Repair of Injury in Freeze-Dried *Salmonella anatum*  

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Repair of injury induced by freeze-drying *Salmonella anatum* in nonfat milk solids occurred rapidly after rehydration. Injury in surviving cells was defined as the inability to form colonies on a plating medium containing deoxycholate. Death was defined as inability to form colonies in the same medium without this selective agent. The rate of repair of injury was reduced by lowering the temperature from 35°C to 10°C and was extremely low at 1°C. Repair was independent of influence of pH between 6.0 and 7.0. Repair did not require synthesis of protein, ribonucleic acid, or cell wall mucoprotein, but did require energy in the form of adenosine triphosphate (ATP) synthesized through oxidative phosphorylation. The requirement for ATP was based on dinitrophenol or cyanide interference with repair. Dinitrophenol activity was pH-dependent; no repair occurred at pH 6.0 and some repair was observed at pH 6.5 and above. Injured cells were extremely sensitive to low concentrations of ethylenedinitrilotetraacetate. This indicated that freeze-drying injury of *S. anatum* may involve the lipopolysaccharide portion of the cell wall and that repair of this damage requires ATP synthesis.

Injury of freeze-dried microorganisms and repair of this damage upon rehydration in a suitable environment has been demonstrated (24, 26). The exact site(s) of this injury has not been specifically determined, but there are indications that cell wall, cell membrane, and ribonucleic acid (RNA) might be involved (26, 29). Freeze-dried cells, with defective permeability due to membrane damage, release RNA fragments and amino acids upon rehydration (26, 29).

The nature of the repair process that occurs upon rehydration of damaged cells has not been fully characterized. After rehydration, injured cells first regain their altered permeability and then initiate RNA synthesis followed by protein synthesis. This repair process ceases as deoxyribonucleic acid (DNA) synthesis begins (26).

In thermally stressed cells, alteration of permeability and degradation of RNA, especially of ribosomal RNA, have been reported (1, 11, 27). During repair, the heat-stressed cells restore their altered permeability and synthesize ribosomal RNA and protein (16, 21). Release of biologically active peptides from frozen damaged cells also has been observed (15).

In this paper, the process of repair of injury in

*Salmonella anatum* freeze-dried in milk is described and characterized. A preliminary report of these findings was presented previously (B. Ray, J. J. Jeżeski, and F. F. Busta, Bacterial Proc., p. 3, 1970).

**MATERIALS AND METHODS**

The test organism, *S. anatum* NF3, was propagated, frozen, and freeze-dried in reconstituted sterile 10% solids nonfat dry milk as described previously (24). The plating media used were xylose-lysine-deoxyagar (XLP) and XLP with 0.25% sodium deoxycholate added (XLDP). Freeze-dried samples obtained from 10 ml of 10% solid nonfat milk containing *S. anatum* cells were stored at 25°C for 24 hr before use.

Each sample was rehydrated rapidly (5 sec) with 10 ml of sterile water at 25°C and mixed for 1 min on a Vortex mixer (Scientific Products, Evanston, Ill.). These samples contained approximately 10⁸ cells/ml. A 1-ml portion was diluted with 9 ml of water and mixed for 30 sec. From this diluted sample, a 1-ml portion was added to 9 ml of test solution in a screw-cap test tube (150 by 25 mm) and was incubated at 25°C. The test solution contained one or more of the chemical agents or 9 ml of water as a control. At indicated time intervals, the test solutions were sampled, serially diluted, and plated on the two media. The first plating time after rehydration was noted for each test system. A 0.1-ml portion was surface-plated in each of three plates of each medium. When fresh cells were used as a control, a 24-hr milk culture incubated at 35°C was diluted with milk to give about 10⁸ cells/ml, and 10-ml test quantities were tested in the same manner. The method for calculating

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the percentage of injury or death has been stated previously (24).

