

Characterization of Mild Thermal Stress in *Pseudomonas fluorescens* and Its Repair

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The exposure of exponentially growing *Pseudomonas fluorescens* P7 cells to heating at 36 C for 2 h in a defined medium, followed by cooling to 25 C and further incubation at this, the optimal growth temperature, resulted in the apparent death of approximately 99% of the cells, as determined by their inability to form colonies on Trypticase soy agar. Continued incubation at 25 C resulted in an extremely rapid increase in the Trypticase soy agar count, demonstrating that the phenomenon observed was not death but rather injury. Presumptive evidence of heat-stimulated ribonucleic acid (RNA) degradation and membrane damage was provided by the observed loss of 260-nm absorbing materials. Confirmation of RNA degradation was obtained by colorimetric analysis. Ribosomal RNA from normal and injured cells, which was electrophoretically separated on polyacrylamide gels, revealed that the 23S and 16S species were only partially destroyed. Inhibitor studies demonstrated, however, that RNA synthesis was necessary for recovery. The unusual accumulation of 17S RNA during recovery pointed to the presence of a heat-induced lesion in the RNA maturation process. A thermally induced membrane lesion is also discussed.

Much work has been devoted to the investigation of thermal injury in mesophilic organisms. Studies of this phenomenon in *Staphylococcus aureus* (1, 3, 13, 14, 20, 23, 24), *Streptococcus faecalis* (2, 5, 28), and *Salmonella typhimurium* (4, 19, 26) have indicated that similar mechanisms are operative in both gram-negative and gram-positive bacteria, although some discrete differences exist. Thermal injury in psychrophilic organisms has enjoyed some discussion during investigations into the biochemical bases for the low maximal growth temperature of these cells (7-9, 17; R. J. H. Gray, M.Sc. thesis, University of Alberta, Canada, 1969). However, direct investigations into the response of psychrophiles to sublethal heat treatment are largely confined to earlier studies which only describe the effect in general terms (10-12, 15, 18).

The present study was undertaken as a part of an investigation into the behavior of *Pseudomonas fluorescens* both during and after exposure to a growth-restricting temperature. The report explores the effect in a system where exposure was at a temperature just above the maximum for growth and where the cells were subjected to a minimal degree of manipulation.

MATERIALS AND METHODS

Organism. The organism used was a laboratory strain of *Pseudomonas fluorescens* P7. This facultative psychrophile has a growth range of 34 C to less than 0 C and has an optimal growth temperature of 25 C.

Media. The defined medium routinely used for growth, injury, and recovery had the following composition (grams/liter): monosodium glutamate, 7.5; sodium citrate, 5.0; tris(hydroxymethyl)aminomethane, 12.0; hydrochloric acid (concentrated), 8.0 ml; potassium chloride, 2.0; sodium chloride, 2.0; sodium phosphate (dibasic), 0.5; magnesium sulfate, 0.08; manganese chloride, 0.01; and ferric chloride, 0.001. The medium was autoclaved in the absence of the glutamate which was filter-sterilized and added as a 30% aqueous solution just prior to inoculation. The final pH of the medium was 7.0. Trypticase soy agar (TSA) (Baltimore Biological Laboratory) was utilized as the standard plating medium for enumeration.

Growth. The culture (200 ml) was grown to the early logarithmic phase in 500-ml Erlenmeyer flasks shaken at 200 rpm. Incubation was at 25 C in a psychrotherm-controlled environment incubator shaker. Growth was monitored by following the change in absorbance at 600 nm in a Spectronic 20 colorimeter and by viable cell count where 1-ml samples were withdrawn at intervals from the culture flask, diluted in 0.1% peptone-distilled water, and

plated at the appropriate dilution on TSA. Colonies were counted after incubation for 48 h at 25 C. The total number of cells present was determined by a direct cell count in a Petroff-Hausser counting chamber and phase-contrast optics ($\times 1,250$).

