Thermal Injury and Recovery of *Bacillus subtilis*

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Exposure of *Bacillus subtilis* NCTC 8236 to sublethal temperatures produced a change in the sensitivity of the organism to salt and polymyxin. After 30 min at 47 °C, 90% of the population was unable to grow on a modified sultite polymyxin sulfadiazine agar containing an added 1% NaCl, 1% glucose, and 1% asparagine. The data presented demonstrate that thermal injury results in degradation of both 16S and 23S ribonucleic acid (RNA) and in damage to the cell membrane, suggested by leakage into the heating menstrum of material absorbing at 260 nm. When the cells were placed in a recovery medium (Trypticase soy broth), complete recovery, indicated by a returned tolerance to salt and polymyxin, occurred within 2 hr. The presence of a protein inhibitor (chloramphenicol) and cell wall inhibitors (vancomycin and penicillin) during recovery had no effect, whereas the presence of an RNA inhibitor (actinomycin D) effectively inhibited recovery. Further data demonstrated that the injured cells were able to resynthesize both species of ribosomal RNA during recovery by using the fragments which resulted from the injury process. Also, precursor 16S and precursor 23S particles accumulated during recovery. The maturation of the precursor particles during recovery was not affected by the presence of chloramphenicol in the recovery medium.

The effects of sublethal heating on nonsporulating organisms, both gram-positive and gram-negative, have been characterized (2, 6, 7, 10, 12, 15, 19). A partial list of these effects includes ribonucleic acid (RNA) degradation, alteration of enzyme activity (4, 24), and damage to the cellular membrane. RNA degradation has been detected by leakage of nucleotides from the cell (1, 9) and by an absence or decrease of a ribosomal RNA (rRNA) species (17, 20, 23). Cellular membrane damage was suggested by loss of cellular material into the heating menstrum (2, 12, 19) and by an increased sensitivity to harsh environments, e.g., a high concentration of salt (5, 6, 12).

Although the nonsporeforming microorganisms tested characteristically exhibited these effects, the types of macromolecular synthesis necessary for recovery were found to depend on the organism injured. In particular, *Staphylococcus aureus* and *Streptococcus faecalis* required RNA synthesis for recovery (7, 12, 20), whereas *Salmonella typhimurium* needed both protein and RNA synthesis (22).

This report describes sublethal thermal injury and its effects on *Bacillus subtilis* NCTC 8236, a gram-positive sporeformer. It also includes a description of the RNA synthesis occurring and the cellular processes involved in recovery.

**MATERIALS AND METHODS**

**Injury procedure.** Cultures of *B. subtilis* NCTC 8236 were grown in Trypticase soy broth (TSB, BBL). Frozen stock cultures for regular use were prepared by first growing the organism in TSB to an optical density at 540 nm of 0.5. A 40-ml amount of this culture was then transferred to 200 ml of TSB and incubated for 3 hr. All incubations were at 37 °C on a rotary shaker. Portions (10 ml) of the 3-hr culture were put into sterile test tubes and frozen at -20 °C. When cells were needed for an experiment, a frozen tube was thawed and added to 200 ml of TSB. After incubation for 16 to 18 hr, 40 ml of this culture was inoculated into a second 200 ml of TSB. This transfer was done to preclude the presence of spores in the culture when it was harvested and injured. After a 3-hr incubation, these cells were harvested by centrifugation for 10 min at 8,000 × g at 0 to 2 °C. The supernatant fluid was decanted, and the cells were washed once in 100 mm potassium phosphate buffer (pH 6.0), centrifuged, and suspended in 10 ml of the same buffer. These cells were heated at 47 °C for 30 min by adding the suspension to 190 ml of 100 mm potassium phosphate buffer (pH 6.0), pretempered under constant agitation.
**Recovery procedure.** After heating, the cells were harvested by centrifugation for 10 min at 8,000 \( \times g \) at 0 to 2 C. The supernatant fluid was discarded; the cells were resuspended in 200 ml of 100 mM potassium phosphate buffer (pH 6.0), and 0.5 ml of this suspension was inoculated into 50 ml of TSB and incubated at 37 C.

