DOE/ER/13204--6

DE92 004033

FINAL PROGRESS REPORT

DOE/ER/13204-6

Title: Genetics of Thermophilic Bacteria

DE-FG02-84ER13204

Project Period: May 1, 1984 to April 30, 1991

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A. Report Period: May 1, 1984 to April 30, 1991

The final report summarizes the entire investigation. Specific technical details were described in the following progress reports:

DOE/ER/13204-1 submitted 12/21/84
DOE/ER/13204-2 submitted 07/11/85
DOE/ER/13204-3 submitted 01/13/87
DOE/ER/13204-4 submitted 12/28/87
DOE/ER/13204-5 submitted 07/14/88

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C. Publications:

1. Chen, Z.-F., S.F. Wojick, and N.E. Welker. 1986. Genetic analysis of Bacillus stearothermophilus by protoplast fusion. J. Bacteriol. 165:994-1001.

2. Welker, N.E. 1988. Transduction in Bacillus stearothermophilus. J. Bacteriol. 170:3761-3764.

3. Wu, L. and N.E. Welker. 1989. Protoplast transformation of Bacillus stearothermophilus NUB36 by plasmid DNA. J. Gen. Microbiol. 135:1315-1324.

4. Vallier, H. and N.E. Welker. 1990. Genetic map of the Bacillus stearothermophilus NUB36 chromosome. J. Bacteriol. 172:793-801.

5. Wu, L. and N.E. Welker. 1991. Cloning and characterization of a glutamine transport operon of Bacillus stearothermophilus NUB36: effect of temperature on regulation of transcription. J. Bacteriol. 173:4877-4888.

6. Wu, L, and N.E. Welker. 1991. Temperature-induced protein synthesis in Bacillus stearothermophilus NUB36. J. Bacteriol. 173:4889-4892.

7. Welker, N.E. (0000). Genetic map of Bacillus stearothermophilus NUB36, p. xxx - xxx. In A.L. Sonenshein, J. Hoch, and R. Losick, Bacillus subtilis and other Gram-positive bacteria; physiology, biochemistry, and molecular biology. American Society for Microbiology, Washington, D.C. (submitted)

Three copies of publications 1-4 were submitted with the previous Progress Reports and three copies each of publications 5 and 6 and of a manuscript (#7) that is submitted for publication are included with this final report.

D. Objectives of the project.

Organisms adapted to high temperature have evolved a variety of unique solutions to the biochemical problems imposed by this environment. Adaptation is commonly used to describe the biochemical properties of organisms which have become adapted to their environment (genetic adaptation). It can also mean the direct response-at the cellular level-of an organism to changes in temperature (physiological adaptation). Thermophilic bacilli (strains of Bacillus stearothermophilus) can exhibit a variety of biochemical adaptations in response to changes in temperature. These include changes in the composition and stability of the membrane, metabolic potential, the transport of amino acids, regulatory mechanisms, ribose methylation of tRNA, protein thermostability, and nutritional requirements. The objectives of the research were to develop efficient and reliable genetic systems to analyze and manipulate B. stearothermophilus, and to use these systems initiate a biochemical, molecular, and genetic investigations of genes that are required for growth at high temperature.

E. Project summary.

Efficient and reliable protoplast fusion techniques were established for prototrophic strain B. stearothermophilus NUB36 and temperate and virulent phages isolated from soil were shown to carry out generalized transduction of this strain. The frequencies of transduction for the temperate phages were similar to or higher than the frequencies obtained for transducing phages of mesophilic bacilli. A repertoire of mutant strains were isolated and a circular genetic map of B. stearothermophilus NUB36 was constructed by transduction and protoplast fusion. Fifty-one loci were tentatively assigned a cognate B. subtilis genes and although the relative position of many genes on the B. stearothermophilus and B. subtilis genetic map appears to be similar, some differences were detected.

An efficient protoplast transformation system was established for B. stearothermophilus NUB36 by using a modification of the protoplasting and regeneration techniques established for protoplast fusion. The efficiency of transformation of NUB3621 Rif^r Hsr^- Hsm^- with mesophilic plasmid pLW05 Cm^r and thermophilic plasmid pTHT15 Tc^r was between 2 x 10^7 to 4 x 10^8 transformants/μg DNA. The transformation frequency (transformants/regenerant) was between 0.5 and 1.0. The efficiency of transformation obtained with NUB3621 was higher than that reported for B. stearothermophilus strains CU21 (2 x 10^7 transformants/μg DNA [Imanaka et al., J. Bacteriol. 149:824-830 1982]) and NRRL1174 (4 x 10^4 transformants/μg DNA [Liao et al., Proc. Natl. Acad. Sci. U.S.A. 83:576-580 1986]). The unique viability of protoplasts of NUB3621 accounts for the high efficiency of transformation observed with this strain.

