

ELECTRON MICROSCOPY OF HEAT-INJURED AND REPAIRED *STAPHYLOCOCCUS AUREUS*

SUSAN B. JONES, SAMUEL A. PALUMBO and JAMES L. SMITH

Eastern Regional Research Center¹
Philadelphia, Pennsylvania 19118

Received for Review January 24, 1983
Accepted for Publication April 7, 1983

ABSTRACT

Changes in the ultrastructure of Staphylococcus aureus 196E during heating at 50°C in 0.1 M phosphate buffer, and during repair in nutrient medium were studied using transmission electron microscopy. Injury was assessed by differential plating on tryptic soy agar (TSA) + 1% pyruvate and on TSA + 7% NaCl. Injury was > 99% in the first 15 min. Mild aldehyde fixation or osmium tetroxide fixation gave good cellular preservation. The observation of ribosome-free areas in all heated cells at all times (≥ 15 min) of injury is consistent with published reports that rRNA destruction is a primary locus of injury in heated bacteria. Cells heated 45 min or longer, as well as cells starved 1-2 h in buffer at 35°C, generated a variety of internal membranes, typically near the DNA region. At 90 and 120 min, extreme alterations of structure were apparent indicators of cell death. Cells heated 90 and 120 min had virtually no ribosomes, exaggerated internal membranes, and surface blebs. During repair of cells injured 30 min, the population assumed normal appearance in 4 h, although some cells clearly were incapable of repair. After 6 h, the number of cells undergoing division increased. The reappearance of normal ultrastructure paralleled re-gaining of salt tolerance in the culture.

INTRODUCTION

Consequences of sublethal heat stress in bacteria have been studied extensively (Hurst 1977; Tomlins *et al.* 1972; Witter and Ordal 1977). When *Staphylococcus aureus* cells are heated at sublethal temperatures (50°-55°C), a specific set of biochemical effects occurs: reduced salt tolerance, destruction of 16S RNA and the 30S ribosomal subunit, leakage of intracellular constituents, altered membrane transport, and loss of many enzyme functions. Sublethal heat damage has been noted

¹ Agricultural Research Service, U.S. Department of Agriculture

in other Gram-positive cocci, *Bacillus*, *Clostridium*, as well as in Gram-negative rods (Hurst 1977). Repair of injured cells can occur under suitable culture conditions (Iandolo and Ordal 1966). While growth characteristics return to normal, enzyme function and active transport may not become fully functional (Bluhm and Ordal 1969; Hurst *et al.* 1973; Tomlins *et al.* 1971).

A few brief reports of the ultrastructural changes resulting from application of heat are available (Allwood and Russell 1969, 1970; Silva *et al.* 1978). One study (Silva and Sousa 1972) is a systematic electron microscopy investigation of the effects of heat on the bacterium *Bacillus cereus*.

In this report, we present the ultrastructure of *S. aureus* cells heated at 50°C for various times and after periods of repair following 30-min heating at 50°C. Two different cell-fixation protocols were used to maximize the expression of injury in the ultrastructure of the cells.

MATERIALS AND METHODS

Culture and Growth Conditions

All culture media were obtained from Difco. *Staphylococcus aureus* 196E was grown for 16 h at 35°C in a TSB shake flask (100 ml tryptic soy broth in a 1000-ml/Erlenmeyer flask) rotating at 200 rpm. Starting cell level was $\sim 5 \times 10^5$ /ml. The cells were in the stationary phase of growth when harvested.

Heat Injury

After 16-h incubation, cells were removed by centrifugation at $16,300 \times g$ in the cold (2°-4°C), washed three times with 0.1 M potassium phosphate buffer (pH 7.2), and resuspended in 5 ml of buffer. The cell suspension was added to 200 ml of the same buffer at 50°C, and was maintained at this temperature with agitation. A control was prepared by inoculating the cell suspension into the same buffer held at 35°C. At intervals, aliquots of the heated suspension and control were removed for plating and electron microscopy. Heated samples were cooled immediately in an ice bath.

Repair

Cells heated 30 min at 50°C were collected by centrifugation and suspended in 200 ml of TSB in a shake flask. The cell suspension was incubated for 11 h at 35°C and agitated at 200 rpm. Aliquots were removed periodically for plating and microscopy.

Measuring Injury

Injury was assessed by surface-plating dilutions made in 0.1% peptone water on tryptic soy agar + 1% Na pyruvate (TSAP) and on tryptic soy agar + 7% NaCl (TSAS). TSAP supports growth of both injured and noninjured cells (Martin *et al.* 1976) while TSAS supports only noninjured cells (Iandolo and Ordal 1966). This method of measuring injury relies on the increased salt sensitivity of heat-injured *S. aureus*.