Injury and recovery procedure. When the absorbance of the shake culture was 0.3 at 600 nm (approximately 2×10^8 cells/ml), the flask was transferred to a 36 C shaking water bath; the equilibration time was less than 3 min. The culture remained at this temperature for 2 h; the flask was then cooled and returned to the 25 C shaker for further incubation. Protein synthesis during recovery was inhibited by chloramphenicol (CM) at a final concentration of 100 $\mu\text{g/ml}$ of defined medium. Similarly, the inhibition of ribonucleic acid (RNA) synthesis was accomplished using rifamycin (RM) at a final concentration of 10 $\mu\text{g/ml}$ of defined medium. The growth of *P. fluorescens* is inhibited by the concentration of CM or RM used above. Inhibition only took place in the culture flask since dilution of the cell population for the purpose of plate counts resulted in the concomitant dilution of the inhibitor to a level beyond its effective inhibitory concentration.

Assay procedure for injury and recovery. The plating technique used to monitor growth (described above) was also used to assay both injury and recovery. Organisms having the ability to produce colonies on TSA were either uninjured or had been repaired subsequent to injury. Recovery, after sublethal heating, was recognized on the basis of rapid increase in plate count numbers. The presence of recovery was a priori evidence that the initial fall in plate count numbers was injury and not death.

Extraction and colorimetric determination of RNA. The method employed for extraction and colorimetric determination of RNA was a modification of the Schmidt and Thannhauser procedure (21). Harvested cells were washed in 0.1 M K_2HPO_4 - KH_2PO_4 buffer (pH 7.2) and then acid-extracted in 0.2 N HClO_4 at 0 to 4 C. The cells were then incubated for 2 h at 37 C in 0.3 N KOH to accomplish RNA hydrolysis, and cooled to 0 C, and 1.2 N HClO_4 was added to precipitate deoxyribonucleic acid (DNA) and protein. The supernatant fluids from this and from the precipitate washed with 0.2 N HClO_4 were combined for RNA estimation by the orcinol reaction (22).

Isotopic labeling of RNA. The ^{14}C -labeled RNA was prepared by the addition of 0.5 μCi of uracil-2- ^{14}C per ml of an actively growing culture of *P. fluorescens* at 25 C. When the culture reached the stage at which it was normally shifted to 36 C (injury and recovery procedure), the cells were harvested and the control RNA was extracted. The preparation of ^3H -labeled RNA was achieved by adding 1.0 μCi of uracil-6- ^3H per ml of culture at the end of the 2-h heating period. After the 1-h sample was removed, the ^3H label was chased by adding an excess of unlabeled uracil.

RNA extraction and fractionation by polyacrylamide gel electrophoresis. RNA was extracted from normal injured and recovered cells by the modified phenol-Duponal method reported by Sypherd and Fansler (25). After extraction, the RNA was separated

by polyacrylamide gel electrophoresis and assayed by methods previously described (26).

RESULTS

When an actively growing culture of *P. fluorescens* was transferred from its optimal growth temperature, 25 C, to a temperature just above its growth maximum, 36 C, the cells immediately stopped growing and exhibited a death phase (Fig. 1). After 2 h of such exposure, the culture was returned to its optimal temperature, but the TSA count continued to fall for an additional hour, dropping to a level of only 1% of the initial numbers exposed. This fall in TSA count represented a typical survivor curve so that the cells comprising the other 99% would normally be classified as dead, thereby implying that the majority of the population had succumbed to the heat treatment.

In the following 2 h, the viable cell count increased, but at a 10-fold greater rate than would be expected for growth at this temperature. Such a rapid increase in numbers after a heat treatment is characteristic of the cell injury-recovery phenomenon (4). This conjecture was further supported by the failure of CM

