COAGULASE PRODUCTION BY HEAT-INJURED *STAPHYLOCOCCUS AUREUS* MF-31

by

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I. INTRODUCTION

Thermal injury of staphylococci is a complex series of events affecting cell permeability (9, 31), ribonucleic acid (31, 58), and cellular metabolism (8). The metabolic imbalance resulting from injury can be repaired in a suitable medium during a recovery period. This recovery period has been aptly defined by Harris (26) as "...the period of 'getting back' organisms from their (sublethal) environment."

Early work on the recovery period indicated that the nutritional requirements for recovery were different from the nutritional requirements for growth (28). Iandolo and Ordal (31) confirmed and expanded these results. They showed that Staphylococcus aureus was more demanding nutritionally during growth than recovery. Bluhm (8) found that various damaged cell functions did not have identical nutritional requirements for recovery.

The rates of recovery for different damaged cell functions were not found to be equal. While repair of cell permeability and RNA (evidenced by return of salt tolerance) seemed to parallel one another (31), return of dehydrogenase activity and oxygen uptake to normal (uninjured) levels preceded them (8).

In characterizing the lesions produced in S. aureus by sub-lethal heat treatment, it was observed that protein synthesis was unnecessary for repair of cell permeability (31, 58). RNA synthesis, however, was essential for recovery of salt tolerance (31, 58). The return of dehydrogenase activity and oxygen uptake to levels approaching normal occurred when RNA or protein synthesis was inhibited (8).

This study was undertaken to investigate the effect of sublethal heat treatment on coagulase synthesis in Staphylococcus aureus MF-31. The diagnostic importance of coagulase to the Food Industry and to Public Health officials is well known. It was found (see Literature Review) that coagulase was produced by normal cells when they were in the lag and logarithmic growth phases, but apparently ceased when the cells entered the stationary growth phase. Coagulase tests were performed on the supernatant fluids of cultures of S. aureus recovering from heat injury to ascertain if coagulase was produced by recovering cells. Partial characterization of the phenomenon was attempted by varying the conditions of injury and recovery.

II. LITERATURE REVIEW

A. Coagulase

Loeb (41) was the first to recognize that staphylococci have the ability to clot normal plasma. He reported this finding in 1903, and suggested that the effect was enzymatic. In 1908, Much (46) confirmed and expanded Loeb's observations. Much found that staphylococci could clot plasma containing citrate or hirudin, both inhibitors of the normal clotting process. He showed that only Staphylococcus aureus, and not S. albus, S. citreus, or other bacterial genera, possessed this ability. In 1920, Gratia (25) names the substance responsible for the clotting phenomenon, "staphylocoagulase".

Coagulase is universally accepted as the best single criterion of potential staphylococcal pathogenicity (17, 57). Evans, et al. (18), reported a high correlation between coagulase and enterotoxin production, and concluded that any food containing coagulase positive staphylococci should be considered dangerous for human consumption. Niven (48) noted that while coagulase positive strains of S. aureus are not necessarily enterotoxigenic, all known strains of S. aureus that do produce enterotoxin are coagulase positive and beta-hemolytic.

The relationship between the in vitro coagulase-plasma clotting reaction and pathogenicity is not clear (50). Although it was reported that coagulase positive staphylococci resisted phagocytosis by virtue of a coagulase catalyzed fibrin envelope surrounding the cells (56), other workers (7, 51) observed that both coagulase positive and coagulase negative staphylococci were readily engulfed by human granulocytes, monocytes and polymorphonuclear leucocytes. Raffel (50) states: "It is difficult to see how clotting can further the

pathogenic ends of a bacterium...." Yet the high correlation between staphylococcal pathogenicity and coagulase production (17, 57), and the similarly high correlation between staphylococcal virulence and coagulase production (67), intuitively leads one to conclude that coagulase is important in staphylococcal infections. There is evidence that the importance of coagulase to pathogenic staphylococci is not in its clot catalyzing capability. Yotis and Ekstedt (69) observed that while coagulase positive staphylococci flourished in normal serum, coagulase negative staphylococci were unable to grow. When coagulase was added to the serum, coagulase negative cells proliferated similarly to coagulase positive cells. Ekstedt (16) showed that this phenomenon was due to the presence of an antistaphylococcal factor in normal serum that was "neutralized" by coagulase. Amano, et al. (2) precipitated an antistaphylococcal factor from acidic guinea pig leucocyte extracts. The factor, "leucozyme A," adsorbed to the cell walls of coagulase negative staphylococci, but not to the cell walls of coagulase positive staphylococci. Treating coagulase negative cells with coagulase rendered them resistant to leucozyme A adsorption. Sall (54) found that under certain conditions soluble coagulase formed a capsule-like structure, "pseudocapsule," around S. aureus cells. He hypothesized that this may occur in vivo thereby protecting the invading staphylococci from host defences. One may conclude that if these observations occur in vivo, and if coagulase itself protects infecting staphylococci, the in vitro coagulase clotting reaction may be incidental to the primary functions of coagulase in pathogenicity. However, this aspect of coagulase activity has yet to be demonstrated.

Several workers have reported methods of purifying coagulase (30, 34, 39, 45, 60, 61, 62). However, investigations of the chemical composition of coagulase have not yielded consistant results. Soru, et al. (59) found

no carbohydrate, glucosamine, or nucleic acid in their coagulase preparations. The amino acid composition consisted of glycine, threonine, aspartic acid, glutamic acid, serine, lysine, histidine, arginine, cystine, and cysteine. Hitokoto (30) reported that his coagulase preparations had a similar amino acid composition except that alanine, valine, and methionine replaced histidine, arginine, cystine, and cysteine. It contained no carbohydrates, lipids, or phosphates. Miller, et al. (45) isolated a coagulase fraction with high activity that was ninhydrin negative and failed to aborb ultraviolet light at 280 mu. Jungerman (39) purified a glycopeptide fraction with coagulase activity. He reported glucose, mannose, xylose, and unidentified hexose, glucosamine, and phosphate were present.

The discrepencies may be partly explained by two observations. Initially, it was shown that different strains of S. aureus produced coagulases that were distinguishable immunologically (29, 32) and electrophoretically (44). Even single strains of S. aureus produced several different protein fractions with coagulase activity (29, 44). Secondly, various contaminating proteins have been found associated with highly purified coagulase. An "egg yolk factor," which acts on egg yolk suspensions in a manner grossly similar to the action of the alpha lysin of Clostridium perfrigens (6, 11, 62), acid phosphatase (11, 34, 62, 70), and deoxyribonuclease (70), were found to be contaminating proteins, separable from the coagulase moiety only by highly sensitive chemical and physical methods. It was reported by Gerheim (22) that coagulase and staphylokinase, formerly thought to be functions of the same protein (21, 64, 65), are separate substances. He demonstrated that staphylokinase, not coagulase, is responsible for clot disintegration.