Electron Microscopy

Cells in either phosphate buffer heating menstruum or TSB recovery medium were mixed with an equal volume of fixative to give these conditions: 1% glutaraldehyde, 1.5% formaldehyde, 50 mM sodium cacodylate, pH 6.2, 10 mM MgCl₂, and 150 mM NaCl for 30 min, followed by a 24-h rinse in the same buffer (Ghosh 1977). Secondary fixation was performed by exposing the cells to 1% osmium tetroxide overnight followed by 0.5% uranyl acetate, 90 min, both in veronal acetate buffer. A second fixation protocol employed osmium tetroxide alone (Ryter and Kellenberger 1958). Sample size was approximately 4000 μ g (dry weight) of cells. Fixed bacterial cells in agar blocks were dehydrated with an ethanol series followed by propylene oxide, and embedded in Epon 812 epoxy resin. Ultrathin sections (silver-gray, 600Å) were cut with a diamond knife and post-stained with uranyl acetate and lead citrate.

Mild conditions of aldehyde fixation (Ghosh 1977) followed by extensive washing were essential for fixing *S. aureus* for electron microscopy. When a 30-min time of fixation was exceeded, or the glutaraldehyde concentration raised, the bacteria in thin section frequently had holes in the centers. Specifically, the DNA regions were poorly retained. This defect did not occur in cells fixed with OsO₄ alone and presumably resulted from crosslinking of the cell wall components by glutaraldehyde (Gorman *et al.* 1980). Such cross-linking may either prevent efficient penetration of fixative into the cell or prevent efficient removal of unbound glutaraldehyde during washing steps.

RESULTS

In injury experiments, the number of viable cells remained constant during 45 min of heating at 50°C, while >99% injury occurred in the first 15 min (Fig. 1). After 45-min heating, the number of viable cells began to fall (upper curve, Fig. 1) so that by 90 min the culture

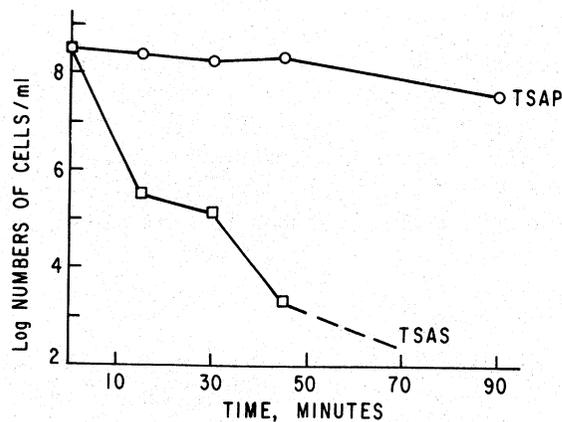


FIG. 1. INJURY OF STAPHYLOCOCCUS AUREUS 196E IN 0.1 M POTASSIUM PHOSPHATE BUFFER, pH 7.2, AT 50°C

Counts in (O) tryptic soy agar + 1% Na pyruvate (TSAP) and (□) tryptic soy agar + 7% NaCl (TSAS). TSAP counts enumerate both injured and noninjured cells, while TSAS enumerates only noninjured cells. The difference between the two counts at any time is the magnitude of injury.

contained a significant percentage of dead cells. No injury occurred in the control held in 0.1 M potassium phosphate, pH 7.2, at 35°C for up to 2 h.

The ultrastructure of control and injured cells is shown in Fig. 2. The normal ultrastructure of *S. aureus* cells (Fig. 2a) fixed as described with aldehydes followed by osmium tetroxide and uranyl acetate showed a uniformly granular cytoplasm containing ribosomes that were poorly contrasted with the background in stationary cells. Even in very thin sections, the ribosomes were not distinct particles, but instead gave a mottled appearance to the cytoplasm. Control cells contained dispersed and undefined DNA regions, characteristic of this fixation (Séchaud and Kellenberger 1972) and a few simple mesosomes at the septum and near the cell periphery.

After 30 min of injury (Fig. 2b), much of the cytoplasm was smooth, structureless, and less intensely stained than the control. These areas appeared to be free of ribosomes. Mesosomes were unchanged, except to stand out more prominently against the lighter background. Under our conditions of fixation, we saw heavy staining particulates within the cytoplasm at all times of heating. This may have been residual ribonucleoprotein, or an artifact of preparation. If such deposits were present in the control, they were obscured by the normal ultrastructure of the cytoplasm-ribosome matrix.