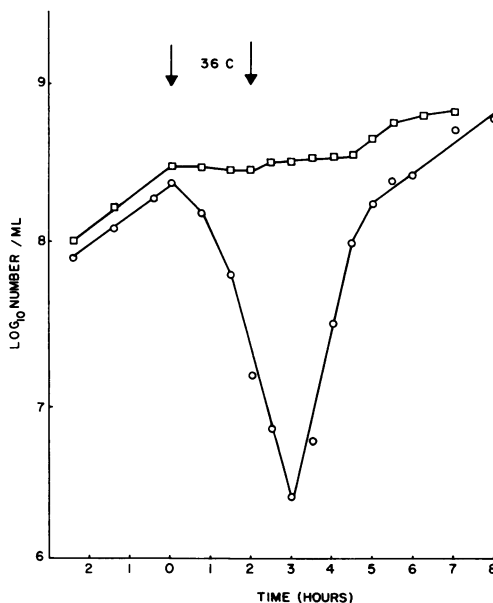


FIG. 1. The changes in total numbers and "viable" numbers during injury and recovery of *Pseudomonas fluorescens*. Exponentially growing cells were heated at 36 C for 2 h in defined growth medium and then recovered in the same medium at 25 C. Effects were monitored by surface plating and by direct microscope count. Symbols: O, surface plating on Trypticase soy agar; □, direct microscope count.

to inhibit the rapid increase in numbers (Fig. 2). The rapid decrease in the conventional plate count on TSA did not, therefore, represent the total number of viable cells in the culture flask. Also the subsequent rapid increase in numbers on TSA did not reflect cell multiplication but, rather, was due to the continuing repair of the heat-treated cells. After this period, the TSA count assumed a rate of increase similar to that of the culture prior to the heat treatment. This change in rate coincided with an increase in the direct count, confirming that multiplication had begun.

Effect of protein and RNA synthesis inhibitors on recovering cells. Injury and recovery systems have been further characterized on the basis of requirements for RNA and protein synthesis. The response depicted in Fig. 2 was obtained when recovery of sublethally heated *P. fluorescens* was examined in the presence of

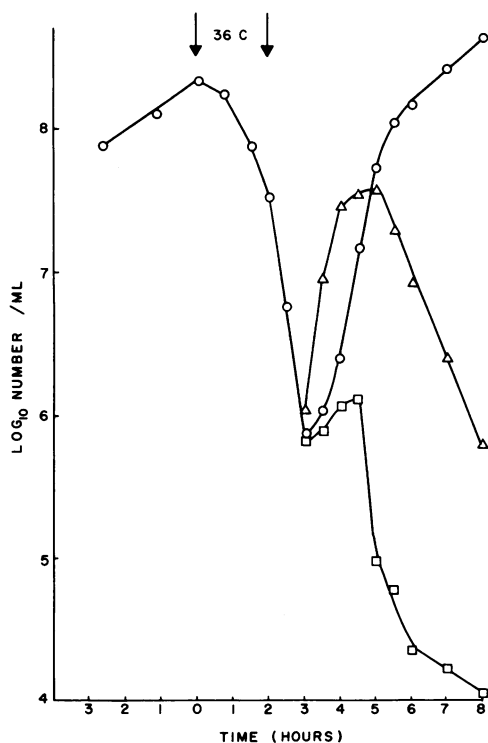


FIG. 2. Effect of chloramphenicol and rifamycin on the recovery of heat-treated *Pseudomonas fluorescens*. The culture was heated for 2 h at 36 C, returned to 25 C for 1 h, subdivided, and allowed to recover in the presence of the antibiotics. The cells were diluted in 0.1% peptone and plated on Trypticase soy agar. Symbols: O, cells recovered in defined medium (DM); Δ, cells recovered in DM plus chloramphenicol (final concentration 100 μg/ml); □, cells recovered in DM plus rifamycin (final concentration 10 μg/ml).

CM, a potent inhibitor of protein synthesis, and RM, an inhibitor whose mode of action is to bind irreversibly to DNA-dependent RNA polymerase. The heated cells were returned to the optimal temperature for 1 h at which point the antibiotics CM and RM were added at final concentrations of 100 and 10 μg/ml, respectively.

The addition of CM to the medium 1 h after completion of sublethal heating did not hinder the subsequent increase in the TSA count normally expressed at this time. In fact, the CM stimulated an even faster recovery during the first hour than that exhibited by the control sample. In the following hour, the plate counts plateaued and then began to decline. These results suggested that protein synthesis was not a dominant factor in the repair of the thermally injured cells and of course substantiate the earlier statements that the increase in TSA counts reflect recovery and not growth. Multiplication in the control culture (Fig. 2) commenced at about the 5-h point from a population considerably less than originally exposed. Hence, at least a portion of the exposed cells was killed by exposure to 36 C and not subject to recovery. At this 5-h point where multiplication would normally resume, the CM-containing medium then showed a decline in viable counts.

