy Doi), B. subtilis 168 (kindly supplied by Dr. W. C. McDonald) and B. subtilis Br 151, tryC2, lys-3 and B. pumilus arg (from Dr. M. Bramucci). B. caldolyticus YTP was obtained from Dr. Welder of Penn State University (the initial request to Dr. Johnson of Tulane University, the keeper of the organism originally used by MacElroy, has not been received as of this date). From this B. caldolyticus YTP we isolated a strain requiring quanine.

The transformation experiments were carried out by the method of Spizizen (4) as described by Lindsay and Creaser (2) using B. caldolyticus YTP qua as the donor and B. subtilis DB-1, 168 and Br 151, tryC2, lys-3 as recipients. Transformation of the lysine or tryptophan markers to the recipient, Br 151, tryC2, lys-3, at 37°C demonstrated the transforming ability of the donor DNA and the competence of the recipient cells. DNA isolated from the donor strain by various phenol-extraction methods were tried and compared. Various concentrations of DNA (2.5ug/ml and greater) were incubated with

the recipient cells for 2-4 hours at 37°C after which DNase was added. After exposure to DNA, cells were grown on nutrient agar or blood agar base for 4 hours at 37°C, 47°C or 56°C. Selection for growth at the high temperature was made by transferring cells to 68°C and incubating for several days. Numerous attempts were made to isolate stable transformants by varying the incubation temperature of the DNA exposed cells.

B. subtilis and B. pumilus mutants were isolated by treating fresh cultures with 30 ug/ml of N-methyl-N-nitro-N-nitrosoquanidine for 30 minutes at 37°C in PBS, washing, plating on nutrient agar, incubating at temperatures of 56°C for 4 hours then incubating at 68°C for 2 to 5 days. Spontaneous mutants were isolated in the same manner but without treatment.

RESULTS

Effect of nutritional supplements on growth temperature. Since B. subtilis, strains 168 and DB-1 (168 wt), can grow in complex media but not in minimal medium at 56°C we presumed that there was a nutritional basis for this discrepancy. We found that aspartate, threonine, or pyridoxine enabled these strains to grow at 56°C in minimal medium (Table 1). In addition, mutants that have been isolated which are able to grow in unsupplemented minimal medium at 56°C. From these data, we conclude that an enzyme responsible for biosynthesis of amino acids by the "aspartate" pathway is unstable at 56°C. Consequently, growth at 56°C does not require a "thermophilic" factor.

Discrepancy in growth in liquid and solid media at elevated temperatures. Unexpectedly, we found that B. subtilis can grow at 60°C on nutrient agar but is unable to grow in nutrient broth or other liquid media (Table 2). Addition of 1M sucrose, 0.1% nitrate, or bubbling air through the broth had no effect. This result suggested that B. subtilis is unable to grow at high temperatures because it lacks the ability to synthesize cell wall materia.

Thus, although we have as yet no explanation for this discrepancy, it has clarified our understanding of the maximum growth temperature of B. subtilis.

Isolation of thermostable mutants of *B. pumilus* and *B. subtilis*. Many spontaneous mutants of the facultative thermophiles, *B. subtilis* DB-1 or 168 able to grow on nutrient agar at 68°C have been isolated. However, none of these strains have ever been shown to grow in broth at this temperature and most appear to lyse after 1 to 3 passages on nutrient agar. Two mutants of *B. subtilis*, one originating from Br 151 tryC2, lys-3 and one from DB-1 wt 56 (able to grow on minimal salts media at 56°C) appear to be stable, can be passed on nutrient agar and do grow in broth or minimal liquid media plus casamino acids at 68°C.

Moreover mutants of *B. pumilus*, a mesophil apparently unable to grow above 50°C under any conditions, produces mutants able to grow at 68°C. One of these mutants *B. pumilus* TR-3 grows well at 68°C on nutrient agar and in various liquid media as shown in Table 4. In contrast to the parent strain TR-3 has a temperature range from 50°C to at least 68°C.

Treating with N-methyl-N-nitro-N-nitrosoquandine only slightly increased the frequency of this mutation to thermophily.

Isolation of transformants of *B. subtilis* which grow at 68°C. DNA from *B. caldolyticus* was used to obtain transformants of *B. subtilis* which grow at 68°C and prototrophs for tryptophan and lysine at 37°C (Table 4). Although stable tryptophan and lysine independent recombinants were readily obtained, there were no transformants which could grow at 68°C. The few colonies obtained at 68°C were considered unstable mutants described in the previous section.

