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Production of Extracellular Enzymes and Enterotoxins A, B, and C by *Staphylococcus aureus*

AUDREY W. JARVIS AND R. C. LAWRENCE
New Zealand Dairy Research Institute, Palmerston North, New Zealand

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Eighty-seven strains of *Staphylococcus aureus* were examined for their ability to produce enterotoxins A, B, and C, deoxyribonuclease, lysozyme, proteinase, lipase, and α-, β-, and δ-hemolysins. Enterotoxigenic strains showed a significant tendency to be high lipase producers, but none of the other enzymes formed were correlated with the ability of the staphylococci to produce enterotoxins A, B, or C. The conversion of ent$^-$ to ent$^+$ strains by lysogenization did not affect significantly the ability of the strains to produce any of the above extracellular enzymes. The formation of enzymes such as deoxyribonuclease and lysozyme by staphylococci is not therefore an indication, necessarily, of their potential enterotoxigenicity.

The detection of enterotoxin production in foods and cultures by strains of *Staphylococcus aureus* is hampered by the difficulties of extracting and concentrating the enterotoxins from food and the increasing number of antigenically distinct enterotoxins which need to be considered. The most widely used criterion for distinguishing enterotoxigenic staphylococci is the coagulase test, even though it is recognized that strains of *S. aureus* may lose this characteristic and still retain enterotoxigenicity (14). A need, therefore, still exists for some easily measured characteristic of enterotoxigenic staphylococci which is not shared by other related organisms. Much interest has been shown in reports that a number of enzymes produced by staphylococci are linked with enterotoxin production, such as heat-stable nuclease (3, 19), and lysozyme (15).

An investigation was undertaken to examine the relation between enterotoxin production and a number of other physiological characteristics. Eighty-seven strains of *S. aureus* of human and bovine origin were studied for the production of enterotoxins A, B, and C, total extracellular protein, and seven enzymes which have been considered by other workers to have been associated with toxigenic staphylococci. A number of strains were also lysogenized with phage from enterotoxin-producing staphylococci to determine whether significant changes occurred in the ability of the organism to produce any of these extracellular enzymes as it acquired the ability to produce enterotoxin.

MATERIALS AND METHODS

Strains. A total of 87 strains of *S. aureus* were studied. Thirty-five were clinical strains derived from lesions in hospital patients, 24 were from mastitic cows, and 25 were NCTC phage-propagating strains. *S. aureus* 100, S-6, and 361 were obtained from M. S. Bergdoll, University of Wisconsin, Madison, Wis.

Strains were cultured and the supernatant fluids were prepared by a cellophane-over-agar technique (7). Cultures were harvested at 22 hr and centrifuged, and the supernatant fluids were standardized to a given number of cells.

Enterotoxins. The supernatant fluids were tested for enterotoxins A, B, and C by the microslide gel diffusion technique (20), and throughout this paper the terms enterotoxigenic and nonenterotoxigenic refer to the production of these three enterotoxins.

Deoxyribonuclease. Determinations for deoxyribonuclease were carried out by a microslide gel diffusion technique (6) in which the activity of the enzyme is estimated by the production of acid-soluble nucleotides in deoxyribonucleic acid (DNA)-agar.

Proteinase. Levels of proteinase were determined by using a microslide gel diffusion technique (10) in which proteinase was estimated by the zone of precipitation in caseinate agar.

Lipase. Activity was estimated by the zone of clearing in tributyrin agar, by using a microslide technique (8).

Lysozyme. Determination of lysozyme was essentially by the method used by Hawiger (5). Substrate consisted of cells of *Micrococcus lysodeikticus* which had been exposed to ultraviolet light (30 w, 14 cm distance) for 10 min with gentle agitation and then heated at 37 C for 1 hr. In later experiments, lysozyme substrate (Difco) was used, and a comparison was

made of the two substrates. Enzyme (0.1 ml) was added to 4 ml of substrate which had been standardized to an optical density (OD) between 0.80 and 0.90. All OD readings were carried out at 37 C at 55 nm in an S.P. 500 spectrophotometer (Unicam Instruments Ltd, Cambridge, England). Lysozyme activity was measured as the percentage decrease in OD after a further 30 min of incubation at 37 C. The activity obtained could be compared to that of known dilutions of egg-white lysozyme (EWL; Worthington Biochemical Corp.) and thus was recorded as equivalent EWL units.