Only a slight increase in the TSA count was observed when RM was added to the medium, and a sharp decline was noted at 4.5 h (Fig. 2). The injury induced by heating *P. fluorescens* was therefore dependent on RNA synthesis for repair.

The same antibiotics were added immediately after the sublethal heat treatment. As in the previous experiment, there was a prompt response to the CM which not only counteracted the expected decrease in TSA count but, indeed, caused a pronounced increase in the count, prior to exerting its effect as a lethal agent (Fig. 3). This provided further evidence that protein synthesis was unnecessary for recovery per se but essential for subsequent growth and multiplication.

When RM was included in the medium after heat treatment, the TSA count immediately fell, indicating that no recovery was taking place. The initial rate of decrease in TSA count was somewhat less than in the control. However, the count continued to drop after the 3-h point when the control exhibited the beginning of normal recovery and the rate of fall increased markedly beyond 4.5 h.

Leakage of 260-nm absorbing material. Cell membrane damage and degradation of

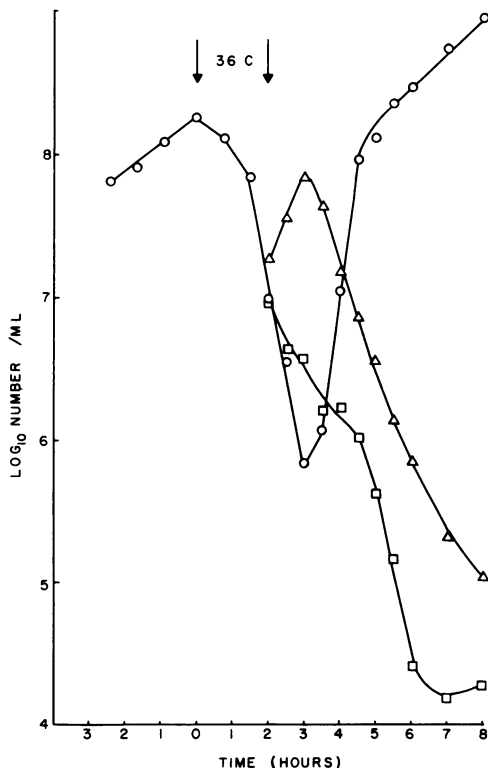


FIG. 3. Effect of chloramphenicol and rifamycin on the recovery of heat-treated *Pseudomonas fluorescens*. The culture was heated for 2 h at 36 C, subdivided, and allowed to recover in the presence of the antibiotics at 25 C. The cells were diluted in 0.1% peptone and plated on Trypticase soy agar. Symbols: ○, cells recovered in defined medium (DM); △, cells recovered in DM plus chloramphenicol (final concentration 100 µg/ml); □, cells recovered in DM plus rifamycin (final concentration 10 µg/ml).

RNA have often been demonstrated indirectly by the leakage of 260-nm absorbing compounds into the suspending menstrium. The release of such material was followed both during and subsequent to the heating of the culture at 36 C (Fig. 4). The exponential release of 260-nm absorbing material, stimulated by exposure to the elevated temperature, was maintained during the complete 2-h heating period. Subsequent to this time, 260-nm absorbing material continued to be released but at a slower rate. Finally, a reduction in the rate of release took place 3 h after the heating period (the 5-h point); significantly, this corresponded to that time at which multiplication resumed in the culture.

Degradation and resynthesis of RNA. The results presented in Fig. 4 were presumptive evidence that RNA degradation was stimulated

