JOURNAL OF BACTERIOLOGY, Aug. 1991, p. 4889–4892
0021-9193/91/154889-04$02.00/0
Copyright © 1991, American Society for Microbiology
Vol. 173, No. 15

NOTES

Temperature-Induced Protein Synthesis in *Bacillus stearothermophilus* NUB36

LIJUN WU† AND NEIL E. WELKER*

Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, Illinois 60208

Received 25 February 1991/Accepted 23 May 1991

Cultures of *Bacillus stearothermophilus* subjected to a temperature shift-up or shift-down of 15°C within the normal temperature range of growth (45 to 65°C) enter a transient adaptation period before exponential growth at the new temperature. The de novo synthesis of some proteins coincides with the adaptation period.

Thermophilic bacilli can exhibit a variety of biochemical adaptations in response to a change in the growth temperature. These include changes in the composition (8, 13, 16, 27) and stability (4, 29) of the membrane, metabolic potential (11, 22), regulatory mechanisms (3, 15, 21), ribose methylation of tRNA (1), protein thermostability (14), and nutritional requirements (6, 18). Studies have contributed little to our understanding of the biochemical mechanisms of thermoadaptation or of the molecular mechanisms for sensing changes in temperature, because the organisms used in these investigations could not be genetically manipulated or analyzed. The progress made in developing genetic techniques for *Bacillus stearothermophilus* NUB36 (7, 28, 30) now makes it possible to carry out detailed biochemical, molecular, and genetic analyses of thermoadaptation. In this study, we define adaptation as the direct response, at the molecular and biochemical levels, of an organism to a sudden change in temperature (12). In this study, we identified cellular proteins of *B. stearothermophilus* NUB36 that were synthesized only at a low temperature or at a high temperature. We report here on the findings of this study.

The bacterial strain used in this investigation was *B. stearothermophilus* NUB36105 *rfm-1 met-2* (26). Temperature shift-up and shift-down experiments were carried out as follows. Cells from an overnight modified Luria-Bertani (LB) medium (7) plate were used to inoculate a tube (18 by 150 mm) containing 5 ml of LB medium or minimal glucose medium supplemented with 0.1% Bacto-Casamino Acids (Difco Laboratories, Detroit, Mich.) (MGCA medium). The tube, capped with a plastic closure (Bellco Glass Inc., Vineland, N.J.) that contained a gas dispersion tube (extra-coarse porosity), was placed into an opening in the cover of a water bath, and cells were grown for 2 h. The temperature was maintained with a constant temperature circulator, and aeration and agitation of the culture were accomplished with 5 cm^3 of filter-sterilized air per min. Cells from a 5-ml culture were used to inoculate a tube (25 by 150 mm) containing 15 ml of LB or MGCA medium to a cell density of 8×10^6 to 2×10^7 CFU/ml. The tube was capped with a plastic closure that contained a gas dispersion tube and a sampling port. The culture was grown as described above. The 5-ml culture was grown at the same temperature as the 15-ml culture. A temperature shift-up or shift-down was accomplished by transferring the culture from one water bath to another. Approximately 1.5 min after a temperature shift-up or shift-down, the temperature of the culture reached the temperature of the water bath. Growth was monitored by determining the CFU per milliliter in samples removed from the culture at intervals. Before being plated, diluted cell suspensions were vigorously agitated to disperse cells that failed to separate after division. Spores, sporulating cells, filaments, and cells per filament were quantitated by direct counts with a phase-contrast microscope. Immediately after the temperature shift, 5 μg of chloramphenicol/ml of ethanol was added to one culture, and an identical volume of 100% ethanol was added to the control culture.

Cultures of *B. stearothermophilus* NUB36105 grown in MGCA or modified LB medium maintained balanced growth between 39 and 75°C, with optimal growth at 67°C. In the middle of this range, between 45 and 65°C, the rate of growth varied as a simple function of temperature. At higher and lower temperatures, the rate of growth decreased progressively. The rate of growth in LB medium was higher than the rate of growth in MGCA medium. Cultures grown in minimal glucose medium exhibited a narrower temperature range of growth (45 to 67°C), a narrower middle temperature range of growth (52 to 60°C), and a lower optimal growth temperature (62°C). The rate of growth in minimal glucose medium at a given temperature was generally 5- to 10-fold lower than the rates observed in MGCA or LB medium.