Despite evidence presented by Miller, et al. (45), that their purified coagulase fraction was ninhydrin-negative and failed to absorb at 280 mμ, it has been shown conclusively that coagulase activity resides in a protein with enzymatic properties (10, 17, 60, 61, 70).

Coagulase is reported to have a $S_{20,w}$ of 1.59S (32) and 4.29S (14), and a molecular weight of 5000-10,000 (47) and 44,000 (14). Tager and Drummond (62) suggest that the larger values are correct, and that the smaller values are due to active fragments resulting from the purification processes.

Various physical and chemical treatments effect the activity of coagulase. It was reported that oxidizing agents inactivated coagulase, while reducing agents had no effect (37, 40, 61). This could explain why attempts to lyophilize coagulase have failed (20), since in the dried state most substances are susceptible to oxidation by small quantities of oxygen. Further, it was found that coagulase was extremely sensitive to surface denaturation (1) which could be amplified by the lyophilization process. It has been observed that crude coagulase is more thermolabile than purified coagulase (61, 66). The reason is not known (17). It was reported that merthiolate, albucid (a blood preservative), and formalin completely inactivated coagulase (33, 38, 68), while phenol reduced the activity of coagulase slightly (33, 38).

Biological materials have been found to affect the activity of coagulase. Deoxyribonucleic acid and ribonucleic acid inactivated coagulase (38, 68). Gamma globulin in a concentration of 9 mg/ml almost completely inhibited coagulase activity (3, 4). The mechanism of inhibition is thought to be complex formation between γ-globulin and the sulfhydryl groups of coagulase.

The activity of coagulase was inhibited completely by several antibiotics in high concentrations: oleandomycin, spiramycin, sulfamethoxypyridazine,

and sulfanilimide at concentrations of 1000 µg/ml (38, 68). It was inhibited slightly by bacitracin, cycloserine (38) and oxytetracycline (49).

The biosynthesis of coagulase is apparently influenced by a number of different factors. The age of the cells seems to be particularly important. Cultures in the lag and logarithmic phases of growth readily produced coagulase, but upon reaching the stationary growth phase coagulase elaboration ceased (15, 19, 43, 52, 54). This indicated that growing cells synthesized coagulase while resting cells did not. Marston, et al. (43) considered these results and concluded: "There seems to be a correlation between the active growth of Staphylococcus aureus and the ability of the organisms to produce coagulase." If this hypothesis is correct, one might expect that those factors which influence growth also influence coagulase production. Among the factors which affect growth are: available nutrients, amount of aeration, pH, ionic strength, and the concentration of inhibitory substances. It was found that the nutrients necessary for optimal growth supported optimal coagulase production (27, 35, 43, 63). Aerobic incubation stimulated coagulase production (43), and it is well known that aerobic incubation favors the growth of staphylococci. The optimal pH for coagulase production was near neutral (13, 19, 27). This pH is also most favorable for growth. The addition of large amounts of sodium chloride to media depressed coagulase synthesis (42), and high concentrations of sodium chloride also retard the growth of S. aureus. Sub-bacteriostatic concentrations of chloramphenicol (1) and oxytetracycline (49) inhibited coagulase production. The presence of antibiotics in culture media depresses growth. In summary, antibiotics, large quantities of sodium chloride, and low pH impair growth. The reports cited indicate that these factors also depressed or inhibited coagulase production. Aerobic incubation and the availability of carbohydrates and vitamins stimulate growth, and these factors

favored coagulase production. These findings appear to support the hypothesis of Marston, et al. (43).

There appears to be one exception to the evidence presented above. Albumin was reported to stimulate coagulase production even though growth was not affected (1. 13). This does not seem to be a general response of S. aureus, as coagulase production by at least one strain was not stimulated by albumin (19).

The biosynthesis of coagulase in vitro has been accomplished with cell-free extracts (36). The system was inhibited by ribonuclease or staphylococcal ribonucleic acid, but was unaffected by staphylococcal deoxyribonucleic acid. This suggests that the destruction of RNA or the competitive inhibition of RNA present in the extract, by the addition of exogenous RNA, inhibited the production of coagulase. The data imply that coagulase synthesis was intimately related to RNA. Since coagulase is a protein (10, 17, 60, 61, 70), and since protein synthesis is under the direction of RNA, these results are to be expected. It is of interest to note, however, that coagulase activity has not been demonstrated in cell extracts (33, 35). Altenbern (1) suggested that coagulase is produced, in vivo, at or very near to the cell surface, and is rapidly released upon synthesis.

The phenomenon of staphylococcal clumping in the presence of normal plasma has been reported by several workers (62). The clumping factor responsible for this observation has been called "bound coagulase" and has been shown to be antigenically distinct from free coagulase (12). Tager and Drummond (62) argue that "bound coagulase" is a misnomer since there is no evidence that the clumping factor is related to free coagulase. Recently, however, evidence was presented indicating that bound coagulase may be the precursor for free coagulase (5).

B. Injury and Recovery

Thermal injury of staphylococci is a complex series of events effecting cell permeability (9, 31), ribonucleic acid (31, 58), and cell metabolism (8). Busta and Jezeski (9) reported that S. aureus cells lost salt tolerance after being heated to 60 C in milk. Iandolo and Ordal (31) confirmed this and found that heat-injured cells leaked soluble cellular components into the surrounding medium. The data indicated that sub-lethally heated cells had difficulty in controlling selective permeability at the cell membrane. Injury also resulted in damage to ribosomal ribonucleic acid. Iandolo and Ordal (31) showed with metabolic inhibitors and radiotracer studies that RNA was particularly involved in heat injury. Sogin (58) confirmed this and found that ribosomal RNA was a specific site of heat injury. Finally, injury can effect the metabolic processes of the cell. Heinmets, et al. (28) showed that Escherichia coli cells injured by chlorine or heat treatment could not survive in complex media, but demanded special nutritional considerations. Bluhm (9), working with S. aureus MF-31, found that dehydrogenase activity and oxygen uptake were effected by heat, and that this damage was independent of cell membrane or RNA damage.