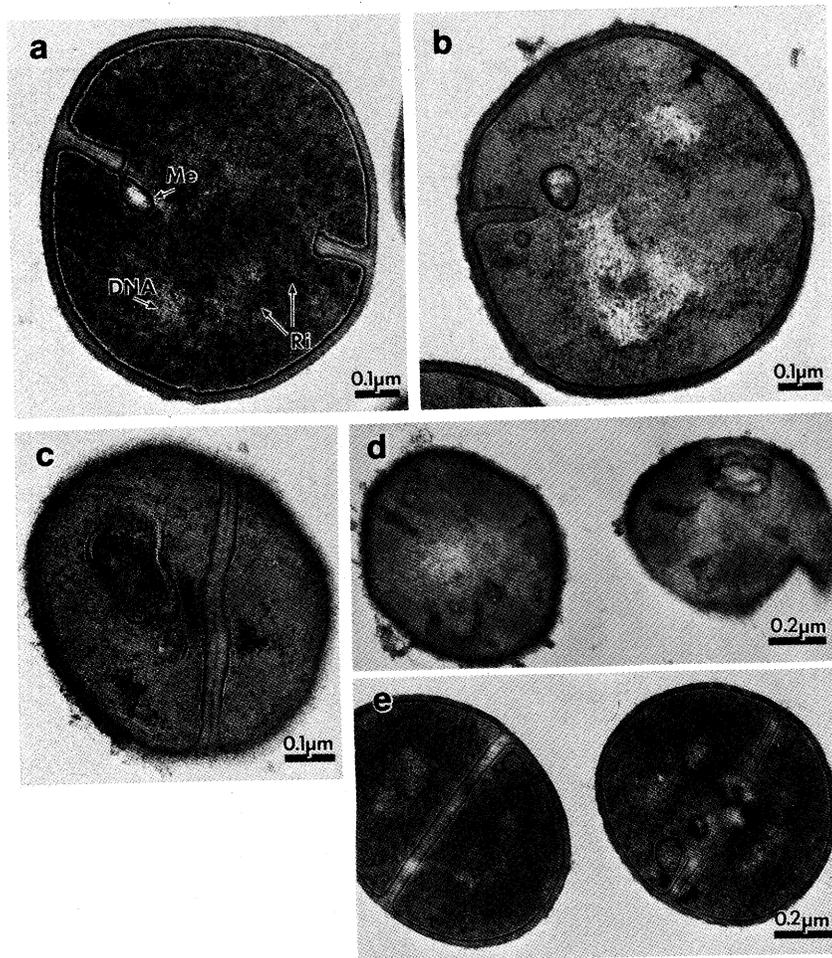


FIG. 2. HEAT-INJURED *STAPHYLOCOCCUS AUREUS* 196E, STATIONARY PHASE, FIXED WITH 1% GLUTARALDEHYDE + 1.5% FORMALDEHYDE, FOLLOWED BY OSMIUM TETROXIDE AND URANYL ACETATE
 Fig. 2a—unheated control; Fig. 2b, c, d—cells heated at 50°C in 0.1 M phosphate buffer for 30, 45, and 90 min, respectively; Fig. 2e—held in 0.1 M phosphate buffer at 35°C for 2 h. Ri = ribosome, Me = mesosome.

The appearance after 45 min of injury was similar to 30 min (Fig. 2c) except that internal membranes were increased in amount and complexity. At 90 min (fig. 2d), with ~50% of the cells dead, the ultrastructure showed exaggerated internal membranes, cytoplasm devoid of