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 is a simple function of temperature but also because shifts in temperature within this range generally result in immediate growth at the normal exponential rate for the new temperature (9).

When cultures of NUB36105 were subjected to a temperature shift-up or shift-down of 15°C but within the normal temperature range of growth for this strain, a lag phase was observed that lasted 2 to 3 h before exponential growth resumed at the new temperature. The growth response of a culture shifted from 45 to 65°C is shown in Fig. 1A. Approximately 15 min after the temperature shift-up, there was a

* Corresponding author.
† Present address: Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.

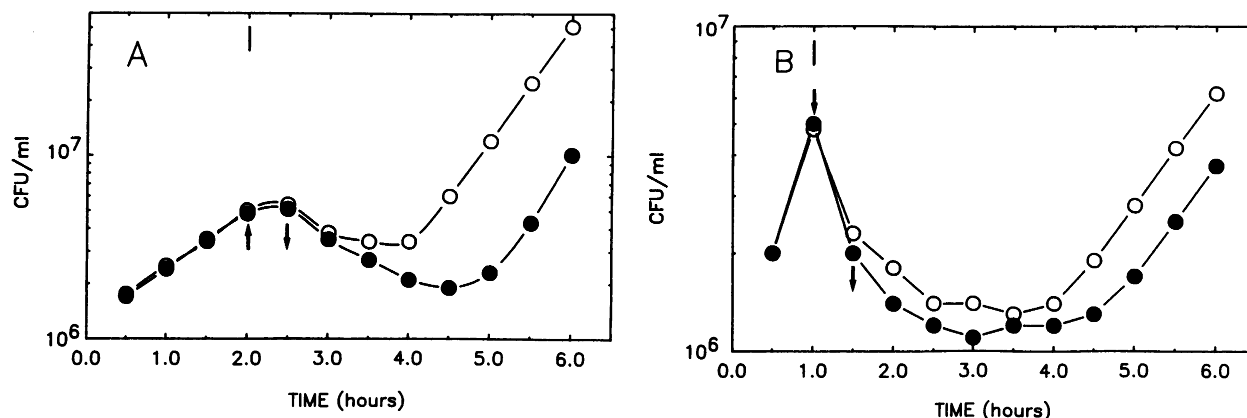

FIG. 1. Effect of temperature on the growth of *B. stearothermophilus* NUB36105. The short vertical line on the upper *x* axis denotes the time when the culture was shifted from 45 to 65°C (A) or from 65 to 45°C (B). Symbols: ●, 5 μg of chloramphenicol/ml of ethanol added; ○, identical volume of 100% ethanol added to control culture, arrow pointing toward time point, chloramphenicol added immediately after sample was removed from the culture; arrow pointing away from time point, cells from the entire culture were collected by centrifugation at 2,000 × *g* for 5 min at room temperature and suspended in fresh MGCA medium without chloramphenicol.

gradual decrease in the CFU per milliliter over a period of 2 h before normal exponential growth at 65°C. Cultures grown at 45°C contained short filaments with completed septa consisting of an average of 6 to 10 cell units. In contrast, only single cells and pairs of cells were detected in cultures grown at 65°C. Single cells and pairs of cells were detected 15 to 20 min after a shift from 45 to 65°C. The addition of chloramphenicol at the time of the shift delayed normal exponential growth at 65°C by a time that was approximately equal to the time that the cells were exposed to chloramphenicol (30 min).

In a converse experiment, a shift from 65 to 45°C resulted in a progressive decrease in CFU per milliliter over a period of 3 h before normal exponential growth at 45°C (Fig. 1B). Filaments were first detected 1.5 to 2 h after the culture was shifted to 45°C. The addition of chloramphenicol at the time of the shift from 65 to 45°C delayed normal exponential growth at 45°C for a time that was equal to the time that the cells were exposed to chloramphenicol (30 min). Cultures exposed to chloramphenicol for 60 and 90 min after a temperature shift-up or shift-down were delayed by 60 and 90 min, respectively, before growth at the new temperature (data not shown). The centrifugation step used to remove chloramphenicol did not significantly alter the growth response of a culture subjected to a temperature shift-up or shift-down.

The growth response of a culture subjected to a temperature shift-up or shift-down was not altered if cells were grown in LB medium or if aeration was increased to 40 cm^3/min. The composition of the plating medium or the incubation temperature did not affect the plating efficiency of the cells before and after a culture was subjected to a temperature shift.