Plasmid pLW05, a deletion derivative of mesophilic plasmid pPL401 Cm^r Km^r, carries the cat gene and the replication region of mesophilic plasmids pUB110 and pC194, respectively. pLW05 was not stable in NUB3621 grown under non-selective conditions at 50°C but NUB3621 (pLW05) would grow under selective conditions at temperatures up to 70°C. These results indicate that the chloramphenicol acetyltransferase (CTase) was active at high temperature.

Plasmid pTHT15, a plasmid isolated from a thermophilic bacillus, transformed strain NUB3621 without undergoing rearrangements or deletions. Plasmid pTHT15 was stable in NUB3621 grown under non-selective conditions at temperatures up to 65°C and cultures of NUB3621 (pTHT15) would grow under selective conditions at temperatures up to 72°C. These combined results indicate that plasmid pTHT15 would be a more useful vector than plasmid pLW05 for molecular cloning in B. stearothermophilus NUB3621. Plasmid pTHT15 contains five unique restriction sites, however, only one site (BstEII) is located outside of the tet gene (Hoshino et al., Can. J. Microbiol. 31:339-345 1985). An unique EcoRI site was generated into this plasmid by removing a 0.5-kb EcoRI fragment. The resulting plasmid was designated pLW06. Plasmid pLW06 was inserted into the EcoRI site of plasmid pIF710. The resulting plasmid, designated pIF711, carries the pUB110 and pBR322 replicons, the amp and tet genes, and a synthetic polylinker. A derivative of plasmid pLW06 was constructed by introducing the 1.35-bp EcoRI fragment (carries the cat gene) from pLW05 into the EcoRI site of pLW06. The resulting plasmid, designated pBS01, has four unique restriction sites in the tet gene (insertional inactivation), and one unique restriction site outside of the tet and cat genes. These plasmids efficiently transform both B.

stearothermophilus NUB3621 and B. subtilis. Plasmid pIF711 also transforms E. coli. Plasmids pLW06, pBS01, and pIF711 are stably maintained in B. stearothermophilus NUB3621 at temperatures up to 73°C

Plasmid pLW06 was used to construct a vector that carries transposon Tn917. A 6.45-kb EcoRI fragment from the E. coli plasmid pAM225, that carries the 5.25-kb Tn917 element, was inserted into the EcoRI site of pLW06. The ligation mixture was used to transform protoplasts of NUB3621 to tetracycline (Tet^r) and MLS resistance (MLS^r). The plasmid in several transformant colonies (pMH01 Tc^r MLS^r) was verified by restriction analyses. In preliminary experiments, we isolated several different auxotrophic insertion mutants. These results indicate that Tn917 inserts randomly in the chromosome.

B. subtilis promoter probe plasmid pMH109 Km^r Tc^r (Hudson and Stewart, Gene 48:93-100 1986) transformed B. stearothermophilus NUB3621to Kan^r. This plasmid carries the promoter-less cat gene from pC194. In preliminary experiments, plasmid pMH109 was used to clone fragments of NUB36 chromosomal DNA that exhibited promoter activity either at low temperature or at high temperature (Wu, Ph.D. Thesis, Northwestern University, 1990). The most important finding from these preliminary experiments was that a promoter probe plasmid developed for use with B. subtilis can also be used with B. stearothermophilus.

The progress that we have made in developing genetic techniques for B. stearothermophilus NUB36 now makes it possible to carry out detailed biochemical, molecular, and genetic analyses of thermoadaptation in this organism. To initiate these studies we used temperature shift-up and shift-down experiments to characterize the response of cells to changes in temperature. Our goal was to identify cellular proteins that were synthesized only at a low temperature or at a high temperature. In nitrogen-rich media strain NUB3621 maintained balanced growth between 39 and 75° with optimal growth between 67 to 69°. The middle temperature range of growth was between 45 and 65°. Cultures grown in a nitrogen-poor medium exhibited a narrower temperature range of growth (45 to 67°C), a lower optimal growth temperature (62 to 63°C), and a narrower middle temperature range of growth (52 to 60°C). The middle or intermediate temperature range of growth of bacteria is generally regarded as the normal or Arrhenius range not only because the rate of growth varies as a simple function of temperature but also because shifts in temperature within this range results in immediate growth at the normal exponential rate for the new temperature. However, cultures of B. stearothermophilus NUB3621 grown in a nitrogen-rich or in a nitrogen-poor medium, subjected to a temperature shift-up or shift-down of 8- to 15-degrees within the normal temperature range of growth, entered a transient adaptation period before exponential growth began at the new temperature. The de novo synthesis of a unique set of cellular proteins coincided with the transient adaptation period. The synthesis of several new proteins was detected after a shift from a low temperature to a high temperature and coincides with the transient adaptation period.