The metabolic imbalance resulting from injury can be repaired during a recovery period in a suitable medium. The recovery period has been aptly defined by Harris (26) as "...the period of 'getting back' organisms from their (sublethal) environment." Early work on the recovery period indicated that the nutritional requirements for recovery differed from the nutritional requirements for growth (28). These results were confirmed and expanded by Iandolo and Ordal (31). They found, with S. aureus MF-31, that growth was more nutritionally demanding than recovery. Later work (8) indicated that damaged cellular functions did not have identical nutritional requirements

for recovery. The nutritional requirements for the return of dehydrogenase activity and oxygen uptake to normal (uninjured) levels were more demanding than the nutritional requirements for the repair of RNA and cell membrane.

The repair of damaged cell membrane (evidenced by the return of salt tolerance) and the resynthesis of RNA appeared to parallel each other (31). While both were inhibited by actinomycin D, neither was affected by chloramphenicol, cycloserine or penicillin. This indicated that RNA synthesis, and not protein or cell wall synthesis, was necessary for return of salt tolerance. Radiotracer studies confirmed that RNA synthesis controlled the recovery of salt tolerance. Bluhm (8) found that the return of dehydrogenase activity and oxygen uptake to normal levels preceded the return of salt tolerance. He further found that the enzymes returned to nearly normal levels in the presence of actinomycin D or chloramphenicol. This indicated that neither RNA nor protein synthesis was necessary for partial return of dehydrogenase activity or oxygen uptake. It was postulated that partial return of enzyme activity may be due to energy-requiring reactions that reverse heat induced denaturation. Such reactions would not be affected by RNA or protein synthesis inhibitors.

III. MATERIALS AND METHODS

A. Test Organism

The test organism used for this study was Staphylococcus aureus MF-31. This strain was originally isolated from cheese implicated in a food poisoning outbreak. It was supplied by the Robert A. Taft Sanitary Engineering Center in Cincinnati, Ohio.

The organism was maintained on stock slants of Trypticase Soy agar (TSA). Frozen starter cultures were prepared from stock cultures by inoculating 200 ml of sterile Trypticase Soy broth (TSB) with a loopful of organisms. The broth culture was incubated on a rotary shaker at 37 C for 18-24 hours. Test tubes, containing 10 ml sterile TSB, were inoculated with 0.1 ml aliquots of this culture and immediately frozen at -20 C. The frozen starter cultures were thawed and inoculated into 90 ml of fresh TSB to prepare cultures used thoughout this study.

B. Media and Buffers

1. TSA and TSB were purchased from the Baltimore Biological Laboratory (BBL).

2. Trypticase Soy agar + 7.5% NaCl (TSAS) was prepared by adding 70g/l NaCl to TSA. TSA normally contains 0.5% NaCl.

3. Semi-defined medium (SDM) was prepared as follows:

Casamino acids.....1.0%
K_2HPO_4.....0.5%
Glucose (autoclaved separately).....0.2%
Tryptophane.....2 mg/100ml
pH 7.4

Semi-defined medium + vitamins (SDMV) was prepared by adding 0.1% (by volume) of MEM vitamins (Grand Island Biological Company) to SDM. MEM vitamins is a vitamin mixture containing the following constituents:

calcium pantothenate	100 mg/l
choline chloride	100 mg/l
folic acid	100 mg/l
i-inositol	200 mg/l
nicotinamide	100 mg/ml
pyridoxal HCl	100 mg/ml
riboflavin	10 mg/ml
thiamine HCl	100 mg/ml

For nutritional studies, separate, sterile 50 ml volumes of casamino acids (5.0%) plus tryptophane (10 mg/ml), K_2HPO_4 (2.5%), glucose (1.0%), and vitamins (0.5% by volume) were prepared. Various combinations were made by mixing 2 ml portions of the desired nutrients together in sterile 125 ml flasks. Enough sterile water was added to bring the volumes to 9 ml. Injured cells were concentrated 10 times, and added in 1 ml samples to the sterile flasks.

4. Stock solutions of 0.1 M NaH_2PO_4 and Na_2HPO_4 were combined as needed to prepare 0.1 M sodium phosphate, pH 7.2 (24). Samples of 0.1 M sodium phosphate, pH 7.2, were diluted 1:2 with distilled water to prepare 0.05 M sodium phosphate, pH 7.2. Solutions of 0.3 M sodium phosphate buffer, pH 7.2, were prepared separately as needed.

C. Preparation of Cultures and Harvesting Procedures

A previously prepared frozen starter culture was thawed and added to 90 ml TSB. The culture was incubated at 37 C for 12, 15, or 21-24 hours. Cells at these ages represented late logarithmic, early stationary, and middle stationary phases of growth (8, 31). The cells were separated from the growth

medium in a Sorvall RC-2B refrigerated centrifuge at 13,100 X g for 10 minutes. The pellet was resuspended in phosphate buffer of the desired molarity, pH 7.2, and re-centrifuged as above.

For nutritional experiments, or when SDM was to be used for incubation or recovery, it was necessary to remove all residual vitamins from the cells. The following procedure was used: The pellet of cells was resuspended in SDM after the second centrifugation. The suspension was added to a volume of SDM such that the final volume of SDM plus cells equaled the volume of media from which the cells had originally been separated. The preparation was incubated at 37 C for 45 minutes on a rotary shaker at 200 rpm. The cells were then harvested and washed twice in fresh buffer as above.

D. Injury Conditions

The washed pellet from section C was re-suspended in 5 ml of the appropriate buffer, and added to buffer pre-tempered at 54 C. The final volume in the injury vessel was equal to the volume of media from which the cells had originally been separated. The cells were injured in a sterile 300 ml flask on a shaking water bath. The cells were shaken for 10 seconds every 5 minutes to ensure even heating (31). Heating of the added cells to 54 C was considered to be instantaneous. The cells were heated for 15 minutes.

At the termination of the heating period, the flask was immersed in an ice bath and swirled continuously for 2 minutes. After cooling, the cells were centrifuged from the heating menstruum as previously described.

E. Recovery Conditions

The pellet of cells from Section D was resuspended in 5 ml of TSB, SDM, or SDMV and added to the appropriate recovery media. The final volume of the inocualted recovery media was equal to the volume of media from which the

cells had originally been separated. When SDM was used for recovery, the cells were resuspended in the appropriate phosphate buffer and washed once before inoculation. Recovering cultures were incubated at 37 C on a rotary shaker. Slow rotation (100 rpm) was indicated because coagulase is extremely labile to surface denaturation (1).

F. Quantitative Estimation of Injury

At various times, 1 ml samples were removed from the recovering cultures of section E and diluted in 99 ml sterile water blanks. Appropriate dilutions were plated in triplicate on TSA and TSAS, and incubated at 37 C for 48 hours. The difference in counts for a particular sample plated on TSA and TSAS gave an estimation of the salt sensitivity and therefore injury induced by sublethal heat treatment (31).