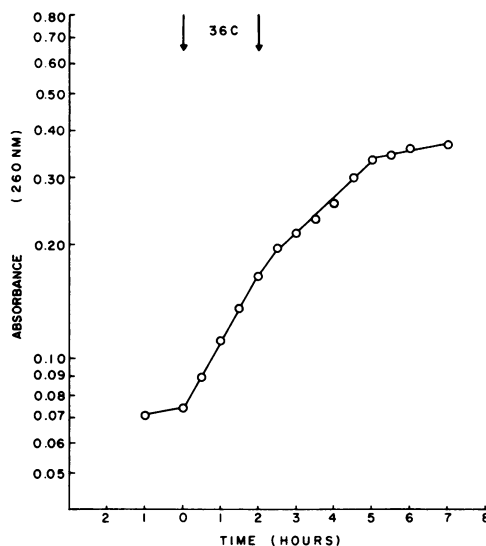


FIG. 4. Cellular leakage of 260-nm absorbing materials into the growth medium both during and after sublethal heating of *Pseudomonas fluorescens*. Exponentially growing cells at 25 C were heated at 36 C for 2 h and then returned to 25 C. The absorbance was determined on the culture medium after the removal of cells using a membrane filter of pore size 0.22 µm.

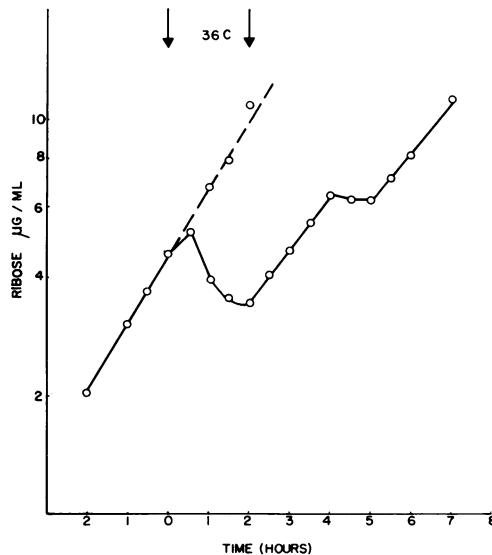


FIG. 5. The in vivo degradation and resynthesis of RNA during heat treatment and recovery of *Pseudomonas fluorescens*. Exponentially growing cells at 25 C were heated at 36 C for 2 h and then returned to 25 C. The RNA content of the cells was assayed colorimetrically using the orcinol reaction with ribose as a standard.