We cloned and sequenced a fragment of chromosomal DNA that contained two ORF's whose products were detected only in cells of cultures grown at high temperature. The nucleotide sequence of the two ORF's exhibited significant identity to the sequence of the glnQ and glnH loci of the glutamine transport system in enteric bacteria. Growth response to L-glutamine, sensitivity to the toxic glutamine analog γ-L-glutamylhydrazide, and

glutamine transport assays with parental strain NUB3621 and mutant strain NUB36500, in which the ORF1 coding segment in the chromosome was interrupted with the cat gene, demonstrated that ORF1 (glnQ) and ORF2 (glnH) encode proteins Q and H, respectively, that are active in a glutamine transport system in this organism. The transcription of glnQH originated from a single promoter segment (-35 and -10 regions) that had a low degree of homology with the various consensus promoter sequences of other prokaryote genes. Transcription of the glutamine transport operon (glnHPQ) of enteric bacteria is activated in cultures grown in a nitrogen-poor medium and inhibited in cultures grown in a nitrogen-rich medium. In contrast, transcription (within the normal temperature ranges of growth) of the glnQH operon of B. stearothermophilus was activated in a nitrogen-rich and in a nitrogen-poor medium at high temperature and inhibited under the same conditions at low temperature. This indicates that the glnQH operon is transcriptionally regulated by temperature. However, the region upstream of the glnQH operon contains sequences that have a low degree of homology to the consensus sequence of nitrogen regulated (Ntr) operons and to the binding site of the nitrogen regulator I (NR_I) protein of enteric bacteria. Thus, under conditions of nitrogen-depravation and temperature stress, the glnQH operon may be weakly driven from this promoter.

To determine the role of glnQH in the physiology of growth at high temperature, mutant strain NUB36500 in which the glnQH operon is transcriptionally inactivated was subjected to a temperature shift from 45 to 65°C and from 65 to 45°C . After a temperature shift-up, the mutant strain, in contrast to the parental strain, did not exhibit a lag period before exponential growth at 65°C. In a temperature shift-down experiment, the mutant responded in the same manner as did the parental strain. Cultures of mutant strain NUB36500 grown in a nitrogen-rich or in a nitrogen-poor medium at 45°C or at 65°C had a final cell density that was 20 to 35 times higher than the cell density of a culture of the parental strain grown under the same conditions. In addition, the rate of growth of the mutant strain at 45 and 65°C was greater than the rate of growth of the parental strain under the same conditions. These results are consistent with reports that cultures of thermophilic bacteria grown at high temperatures exhibit a low cell density. For example, Coultate and Sundarum (J. Bacteriol. 121:55-64 1975) reported that the molar growth yield of a prototrophic strain of B. stearothermophilus progressively decreases at higher growth temperatures. The molar growth yields of this strain appear to be inversely related to the growth rate and high temperature. At the higher temperatures, a larger proportion of the glucose carbon remains incompletely utilized in the medium and energy production was uncoupled from respiration. Using another strain of B. stearothermophilus, de Vrig et al. (FEMS Microbiol. Rev. 75:183-200 1990) reported that the efficiency of energy transduction was decreased at high temperature. In addition, most strains of B. stearothermophilus (including NUB36) when grown at their optimal temperature for growth or higher, generally reached cell densities that were lower than their cell densities when grown under the same conditions a lower temperatures (Zhang et al., Appl. Environ. Microbiol. 54:3162-3164 1988). Protoplasts prepared from another strain of B. stearothermophilus grown at high temperature were more stable than protoplasts prepared from cells grown at low temperature (Bodman and Welker, J. Bacteriol. 97:924-935 1969; Wisdom and Welker, J. Bacteriol. 114:1336-1345 1973). The thermostable membranes contained more protein than

did the thermolabile membranes. Protoplasts prepared from cells of strain NUB3621 grown at 65°C were also more stable than protoplasts prepared from cells grown at 65°C. Protoplasts prepared from cells of mutant strain NUB36500 (glnQH null mutant) grown at high temperature were less stable than protoplasts prepared from the parental strain NUB3621 grown under the same conditions. Since proteins Q and H of the glutamine transport system are located in the membrane, they may contribute to the enhanced stability of the membrane at high temperature. Thus, the glnQH operon may have at least two roles in the physiology of growth at high temperature - nitrogen metabolism and membrane stability.