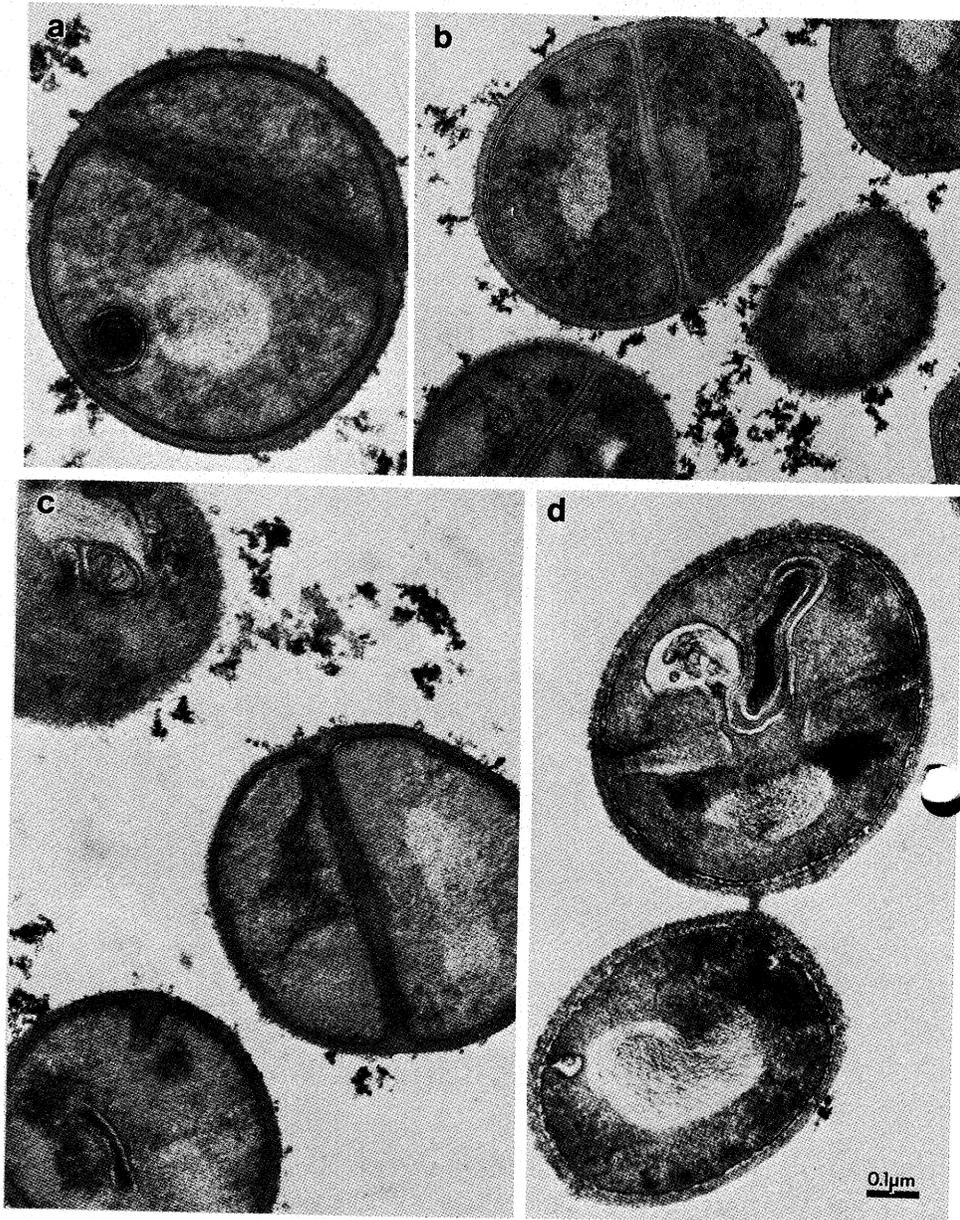


FIG. 3. INJURED CELLS FIXED WITH OSMIUM TETROXIDE FOLLOWED BY URANYL ACETATE
Fig. 3a—control cells. Fig. 3b, c, d—cells injured 15, 90, and 120 min, respectively.

ribosomes, clusters of granular deposits in the cell, surface blebs, and many misshapen cells. There was no appreciable lysis during injury.

Cells held up to 2 h in buffer at 35°C (Fig. 2e) did not acquire the structural characteristics of injury. However, compared to the normal control, these starved cells had a more granular cytoplasm, and atypical internal membranes.

The same effects of applied heat were seen in cells fixed with osmium tetroxide alone (Fig. 3). This fixation renders the nucleoid as unified and centrally located and gives rise to (or preserves) elaborate mesosomes associated with the DNA region (Fig. 3a). After 15-min injury (Fig. 3b), ribosome-free areas were clearly seen. At 90 min (Fig. 3c), cytoplasm had lost considerable electron density. Heavy-staining deposits were present and internal membranous forms were increased. At 120 min of heating (Fig. 3d), DNA was fibrillar and mesosomes were exaggerated multilamellar and vesicular forms.

Cells injured 30 min (>99% injury) were incubated for repair in TSB at 35°C. Differential plating (Fig. 4) showed virtually complete recovery of salt tolerance in 4 h.