The combined results indicate that cells of NUB36105 subjected to a temperature shift-up or shift-down of 15°C within the normal temperature range of growth enter a transient adaptation period before exponential growth at the new temperature. The results obtained with cultures exposed to chloramphenicol indicate that de novo synthesis of some proteins may be required for exponential growth at the new temperature.

Exponential-phase cultures of NUB36105 grown in MGCA medium at 45, 50, 60, and 65°C were pulse-labeled with [^{35}S]methionine. Cells for [^{35}S]methionine labeling experiments were obtained from cultures grown as described by Chen et al. (7). Cells from 5 ml of an exponential-phase culture grown in MGCA medium (1×10^8 to 2×10^8 CFU/ml) were collected by centrifugation at room temperature and suspended in 0.5 ml of minimal glucose medium containing 0.02% Casamino Acids. The cells were incubated for 5 min on a gyratory shaker, 75 μCi of L-[^{35}S]methionine (>800 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was added, and the cells were incubated for 5 min. The 5-min pulse was terminated by the addition of 400 μg of L-methionine per ml. The cells were transferred to a 1.5-ml microcentrifuge tube, collected by centrifugation at room temperature, washed three times with minimal glucose medium, and suspended in 10 μl of 10 mM Tris HCl buffer (pH 8) containing 20 μg of lysozyme per ml to which was added 5 μl of 1-mg/ml DNase, 2 μl of 10 mM phenylmethylsulfonyl fluoride, and 1 μl of 75 mM EDTA (pH 8) (lysing buffer). The mixture was incubated for 30 min at 37°C. Seventy-five to one hundred microliters of an isoelectric focusing sample solution (1.6% Bio-Lyte 5/7 and 0.4% Bio-Lyte 3/10 ampholytes [Bio-Rad Laboratories, Richmond, Calif.], 9.5 M urea, 4% Triton X-100, 0.3% sodium dodecyl sulfate, and 100 mM dithiothreitol) was added, and the mixture was incubated for 30 min at room temperature. The debris was removed by centrifugation, and the ^{35}S-labeled cellular protein samples were stored at −70°C.

Figure 2 shows autoradiograms of the two-dimensional gel electrophoresis of proteins from cells grown at 45 and 65°C. A visual comparison of the protein spots on the two panels revealed that a number of proteins were present in relatively high levels only at 45°C (proteins synthesized at low temperature; identified as L1 through L5 in Fig. 2) or at 65°C (proteins synthesized at high temperature; identified as H1 through H4 in Fig. 2). Proteins L1 through L5 and H1 through H4 were detected at 50 and 60°C, respectively. However, the levels of these proteins were less than their levels at 45 or 65°C (data not shown). Several minor proteins were also detected only at 45 or 65°C, but they were not included in these studies.

Several major proteins were more abundant at 45°C than at 65°C (proteins L6 through L10) or more abundant at 65°C than at 45°C (proteins H5 through H12 and proteins HS1

