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Synthesis of Catalase in *Staphylococcus aureus* MF-31

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During the growth of *Staphylococcus aureus* MF-31, initial catalase activity dropped to a reduced level at the onset of exponential phase before increasing. When *S. aureus* was grown at 25, 32, or 37°C, catalase activity was found to decrease by 80 to 90% within 1 h of inoculation. Two catalase-negative mutants and wild-type *S. aureus* MF-31 cells were exposed to exogenous 20 mM H_2O_2 for 15 min. For wild-type *S. aureus*, there was no effect from H_2O_2 until min 15, at which time a 10% decrease in CFU was observed. Both mutants showed increased sensitivity to the H_2O_2, with 56 and 71% reductions in the CFU for mutants C3 and C4, respectively, after a 15-min exposure. Cells of mutant and wild-type *S. aureus* were subjected to sublethal heating at 52°C for 20 min. The lack of catalase activity in the mutants resulted in large decreases in enumeration.

Staphylococcal selective media allow the growth of *Staphylococcus aureus* while inhibiting the growth of other microorganisms. Many of these selective media are satisfactory for the enumeration of normal (unstressed) cells but fail to give total enumeration of stressed staphylococci. Sodium chloride is a common selective agent used in many selective media. The synergistic effects of heat and NaCl in selective media have been shown to decrease the activity of catalase in heat-stressed *S. aureus* cells (4). The accumulation of hydrogen peroxide in or around recovering stressed cells has been implicated as a factor in decreased enumeration on many selective media (1, 7, 15). The addition of catalase to selective media has been demonstrated to increase the enumeration of thermally stressed *S. aureus* (7).

Andrews and Martin (1) examined the effects of heat on catalase from *S. aureus* MF-31 lysates. They reported that catalase activity increased with increasing concentrations of potassium phosphate when the lysates were heated (10 min) at temperatures between 50 and 65°C. When NaCl was added to the lysates, inactivation was observed. Heating lysates at 62°C caused a decrease in catalase activity but not complete inactivation. When Andrews and Martin (2) examined catalase activity in lysates derived from cells recovering from heat injury, they found an 85% decrease. Galligan et al. (8) found that, even in the absence of heat injury, catalase activity decreased in lysates derived from cells upon inoculation of the cells into fresh growth medium.

The objectives of this study were to examine catalase production during the growth of *S. aureus*, the sensitivity of catalase-negative mutants to H_2O_2, and the heat sensitivity of catalase-negative mutants.

MATERIALS AND METHODS

Organisms and growth conditions. Wild-type *S. aureus* MF-31 and catalase-negative mutants (designated C3 and C4) of *S. aureus* MF-31 were obtained from the University of Illinois Department of Food Science culture collection. Catalase-negative mutants were originally obtained by exposing wild-type *S. aureus* MF-31 cells to diethylsulfonate. Catalase-negative mutants were isolated by replicate plating on tryptic soy agar (TSA) and selection of colonies that did not produce O_2 upon exposure to 30% H_2O_2. Catalase-negative mutants were characterized as follows: gram-positive cocci, coagulase positive, showing thermostable nuclease production, lysostaphin sensitive, and capable of fermenting mannitol anaerobically. All mutants were catalase negative and were shown by a modified benzidine test (5) to possess a functioning cytochrome system. Frozen stock cultures were prepared by inoculating 10 ml of tryptic soy broth (TSB) with 0.1 ml of an overnight stationary-phase inoculum, mixing the two, and freezing the mixture at −20°C.

Frozen stocks were thawed and inoculated into TSB and incubated overnight (approximately 12 h) at 35°C. The resultant stationary-phase culture (approximately 2×10^9 cells per ml) served as the inoculum for these studies. As required, samples were removed and cells were pelleted by centrifugation at 16,000 × *g* for 10 min. The supernatant was discarded, and the cell pellets were stored frozen (−20°C).

Cell lysis and catalase assay. Cells were lysed with lysostaphin, and catalase activity was determined by the reduction of dichromate in acetic acid to chromic acetate when the mixture was heated in the presence of H_2O_2 (1).

Exogenous hydrogen peroxide. Wild-type and mutant *S. aureus* cells were grown overnight in TSB at 35°C and diluted in 0.1% sterile peptone water. One portion (1 ml) of the diluent (approximately 10^6 cells) was transferred to 9 ml of 0.1% peptone with a total concentration of 20 mM H_2O_2. A second portion was transferred to 0.1% sterile peptone water containing 0.02% catalase (34,000 U/mg). The mixtures were held at 25°C, and at appropriate time intervals, samples were removed, added to 0.1% peptone water containing 0.02% catalase, and spread plated on TSA. Plates were incubated at 35°C for 24 h.