DISCUSSION

Growth of B. subtilis occurs at 56°C either in complex medium, or in minimal medium supplemented with aspartate, threonine or pyridoxine. A single mutation results in growth on minimal salts media.

Thus it appears that most strains of B. subtilis grow at 56°C, however, some may require nutritional supplements for growth at this temperature. The strains of B. subtilis used by McDonald and Matney (3) presumably contained temperature sensitive mutations and were able to grow at 55°C even in complex media. Using other B. subtilis strains not containing this mutation they obtained transformants of these strains which were able to grow at 55°C. However, it is doubtful that this mutation represents the thermophily gene. Instead, it appears that the recipient cells harbored a gene which coded for a heat-labile product. Since this gene was linked to streptomycin resistance, one might suspect that the defect lies in the protein synthesis apparatus.

The transformants of B. subtilis reported by Lindsay and Creaser (2) grew at extreme temperatures (above 68°C) which implies that there is a gene(s) responsible for thermophily and this gene is linked to streptomycin. Likewise, Friedman and Mojica (1) obtained transformants of B. subtilis

which grew at 65°C but they found these recombinants exhibited instability.

Although we have been unsuccessful in isolating thermophilic transformants in the present study, we have obtained unstable and stable mutants of B. subtilis which grow at 68°C. Furthermore, relatively stable mutants of B. pumilus which grow at 68°C have been isolated. We believe that this finding is consistent with the idea that there is a gene(s) responsible for thermophily.

Since the mutation in B. pumilus results in a shift in growth temperature from mesophilic, growth up to 50°C to thermophilic, growth from 50°C to at least 68°C, we believe this might represent a true gene for thermophily.

We anticipate that the wild type B. pumilus mesophil, and the mutant B. pumilus tr3 thermophil will provide an excellent system for studying the genetics of thermophily.

Table 1: Growth of two wild type B. subtilis strains on various media at 56°C*

Media	<u>B. subtilis</u> DB-1	<u>B. subtilis</u> 168
Minimal media	-	-
Nutrient broth	+++	+++
Aspartic acid and threonine	++	++
Aspartic acid	+	+
Homoserine	+	-
Pyridoxine	+	+

*In all cases except nutrient broth, cell lines sporulated and would not grow after 2-3 passages on the defined media.

Table 2: Maximum growth temperature of B. subtilis and B. pumilus under different conditions.

Media	Temperature	Strain		
		<u>B. subtilis</u> DB-1 (160 wt)	<u>B. subtilis</u> 168	<u>B. pumilus</u>
Minimal	37°C	+	+	+
Liquid	50°C	+	+	+
Media	56°C	-	-	-
Nutrient	50°C	+	+	+
Broth	56°C	+	+	-
	60°C	-	-	-
Brain Heart	50°C	+	+	+
Infusion	56°C	+	+	-
Broth	60°C	-	-	-
Antibiotic	50°C	+	+	+
III	56°C	+	+	-
Broth	60°C	-	-	-
L Broth	47°C	+	+	+
	56°C	+	+	-
	60°C	-	-	-
Nutrient	50°C	+	+	+
Agar	56°C	+	+	-
Plates	60°C	+	+	-
	63°C	-	-	
	68°C	-	-	
Minimal	50°C	+	+	+
Agar plus	56°C	+	+	-
Casamino	60°C	+	+	-
Acids	63°C	-	-	
	68°C	-	-	

Table 3: Characterization of a *B. pumilus* mutant able to grow well at high temperatures.

Temperature	Minimal Media plus Casamino Acids	Nutrient Broth	L. Broth	Nutrient Agar
43°C	-	-	-	-
50°C	-	+	+	+
56°C	+	+	+	+
63°C	+	+	+	+
68°C	+	+	+	+

Table 4: Transformation of B. subtilis with B. caldolyticus DNA.

Growth Condition	DNA (<u>B. caldolyticus</u>) ¹	DB-1	Strains of <u>B. subtilis</u>	
			168	Br151, <u>tryC2</u> , <u>lys-3</u>
			<u>No. of colonies</u>	
68°C	- ²	1	0	0
68°C	+	0	1	1
37°C Lysine prototrophy	+			1071
37°C Tryptophane + prototrophy				997

¹No colonies were present in DNA control

²-, culture not treated with DNA + culture treated with DNA

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