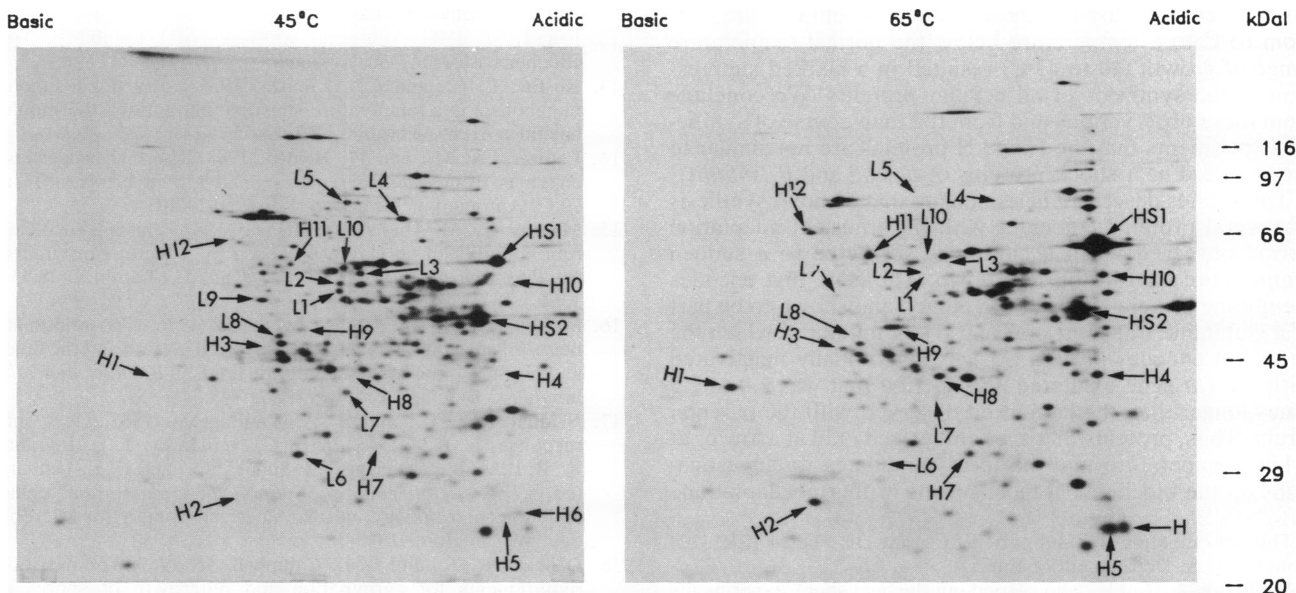

FIG. 2. Two-dimensional gel electrophoresis of ^{35}S-labeled proteins from cells grown at 45 and 65°C. A concentrated cell suspension (1 $\times$ 10^9 to 2 $\times$ 10^9 CFU/ml) of *B. stearothermophilus* NUB36105 grown in MGCA medium at 45 and 65°C was exposed to a 5-min pulse of [^{35}S]methionine. The ^{35}S-labeled proteins (total counts applied to each gel were approximately 5 $\times$ 10^5 to 8 $\times$ 10^5 cpm) were resolved by two-dimensional gel electrophoresis (19). The first dimension was isoelectric focusing (8.1 M urea, 2% Triton X-100, 1.6% Bio-Lyte 5/7 and 0.4% Bio-Lyte 3/10 ampholytes, 3.5% polyacrylamide) to equilibrium for 14 h at 500 V, and the second dimension was on a 1% sodium dodecyl sulfate–10% polyacrylamide gel for 5 to 6 h at 35 mA. Fluorography was carried out as described by Bonner and Lasky (5). The upper left (basic) and right (acidic) corners of the abscissa represent isoelectric pHs of 9.0 and 5.4, respectively. The arrows identify those proteins whose syntheses were markedly changed by temperature (proteins L1 through L10, H1 through H12, and HS1 and HS2). Positions of molecular mass markers in kilodaltons (kDal) are shown on the right.

[heat shock protein 1] and HS2). The estimated molecular weights (65 and 53 kDa) and relative abundance of proteins HS1 and HS2 compared with those of other cellular proteins indicate that HS1 and HS2 may be similar to the heat shock proteins encoded by *dnaK* and *groEL*, respectively, of *Escherichia coli* (17) and *Bacillus subtilis* (2).

No attempt was made to resolve the more basic proteins in the ^{35}S-labeled protein samples by two-dimensional non-equilibrating pH gradient electrophoresis (20).

The time course of the synthesis of the L and H proteins was monitored in cells shifted from 45 to 65°C or from 65 to 45°C. ^{35}S-labeled cellular proteins were prepared from cells at various times after the shift from 45 to 65°C or from 65 to 45°C and analyzed by two-dimensional gel electrophoresis (data not shown). Proteins L1 through L5 and H1 through H4 were first detected 20 to 30 min after a temperature shift-up or shift-down, respectively, and reached levels that were characteristic of the new temperature after 1.5 h. The levels of proteins L1 through L5 and H1 through H4 in cells shifted from 45 to 65°C and 65 to 45°C, respectively, gradually decreased, and the proteins were not detected after 1.5 h at the new temperature. Thus, de novo synthesis of the L and H proteins coincides with the transient adaptation period observed when a culture is subjected to a temperature shift-up or shift-down. The cellular locations of the L and H proteins were determined by separating the ^{35}S-labeled proteins into membrane and cytosol fractions and resolving the proteins by two-dimensional gel electrophoresis. Proteins H2, H3, L1, and L2 were detected only in the membrane fraction. The other L and H proteins were detected only in the cytosol fraction (data not shown).