Chemicals used in test solutions were potassium penicillin G (E. R. Squibb & Sons, New York, N.Y.); d-cycloserine, actinomycin D, 2,4-dinitrophenol, and adenosine-5'-triphosphate disodium (all from Mann Research Laboratories, New York, N.Y.); chloramphenicol (Parke-Davis & Co., Detroit, Mich.); tetracycline HCl (Lederle Laboratories Division, Pearl River, N.Y.); sodium cyanide (J. T. Baker Chemical Co., Phillipsburg, N.J.); sodium deoxycholate (Difco); sodium dodecyl sulfate (K & K Laboratories, Plainview, N.Y.); and ethylenedinitrilotetraacetate (EDTA; Eastman Organic Chemicals, Rochester, N.Y.).

All of the chemicals were dissolved in sterile water in 10 times concentration, and 1-ml portions were used in the test solutions. The required amount of sterile distilled water was added to make a 9-ml volume of test solution. The test solutions with the sample contained about 0.1% milk solids (except for the control, which contained 9 ml of water). The stock solutions were stored at 5°C for no more than 7 days. Dinitrophenol (DNP) was dissolved in water by mild heat treatment. EDTA was prepared by dissolving disodium (2H2O) and tetrasodium (anhydrous) salts separately and then mixing them in proportion to give pH 7.0. The test solution of EDTA contained about 0.3 mm calcium (from 0.1% milk solids); thus, when 0.4 mm EDTA was used, only about 0.1 mm EDTA was available and not complexed with calcium.

RESULTS

Effect of incubation temperature on repair. Portions of a rehydrated and diluted freeze-dried sample were transferred to test solutions pretempered at 1 to 35°C. The test solutions were incubated at these temperatures for the 2-hr test period. Apparent populations on XLP remained essentially constant up to 2 hr at all test temperatures except for a gradual reduction in counts observed at 1°C (Fig. 1). The counts on XLDP increased at different rates at all temperatures. The slowest rate of increase was observed at 1°C. After 2 hr of incubation, only about 15 to 20% of the viable cells remained injured at 35 and 25°C, whereas about 80% remained damaged at 1°C. At 10 and 15°C, the rate and extent of repair was slower than at 25 and 35°C.

Effect of pH on repair. Repair was evaluated at four pH levels from 5.5 to 7.0 by adjustments of the test solutions with predetermined amounts of 0.1 N HCl and 0.1 N NaOH. The data in Fig. 2 show that, during a 2-hr test period, numbers observed on XLP remained constant at pH levels of

![Fig. 1. Effect of incubation temperature on repair of injury of freeze-dried Salmonella anatum. Times of first plating after rehydration were 3 min for 35°C sample, 5 min for 25°C, 7 min for 15°C, 9 min for 10°C, and 11 min for 1°C. The samples were plated on xylose-lysine-peptone-agar (XLP) and XLP with 0.25% sodium deoxycholate (XLDP). The test solutions contained about 0.1% milk solids.](http://aem.asm.org/)

![Fig. 2. Effect of pH on repair of injury of freeze-dried Salmonella anatum. Times of first plating after rehydration were 3 min for pH 7.0, 5 min for pH 6.5, 7 min for pH 6.0, and 9 min for pH 5.5. The samples were plated on xylose-lysine-peptone-agar (XLP) and XLP with 0.25% sodium deoxycholate (XLDP). The test solutions contained about 0.1% milk solids. Incubation was at 25°C.](http://aem.asm.org/)
6.0, 6.5, and 7.0, but dropped gradually at pH 5.5. The increase in numbers of colonies on XLD P, indicating the rate and extent of repair, was rapid and essentially the same at pH levels of 6.0, 6.5, and 7.0 but relatively less at pH 5.5. The initial high recovery on XLD P at pH 5.5 and 6.0 was due to difference in time elapsed between rehydration and first plating.