Total extracellular protein. Measurement of extracellular protein was by a modification of the method of Stadtman et al. (17). Thirty per cent trichloroacetic acid (0.5 ml) was added to a mixture of 1.0 of supernatant fluid and 2.0 ml of 0.85% saline; the OD at 600 nm was read after 7 min. After the addition of trichloroacetic acid, the OD increased rapidly but had generally stabilized after 7 min. The extinction values obtained were expressed in terms of equivalent amounts of bovine albumin. A standard curve was obtained by using dilutions of bovine albumin, OD being a linear function of albumin up to 3.0 mg/ml.

Lysogenization of strains. Lysogenization was carried out by the method of Blair and Carr (1). Lysogenization was confirmed by immunity to the lysogenizing phage, ability to release phage which lysed the parent strain, and change in phage typing pattern.

Penicillin sensitivities. Sensitivity to penicillin was determined by the disc method (Biolab sensitivity discs 10 units). Changes in sensitivity were confirmed by growth on agar containing 10 units of penicillin per ml.

RESULTS

Effect of incubation time on enzyme production. For the enterotoxin and extracellular enzyme determinations carried out on the same supernatant fluids to be meaningful, it was necessary to standardize on an incubation time that would give as near maximum levels of all products as possible. The levels of enterotoxin and extracellular enzymes were, therefore, determined for incubation periods of 10 to 72 hr (Fig. 1).

All three enterotoxins reached maximum level of production by 20 hr and showed no decrease in activity after 72 hr of incubation. Lipase activity reached maximum level by 20 to 24 hr with all strains tested and remained stable as long as 72 hr. Nuclease production reached a peak at 16 to 24 hr but had decreased by 30% by 48 hr. Optimum lysozyme production was reached by 12 to 48 hr, but thereafter activity decreased markedly and by 72 hr had decreased by approximately 40% for all strains examined. It may be that the decrease in nuclease and lysozyme activity on prolonged incubation was accelerated by the method of culture used. This results in an

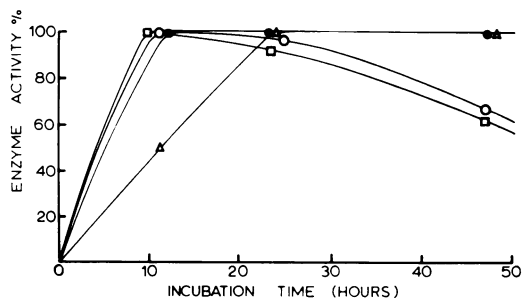

FIG. 1. *Concentrations of some extracellular enzymes in the supernatant fluids of cellophane-over-agar cultures of S. aureus after various incubation times at 37 C. Nuclease* (○), *enterotoxin A* (△), *and lipase* (●) *were recorded as zones of activity (mm) by microslide gel diffusion tests. Lysozyme* (□) *was determined relative to the activity of egg-white lysozyme (μg/ml).*

enzyme preparation comparatively free from medium constituents, and there is some evidence that the presence of compounds such as peptone (9) delays the denaturation of some enzymes. However, these preliminary investigations showed that for all strains examined 20 to 24 hr of incubation gave yields very close to maximum activity for enterotoxins, lipase, lysozyme, and deoxyribonuclease, and 22 hr of incubation was thus used in all subsequent experiments.

Proteinase activity was also measured but was found to vary markedly with incubation time. Maximum activity was reached between 12 and 20 hr for four strains tested in 10 time sequences, and activity decreased rapidly on longer incubation. Further experiments showed that staphylococcal proteinases were extremely heat-labile, the activity of supernatant fluids decreasing by 85% after 20 hr of incubation at 37 C.

Correlation of extracellular enzyme production and enterotoxigenicity. All enzyme and enterotoxin assays for any one strain were carried out on the same supernatant fluid. The relation between the production of enterotoxins A, B, and C by various strains and the levels of extracellular enzymes found is shown in Table 1. There was a significant tendency for enterotoxigenic strains to be more highly lipolytic than nonenterotoxigenic strains. None of 23 enterotoxigenic strains produced zones of activity of less than 8.0 mm, whereas 17 of 64 nonenterotoxigenic strains were in this group. There was a significant ($P < 0.01$) correlation between enterotoxigenicity and lipolytic activity ($\chi^2 = 7.7$, 1 degree of freedom).