Finally, it could be argued that adaptation studies should be carried out with organisms that are genetically well-characterized (B. subtilis or E. coli). To investigate this possibility, chromosomal DNA was isolated from B. subtilis BR151 and E. coli JM109 and digested with HindIII. Southern hybridizations were performed using a ^{32}P-labeled 2.0-kb EcoRI fragment that contains the B. stearothermophilus glnQH. The probe hybridized to a single fragment in each DNA. These results were expected for E. coli and also indicate that glnQH has significant identity to a corresponding locus in B. subtilis. Total RNA, isolated from E. coli and B. subtilis grown in a nitrogen-rich medium and in a nitrogen-poor medium at 22°C and at 38°C and at 30°C and at 50°C, respectively, was probed with the 2.0-kb EcoRI fragment. A single 1.7-kb and a 2.6-kb mRNA species was detected in the RNA of B. subtilis and E. coli, respectively, grown in a nitrogen-rich medium at low and high temperature. As expected, the relative amounts of probe-specific mRNA detected in cells of each organism grown in a nitrogen-poor medium was less than the amount of mRNA detected in cells grown in a nitrogen-rich medium at low and high temperatures. These results indicate that the cognate glutamine transport operons of the two organisms are not regulated by temperature.

F. SIGNIFICANCE

Although there has been some progress in the development of genetic technology for the manipulation and genetic analysis of strains of the genus Thermus and Methanobacterium. thermoautotrophicum, the lack of efficient and reliable genetic transfer systems, a repertoire of mutants, and of plasmids that express useful genetic markers have hampered the exploitation of these organisms for basic and applied research. In contrast, considerable progress has been made in developing genetic technology for Bacillus stearothermophilus.

We have made considerable progress in developing genetic techniques for B. stearothermophilus NUB36. We have already used this system to investigate the glnQH operon (glutamine transport) that is transcriptionally regulated by temperature. Additional molecular and genetic techniques will be required if we are to understand how organisms have adapted to high temperature. B. stearothermophilus NUB36 is particularly suited for these studies because it appears that the molecular and genetic technology developed for B. subtilis can easily be adapted for use with this strain. Mesophilic hosts (B. subtilis or E.

coli) can not be used for investigations of thermophile gene regulation at high temperature or the genetic and functional dissection of complex enzyme systems of thermophiles. The molecular and genetic techniques and vectors developed for strain NUB36 will be of particular importance to researchers who are investigating the biochemical, physiological, and genetic properties of thermophilic bacilli. Thus, it will be possible to carry out detailed biochemical, molecular, and genetic analyses of the structural and regulatory genes encoding important enzymes and metabolic systems in a thermophilic species, and to develop systems for improving their synthesis and thermostability.

This research will also have an impact on other areas of endeavor. Desired genetic traits from other thermophiles that lack a system for genetic analysis or have growth characteristics that make them unsuitable for research can be cloned in B. stearothermophilus. These studies may uncover some unique features that distinguish gene organization and function in thermophiles from that in mesophiles.

A detailed investigation of the regulation of glnQH expression will make it possible to develop molecular models of the sensing mechanisms that respond to temperature. In addition, these investigations will not only contribute to our understanding of the various adaptation mechanisms employed by organisms that grow over a relatively broad temperature range but will also add to our understanding of how organisms respond to temperature.

The results obtained from these studies will play an important role in the future exploitation of thermophiles in biotechnology. Much of the industrial technology that uses enzymes operate at relatively high temperatures. The main advantages of using thermostable enzymes in these processes are increased reaction rates and low bacterial contamination. Thus, the development of microbial strains with these enzymes is of primary importance in these systems. In the past 10-15 years, most of the research has been on enzymes from thermophiles of the genera Bacillus, Thermus, and Clostridium. Most of the strains used as a source of thermostable enzymes were isolated by enrichment culture methods. These strains have been extensively mutated to obtain the utmost in enzyme yields. Unfortunately, there have been no reports on the improvement of enzyme yield or enzyme thermostability by molecular and genetic methods. This is because the industrial thermophilic strains could not be genetically manipulated or analyzed. We have made major progress in developing genetic techniques for B. stearothermophilus NUB36. It is now possible to carry out detailed biochemical, molecular, and genetic analyses of the structural and regulatory genes encoding important enzymes and metabolic systems in a thermophilic species and to use these systems for improving their synthesis and thermostability. For example, the ability to improve the cell yield of appropriately engineered strains (eg. mutant strain NUB36500 glnQ:cat) will greatly facilitate the isolation of useful enzymes and other cellular components. Genes encoding thermolabile enzymes can be cloned in B. stearothermophilus and more thermostable forms of these enzymes can be isolated by growing cultures of the plasmid-carrying strain at progressively higher temperatures under the appropriate selective conditions. Finally, it will be possible to characterize genes from other thermophiles that either lack a system for genetic analysis or possess growth characteristics that make them unsuitable for basic or applied research.

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