**Effect of antimicrobial agents on repair and growth.** Several antimicrobial agents which either prevent synthesis of specific cell components or interfere with cellular normal functions were used in the test solutions (Table 2). The pH levels of the test solutions after the addition of the sample were between 6.0 and 7.0. Since repair was unaffected in this pH range, no adjustment was made. The time of the first plating, the pH of the test solution, and the concentration of each agent are given in Table 1. The variation in the percentage of injury with different compounds at 0 hr of plating was due largely to the difference in time of their initial plating.

In water (containing 0.1% milk solids), the initial 90% injury was reduced to 17% after 2 hr. In the presence of penicillin and n-cycloserine, there was reduction in injury at 1 hr followed by an increase at 2 hr. Considerable reduction of injury occurred in the presence of actinomycin D, chloramphenicol, and tetracycline.

Sodium deoxycholate, when present in the XLD P, inhibited the colony-forming ability of the injured cells. However, in the test solution it did not prevent repair. Sodium dodecyl sulfate produced similar results.

In the presence of about 0.1 mM available EDTA, partial repair occurred as the original 89% injury was reduced to 55% after 1 hr. The percentage of injury changed little in the next hour; however, about 47% of the freeze-dried cells were killed by EDTA. Under similar conditions with fresh cells, only 18% were killed, but about 60% of the survivors were injured from exposure to EDTA.

In the presence of DNP, no reduction in injury occurred during 2 hr. About 25% of the cells were killed during this test period. Partial recovery was observed in the presence of sodium cyanide.

To test the effect of actinomycin D, chloramphenicol, DNP, and cyanide on fresh and freeze-dried S. anatum cells, growth of cells incubated at 25°C for 7 hr was measured on XLD. The results presented in Fig. 3 show that the growth of control fresh and freeze-dried cells had lag phases of 2 and 3 hr, respectively. With actinomycin D, the fresh cells had about a 2 hr lag, whereas the freeze-dried cells did not start growth until after 4 hr. In the presence of chloramphenicol, both the fresh and freeze-dried cells started growth after about 3 and 4 hr, respectively. Growth rates in the presence of chloramphenicol were slower than with control fresh or freeze-dried cells. Both fresh and freeze-dried cells showed no initiation of growth in the presence of DNP or sodium cyanide during the 7-hr test period.
Effect of deoxycholate on repair. The influence of deoxycholate on repair was studied with XLP and XLD broths (identical to solid plating media but without agar) and with deoxycholate in water (Fig. 4). XLD broth was prepared by adding 0.25% deoxycholate to XLP broth immediately before adding cells, and this broth or water was used as the test solution (Fig. 4). The number of cells on XLP remained essentially constant during the 2-hr test period. The initial injury in the test solution was about 90%, but within 2 hr was reduced to about 20%. In 0.25% sodium deoxycholate-water solution, the initial number on XLP was less, and some reduction in population occurred during the test period. Initial counts on XLDP appeared to be higher with the deoxycholate solutions than those with broth or water, because the first plating from the XLP broth took place within 3 min after rehydration and after 6 min with the deoxycholate solution. Repair of the cells occurred in the deoxycholate solution. However, when XLDP broth was used as the test solution, the initial numbers on XLP indicated 90% death. Little injury was observed in XLPD broth, because the initial counts obtained on XLP and XLDP were similar. These counts remained constant during the 2-hr test period.
Effect of DNP and sodium cyanide on repair.
Both DNP and cyanide interfered with repair of
injury in freeze-dried S. anatum cells (Fig. 5).
This inhibition of the repair process by DNP was
pH-dependent. Repair in test solutions adjusted
with 20 mm sodium phosphate buffer indicated
that pH levels from 6.0 to 7.0 did not influence
the rate of repair, and at the end of 2 hr about
20% of the cells remained injured (Fig. 5). DNP
inhibited completely the repair process at pH 6.0
but at pH 6.5 and 7.0 about 20 and 40% of
the cells were repaired in 2 hr. No pH dependency
was observed with cyanide. Between pH 6.0 and
7.0, about 50% of the cells remained injured after
2 hr in the presence of cyanide. However, in the
presence of both DNP and cyanide at pH 7.0,
about 75 to 80% remained injured. The repair
process appeared to require energy in the form of
adenosine triphosphate (ATP); however, ATP
supplementation (75 μg/ml) did not counteract
the DNP inhibition of the repair process (data
not shown).