Nuclease and lysozyme levels showed little correlation with enterotoxigenicity of strains. One of 23 ent$^+$ and 4 of 64 ent$^-$ strains produced very little deoxyribonuclease, whereas 8 of 23 ent$^+$

TABLE 1. *Enzyme activity of standardized supernatant fluids from enterotoxigenic and nonenterotoxigenic strains[a] of staphylococci*

Enzyme[b]	Strains	No. with zone of activity (mm)			
		0-7.9	8.0-11.9	12.0-15.9	>16.0
Lipase	Enterotoxigenic	0	8	7	8
	Nonenterotoxigenic	17	14	18	15
Deoxyribonuclease	Enterotoxigenic	1	7	7	8
	Nonenterotoxigenic	4	24	20	16
Proteinase	Enterotoxigenic	15	5	2	1
	Nonenterotoxigenic	48	8	3	5
Lysozyme	Enterotoxigenic	4[c]	5[d]	14[e]	
	Nonenterotoxigenic	15[c]	12[d]	37[e]	

[a] Enterotoxigenic and nonenterotoxigenic refer to the production of enterotoxins A, B, or C.
[b] Lipase, nuclease, and proteinase levels in supernatants were determined by as in the text. Lysozyme is recorded in units equivalent to the activity of known dilutions of egg-white lysozyme (μg/ml).
[c] Values for lysozyme are <3 μg/ml.
[d] Activity in range of 3.0 to 10.0 μg/ml.
[e] Activity of >10 μg/ml.

and 16 of 24 ent$^-$ produced large amounts. Similarly, 4 of 23 ent$^+$ and 15 of 64 ent$^-$ strains produced little or no lysozyme, whereas 14 of 23 ent$^+$ and 37 of 64 ent$^-$ strains produced large amounts.

All enterotoxigenic strains and the majority of of ent$^-$ strains produced α- and δ-hemolysins (Table 2). However only 6 of 23 ent$^+$ strains produced β hemolysin compared with 29 of 66 ent$^-$ strains. This may reflect the lact of enterotoxigenicity in bovine strains which are generally β-hemolytic, rather than a negative correlation between enterotoxigenicity and the production of β-hemolysin.

No correlation could be found between enterotoxin and proteinase activity in the supernatant fluids (Table 1) although the recent findings (11) suggested that high enterotoxin levels might be associated with low proteinase activity. However proteinase was shown to be extremely unstable when incubated at 37 C, so that the level in the supernatant fluid did not necessarily reflect the ability of a strain to produce proteinase. A measure of this ability was, however, obtained by using the method of Martley et al. (12). This measures proteolytic activity by the zones of precipitation of *p*-caseins around a point inoculation on caseinate agar after 48 hr of incubation at 37 C. There was considerable variation between strains in both the pattern and size of zone produced. Zone diameter varied from 0 to 25 mm, but again no correlation was apparent between the enterotoxigenicity of strains and zone sizes or patterns of proteolysis. This part of the investigation has been reported in full (13). All strains which showed a high level of proteinase activity in the supernatant fluids produced large zones of activity on caseinate agar, but, owing to the instability of the enzyme in the supernatant fluids, the reverse was not true.

Total extracellular protein determinations on culture supernatant fluids varied from 0.0 to 3.7 mg/ml. Of 20 enterotoxigenic strains examined, supernatant fluids of seven strains contained less than 1.0 mg/ml, five contained 1.0 to 1.9 mg/ml, four contained 2.0 to 2.9 mg/ml, and four contained more than 3.0 mg/ml of total extracellular protein. Of 18 nonenterotoxigenic strains examined, nine contained less than 1.0 mg/ml, seven contained 1.0 to 1.9 mg/ml, and two contained more than 3.0 mg/ml of total extracellular protein. As with the individual enzymes detailed above, there was a small tendency for ent$^+$ strains to produce slightly more extracellular protein than ent$^-$ strains, but no correlation between the enterotoxigenicity of strains and the total extracellular protein content of the supernatant fluids could be determined.

Total extracellular protein determinations in the supernatant fluids showed a wide variation which did not correlate with the source of strains or any of the extracellular enzymes or enterotoxins studied. This was in contrast to Richmond (16), who found a direct correlation between lysozyme and total extracellular protein in culture extracts of *Bacillus subtilis*. Thus, none of the enterotoxins or enzymes studied formed a constant proportion of the total extracellular protein.

Correlations between the production of extracellular enzymes and the source of staphylococcal strains. Enzyme production by staphylococcal strains from various sources is shown in Table 3. Bovine strains tended to be low and clinical strains high lipase producers. No correlation was

TABLE 2. *Production of hemolysins by enterotoxigenic and nonenterotoxigenic[a] strains of staphylococci*

Strain	Hemolysins produced[b]							No. of strains tested
	αβδ	βδ	αδ	α	β	δ	Nil	
Enterotoxigenic	6	0	17	0	0	0	0	23
Nonenterotoxigenic	23	3	25	1	1	8	3	64

[a] Enterotoxigenic and nonenterotoxigenic refer to the production of enterotoxins A, B, or C.
[b] Hemolysins (α and δ) were detected as lysis of rabbit and human erythrocytes (5% in nutrient agar), respectively, at 37 C. β-Hemolysin was detected as hot-cold lysis of sheep erythrocytes.