The ultrastructure of cells harvested during repair is shown in Fig. 5. After 2 h (Fig. 5a), cells still contained areas of low-stain uptake and

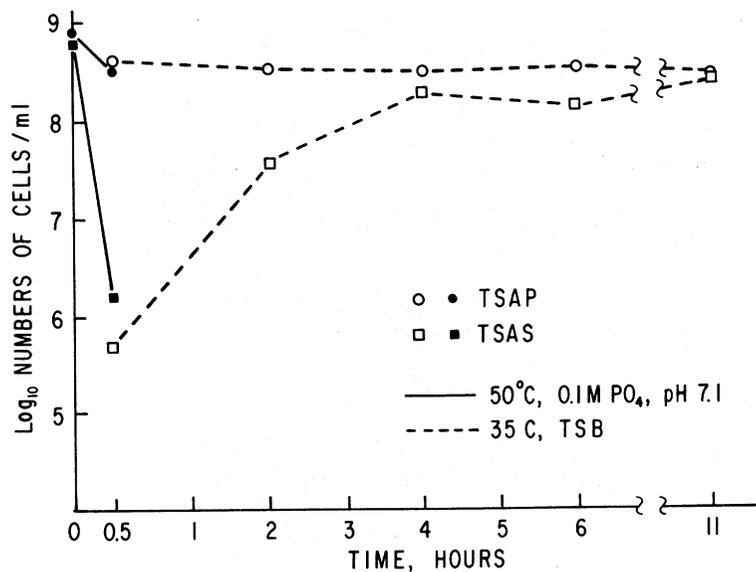


FIG. 4. INJURY(—) OF *S. AUREUS* 196E HEATED AT 50°C IN 0.1 M POTASSIUM PHOSPHATE, pH 7.1, FOR 30 MIN, AND REPAIR (---) IN TRYPTIC SOY BROTH (TSB) AT 35°C
Salt tolerance was largely recovered by 4-h incubation in repair medium.

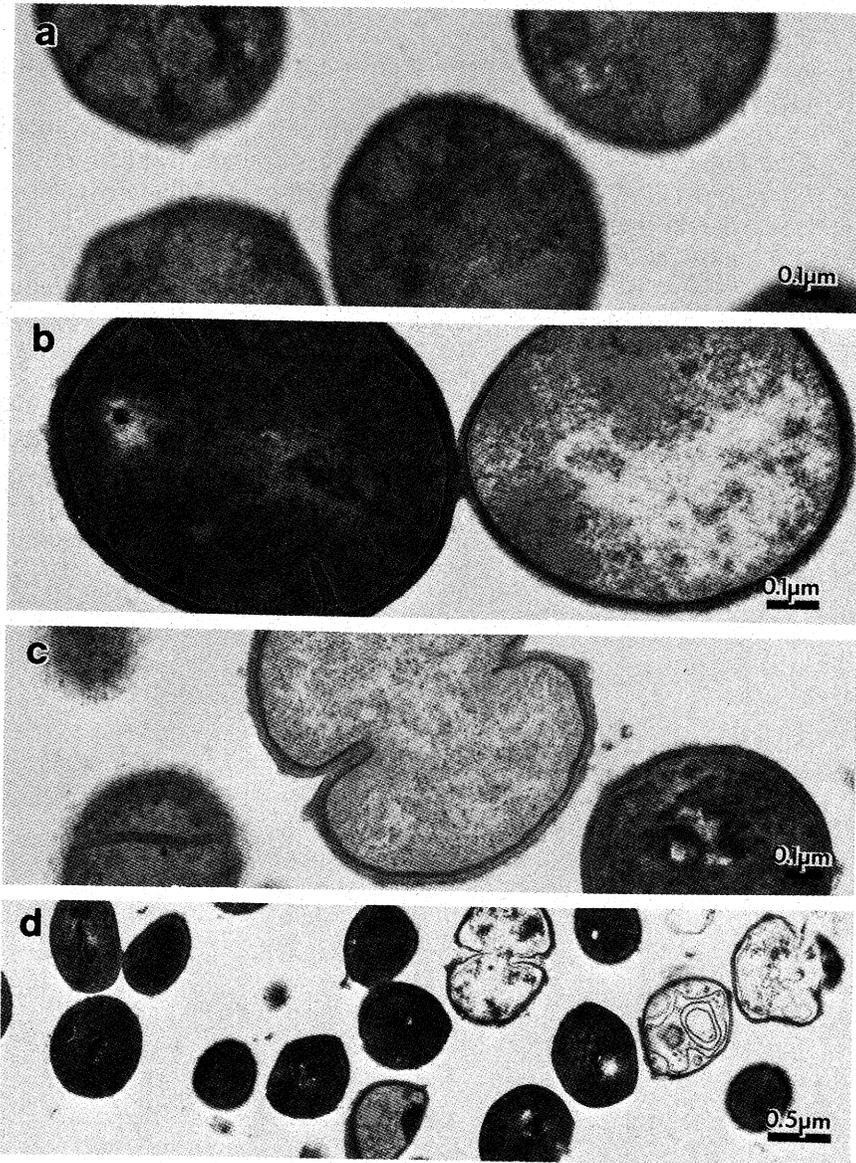


FIG. 5. REPAIR OF HEAT-INJURED *S. AUREUS* 196E IN TSB AT 35°C
Fixed with 1% glutaraldehyde + 1.5% formaldehyde, followed by osmium tetroxide and uranyl acetate. Figures 5a, b, c, d—cells repaired for 2, 4, 6, and 11 h, respectively.