To demonstrate that the L and H proteins synthesized at one temperature were not digested by proteolytic enzymes synthesized at another temperature, cultures of NUB36105 grown in MGCA medium at 45 and 65°C were pulse-labeled with [^{35}S]methionine for 5 min, unlabeled methionine was added, and the 45 and 65°C cultures were grown for 40 min at 65 and 45°C, respectively. Protein extracts were prepared and analyzed by two-dimensional gel electrophoresis. Visual analysis of the two-dimensional gels revealed that the estimated molecular weights and isoelectric points and the relative abundance of the L and H proteins in extracts treated as described above were unchanged. These results indicate that the L and H proteins are not products of proteolytic enzymes whose syntheses are regulated by temperature.

The induction of specific proteins by heat shock or stress has been extensively studied in *E. coli* (17) and has been demonstrated in *B. subtilis* (2, 23–25). The induction of specific proteins in response to low temperature (cold shock) has been described for *E. coli* (10). In the studies with *B. subtilis*, cultures were grown at a temperature within the normal temperature range of growth and shifted to a temperature above the normal temperature range of growth or to a nonphysiological temperature. In a similar approach, a culture of NUB36105 grown at 45°C was shifted to a temperature above the normal temperature range of growth (70 to 73°C). There was a marked suppression in the synthesis of cellular proteins, including the L and H proteins, and a marked enhancement of HS1, HS2, and a small number of other proteins. This response is characteristic of the heat shock response demonstrated in *B. subtilis*. Streips and Polio reported that *B. stearothermophilus* NUB36 exhibited a strong heat shock response (24), but they did not specify

the temperatures used in these studies. A temperature shift from 65°C to a temperature below the normal temperature range of growth (40 to 43°C) resulted in a marked suppression in the synthesis of all cellular proteins. We conclude from these observations and from the time course of synthesis experiments that the L and H proteins are not similar to the standard heat shock proteins of *E. coli* and *B. subtilis*.

The studies described here indicate that de novo synthesis of several proteins coincides with the transient adaptation period observed when a culture is subjected to a sudden temperature shift-up or shift-down. The gene that encodes membrane protein H2 has been cloned and shown to be part of a glutamine transport operon (31). A mutant in which the gene that encodes protein H2 was insertionally inactivated with the *cat* gene exhibited a lag period that was 2.0 to 2.5 times longer than the lag period observed with the parental strain. Thus, proteins that are synthesized only at a low or at a high temperature may be ideal candidates for investigations on the biochemical mechanisms of thermoadaptation.

This investigation was supported by grant DE-FG02-84ER13204 from the U.S. Department of Energy.

We thank M. Hinkle, who carried out the heat shock experiments with NUB36105.