Heat injury. An early-stationary-phase culture was heat injured as described by Andrews and Martin (2) except that the injury menstruum was 10mM potassium phosphate buffer (pH 7.2). The dual-plating procedure of Iandolo and Ordal (9) was used to determine the amount of heat injury. Samples were diluted in 0.1% sterile peptone water dilution blanks and spread plated in triplicate on TSA and on TSA plus 7% NaCl (TSAS). Enumeration on TSA, a nonselective medium, represented the total CFU; enumeration on TSAS, a selective medium, represented the number of uninjured cells. The difference between the two counts was defined as the number of injured cells.

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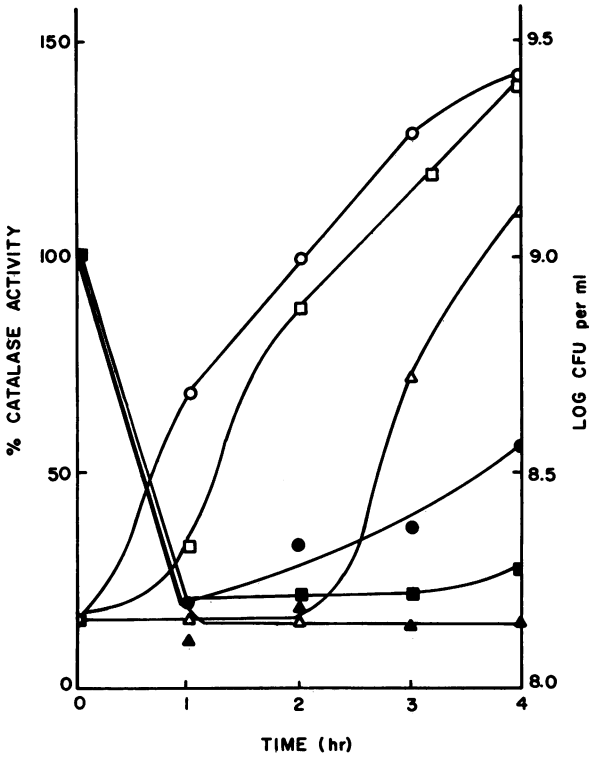

FIG. 1. Growth and catalase activity of *S. aureus* MF-31 at 25, 32, and 37°C. One volume of stationary-phase cells grown in TSB was used to inoculate 200 volumes of fresh TSB tempered to 25, 32, and 37°C. At appropriate time intervals, samples were removed, suspended in dilution blanks, and plated on TSA to determine CFU. Additional samples were removed, and cells were pelleted by centrifugation and frozen. Catalase activity is plotted as a percentage of the activity of the inoculum. Open symbols represent log CFU per milliliter; closed symbols represent activity. ○ and ●, 37°C; □ and ■, 32°C; △ and ▲, 25°C.

Protein assay. The protein concentration of the cell lysate was determined by the method of Lowry et al. (14), with lysozyme as the standard.

RESULTS

Catalase activity during growth of *S. aureus*. When a stationary-phase culture served as the source of inoculum for fresh medium at 37°C, the initial catalase activity was high (Fig. 1). Catalase activity then dropped to a low level at the onset of, or early in, exponential phase before increasing. In an effort to examine this phenomenon further, the decrease in catalase activity during the lag phase of growth was investigated with lower-than-optimum growth temperatures to extend lag phase. At 37°C, lag phase was not observed. At 32°C, exponential phase was initiated within 1 h, and at 25°C, lag phase was extended for 2 h (Fig. 1). Under all growth conditions, catalase activity was found to decrease by 80 to 90% within 1 h of inoculation. At 37°C, catalase activity increased at the onset of, or early in, exponential phase, while at 32°C, catalase activity remained low and did not increase until mid-exponential phase. At 25°C, catalase activity remained at 15% of the initial level throughout the 4 h of the study.

Catalase-negative *S. aureus* MF-31 mutants. Two catalase-negative mutants and wild-type *S. aureus* MF-31 cells were exposed to exogenous 20 mM H_2O_2 for 15 min (Table 1). For wild-type *S. aureus*, there was no effect of H_2O_2 until min 15, at which time a 10% decrease in CFU was observed. Both mutants showed increased sensitivity to H_2O_2, with 56 and 71% reductions in the CFU for mutants C3 and C4, respectively, after a 15-min exposure.