G. Coagulase Assay

1. Plasma. Whole blood was taken from the ear veins of six albino New Zealand rabbits. The blood was collected in sterile glass bottles containing potassium oxalate. The final oxalate concentration was 0.4%. The blood was clarified by centrifugation at 13,100 X g for 10 minutes in a Sorvall RC-2B refrigerated centrifuge. The plasma was pooled and stored at -20 C until used.

2. Preparation of samples. At various times 5 ml samples were withdrawn from recovering cultures (Section E) and clarified as previously described. The supernatant fluid was stored in sterile test tubes at 4 C until assayed.

3. Assay. Dilutions of the clarified broth samples were prepared in 0.01 M Tris buffer plus 10^{-4} M magnesium acetate, pH 7.4 (TM-4).

Aliquots of 0.3 ml were removed from the diluted samples and pipetted into shell vials (9 X 30 mm). Chloramphenicol from a stock solution (450 µg/ml) was added to each vial in 0.02 ml aliquots. Previous studies showed this

effective in preventing coagulase production by residual staphylococci (1). Plasma was added to each vial in 0.3 ml aliquots. The mixture was thoroughly blended by twirling a sterile loop in the fluid for 10-15 seconds.

The tubes were incubated in wooden blocks pre-heated to 37 C for 3 hours. The endpoint was the highest dilution showing any visible sign of coagulation. It was subjectively fixed to be a small thread of fibrin usually extending downward vertically from the meniscus of the fluid. It was preceded by a vial containing a larger fibrin thread, and followed by a vial containing clear liquid with no discernible clot. The reciprocal of the end point was considered the number of units of coagulase/ml in the sample.

Occasionally during this investigation it was necessary to ascertain if very small amounts of coagulase were produced. In these cases, coagulase tests were performed on undiluted samples for longer periods of time, ranging from 15 hours to 48 hours. The results were recorded as positive or negative.

IV. RESULTS AND DISCUSSION

Thermal injury of Staphylococcus aureus results in damage to cellular activities involving membrane integrity, ribosome function, enzyme synthesis and enzyme activity (8, 31, 58). These cellular activities are repaired when the injured cells are incubated in a suitable recovery medium (8, 31). However, the individual cell systems may exhibit varying nutritional requirements during recovery to normal levels (8).

This investigation was undertaken to study the effect of heat injury on coagulase production. Coagulase is thought to be a "non-essential" protein in that it has not been shown to be active in cell metabolic processes. On the other hand, coagulase is an extremely important protein because it is the best single criterion for implicating potential enterotoxigenic staphylococci (18, 48), and for demonstrating potential staphylococcal pathogenicity (17, 57).

Experiments were conducted comparing the sensitivities of bovine, sheep and rabbit plasma in detecting coagulase. Bovine and sheep plasma were collected and stored as previously described for rabbit plasma in Materials and Methods. The data, complied in Table I, indicate that undiluted rabbit plasma was superior to undiluted bovine or sheep plasma in detecting high dilutions of coagulase. Rabbit plasma was clotted by coagulase in a sample diluted 1:21 while bovine and sheep plasma were not clotted by 1:5 dilutions of the same sample. It was evident from these data that rabbit plasma was more sensitive in detecting small amounts of coagulase and small changes in coagulase concentration than bovine or sheep plasma. For this reason, rabbit plasma was selected for use in coagulase assays in this study.

TABLE I

COMPARISON OF UNDILUTED BOVINE, SHEEP AND RABBIT PLASMA
FOR SENSITIVITY IN COAGULASE ASSAY

	Dilution in TM-4 of Coagulase Sample						
	1:1	1:5	1:10	1:15	1:20	1:21	1:22
Rabbit Plasma	+	+	+	+	+	+	-
Bovine Plasma	+	-	-	ND	ND	ND	ND
Sheep Plasma	ND	-	-	-	-	ND	ND

The reaction mixture contained 0.3 ml undiluted plasma, 0.3 ml diluted cell-free supernatant fluid. The mixture was incubated at 37 C for 3 hours.

\+ Visible clot in 3 hours

\- Negative test, (no visible clot in 3 hours)

ND Dilution not done

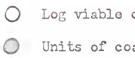
The production of coagulase by uninjured S. aureus MF-31 proliferating in TSB is shown in Figure 1. Coagulase was produced during the logarithmic growth phase, but not during the lag or stationary growth phases. During growth, 3 units of coagulase were produced. The viable population level at stationary phase was approximately 2.8×10^9 cells/ml.

The data of Figure 1 indicate that coagulase, detectable in a 3 hour assay, was produced during the logarithmic growth phase, but not during the lag phase. These data sharply contrast with the observations of Duthie (13). He reported that coagulase was produced only during the lag phase, prior to cell multiplication.

In addition, these data show that coagulase was not produced during the stationary growth phase, thus confirming other reports (15, 19, 43, 52, 54). This finding suggested that there was a correlation between coagulase synthesis and cell multiplication. To test this hypothesis, two experiments were performed. Uninjured cells were inoculated into TSB at a population level too high to permit growth (Table II). Coagulase was not detected even after extended incubation for 23 hours at 37 C. Uninjured cells were also inoculated into SDM at a lower population level (6.0×10^8 cells/ml) which encouraged limited growth (Table II). SDM, however, contained no vitamins (see Materials and Methods) and did not support proliferation of the fastidious Staphylococcus aureus. No coagulase was detected after 20.5 hours of incubation at 37 C in SDM. The results of these experiments indicate that cells which are resting because of population density or nutritional deficiency do not elaborate coagulase. They support the hypothesis that there is a correlation between coagulase synthesis and cell multiplication.

In contrast to the above data, injured cells produced coagulase during recovery (Figures 2, 3, and 4). Since cell counts on TSA did not increase

Figure 1. Growth and coagulase production by S. aureus MF-31 in TSB.