REFERENCES

1. **Agris, P. E., H. Koh, and D. Soll.** 1973. The effect of growth temperatures on the in vivo ribose methylation of *Bacillus stearothermophilus* transfer RNA. Arch. Biochem. Biophys. **154:**277–282.
2. **Arnosti, D. N., V. L. Singer, and M. J. Chamberlin.** 1986. Characterization of heat shock in *Bacillus subtilis*. J. Bacteriol. **168:**1243–1249.
3. **Balerna, M., and H. Zuber.** 1974. Thermophilic aminopeptidase from *Bacillus stearothermophilus*. IV. Aminopeptidases (API_m, APIII) in mesophilic (37°C) cultures of the thermophilic bacterium *B. stearothermophilus*. Int. J. Peptide Protein Res. **6:**499–514.
4. **Bodman, H., and N. E. Welker.** 1969. Isolation of spheroplast membranes and stability of spheroplasts of *Bacillus stearothermophilus*. J. Bacteriol. **97:**924–935.
5. **Bonner, M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46:**83–88.
6. **Campbell, L. L., Jr., and O. B. Williams.** 1953. The effect of temperature on the nutritional requirements of facultative and obligate thermophilic bacteria. J. Bacteriol. **65:**141–145.
7. **Chen, Z.-F., S. F. Wojcik, and N. E. Welker.** 1986. Genetic analysis of *Bacillus stearothermophilus* by protoplast fusion. J. Bacteriol. **165:**994–1001.
8. **Hasegawa, Y., N. Kawada, and Y. Nosoh.** 1980. Change in chemical composition of membrane of *Bacillus caldotenax* after shifting the growth temperature. Arch. Microbiol. **126:**103–108.
9. **Ingraham, J.** 1987. Effect of temperature, pH, water activity, and pressure on growth, p. 1543–1554. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
10. **Jones, P. G., R. A. VanBogelen, and F. C. Neidhardt.** 1987. Induction of proteins in response to low temperature in *Escherichia coli*. J. Bacteriol. **169:**2092–2095.
11. **Jung, L., R. Jost, E. Stoll, and H. Zuber.** 1974. Metabolic differences in *Bacillus stearothermophilus* at 55°C and 37°C. Arch. Microbiol. **95:**125–138.
12. **Kogut, M.** 1980. Microbial strategies of adaptability. Trend Biochem. Sci. **5:**47–50.
13. **Kostiw, L. L., and K. A. Souza.** 1976. Altered phospholipid metabolism in a temperature-sensitive mutant of a thermophilic bacillus. Arch. Microbiol. **107:**49–55.
14. **Lauwers, A. M., and W. Heinen.** 1983. Thermal properties of enzymes from *Bacillus flavothermus*, grown between 34 and 70°C. Antonie van Leeuwenhoek **49:**191–201.
15. **Majima, E., M. Abe, and S. Higashi.** 1982. Varying electrophoretic mobility of an esterase caused by a factor in extracts of *Bacillus stearothermophilus* cells. Can. J. Microbiol. **28:**1355–1364.
16. **McElhaney, R. N., and K. A. Souza.** 1976. The relationship between environmental temperature, cell growth and the fluidity and physical state of the membrane lipids in *Bacillus stearothermophilus*. Biochim. Biophys. Acta **443:**348–359.
17. **Neidhardt, F. C., and R. A. VanBogelen.** 1987. Heat shock response, p. 1334–1345. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
18. **O'Brien, R. T., and L. L. Campbell, Jr.** 1957. The nutritional requirements for germination and outgrowth of spores and vegetative cell growth of some aerobic spore forming bacteria. J. Bacteriol. **73:**522–525.
19. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250:**4007–4021.
20. **O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell.** 1977. High resolution two-dimensional electrophoresis of basic as well as acid proteins. Cell **12:**1133–1142.
21. **Pederson, D. M., and R. E. Goodman.** 1980. Isozymes of α-galactosidase from *Bacillus stearothermophilus*. Can. J. Microbiol. **26:**978–984.
22. **Reizer, J., I. Epstein, and N. Grossowicz.** 1977. Temperature-induced metabolic alterations in a thermophilic bacillus. Eur. J. Biochem. **77:**463–470.
23. **Richter, A., and M. Hecker.** 1986. Heat-shock proteins in *Bacillus subtilis*: a two-dimensional gel electrophoresis study. FEMS Microbiol. Lett. **36:**69–71.
24. **Streips, U. N., and F. W. Polio.** 1985. Heat shock proteins in bacilli. J. Bacteriol. **162:**434–437.
25. **Todd, J. A., T. J. P. Hubbard, A. A. Travers, and D. J. Ellar.** 1985. Heat-shock proteins during growth and sporulation of *Bacillus subtilis*. FEBS Lett. **188:**209–214.
26. **Vallier, H., and N. E. Welker.** 1990. Genetic map of the *Bacillus stearothermophilus* NUB36 chromosome. J. Bacteriol. **172:**793–801.
27. **Weerkamp, A., and W. Heinen.** 1972. Effect of temperature on fatty acid composition of the extreme thermophiles, *Bacillus caldolyticus* and *Bacillus caldotenax*. J. Bacteriol. **109:**443–446.
28. **Welker, N. E.** 1988. Transduction in *Bacillus stearothermophilus*. J. Bacteriol. **170:**3761–3764.
29. **Wisdom, C., and N. E. Welker.** 1973. Membranes of *Bacillus stearothermophilus*: factors affecting protoplast stability and thermostability of alkaline phosphatase and reduced nicotinamide adenine dinucleotide oxidase. J. Bacteriol. **114:**1336–1345.
30. **Wu, L., and N. E. Welker.** 1989. Protoplast transformation of *Bacillus stearothermophilus* NUB36 by plasmid DNA. J. Gen. Microbiol. **135:**1315–1324.
31. **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.