TABLE 1. Effects of H_2O_2 on the survival of *S. aureus*[a]

Exposure time (min)	% Enumeration in strain:		
	MF-31	C3	C4
0	100[b]	100	100
5	99	80	80
10	98	80	70
15	90	44	29

[a] Cells were exposed to exogenous H_2O_2 as described in the text and were enumerated on TSA.
[b] Time zero count defined as 100%.

Heat injury and catalase. Cells of mutant and wild-type *S. aureus* were subjected to sublethal heating at 52°C for 20 min. After heat injury, the cells were removed and rapidly cooled. The lack of catalase activity in the mutants resulted in large decreases in enumeration on TSAS (Table 2). A 10- to 100-fold decrease in enumeration was found between counts on TSA and TSAS. In all cases, addition of exogenous catalase to the agar surface increased enumeration on the salt-containing medium.

DISCUSSION

The synthesis of catalase has been studied in many microorganisms (6, 8, 10, 12, 16). In *S. aureus* and many other organisms, the specific activity of catalase is low during the initial exponential phase of growth and increases steadily with cell growth to a maximal level at the onset of, or during, stationary phase. Finn and Condon (6) attributed the initial high level of catalase activity to that already present in the stationary-phase inoculum.

When 1 volume of a stationary-phase culture grown in TSB (37°C) was transferred to 20 volumes of fresh TSB at 0 or 37°C, no decrease in activity was observed (data not shown). These results suggest that transfer of a stationary-phase culture to fresh medium or the change from a high cell density to a low cell density is not involved in the rapid reduction of catalase activity.

Lag phase of growth is the state in which the cell undergoes adaptation to a new environment in preparation for division. Some of the factors known to influence the length of lag phase include the age of the inoculum, previous nutritional conditions, inoculum size, and temperature. As the temperature is lowered from the optimum, the growth rate of a microorganism slows, growth eventually stops, and lag phase is extended. In these studies at lowered tempera-

TABLE 2. Comparative heat injury[a] of wild-type and catalase-negative mutants of *S. aureus* MF-31

S. aureus strain	Log CFU/ml (% enumeration[b]) on:		
	TSA	TSAS	TSASC[c]
Wild type	9.0 (100)	7.7 (5)	8.1 (13)
C3	8.1 (100)	6.9 (6)	7.5 (24)
C4	8.6 (100)	6.8 (2)	8.6 (100)

[a] Heated at 52°C for 20 min in 10 mM potassium phosphate buffer (pH 7.2).
[b] Percent enumeration on TSA defined as 100.
[c] TSASC, TSAS plus 780 U of catalase per plate.

tures, catalase activity was found to decrease at 32°C and remained low until mid-exponential phase, while at 25°C, activity remained at 15% of initial activity for the 4 h of this study. These findings suggest that the decrease in catalase activity was not due to cellular division or protein dilution, but rather to events that precede cellular division.

Kovacs and Marazean (11) suggested that some staphylococcal catalase is bound to the membrane in an unfolded, inactive state. The increase in activity during stationary phase may be due to detachment with subsequent conformational changes, resulting in enzyme activation. During lag phase, a reversal of catalase conformation may occur, resulting in enzyme inactivation.

Two catalase-negative mutants of *S. aureus* were examined. When grown in TSB, both of the mutants and the wild type demonstrated similar growth rates, with the mutants growing to slightly higher cell densities (data not presented). When exogenous catalase (0.002%, 34,000 U/mg) was added to TSB, no enhancement of growth of the strains occurred. In agreement, Loewen (13) found that catalase-negative mutants of *Escherichia coli* grew normally, with no apparent difference in growth rate or extent of growth compared with those of the parental strain.

As expected, catalase-negative mutants of *S. aureus* displayed greater sensitivity to exogenous H_2O_2, confirming the protective role of catalase in the cell defense system. Loewen (13) found that the only physiological difference between catalase-negative mutants of *E. coli* and the wild type was the 50- to 60-fold-greater sensitivity of the catalase-negative mutants to killing by H_2O_2. Barbado et al. (3) suggested that catalase deficiency leads to H_2O_2 sensitivity.

The lack of catalase in the catalase-negative mutants resulted in large decreases in enumeration on the selective medium TSAS. The addition of exogenous catalase was shown to increase enumeration on TSAS. This result is suggestive of the accumulation of H_2O_2 in or around cells of the catalase-negative mutants. Growth on a solid medium would allow greater exposure to atmospheric oxygen, resulting in increased respiration and production of H_2O_2.

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