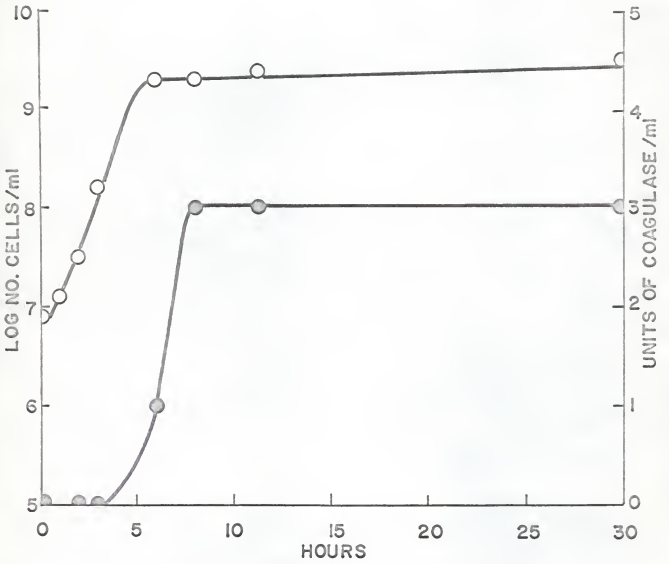

LOG NO. CELLS/ml
UNITS OF COAGULASE/ml
HOURS
10
9
8
7
6
5
4
3
2
1
0
0
5
10
15
20
25
30

TABLE II

COAGULASE PRODUCTION BY UNINJURED RESTING CELLS

Culture Medium	Hours of Incubation	Log Viable Cells/ml, TSA	Coagulase in 24 Hour Assay	Coagulase in 48 Hour Assay
TSB	0	10.0		
	1	10.0	negative	
	2	----	negative	
	6.5	10.0	negative	
	23	10.1	negative	
SDM*	0	8.7	negative	negative
	4	8.7	negative	negative
	7	8.8	negative	negative
	20.5	8.8	negative	negative

*Resuspended in SDM at 0.1 concentration of origional cell suspension.

during the recovery period, multiplication was not implicated in recovery (31) and coagulase production during recovery. Furthermore, data presented elsewhere in this thesis (Table III) indicates that coagulase was produced by injured cells recovering in SDM, a medium which cannot support multiplication (Table II).

To assess the effect of injury on coagulase production, the injury conditions were varied by altering the molar strength of the phosphate buffer in which the cells were injured, and the age of the cells prior to injury. Phosphate buffer (pH 7.2) was used at concentrations of 50, 100 and 300 mM. The cell ages chosen were 12, 15 and 21-24 hours. These ages represented late log, early stationary and middle stationary phases of growth (8, 31).

The data of Figures 2, 3 and 4 indicate that the number of cells injured may have been affected by the molar strength of the phosphate buffer in which the cells were injured. Heating 12, 15 or 21-24 hour cells at 54 C for 15 minutes in 300 mM phosphate buffer, pH 7.2, resulted in injury of less than 90% of the total population. Heating 12 or 21-24 hour cells in 100 mM buffer, and 15 or 21-24 hour cells in 50 mM buffer, resulted in injury of 99% or more of the total population. Although no conclusive evidence was indicated by these observations, the data imply that 300 mM phosphate buffer, pH, 7.2, partially protected the cells from injury because less cells were injured with 300 mM buffer than with 50 or 100 mM phosphate buffers, pH 7.2. In contrast to this, neither the molar strength of the buffer in which the cells were injured, nor the age of the cells prior to injury appeared to effect coagulase production during recovery. Although the amount of coagulase produced during recovery varied from 5 to 13 units, there was no discernible pattern of increased or decreased coagulase production dependent upon the injury conditions employed.

Figure 2. Recovery and coagulase production by injured cells of S. aureus MF-31.

The organism was grown for 12 hours in TSB, injured at 54 C for 15 minutes in phosphate buffer, pH 7.2. Recovery was carried out in TSB.

A Injured in 50 mM buffer

B Injured in 100 mM buffer

C Injured in 300 mM buffer

○ Log number cells/ml, TSA

◎ Log number cells/ml, TSAS

△ Units of coagulase/ml

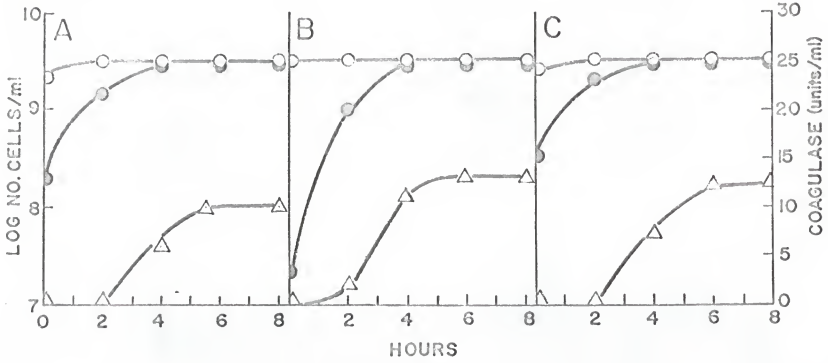

A
B
C
LOG NO. CELLS/ml
COAGULASE (units/ml)
HOURS
10
9
8
7
30
25
20
15
10
5
0
0
2
4
6
8

Figure 3. Recovery and coagulase production by injured cells of S. aureus MF-31.

The organism was grown for 15 hours in TSB, injured at 54 C for 15 minutes in phosphate buffer, pH 7.2. Recovery was carried out in TSB.

A Injured in 50 mM buffer

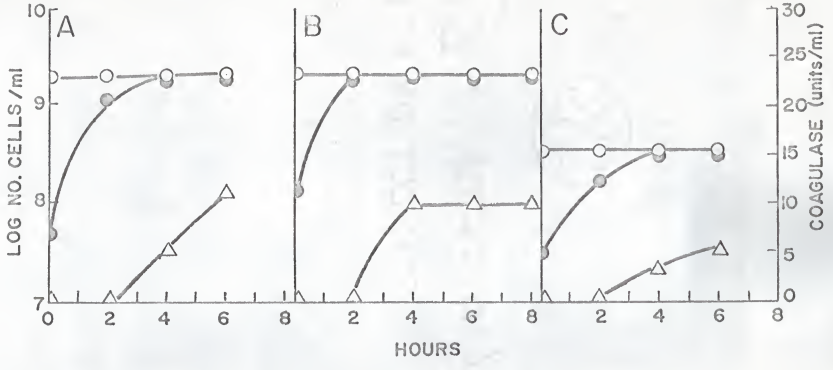
B Injured in 100 mM buffer

C Injured in 300 mM buffer

○ Log number cells/ml, TSA

◎ Log number cells/ml, TSAS

△ Units of coagulase/ml


A
B
C
LOG NO. CELLS/ml
COAGULASE (units/ml)
HOURS
10
9
8
7
30
25
20
15
10
5
0
0
2
4
6
8