the dark deposits characteristic of injury. However, mottling that suggested reappearance of ribosomes was clearly superimposed on the injury pattern. At 4 h (Fig. 5b), the population as a whole had regained normal appearance. Those cells incapable of repair could be easily distinguished, for example, the cell on the right in Fig. 5b. At 6 h (Fig. 5c), approximately 20% of the cells were actively dividing. Active division was defined operationally as septated cells that showed some evidence of separation. However, the observed divisions were usually abnormal, characterized by inaccurate septations and separations, death and lysis of daughter cells. At 11 h repair (Fig. 5d), the probable result of abnormal cell divisions was observed as lysed cells in an otherwise structurally normal population.

DISCUSSION

Ribosomal breakdown, substantiated repeatedly as a primary locus of bacterial injury from heat stress (Miller and Ordal 1972; Rosenthal *et al.* 1972; Tomlins and Ordal 1971; Weiss and Tal 1973) was visualized in the ultrastructure of *S. aureus* cells as smooth, structureless patches within the cytoplasm. This structural alteration was already well advanced after 15 min of heating at 50°C. Published kinetic data show that 90% of isolated *S. aureus* 30S ribosomal subunits are destroyed in 7.25 min of heating in phosphate buffer at 50°C (Iandolo 1974). While destruction *in vivo* is expected to be slower, nevertheless the injury lesions are established at 15 min. Cell injury data (Fig. 1) show 99.9% injury effected in the first 15 min. Decimal reduction times (times at which 90% injury is achieved) for *S. aureus* are 3 min at 55°C (Walker and Harmon 1966) and 9 min at 52°C (Hurst *et al.* 1974).

In an ultrastructural study of heat-injured *Bacillus cereus*, Silva and Sousa (1972) observed "dense blocks" in the cytoplasm of cells heated at 65°C for 2 min or longer. After 15 min at 65°C or 5 min at 100°C, these dense areas could be dissolved by pronase and thus were inferred to be coagulated protein. The cells in question were nonviable. It is unlikely that the cytoplasmic patches we observed in cells heated at 50°C were coagulated protein. Allwood and Russell (1968, 1969), from examination of metabolic pools and physical properties of heated *S. aureus*, concluded that coagulation was a factor at 60°C but not at 50°C. Moreover, from differential plating, 99% of the cells heated 30 min in our experiments were viable and did repair subsequently. During repair, ribosomal subunit assembly in the first 90 min is accomplished with preexisting protein (Iandolo 1974; Rosenthal *et al.* 1972). This would be improbable if coagulation or irreversible denatu-

ration of cellular proteins had occurred. The patches that we observed suggest that ribosomal destruction caused by short exposure to sublethal heat (e.g., 15 min at 50°C) may occur in areas localized at the periphery of the cell, in contact with the cell membrane, and may emanate from multiple initiation centers along the membrane. One possible explanation of these observations is that leakage of a cellular constituent, e.g., Mg^{++} , may occur initially at a small number of sites. Loss of cellular Mg^{++} accompanies, and may govern, the breakdown of RNA (Hurst 1977; Hurst *et al.* 1974).

During heating, considerable lipid is lost. Hurst *et al.* reported 30% reduction in fatty acid content (1973) and a 40%-50% reduction in membrane polar lipids (Hurst *et al.* 1975) in *S. aureus* after 15 min at 52°C. It is interesting to note that during fixation of cells with aldehydes + osmium, the color of osmium-treated cells indicated that heated cells took up progressively less osmium tetroxide as the time of heating increased and the trend was reversed during repair. Osmium tetroxide is reduced to osmium black principally by cellular lipid, but also by ribonucleic acid (Stockert and Colman 1974), and to a limited extent, by protein (Nielson and Griffith 1979). Failure to take up osmium might be explained by the loss of both lipid and RNA from the cells during heating.