**Assay procedure.** Assays of injury and recovery were done by using a differential plate counting system. Samples (1 ml) were withdrawn at various intervals from the injury vessel, or from the recovery flask, and were diluted in 0.1% peptone-distilled water blanks. The samples were taken from common dilution bottles and pour-plated on Trypticase soy agar (TSA; BBL) and modified polymyxin sulfadiazine (SPS) agar (BBL) containing an added 1% NaCl, 1% glucose, and 1% asparagine. A series of exploratory experiments demonstrated that this medium would serve the desired purpose. The plates were incubated at 37 C for 48 hr. The TSA counts gave a measure of all viable cells, both injured and uninjured. The modified SPS (m-SPS) agar count gave an estimation of the uninjured cells.

The suspension from the injury vessel was also tested for the release of material absorbing at 260 nm. The cells were removed by filtration through membrane filters (pore size, 0.22 \( \mu m \); Millipore Corp., Bedford, Mass.). The resulting supernatant fluid was cooled in an ice-water bath and analyzed for material absorbing at 260 nm (Beckman model DU spectrophotometer with a dual lamp source and modified with a Gilford 22 photometer). Uninoculated buffer was used as the control.

**Inhibitors.** Metabolic inhibitors were added to the TSB recovery vessel during recovery from heat injury at the following concentrations: chloramphenicol (CAP; Calbiochem, Los Angeles, Calif.), 1.5 \( \mu g/ml \); vancomycin (Vancocin HCl; Eli Lilly & Co., Indianapolis, Ind.), 10 \( \mu g/ml \); penicillin G (Calbiochem), 1,585 units/mg (5 \( \mu g/ml \)); actinomycin D (Calbiochem), 1.5 \( \mu g/ml \); and nalidixic acid (Calbiochem), 5 \( \mu g/ml \).

**Direct count.** Direct counts of bacterial suspensions in a Petroff-Hauser counting chamber were made by use of phase-contrast microscopy. The original suspension was diluted with 10% glycerol-90% water, thereby limiting Brownian movement. At least two counts were made at each dilution, and at least two dilutions of each suspension were counted.

**Incorporation of radioactive RNA.** To prepare \(^{14}C\)-labeled RNA, 0.2 ml of uracil-2-\(^{14}C\) (0.1 mCi/ml) and 40 ml of a 16- to 18-hr culture of \( B. subtilis \) were added simultaneously to 200 ml of TSB. After 3 hr of incubation at 37 C, the cells were harvested and the control RNA was extracted.

To prepare \(^{3}H\)-labeled cells, 0.2 ml of uracil-6-\(^{3}H\) (1.0 mCi/ml) was added to the culture either 3 hr prior to thermal shock or simultaneously with the addition of the injured cells to the recovery flask. After various lengths of incubation (depending on the experiment), the cells were harvested and the RNA was extracted.

**RNA extraction and analysis.** RNA was extracted from normal, heat-injured, and recovered cells by the phenol method originally described by Kirby (14), was separated by polyacrylamide-gel (PAG) electrophoresis, and was assayed by methods previously described (23).

**RESULTS**

**Procedures to estimate injury and recovery.** When cells of \( B. subtilis \) NCTC 8236 were placed in 100 mM potassium phosphate buffer (pH 6.0) at 47 C, an increasing sensitivity to m-SPS agar was noted with an increase in time (Fig. 1). As the heating time continued, the count on the m-SPS agar decreased, whereas the count on TSA remained relatively stable. The difference between the TSA and the m-SPS count provided a measure of the injured cell population. In this system, after 30 min of heating, significant injury was observed. Some death was evident, as seen by the decrease in TSA counts. Corresponding to the degree of injury, as indicated by the change in sensitivity to m-SPS agar, was a continuous increase with time of the amount of material absorbing at 260 nm present in the heating menstruum.

These data indicate that the thermal stress placed on the cell was adequate to permit a detailed study of the changes in the treated cells. When the thermally stressed cells were transferred to a recovery medium (TSB), they demonstrated an extended lag period (Fig. 2), a response characteristic of heat-stressed cells (12, 13, 22). During the extended lag period,
in the recovery of thermally stressed cells of *B. subtilis*.

**Effect of metabolic inhibitors during recovery.** Metabolic inhibitors were used to characterize further cellular events involved in the recovery process. CAP, vancomycin, penicillin, and actinomycin D were added to four separate recovery flasks simultaneously with the introduction of the thermally stressed *B. subtilis* cells to the recovery media (TSB). CAP inhibits protein synthesis without affecting RNA synthesis (25). Penicillin and vancomycin inhibit cell wall and muropeptide synthesis (3, 21). Actinomycin D blocks synthesis of RNA on a DNA template (16).