DISCUSSION

About 90% of the surviving freeze-dried S.
anatum cells exhibited injury when plated within
3 min after rehydration, but these damaged cells
were repaired rapidly after rehydration in a suit-
able medium. At 25 C, most of the cells were re-
paired within 2 hr and grew after about 3 hr.
Sinsky and Silverman (26) reported that rehy-
drated freeze-dried Escherichia coli cells incu-
bated at 37 C started repair after about 5 hr and
had about an 8-hr lag period. This delayed repair
and extended lag may have been due to the mini-
mal broth used by these workers. They observed
only about 25% injury among the survivors. This
was low in comparison to the 90% injury that
was observed in freeze-dried S. anatum cells used
here. These apparent differences could be due to
the use of different plating media in determining
the amount of injury. Sinsky and Silverman (26)
used a minimal agar medium and Trypticase Soy
Agar with yeast extract as a complete medium.
In the present study, a selective agent added to
the medium was used to determine damage, and
this may have affected more cells with different
degrees of injury than did a minimal medium.

The rate of repair was dependent on the tem-
perature and pH of the medium. At low tempera-
tures and low pH, the process was relatively
slow.

The repair of freeze-drying injury in S. anatum
apparently did not involve the synthesis of muco-
peptide, proteins, or RNA. The freeze-dried cells
were repaired in the presence of penicillin (12)
and d-cycloserine (18), which are inhibitors of
cell wall mucopeptide synthesis. S. anatum cells
were susceptible to these two agents, as about
90% of the fresh cells were killed within 2 hr in
the presence of penicillin and d-cycloserine.
These fresh and uninjured cells (with a lag period
of about 2 hr at 25 C) were probably synthesizing
different cell constituents, including mucopeptide,
during this lag period and became sensitive to
penicillin and d-cycloserine. Most of the freeze-
dried cells were repairing their injury during this
same time period. This repair did not appear to
involve mucopeptide synthesis, and thus the cells
remained unaffected by these two agents.

The cells repaired their injury in the presence
of actinomycin D, an inhibitor of RNA synthesis
(6). This indicated no involvement of RNA syn-
thesis in repair. Synthesis of RNA during repair
of freeze-dried E. coli cells was reported by
Sinsky and Silverman (26). These contradicting
observations could be due to differences in the
composition of test solution, methods of deter-
mination of injury, and test organisms. Although
fresh S. anatum cells were not affected by actino-
mycin D, freeze-dried cells initially were sensitive
to this antimicrobial material. However, this sen-
sitivity was lost as repair of the cells occurred.
Actinomycin D added 1 and 2 hr after rehydra-
tion of freeze-dried S. anatum cells did not have
an effect (data not presented).

The data also indicated that freeze-drying of
S. anatum caused some surface alteration of the
cells so that permeability was impaired. The cells
became permeable to molecules like actinomycin
D, which otherwise could not enter the cells.
However, this surface alteration was repaired
quickly, and the repaired cells again became im-
permeable to these molecules. Similar permeab-
ility losses due to different sublethal stresses in-
cluding freeze-drying have been reported by many
workers (4, 13, 15, 20, 26, 29). Chloramphenicol
and tetracycline inhibit protein synthesis (25).
Other workers have observed protein synthesis
during repair of freeze-drying injury (26) or heat
injury (16). However, our data showing repair in
the presence of these agents indicated a lack of
involvement of protein synthesis.