Cells heated 45 min or longer, as well as cells starved 1-2 h in buffer at 35°C, generated a variety of internal membrane-like forms, typically in or near the DNA region. Such structures appeared to be a cellular response to metabolic stress. At 90 and 120 min heating they developed into heavy-staining, vesiculated forms that probably indicated cell death. In no samples did we observe gaps in the plasma membranes such as were reported for *B. cereus* (Silva and Sousa 1972).

Repair in TSB is characterized by a 4-6-h lag period that coincides with complete regain of salt tolerance, regardless of the degree of injury (<90% to >99.5%) (Iandolo 1974). Lipid biosynthesis (Hurst *et al.* 1973), RNA synthesis, and assembly of ribosomal subunits (Rosenthal *et al.* 1972; Sogin and Ordal 1967) commence immediately. Protein synthesis (and therefore functional ribosomes) has been measured after 90 min of repair (Hurst *et al.* 1973). Our results are in complete accord with these biochemical studies. Partial restoration of ribosomes was evident at 2 h and the ultrastructure appeared normal by 4 h.

At the end of the extended lag period, exponential growth resumes at a rate equivalent to that of a control culture (Iandolo 1974). In our experiments, the culture could not achieve exponential growth because the cell numbers were already at limiting levels (10^9 /ml). What we observed, instead, at the end of the lag period, was a spurt of abortive cell division in those cells already committed to divide. DNA in *S.*

aureus is unaffected by heating and remains virtually constant through injury and most of repair (Allwood and Russell 1968, 1969). A net increase in DNA occurs late in the extended lag phase of repair, as it does in the late part of a normal lag phase of growth (Allwood and Russell 1969). The amount of active division we observed was similar to that usually observed in a culture just entering exponential growth, with the striking difference that in the repaired culture, much of the active division clearly resulted in cell death. The phenomenon was possibly a reflection of the limiting culture conditions or some remaining functional incompetence in the dividing cells.

In conclusion, our results show that sublethal heat treatment brings about changes on the ultrastructure of the *S. aureus* cell and that the changes are reversed during repair. The most evident and immediate difference between control and heated was in the appearance of the cytoplasm-ribosome matrix. Changes in the ultrastructure appeared to be progressive and paralleled changes in the salt tolerance of the culture during injury and repair.

ACKNOWLEDGMENTS

The authors wish to thank Dr. George Somkuti and Dr. Thomas Montville for helpful discussion of this manuscript, and Ruth Zabarsky, Tim Dobson, and Karen Sworen for their excellent technical assistance.

Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

REFERENCES

- ALLWOOD, M.C. and RUSSELL, A.D. 1968. Thermally induced ribonucleic acid degradation and leakage of substances from the metabolic pool in *Staphylococcus aureus*. *J. Bacteriol.* *95*, 345-349.
- ALLWOOD, M.C. and RUSSELL, A.D. 1969. Thermally induced changes in the physical properties of *Staphylococcus aureus*. *J. Appl. Bacteriol.* *32*, 68-78.
- ALLWOOD, M.C. and RUSSELL, A.D. 1969. Growth and metabolic activities of heat treated *Staphylococcus aureus*. *J. Appl. Bacteriol.* *32*, 79-85.
- ALLWOOD, M.C. and RUSSELL, A.D. 1970. Mechanisms of thermal injury in nonsporulating bacteria. *Adv. Appl. Microbiol.* *12*, 89-119.
- BLUHM, L. and ORDAL, Z.J. 1969. Effect of sublethal heat on the metabolic activity of *Staphylococcus aureus*. *J. Bacteriol.* *97*, 140-150.