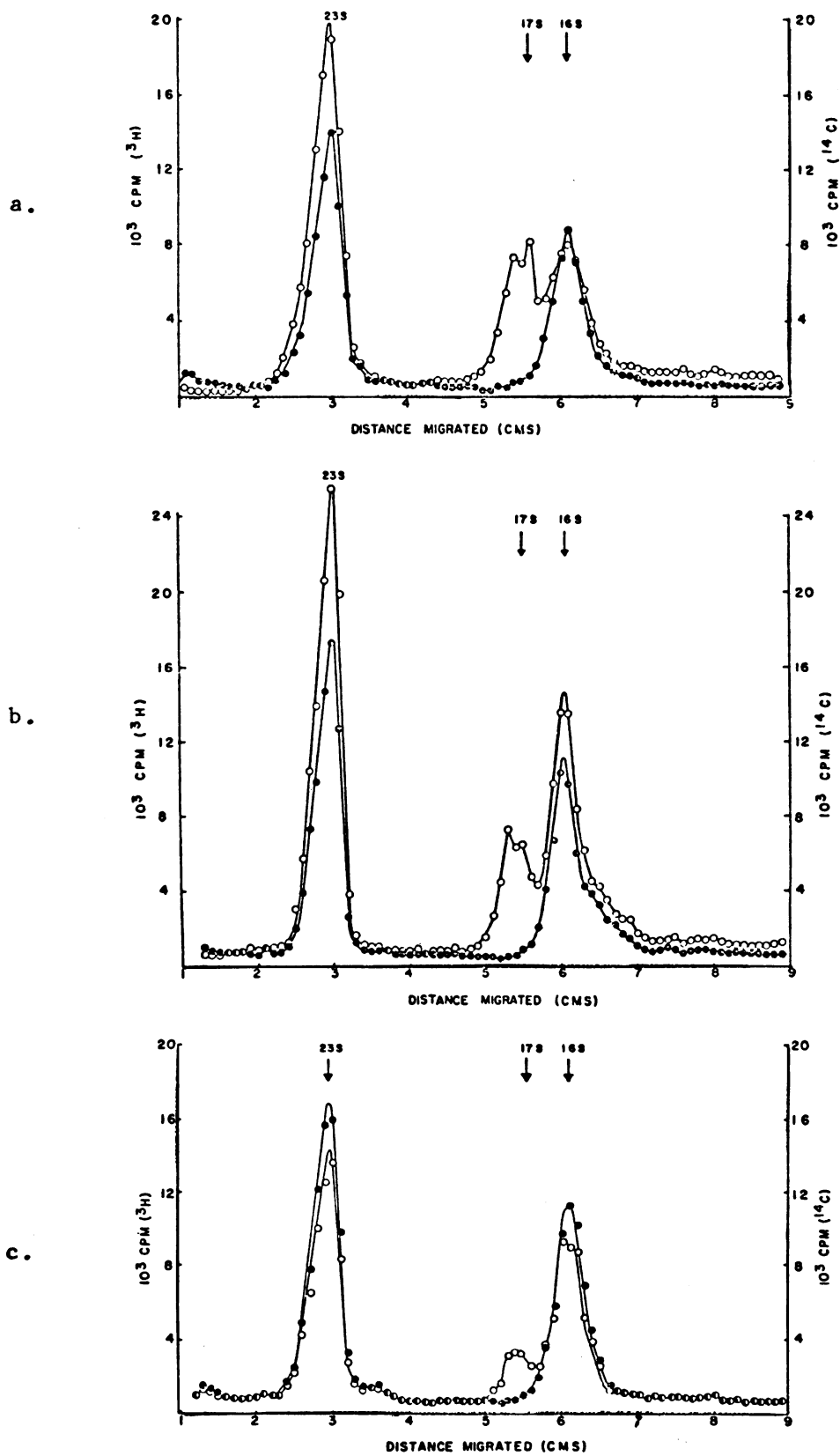


FIG. 6. Synthesis of rRNA in *Pseudomonas fluorescens* after heating, and the maturation of precursor RNA during recovery and subsequent growth. Normal cells were steady-state labeled with uracil-2-¹⁴C for 10 h prior to harvesting and extraction of RNA. Unlabeled cells were heated at 36 C for 2 h and then returned to 25 C in the

by the heat treatment. That RNA degradation, in fact, took place in this system was demonstrated by colorimetric analysis. The *in vivo* degradation of RNA during heat treatment and resynthesis during recovery is shown in Fig. 5. There was an immediate effect on the RNA component with a net loss of approximately 30% during the heating period. Net synthesis is evident as soon as the culture was returned to the optimal temperature and at a rate only marginally lower than that exhibited by the control cells. The results in Fig. 5, therefore, demonstrate that not only was RNA degradation stimulated by the heat treatment but also that RNA biosynthesis occurred subsequent to this time and was involved in the recovery of the cells. There is, however, a dramatic change in the rate of RNA biosynthesis in the 4- to 5-h region. This is the period just prior to cell multiplication.

The resynthesis of RNA after heat treatment was examined more closely. Specifically, resynthesis of rRNA was investigated since this is the major RNA component of the cell. The heat-treated cells were allowed to recover in the presence of uracil-6-³H at 25 C. A suitable fraction of cells was harvested after 60 min, a chase was instituted, and samples were then taken after a further 90 and 240 min. The extracted RNA was then subjected to coelectrophoresis with ¹⁴C-labeled control RNA (Fig. 6a, b, and c). Both mature 23 and 16S RNA species were synthesized during the first 60 min following heat treatment (Fig. 6a), even though the TSA count was still falling during this period. In addition to the presence of the two mature species, the profile of ribosomal RNA (rRNA) from the recovering cells is conspicuous in each case in that it contains a third peak in the 17S region. There is a definite movement of label from the 17 to the 16S region after the chase with cold uracil (Fig. 6b and c), indicating that the rRNA in the 17S region was a functional precursor form of the mature 16S species. This unusual accumulation of precursor 16S RNA demonstrated the presence of a temperature-sensitive step in the rRNA maturation process. Some 17S RNA still persists even after growth has resumed in the culture (Fig. 6c), suggesting that complete repair failed to occur or that the precursor species was accumulated in excess during recovery.

presence of uracil-6-³H. Cells were harvested after 60 min, a chase was instituted, and samples were then taken after a further 90 and 240 min. The RNA samples extracted from the heat-treated cells were mixed with the ¹⁴C-labeled control RNA and subjected to coelectrophoresis on 3% polyacrylamide gels for 6 h at 5 mA/gel. Gels were then frozen, sliced into 1-mm slices, digested, and counted. (a) RNA from cells harvested 60 min after heat treatment. (b) RNA from cells harvested 150 min after heat treatment. (c) RNA from cells harvested 300 min after heat treatment; ●, uracil-2-¹⁴C counts; ○, uracil-6-³H counts.