As is evident in Fig. 3, vancomycin and penicillin did not affect recovery but did dramatically inhibit subsequent multiplication,
and in fact caused death of the multiplying cells. It is interesting to note that the recovered cells again showed a sensitivity to the m-SPS agar, whereas two inhibitors exerted their effect on the multiplying cells. CAP permitted a significant amount of recovery, and the subsequent inhibition of growth was effective but less dramatic (data not presented). In contrast to the above-mentioned inhibitors, a low concentration of actinomycin D not only arrested recovery but also initiated a definite loss in viability of cells (Fig. 4). These inhibitor data indicate that neither protein nor cell wall synthesis was required but rather that RNA synthesis was necessary for injured cells to recover and to regain their tolerance to the m-SPS agar. These results suggested that a characterization of the RNA changes occurring during injury and recovery would be informative.

**rRNA degradation during thermal injury.** To determine the nature and extent of the rRNA degradation occurring during heating, four cultures of ³H-labeled cells were thermally injured for 5, 10, 20, and 30 min; the RNA was extracted and was subjected to co-electrophoresis on PAG with control RNA extracted from cells steady-state-labeled with ¹⁴C-uracil. Typical results are presented in Fig. 5.

This procedure distinctly separates the 16S and 23S RNA peaks, as evidenced by the rRNA profile for the normal cells (Fig. 5, open circles). When the RNA profiles were determined on cells which had been held at 47 C for 5, 10, 20, and 30 min, it was noted that there were pronounced changes in the two rRNA peaks. The 16S peak was undetectable after 5 min of heating, but the 23S peak degraded more slowly. This peak was detectable after 20 min of heating, but appeared to be gone after 30 min of heating (Fig. 5). Even after this dramatic loss of both rRNA species resulting from 30 min at 47 C, these cells were still able to recover as demonstrated in Fig. 2.

The degradation of the 23S RNA is in contrast to what has been previously reported for rRNA changes in thermally stressed cells. In S. aureus, the 16S RNA is extensively degraded, whereas the 23S is retained except for some possible changes in secondary structure (17, 20). Similarly with S. typhimurium, a sublethal thermal treatment caused a pronounced loss of the 16S RNA but only a slight degradation of the 23S RNA (23).

**Characterization of the rRNA synthesized during recovery.** The demonstration that a low concentration of actinomycin D inhibited recovery and that both the 16S and the 23S RNA were degraded during the heat treatment emphasized that RNA synthesis would be required for recovery. To follow the de novo rRNA synthesis, RNA was extracted from cells recovered in the presence of ³H-uracil and subjected to co-electrophoresis on PAG with ¹⁴C-RNA from an untreated culture. Cells were analyzed after 30, 60, 90, or 120 min in the recovery medium. In each profile, the ¹⁴C-RNA (control) clearly separated into two peaks corresponding to the 23S and 16S RNA. The profiles from the recovering cells showed a sequential change with recovery time in the synthesis of the typical 23S and 16S RNA. The ³H-RNA from cells recovered for 30, 60, or 90 min exhibited four peaks corresponding to 24S, 23S, 17.5S, and 16S RNA (Fig. 6). The 24S and the 17.5S peaks represent an accumulation of precursor 23S RNA and precursor 16S RNA (11). This is similar to the accumulation of precursor particles seen in recovering cells of thermally injured S. typhimurium (23). After a recovery time of 120 min, both the 23S and 16S RNA

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**Fig. 4.** Recovery and growth of heat-injured B. subtilis NCTC 8236 in Trypticase soy broth plus 1 µg of actinomycin D per ml. The cells were heated in 100 mm phosphate buffer, pH 6.0, at 47 C for 30 min. For recovery, the cells were transferred to the recovery medium alone (C, plated on Trypticase soy agar; O, plated on modified sulfite polymyxin sulfadiazine agar) or the recovery medium plus actinomycin D (D, plated on Trypticase soy agar; L, plated on modified sulfite polymyxin sulfadiazine agar). All incubations were at 37 C.
Fig. 5. Profile of rRNA extracted from heat-injured cells of B. subtilis NCTC 8236. Cells, steady-state-labeled with $^3$H-uracil, were heat-injured at 47 C for 30 min. The RNA was extracted from these cells and subjected to co-electrophoresis on polyacrylamide gels with control RNA extracted from cells steady-state-labeled with $^{14}$C-uracil. Symbols: O, uracil-2-14C counts; ●, uracil-6-3H counts.

peaks closely resembled the RNA peaks of the untreated cells (Fig. 6).