TABLE 3. *Number of staphylococcal strains from various sources showing different levels of enzyme activity*

Enzyme[a]	Strain	No. with zone of activity (mm)			
		0–7.9	8.0–11.9	12.0–15.9	>16.0
Lipase	Clinical	1	5	14	15
	NCTC[b]	0	8	12	5
	Bovine	15	9	0	0
	Special[c]	0	0	0	3
Deoxyribonuclease	Clinical	0	14	11	10
	NCTC[b]	2	8	7	8
	Bovine	3	8	8	5
	Special[c]	0	1	1	1
Proteinase	Clinical	1	6	17	11
	NCTC[b]	6	5	6	8
	Bovine	4	4	1	15
	Special[c]	0	0	1	2
Lysozyme	Clinical	1[d]	5[e]	29[f]	
	NCTC[b]	8[d]	4[e]	11[f]	
	Bovine	9[d]	4[e]	11[f]	
	Special[c]	1[d]	2[e]	0[f]	

[a] Lipase, deoxyribonuclease, and lysozyme levels in supernatants were determined as in Table 1. Proteinase activity was measured as the diameter of zone of precipitation on caseinate agar after 48 hr of incubation at 37 C.
[b] NCTC strains were phage-propagating strains.
[c] Special strains were known enterotoxigenic strains 100, S-6, and 361.
[d] Activity of <3.0 μg/ml.
[e] Activity of 3.0 to 10.0 μg/ml.
[f] Activity of >10 μg/ml.

apparent between nuclease or proteinase activity and the origin of the strains examined.

There was a marked tendency for clinical strains to be high lysozyme producers, 29 of 35 clinical strains producing more than the equivalent of 10 μg of EWL/ml. By comparison, out of 24 bovine strains investigated, nine produced the equivalent of 3 μg of EWL/ml, four produced 3 to 10 μg/ml, and 11 produced more than 10 μg of EWL/ml.

Effect of lysogenization on production of enterotoxin and extracellular enzymes. Donor, recipient, and lysogenized strains were tested for enterotoxin production (Table 4). All isolates of lysogenized strains gave similar results. By using phage from PS42D, strains 60 and 95 were converted to enterotoxin A production. The reproducibility of this conversion to enterotoxigenicity was shown in repeated experiments. Five isolates of 60 (L-PS42D) and 95 (L-PS42D) were again shown to have been lysogenized and to have been converted to enterotoxin A production, with the same alterations in phage type. In every experiment, every clone of strains 60 and 95 which was lysogenized by phage from PS42D acquired the ability to produce enterotoxin A. Strains lysogenized with phage from PS53 and PS77 remained nonenterotoxigenic.

Lipase, nuclease, proteinase, and lysozyme determinations were carried out with the donor,

TABLE 4. *Effect of lysogenization on the production of enterotoxins, extracellular enzymes, and on penicillin sensitivity*

Strain	Zone of activity (mm)[a]			Lysozyme activity	Penicillin sensitivity	Enterotoxin
	Lipase	Nuclease	Proteinase			
PS53	10.1	16.5	19.0	0.2	R	AB
23	14.5	13.0	9.6	1.9	S	Nil
23 (L-PS53)	14.5	12.5	Tr[b]	3.4	R	Nil
41	15.2	12.1	12.8	5.3	S	Nil
41 (L-PS53)	14.8	12.0	9.3	10.0	R	Nil
PS77	15.3	17.2	17.4	0	R	AB
64	8.3	14.2	14.6	9.4	R	Nil
64 (L-PS77)	8.4	13.8	Tr	7.9	R	Nil
PS42D	15.5	11.4	Tr	9.5	S	A
60	Tr	14.0	15.6	8.5	R	Nil
60 (L-PS42D)	Tr	14.5	14.6	6.5	R	A
95	Tr	13.0	10.0	5.1	R	Nil
95 (L-PS42D)	Tr	13.5	7.5	5.3	R	A

[a] Lipase and nuclease activities determined as in the text. Proteinase activity recorded as zones of precipitation on caseinate agar. Lysozyme activity is recorded in units equivalent to the activity of known dilutions of egg-white lysozyme (μg/ml).