The data depicted in Figures 1, 2, 3 and 4 indicate that the quantity of coagulase produced by the injured cultures during recovery (5 to 13 units) was greater than that produced by the uninjured culture multiplying in similar media (3 units). The number of viable cells/ml in the recovering cultures were, except in one instance, similar to the number of viable cells/ml in the uninjured culture at stationary phase. Although it would appear that uninjured S. aureus is stimulated to produce coagulase at a rapid rate, it cannot be concluded from these data that recovering cells exceed normal mutiplying cells in coagulase production per cell per unit time. Coagulase production during proliferation is a function of an increasing number of cells which cease coagulase elaboration upon reaching maximum population level. On the other hand, coagulase production during recovery is a function of a large, constant number of cells synthesizing coagulase over a period of several hours, during which time recovery occurs. It is possible, for instance, that the rate of coagulase production by uninjured, multiplying cells exceeded that of recovering cells. Greater quantities of coagulase could have been synthesized by the recovering cultures because the large, constant numbers of cells synthesized coagulase for several hours at a depressed rate. Therefore, it cannot be ascertained from these data whether the rate of coagulase production by injured cells was greater than, equal to, or less than, that of uninjured cells.

The results presented in Figures 2, 3 and 4 indicate that coagulase production commenced between 1 and 4 hours after injury, and ceased between 4 and 6 hours after injury. Iandolo and Ordal (31) found that injured cells recovered the ability to multiply after 4-6 hours of incubation in a suitable medium. They concluded that the return of the ability to multiply coincided with complete recovery from heat injury. If this hypothesis is correct, one would expect injured cells that have recovered the ability to multiply to

elborate coagulase during proliferation as normal, uninjured cells. Unfortunately, this prediction could not be tested with the assay employed in this study. As is evident from Figure 1, more than 5×10^8 cells/ml were required before coagulase could be detected during growth. Iandolo and Ordal (31) injured approximately 10^6 cells/ml and followed recovery and the subsequent growth in TSB. In this manner, they were able to demonstrate injured cells that had recovered the ability to multiply could grow as normal cells. The data of Figure 1 indicate that using a cell population of 10^6 cells/ml did not result in the production of enough coagulase to be detected with the assay system employed. An approach to this problem would be the incorporation of labeled amino acids into media inoculated with injured cells that have recovered the ability to multiply. Coagulase fractions could be partially purified and concentrated (70), and assayed with appropriate radioactivity detection equipment. The results would be compared with data obtained from uninjured cells handled similarly.

Summarizing the data of Figures 1, 2, 3 and 4, it appears that injured cells gained the ability to synthesize coagulase after a short incubation period in suitable media. This occurred before salt tolerance was fully restored. When recovery was complete, injured cells seemed to lose coagulase producing capability and thereafter behaved as uninjured cells in that coagulase was not elaborated in the absence of multiplication.

Studies were conducted to assess the differences in the nutritional requirements for recovery of salt tolerance and coagulase production by injured cells. Separate stock solutions of casamino acids plus tryptophane, K_2HPO_4, glucose, and vitamins, were prepared so that upon 5 fold dilution the concentrations would be 1.0% casamino acids plus 2 mg/100ml tryptophane, 0.5% K_2HPO_4, 0.2% glucose, and 0.1% vitamins (see Materials and Methods).

Figure 4. Recovery and coagulase production by injured cells of S. aureus MF-31.

The organism was grown for 21-24 hours in TSB, injured at 54 C for 15 minutes in phosphate buffer, pH 7.2. Recovery was carried out in TSB

A Injured in 50 mM buffer

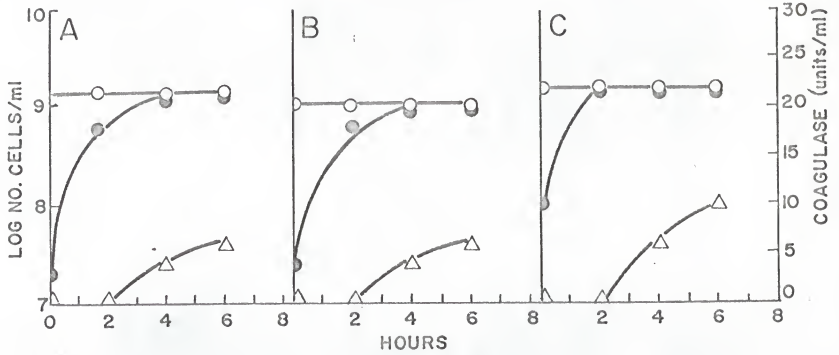
B Injured in 100 mM buffer

C Injured in 300 mM buffer

○ Log number cells/ml, TSA

◎ Log number cells/ml, TSAS

△ Units of coagulase/ml


A
B
C
LOG NO. CELLS/ml
COAGULASE (units/ml)
HOURS
10
9
8
7
30
25
20
15
10
5
0
0
2
4
6
8

Various combinations of the solutions were tested for their abilities to support recovery of salt tolerance and/or coagulase production by injured cells. The preparations were inoculated with samples from a 14 hour culture injured at 54 C for 15 minutes in 100 mM sodium phosphate buffer, pH 7.2. Prior to injury, the vitamin pool had been chased from the cells by incubation in vitamin-free SDM.

The data, presented in Table III, show that coagulase was not produced by injured cells unless they were incubated in a medium that supported the complete return of salt tolerance. Further, the nutritional factors essential for the return of salt tolerance and the initiation of coagulase synthesis were the consitiuents of SDM: casamino acids plus tryptophane, K_2HPO_4, and glucose. These results confirm earlier reports (8, 31) that recovery of salt tolerance required several different amino acids, an inorganic phosphate source, and an energy source.

The data of Table III show that SDM, a medium which did not support multiplication (Table II) supported coagulase production during recovery. This evidence contradicts the contentions of Jacherts (35) and Tamini (62) that the nutrients required for growth were also required for coagulase production. It should be noted, however, that coagulase production during recovery is not optimal in SDM. Only 2 units of coagulase were produced by injured cells during 6 hours of recovery in DDM, while 12 units of coagulase were produced by injured cells from the same culture during 6 hours of recovery in SDM plus 0.1% (by volume) vitamins (SDMV) or TSB (Table III).

Table III shows that identical amounts of coagulase were produced during recovery in SDMV and TSB. From these data, it appears that there are no unspecified nutritional factors present in TSB that are necessary for coagulase production during recovery.