- GHOSH, B.K. 1977. Techniques to study the ultrastructure of microorganisms, pp. 31-38. In *CRC Handbook of Microbiology*, 2nd edition, (A.I. Laskin and H.A. Lechevalier, eds.) CRC Press, Inc. Cleveland, Ohio.
- GORMAN, S.P., SCOTT, E.M. and RUSSELL, A.D. 1980. Antimicrobial activity, uses, and mechanism of action of glutaraldehyde. *J. Appl. Bacteriol.* *48*, 161-190.
- HURST, A. 1977. Bacterial injury: A review. *Can. J. Microbiol.* *23*, 935-944.
- HURST, A., HUGHES, A., BEARE-ROGERS, J.L. and COLLINS-THOMPSON, D.L. 1973. Physiological studies on the recovery of salt tolerance by *Staphylococcus aureus* after sublethal heating. *J. Bacteriol.* *116*, 901-907.
- HURST, A., HUGHES, A. and COLLINS-THOMPSON, D.L. 1974. The effect of sublethal heating on *Staphylococcus aureus* at different physiological ages. *Can. J. Microbiol.* *20*, 765-768.
- HURST, A., HUGHES, A., COLLINS-THOMPSON, D.L. and SHAH, G. 1974. Relationship between loss of magnesium and loss of salt tolerance after sublethal heating of *Staphylococcus aureus*. *Can. J. Microbiol.* *20*, 1153-1158.
- HURST, A., HUGHES, A., DUCKWORTH, M. and BADDILEY, J. 1975. Loss of D-alanine during sublethal heating of *Staphylococcus aureus* S6 and magnesium binding during repair. *J. Gen. Microbiol.* *89*, 277-284.
- IANDOLO, J.J. 1974. Repair of stress-induced macromolecular alterations in *S. aureus*. *Ann. N.Y. Acad. Sci.* *236*, 160-174.
- IANDOLO, J.J. and ORDAL, Z.J. 1966. Repair of thermal injury of *Staphylococcus aureus*. *J. Bacteriol.* *91*, 134-142.
- MARTIN, S.E., FLOWERS, R.S. and ORDAL, Z.J. 1976. Catalase: Its effect on microbial enumeration. *Appl. Environ. Microbiol.* *32*, 731-734.
- MILLER, L.L. and ORDAL, Z.J. 1972. Thermal injury and recovery of *Bacillus subtilis*. *Appl. Microbiol.* *24*, 878-884.
- NIELSON, A.J. and GRIFFITH, W.P. 1979. Tissue fixation by osmium tetroxide. A possible role for proteins. *J. Histochem. Cytochem.* *27*, 997-999.
- ROSENTHAL, L.J., MARTIN, S.E., PARIZA, M.W. and IANDOLO, J.J. 1972. Ribosome synthesis in thermally shocked cells of *S. aureus*. *J. Bacteriol.* *109*, 243-249.
- RYTER, A. and KELLENBERGER, E. 1958. Etude au microscope électronique de plasmas contenant de l'acid desoxyribonucleique. *Z. Naturforsch. Teil B.* *13*, 597-605.
- SÉCHAUD, J. and KELLENBERGER, E. 1972. Electron microscopy of DNA-containing plasms. IV. Glutaraldehyde-uranyl acetate fixation of virus-infected bacteria for thin-sectioning. *J. Ultrastruct. Res.* *39*, 598-607.
- SILVA, M.T. and SOUSA, J.C.F. 1972. Ultrastructural alterations induced by moist heat in *Bacillus cereus*. *Appl. Microbiol.* *24*, 463-476.

- SILVA, M.T., SOUSA, J.C.F. and BALASSA, G. 1978. Ultrastructural effects of chemical agents and moist heat on *Bacillus subtilis*. I. Effects on vegetative cells. *Ann. Microbiol. (Inst. Pasteur)* 129B, 363-375.
- SOGIN, S.J. and ORDAL, Z.J. 1967. Regeneration of ribosomes and ribosomal ribonucleic acid during repair of thermal injury to *Staphylococcus aureus*. *J. Bacteriol.* 94, 1082-1087.
- STOCKERT, J.C. and COLMAN, O.D. 1974. Observations on nucleolar staining with osmium tetroxide. *Experientia* 30, 751-752.
- TOMLINS, R.I. and ORDAL, Z.J. 1971. Precursor ribosomal ribonucleic acid and ribosome accumulation *in vivo* during the recovery of *Salmonella typhimurium* from thermal injury. *J. Bacteriol.* 107, 134-142.
- TOMLINS, R.I., PIERSON, M.D. and ORDAL, Z.J. 1971. Effect of thermal injury on the TCA cycle enzymes of *Staphylococcus aureus* MF31 and *Salmonella typhimurium* 7136. *Can. J. Microbiol.* 17, 759-765.
- TOMLINS, R.I., VAALER, G.L. and ORDAL, Z.J. 1972. Lipid biosynthesis during recovery of *Salmonella typhimurium* from thermal injury. *Can. J. Microbiol.* 18, 1015-1021.
- WALKER, G.C. and HARMON, L.G. 1966. Thermal resistance of *Staphylococcus aureus* in milk whey and phosphate buffer. *Appl. Microbiol.* 14, 584-590.
- WEISS, A. and TAL, M. 1973. *In vivo* thermal stability and activation of *Escherichia coli* ribosomes. *Biochemistry* 12, 4534-4540.
- WITTER, L.D. and ORDAL, Z.J. 1977. Stress effects and food microbiology. In *Antibiotics and Antibiosis in Agriculture*, (M. Woodbine, ed.) Butterworths, Inc., London.