[b] Tr, trace.

recipient, and lysogenized strains (Table 4). No significant changes in lipase, nuclease, and lysozyme activities or hemolytic patterns were found after lysogenization. Strains 23 and 41 were converted to penicillin resistance by phage from penicillin-resistant PS53.

Intercolony variation. At an early stage in this investigation, it was necessary to determine whether the intercolony variation reported for the production of enterotoxin B (18) and hemolysin (4) invalidated the general approach adopted.

A fluctuation test was carried out to determine the variation within strains 100, S-6, and 489 in the production of nuclease, lipase, and lysozyme. One colony was used to inoculate a broth from which subsequently 16 cellophane-over-agar plates were inoculated. At the same time, 16 colonies from the same subculture were transferred to 16 broths, each of which was used to inoculate one cellophane-over-agar culture. The resultant supernatant fluids were tested for lipase, nuclease, and lysozyme, and variance determinations were carried out. For the strains tested, nuclease and lipase production did not vary significantly between colonies. Strain 489 showed considerable variation in lysozyme production, but this variability was not found with strains 100 and S-6.

DISCUSSION

Lipase was the only enzyme investigated which showed a statistically significant correlation with the production of enterotoxins A, B, and C, ent$^+$ strains having a higher lipolytic activity than ent$^-$ strains. However, 47 of 64 ent$^-$ strains also produced lipase, and two lipase-negative ent$^-$ strains were converted to enterotoxin A production without change in lipolytic activity. This indicates that lipolytic activity is not an essential character of enterotoxigenic strains.

Deoxyribonuclease was produced in various amounts by ent$^+$ and ent$^-$ strains of staphylococci alike, and no correlation could be detected between the quantitative production of deoxyribonuclease and enterotoxins. Although Chesbro and Auborn (3) claimed that the nuclease test was as sensitive as the serological test for detection of enterotoxin A, the findings of this present investigation have shown that whereas the detection of heat-resistant nuclease in food would suggest growth of large numbers of staphylococci has taken place, it is not specific for enterotoxigenic strains. However, as further new enterotoxins are continually being discovered, it may be that an increasing number of deoxyribonuclease-positive strains will be found to be enterotoxigenic, and a correlation between the two may become more evident.

The destruction of bacterial toxins by proteinases produced by the same strain has been reported for some species (11). This does not appear to be true of staphylococcal enterotoxins, no correlation being found between proteinase activity and enterotoxigenicity. Lysozyme has been proposed as an index of enterotoxigenicity

(15), but in the present study 4 of 23 enterotoxigenic strains produced very little lysozyme, one of them being lysozyme negative. These findings suggest that lysozyme activity is of little value in the detection of enterotoxigenic strains, particularly since 63 of 64 nonenterotoxigenic strains were also found to produce lysozyme.

The production of lipase and lysozyme appeared to be related to the source of strains, clinical strains producing higher amounts of both these enzymes than bovine strains. The hemolysin pattern also showed some correlation with the source of strains. β-Hemolysin was produced by the majority of bovine strains but by only a smaller number of human strains, whereas α- and δ-hemolysins were produced by the majority of strains in both groups. It is therefore suggested the production of lipase, lysozyme, and β-hemolysin may give a tentative indication of the likely human pathogenicity of staphylococcal strains.

Attempts were made to lysogenize strains of *S. aureus* with phage from staphylococci in phage groups I, II, III, and IV. The only phage which successfully lysogenized strains were from PS53, PS77, and PS42D. These are the strains with which Casman (2) achieved lysogenization, and as in the results presented here Casman found that phage from PS42D conferred the ability to produce enterotoxin A. Although it seems unlikely that enterotoxin A is phage-dependent, it appears that the phage from PS42D carries in its genome information which confers the ability to produce enterotoxin A. The lack of significant changes in the production of enzymes investigated confirms the lack of correlation between these characters and enterotoxin A production.

This investigation suggests that formation of any of the extracellular enzymes examined will not indicate whether a particular strain of *S. aureus* produces enterotoxin A, B, or C. Whereas it must be emphasized that some of the strains examined may produce enterotoxins D or E, or unidentified enterotoxins, the wide range of enzyme activity exhibited by A, B, and C strains suggests that a correlation between the production of any enterotoxins and the enzyme examined here is unlikedly. As enterotoxins and their antisera become increasingly more available, so the direct detection of enterotoxins by gel diffusion tests on a routine basis will become more feasible. Such tests appear to be the only satisfactory method of detecting enterotoxigenic staphylococci but have the limitation that the number of antisera required will increase as new antigenically distinct enterotoxins are discovered

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