TABLE III

THE EFFECT OF VARIATIONS IN THE RECOVERY MEDIUM
ON COAGULASE PRODUCTION BY S. AUREUS MF-31

Recovery Media	% Recovery in 6 hours	Units of Coagulase Produced	Coagulase Detected in 24 Hour Assay
A, P, G, V (SDMV)	100%	12	
A, P, G (SDM)	100%	2	
A, P, V	8.0%	0	negative
A, G, V	17.0%	0	negative
P, G, V	2.2%	0	negative
TSB	100%	12	

A Casamino acids (1.0%) + tryptophane (2 mg/ml)

P 0.5% K_2HPO_4

G 0.2% glucose

V 0.1% (by volume) MEM vitamins

Figure 5. Growth and coagulase production by uninjured cells of S. aureus MF-31 in SDMV

Log viable cells/ml, TSA

Units of coagulase/ml

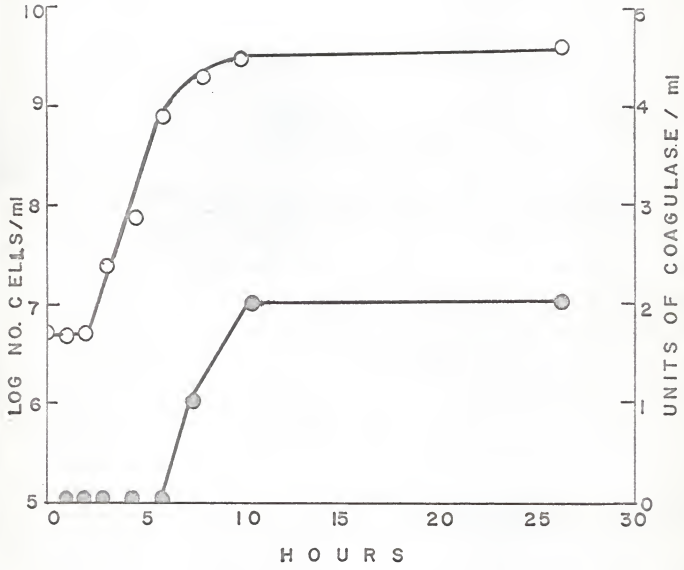

LOG NO. CELLS/ml
UNITS OF COAGULASE / ml
HOURS
10
9
8
7
6
5
0
5
10
15
20
25
30
6
4
3
2
1
0

TABLE IV

THE EFFECT OF PENICILLIN AND CHLORAMPHENICOL ON
COAGULASE PRODUCTION BY INJURED CELLS OF S. AUREUS MF-31

	Coagulase Assay, 3 hour test	Coagulase Assay, 15 hour test
Injured Cells, recovered 6 hours in TSB + Penicillin (833 µg/ml)	+	
Injured Cells, recovered 3 hours in TSB + Chloramphenicol (50 µg/ml)	-	-
Injured Cells, recovered 3 hours in TSB (Chloramphenicol Control)	+	+

\+ Coagulase detected

\- Coagulase not detected

The data of Table III indicate that identical quantities (12 units) of coagulase were produced by injured cells recovering in SDMV and TSB. It was of interest to study coagulase production by uninjured cells growing in SDMV, and to compare the results with Figure 1. The data are depicted in Figure 5 and indicate that the growth rate in SDMV is slightly less than the growth rate in TSB (Figure 1). The logarithmic phase in SDMV was about 7.5 hours in length, while the logarithmic phase in TSB was about 6 hours in length. The lag phase in SDMV was about 1.5 hours as opposed to a 1 hour lag phase in TSB. The cell population at stationary phase was 3.4×10^9 cells/ml in both media indicating that the final population levels in both media were essentially equal. Two units of coagulase were produced in SDMV and 3 units of coagulase were produced in TSB. It appears, from the data of Figure 1 and Figure 5, that the overall growth parameters and coagulase production by uninjured cells in SDMV and TSB are similar, although not identical. Growth rate and coagulase production by uninjured cells appears to be slightly greater in TSB.

The metabolic inhibitors penicillin and chloramphenicol were used to partially clarify the nature of coagulase production during recovery. The results, shown in Table IV, indicate that cell wall synthesis (inhibited by penicillin) was unnecessary for coagulase elaboration by injured cells. Protein synthesis (inhibited by chloramphenicol) was necessary for coagulase production during recovery.

Iandolo and Ordal (31) showed that recovery of salt tolerance by S. aureus MF-31 occurred in the presence of penicillin and other cell wall inhibitors. They indicated that this was to be expected since there was no evidence that heat treatment affected the bacterial cell wall. The results in Table IV indicate that coagulase production during recovery occurred in

the presence of penicillin. This also was expected since coagulase, a protein with enzymatic properties (10, 17, 60, 61, 70), should not be affected by a cell wall inhibitor.

It was shown (8, 31, 58) that recovery of salt tolerance by heat-injured S. aureus MF-31 occurred in thr presence of chloramphenicol, 5 methyl-tryptophane, and other protein synthesis inhibitors. Amino acids were required for recovery of salt tolerance as carbon skeletons for the resynthesis of r-RNA. The results of Table IV, however, indicate that coagulase production during recovery was inhibited by chloramphenicol. This result is expected because coagulase is a protein with enzymatic properties (10, 17, 60, 61, 70). The results imply that coagulase synthesis was not essential for recovery of salt tolerance. If coagulase synthesis had been essential for recovery, recovery could not have occurred in the presence of chloramphenicol since chloramphenicol inhibited coagulase synthesis. It is noted that while coagulase synthesis was not essential for recovery, it did occur during recovery and may indicate that other protein synthesis occurred during the recovery period.

Bluhm (8) observed the return of some protein activity in the presence of chloramphenicol by injured cells during recovery. He concluded that this was not de novo synthesis, but rather, a reversal of heat-induced denaturation by an energy requiring reaction. Such a reaction would not be affected by protein synthesis inhibitors. The finding that chloramphenicol completely inhibited the elaboration of coagulase by injured cells during recovery indicated that coagulase is synthesized de novo by injured cells during recovery.

Studies on the relationship between coagulase synthesis during recovery and the re-synthesis of ribosomes during recovery were performed. Three liters of 12 hour cells were injured at 54 C for 15 minutes in 50 ml of 50 mM sodium

phosphate buffer, pH 7.2. The cells were recovered in 100 ml TSB containing uracil-6-^{3}H. Coagulase tests were performed at 0, 1, 2, 3 and 4 hours. Samples were removed for ribosome analysis and cell counts on TSA and TSAS at 0, 2 and 4 hours. Cell counts on TSA and TSAS, and coagulase production during recovery are shown in Figure 6a. The ribosome profile at 0 and 2 hours recovery, prepared by L. J. Rosenthal (53), is shown in Figure 6b.