Two surface-active agents, deoxycholate and
dodecyl sulfate, were used in the test solutions
on the basis that if injury involved the lipoprotein
of the cell membrane (7), the repair process might
be affected (19). The inability of the injured cells
to repair themselves and grow on XLDP implied
action by deoxycholate; however, repair occurred
in solutions that contained deoxycholate and
dodecyl sulfate. These results did not eliminate
the possibility of damage in the cell membrane.
However, these data indicated that deoxycholate
alone was not responsible in itself for preventing
repair and colony formation by the injured cells.
Studies with XLDP broth confirmed this observation. Injury repair in the presence of EDTA indicated that injured freeze-dried cells, although able to repair themselves, were extremely sensitive to a low concentration of EDTA. Under similar conditions, the fresh cells of *S. anatum* showed little death but a large amount of apparent injury. When the effect of EDTA was neutralized with calcium, the repair of EDTA-injured *S. anatum* cells occurred rapidly. This repair process was also retarded by DNP at pH 6.2 (data not presented). EDTA caused solubilization of the lipopolysaccharide part of the cell wall in gram-negative bacteria, and in low concentration also produced cell injury by a steric or chemical change in the cell surface of gram-negative bacteria (9, 31). This injury was reversible and did not require synthesis of protein, RNA, or cell wall mucopeptide, but required energy and was inhibited by DNP (14). The similarities between the findings of Leive (14) in EDTA-injured gram-negative bacteria including salmonellae and the findings of the present investigations with freeze-dried *S. anatum* suggested that the nature of injury from either stress was similar. The damage may be located in the lipopolysaccharide part of the cell wall. A similar suggestion was made by Bretz and Kocka (4) working with frozen cells.

Repair of freeze-dried injury in *S. anatum* was completely or partially inhibited in the presence of DNP and sodium cyanide. Both DNP and cyanide interfere with ATP synthesis via oxidative phosphorylation in the electron-transport system (3, 10). This inhibition of injury repair by DNP was pH-dependent. At pH 6.0 no repair occurred, whereas at pH 6.5 and above partial repair occurred. A similar pH dependency of DNP inhibition of energy-requiring processes in *Staphylococcus aureus* has been reported (8). This pH dependency may have not been due to the inhibitory effect of undisassociated DNP ions (32). Rather, the net ATP synthesis in the presence of DNP may have been reduced by DNP stimulation of adenosine triphosphatase which otherwise remained in a "latent state" (23) or simply by pH dependency of adenosine triphosphatase (17). DNP-stimulated adenosine triphosphatase activity in oxidative phosphorylation also has been reported in bacteria (5, 22). The partial inhibition of repair of injury by cyanide also suggested the involvement of energy synthesis through the electron-transport system. In the presence of cyanide, electron transport can be maintained through inorganic ions, e.g., nitrate or sulfate (10). In test solutions containing 0.1% milk solids, such inorganic ions may be present, and consequently some ATP might be synthesized. This could then produce the partial repair. In the presence of potassium nitrate and cyanide, injured freeze-dried *S. anatum* cells reduced nitrate in 1 or 2 hr after rehydration (data not presented).

Supplementation with ATP from an external source did not counteract DNP inhibition of repair at pH 6.0. This might result from inability of the highly charged ATP molecules to enter the cells; however, ATP has entered EDTA-injured *E. coli* cells (14). Repair of injury produced by EDTA in gram-negative bacteria (14) or produced by aerosol dehydration of *E. coli* (30) has been reported to be energy-dependent. Energy metabolism during repair of injury was also suggested in heat-stressed *S. aureus* cells (2), heat-stressed *S. typhimurium* cells (28), and freeze-dried *E. coli* cells (26).

The injury in freeze-dried *S. anatum* cells seemed to be located, at least partially, in the lipopolysaccharide part of the cell wall and was reversible. After rehydration, this injury was repaired rapidly, and the repair required energy in the form of ATP from the electron-transport system.

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