DISCUSSION

The effect of thermal stress was investigated under conditions of minimal manipulation in an effort to achieve a system where the temperature shift would constitute the only variable. The culture was therefore grown, heated, and allowed to recover in the same menstruum. Any additional stresses which would be imposed on the injured cells due to further variation in temperature during centrifugation or due to the use of different media for washing and resuspension were therefore averted. Measurable injury was achieved by shifting the culture to a growth-restricting temperature close to that of the maximum for growth. This provided a stress condition which met the objectives outlined above.

Both injury and recovery could be assayed on a complex medium (TSA) commonly used in the quantitation of microorganisms. With unstressed cells, the direct count closely corresponded to the viable plate count on TSA. However, when cells received the heat treatment, the TSA plate counts exhibited a dramatic change in the numbers which developed, while the direct count remained essentially constant. In effect, the system conventionally used to determine the viability of a culture has been reported in this study to reflect injury of cells rather than loss of viability. In addition, since injury was demonstrable under such mild stress, the legitimacy of counts in any viability study should be carefully scrutinized. It must be remembered that such a mechanism may not necessarily occur in all bacterial species. Reasons for the failure of such a medium as TSA to permit the enumeration of heat-damaged cells of *P. fluorescens* are currently under investigation.

The inhibitor studies revealed that, while RNA synthesis was necessary for the recovery of *P. fluorescens*, the repair process was independent of protein synthesis. Variation is documented in this regard in that both RNA and protein synthesis are required for the recovery of *S. typhimurium* from thermal injury (27) while the recovery of *S. aureus*, *S. faecalis*, and *Bacillus subtilis* has been reported to be independent of protein synthesis (5, 13, 17, 23). In *P. fluorescens*, if the CM had a protective effect for injured cells (Fig. 2 and 3), in addition to the inhibition of protein synthesis, then it could be

suggested that protein synthesis was necessary for the latter stages of recovery. But substantial recovery did occur in the presence of CM. In *S. typhimurium*, one role of the necessary protein synthesis was the maturation of 17S RNA to 16S RNA during the latter stages of the recovery process (27). However, in the presence of CM, *S. typhimurium* showed no recovery as measured by increase in counts. Repair in the presence of CM for *P. fluorescens* therefore reflected the post-treatment synthesis of RNA shown in Fig. 5. Thus after dilution, the cells were able to synthesize protein and subsequently produce colonies on the plates. Even when the requirement for RNA synthesis was fulfilled (the 4-h point), protein synthesis was not possible in the flask since CM was still present, hence the subsequent decline in TSA count.

Degradation of RNA was evident not only from the release of 260-nm absorbing material into the medium but also from the reduction in the cellular content of this macromolecular species. The marked reduction in the rate of RNA synthesis 1 h prior to the commencement of cell division made it apparent that recovery involved repair at a site other than that involving resynthesis of degraded RNA. In other words, although RNA synthesis was necessary for repair, as demonstrated by the use of RM, mere synthesis of RNA by the injured cells was not sufficient to achieve recovery. However, loss of intracellular constituents in itself indicated that the cell membrane had been damaged. Thus growth presumably could only commence when repair to this cell membrane lesion was accomplished.

Abnormalities in the synthesis of rRNA were evident from the accumulation of precursor 16S RNA after the heating of the culture. The temperature-induced lesion in the maturation process was not absolute since both mature 23 and 16S RNA were also synthesized in the first hour following heat treatment, and yet injury, as assayed by the plate count on TSA, continued to take place during this period. However the initial stages of repair would not be reflected immediately in an increased TSA count if more than one lesion was involved, such as the cell membrane lesion referred to above.

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