Figure 6b indicates that the 30S ribosome peak is destroyed by sub-lethal heat treatment. The 50S ribosome peak, however, is apparently unaffected. In Figure 6, the reappearance of the 30S peak at 2 hours coincided with the commencement of coagulase synthesis between 1 and 2 hours after injury. There is evidence (53) that the resynthesized ribosomes at 2 hours are immature. If this hypothesis is confirmed, it will imply that coagulase, a protein with enzymatic properties (10, 17, 60, 61, 70), is synthesized on immature ribosomes.

Figure 6. Recovery, coagulase production, and ribosome profile of 12 hour cells injured at 54 C for 15 minutes in 50 M phosphate buffer, pH 7.2, and recovered in TSB plus 0.1 uc ^{3}H uracil/ml.

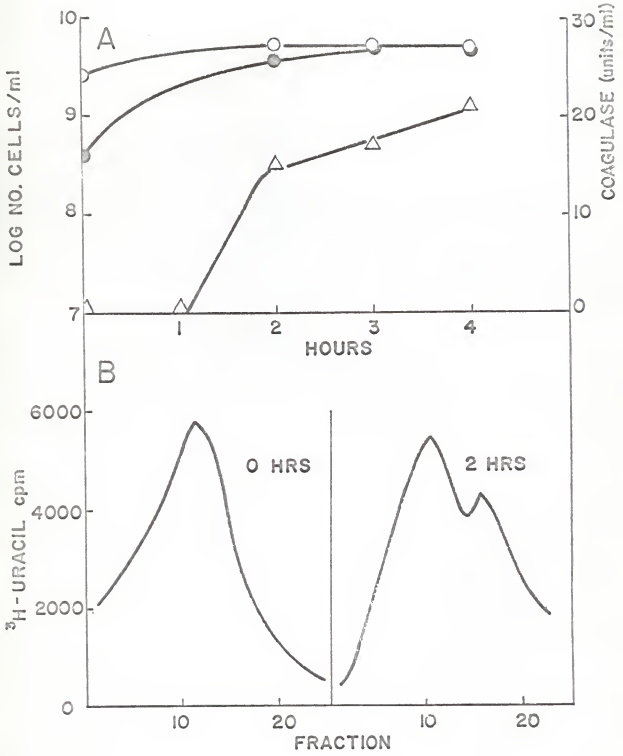
A. Recovery and coagulase production

Log numbers cells/ml, TSA

Log number cells/ml, TSAS

Units of coagulase/ml

B. Ribosome profile. Ribosomes were isolated according to Rosenthal (53) and analyzed by sucrose sensity gradient (5%-20%).


A
LOG NO. CELLS/ml
COAGULASE (units/ml)
HOURS
B
^{3}H-URACIL cpm
0 HRS
2 HRS
FRACTION

V. SUMMARY AND CONCLUSIONS

It was found that uninjured cultures of S. aureus MF-31 produced coagulase only during the logarithmic growth phase. Further experimentation revealed that resting cells did not elaborate coagulase. These data indicate that there was a correlation between multiplication and coagulase production.

In contrast, cells that were injured at 54 C for 15 minutes in sodium phosphate buffer, pH 7.2, produced coagulase during recovery. Cell multiplication did not occur during recovery because cell counts on TSA did not increase (31). Furthermore, coagulase was produced by injured cells during recovery in SDM, a medium in which cell multiplication was not possible. Coagulase elaboration during recovery was independent of the molar strength of the buffer in which the cells were injured, the number of cells injured, or the age of the cells prior to injury.

The quantity of coagulase produced by injured cultures exceeded that produced by uninjured, multiplying cultures. The number of viable cells/ml in recovering and multiplying cultures were similar. No conclusive statement concerning the relative rates of coagulase production by injured and uninjured cells could be made from the data.

Coagulase production during recovery commenced between 1 and 4 hours after injury, and ceased between 4 and 6 hours after injury. The ability to multiply was restored between 4 and 6 hours after injury (31). It was hypothesized that injured cells began producing coagulase after a short incubation period, before salt tolerance was fully restored. Injured cells lost the ability to produce coagulase in the absence of multiplication coincident with the return of the ability to multiply.

Nutritional studies indicated that the nutrients required for complete recovery of salt tolerance were also required for coagulase synthesis during recovery. This finding refutes earlier work (35, 62) that the nutrients required for growth were also required for coagulase production. Vitamins did not effect the recovery of salt tolerance, but the addition of vitamins to recovery media stimulated the production of coagulase during recovery. SDMV and TSB were found to be similar in supporting growth, coagulase production during growth, and coagulase production during recovery.

Coagulase synthesis during recovery was not inhibited by penicillin (833 µg/ml) indicating that cell wall synthesis was not involved in coagulase production during recovery. Chloramphenicol (50 µg/ml), however, completely inhibited the production of coagulase. This implied that coagulase synthesis was not essential for recovery of salt tolerance because recovery of salt tolerance occurred in the presence of chloramphenicol (8, 31). The finding that coagulase synthesis, while not essential for recovery, occurred during recovery indicates that other protein synthesis may occur during recovery. The data showed that coagulase was synthesised de novo by injured cells during recovery.

Studies on coagulase production during recovery and ribosome resynthesis during recovery indicated that the initiation of coagulase synthesis by injured cells paralleled the reappearance of the 30S ribosome peak.

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COAGULASE PRODUCTION BY HEAT INJURED *STAPHYLOCOCCUS AUREUS* MF-31

by

MICHAEL WILLARD PARIZA

B. S., University of Wisconsin, 1967

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Division of Biology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1969

ABSTRACT

Normal (uninjured) cultures of S. aureus MF-31 produced coagulase only during the logarithmic growth phase. In contrast, cells that were injured at 54 C for 15 minutes in sodium phosphate buffer at pH 7.2, produced coagulase during recovery in suitable media. Since cell counts on Trypcase Soy Agar (TSA) did not increase during the recovery period, cell multiplication was not implicated in recovery (8). Furthermore, coagulase was produced by injured cells during recovery in a medium which did not support cell multiplication. Coagulase synthesis during recovery was independent of the molar strength of the buffer in which the cells were injured, the number of cells injured, or the age of the cells prior to injury. Nutritional studies indicated that the nutrients required for complete recovery of salt tolerance were also required for coagulase synthesis during recovery. Coagulase elaboration by injured cells during recovery was not inhibited by penicillin, but chloramphenicol completely inhibited the production of coagulase during recovery. Studies on coagulase elaboration during recovery and ribosome resynthesis during recovery indicated that the initiation of coagulase synthesis by injured cells paralleled the reappearance of the 30S ribosome peak.