To determine whether the degraded RNA was reused for RNA synthesis during recovery, $^3$H-labeled cells were heat-injured, and were allowed to recover without an additional label. After 30, 60, 90, or 120 min in the recovery medium, the cells were harvested; the RNA was extracted and subjected to co-electrophoresis with $^{14}$C-labeled control RNA. The RNA extracted (data not presented) contained the $^3$H-label and had RNA profiles on PAG which were similar to RNA profiles from cells recovered in the presence of $^3$H-uracil for comparable lengths of time (Fig. 6). Since the precursors contained $^3$H-label, it was concluded that the degraded material could be resynthesized into RNA.

rRNA synthesized during CAP-inhibited recovery. Since the recovery of thermally injured cells of B. subtilis was demonstrated to be independent of protein synthesis, as measured by the increased viability on m-SPS agar of cells recovered in the presence of CAP, the synthesis and maturation of RNA should not be affected by the presence of CAP during recovery. To verify this relationship, rRNA was extracted from injured cells which had been permitted to recover for 90 min in the presence of CAP (1.5 μg/ml) and $^3$H-uracil. The PAG profile of this rRNA (Fig. 7) was almost identical to PAG profile for cells recovered in the absence of CAP (Fig. 6).

DISCUSSION

The data presented in this report demonstrate similarities as well as differences when the effects of thermal stress on B. subtilis are compared with those reported for other bacteria. Injury as well as recovery could be followed by the use of a selective medium. The m-SPS agar allowed a reasonable indication of the extent of injury and also provided a means to indicate when recovery was complete. The loss of material absorbing at 260 nm during heating implied membrane damage. This type of lesion was not further characterized, as the recovery experiments implied that it was quickly repaired. The use of selective inhibitors during recovery demonstrated that the cell wall inhibitors (penicillin and vancomycin) and the protein inhibitor (CAP) were without effect, whereas the RNA inhibitor (actinomycin D) effectively stopped recovery of the thermally injured cells. Previous thermal injury studies with S. aureus indicated that protein synthesis was not necessary for recovery (12), and likewise that ribosomal particles were reassembled in the presence of CAP (18). In contrast, thermally injured cells of S. typhimurium were unable to recover in the presence of CAP (22). It was further demonstrated that CAP inhibited the maturation of the precursor 16S RNA (23).

The detailed studies on the fate of the RNA during injury and recovery demonstrated that both 23S and 16S RNA were degraded during the heat treatment, but the injured cells had the
Fig. 6. rRNA profiles from heat-injured B. subtilis NCTC 8236 recovered for various lengths of time. rRNA, extracted from heat-injured cells which were recovered in the presence of \(^{3}H\)-uracil for 30, 60, 90, or 120 min, was subjected to co-electrophoresis on polyacrylamide gels with control RNA extracted from cells steady-state-labeled with \(^{14}C\)-uracil. Symbols: O, uracil-2-\(^{14}C\) counts; ●, uracil-6-\(^{3}H\) counts.

Fig. 7. Profiles of rRNA extracted from heat-injured cells of B. subtilis NCTC 8236 after recovery in the presence of chloramphenicol. Heat-injured cells were recovered in the presence of \(^{3}H\)-uracil and 1.5 µg of chloramphenicol per ml. After 90 min, the RNA was extracted from the \(^{3}H\)-labeled cells and subjected to co-electrophoresis on polyacrylamide gels with control RNA extracted from cells steady-state-labeled with \(^{14}C\)-uracil. Symbols: O, uracil-2-\(^{14}C\) counts; ●, uracil-6-\(^{3}H\) counts.

ability to resynthesize both species of rRNA by using the fragments which resulted from the injury process. The data further demonstrated that 24S and 17.5S RNA particles accumulated during recovery, and that the maturation of these particles during recovery was not affected by the presence of CAP in the medium.

The events discussed above suggest certain
effects attributable to heat. Thermal injury produced a weakening of the cell membrane, resulting in a loss of material absorbing at 260 nm and in an increased sensitivity to the salt and the polymyxin in the SPS agar. Also, complete loss of both 23S and 16S RNA species occurred after 30 min of heating. Recovery from thermal stress occurred within 2 hr and resulted in a repair of the weakened membrane and resynthesis of both rRNA species.

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