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Pleiotropic Alteration of Activities of Several Toxins and Enzymes in Mutants of *Staphylococcus aureus*

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Pleiotropic alteration of several genetic characters including toxin production was quantitatively shown with a strain of *Staphylococcus aureus* of phage type 80, 81 which had been given a very specific genetic marker (temperature sensitivity of mannitol fermentation) to avoid confusion by contamination. Thus, alpha-hemolysin hyperproducers obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) mutagenesis were very often hyperproducers of DNase, coagulase, and protease. Their colonies were less yellow than the parent. DNase hyperproducers obtained after NTG mutagenesis were also often hyperproducers of alpha-hemolysin, coagulase, and protease, with colonies less yellow than the parent. Almost all of the revertants obtained by mutagenesis with ethyl methane sulfonate with respect to alpha-hemolysin or DNase were shown to have simultaneously become hypoproducers of alpha-hemolysin, DNase, and protease. Since the pleiotropic alteration of multiple functions was thus quantitatively confirmed, the mechanism underlying this phenomenon should probably be related to a regulatory mechanism common to them.

Several extracellular toxins and enzymes of *Staphylococcus aureus* such as hemolysins, coagulase, DNase (or nuclease), protease, gelatinase, hyaluronidase, and leucocidine may be important for their possible roles in the pathogenesis of staphylococcal infections. Some of them are also important for their taxonomical usefulness in clinical medicine. However, production of these substances is subject to a high frequency of change. Furthermore, it has been widely reported that production of several toxins and enzymes are lost or recovered together by treatment with "noxious substances" (7) or with mutagenic agents (1-5, 8). The combinations of the characters lost or recovered together are different from report to report. It is possible that these observations are concerned with a large number of different mechanisms of genetic changes. If the simultaneous loss or recovery of these characters is due to a mutation of a regulatory mechanism common to all of these characters as was supposed by some investigators (1, 5), this apparent pleiotropism should be observed in terms of quantitative changes of these characters.

This report deals with quantitative observations on pleiotropic alteration of several genetic characters, including toxin production. Confusion of the results by contamination with air-borne staphylococci was excluded by the use of a parent strain with a specific genetic marker (temperature sensitivity of mannitol fermentability) and, for future genetic study by transduction, a strain of *S. aureus* of phage type 80, 81 was used.

MATERIALS AND METHODS

Bacterial strains. The initial parent strain of *S. aureus* S-800 was isolated from a clinical specimen and kindly supplied by S. Mitsuhashi, Department of Microbiology, School of Medicine, Gunma University. It produces alpha-hemolysin, coagulase, DNase, gelatinase, protease, and a golden-yellow pigment. It grows well on Chapman Staphylococcus No. 110 (Eiken, Tokyo) and on a mannitol-salt agar (Eiken, Tokyo) with positive mannitol fermentability and is of phage type 80, 81. From this strain a mutant in which mannitol fermentability was temperature sensitive was obtained as described in Results and used throughout the experiments as the immediate parent to avoid confusion by air-borne contaminant staphylococci.

Assay for alpha-hemolysin. For screening of alpha-hemolysin mutants, blood agar plates prepared from 18 ml of nutrient agar (Difco) supplemented with 0.09 g of NaCl (0.5%) and washed rabbit erythrocytes from 2 ml of blood were used. The quantitative assay of alpha-hemolysin was done by the twofold dilution method. Overnight Penassay (PA) broth (Difco) culture with shaking was diluted serially

twofold with fresh PA broth and mixed with an equal amount of a 4.0% suspension of washed rabbit erythrocytes suspended in PA broth containing 200 μg of chloramphenicol per ml. The tubes were incubated at 37 C for 18 h. At that time, the highest dilution giving complete lysis was determined. Although the concentration of alpha-hemolysin is best designated in HU 50 (50% hemolytic units of hemolysin), we abandoned this method because alpha-hemolysin in the wild-type strain is too weak to be detected by it. To eliminate the possibility of lysis of erythrocytes by extracellular proteins other than alpha-hemolysin, the overnight culture of the parent and the mutant strains was pretreated with anti-alpha-hemolysin antibody, followed by tests for the activities of alpha-hemolysin. This anti-alpha-hemolysin preparation (anti-staphylococcal alpha-hemolysin MR 21, Wellcome Reagents Ltd.) completely abolished the ability to lyse erythrocytes under the experimental condition described above.

Assay for coagulase. Coagulase was also examined by the serial dilution method. Twofold-diluted rabbit plasma containing 200 μg of chloramphenicol per ml was used instead of the erythrocyte suspension used in the alpha-hemolysin assay. The highest dilution giving visible coagulation was determined.

Assay for DNase. For screening of DNase mutants, a DNase agar (Eiken, Tokyo) was used and examined according to the instructions provided by the manufacturer. The quantitative assay of DNase activity was conducted by counting radioactivity rendered soluble with 5% trichloroacetic acid. The DNA sample was prepared by propagating phage T6 on a thymine-requiring mutant of *Escherichia coli* K-12 in the presence of [^{3}H]thymine, followed by the phenol extraction. The radioactive DNA was suspended in physiological saline containing sodium citrate at 5×10^{-4} M and kept at −20 C. Just before use, 0.12 ml of 3.0 M sodium acetate (pH 8.0) was added to 1 ml of the DNA solution, followed by the addition of 1.0 ml of isopropanol. Then DNA was precipitated by centrifugation and resuspended in 1 ml of 0.2 M Tris buffer (pH 9.0). The reaction mixture consisted of 0.2 ml of overnight PA broth culture, 0.1 ml of radioactive DNA, 0.05 ml of chloramphenicol solution (1 mg/ml), and 0.05 ml of 0.2 M Tris buffer (pH 9.0) containing $CaCl_2$ at 4×10^{-3} M. The tubes were kept at 37 C for 2 h, and the reaction was stopped by the addition of 0.05 ml of 50% trichloroacetic acid. As a carrier, 0.05 ml of 1% crystalline bovine serum albumin was added. After centrifugation, a sample of the supernatant was collected on a filter paper, which was then dried and rinsed in ether for 30 min to remove trichloroacetic acid. After drying, the radioactivity was counted in a liquid scintillation counter (Beckman). The DNase activity of our strain was shown to be dependent on the presence of Ca^{2+} and was inhibited by Mg^{2+}, in accord with the report by Uchida (6).

Assay for protease. Protease activity was determined by measuring the decrease of the turbidity of skim milk broth. A 4-ml sample of overnight PA broth cultures was mixed with 0.5 ml of 4% skim milk solution and 0.5 ml of chloramphenicol solution (1 mg/ml). The tubes were kept at 37 C for 18 h, at which time the decrease in turbidity at 540 nm was measured with a Klett-Summerson photometer.

Tests for temperature sensitivity in mannitol fermentability. Freshly grown cultures were inoculated on two mannitol-salt agar plates with a loop. One of them was incubated at 30 C for 2 days and another at 42 C for 2 days. Those strains that changed agar to a yellow color at 30 C but not at 42 C were adopted.

RESULTS

Isolation and characterization of temperature-sensitive mutants in mannitol fermentability. To avoid confusion from contaminating staphylococci, it was necessary to use a strain with a very specific genetic marker seldom encountered among contaminants. Therefore, temperature sensitivity in mannitol fermentation was adopted since only two out of 56 contaminant staphylococci examined on mannitol-salt agar plates kept open in our laboratory during the day were shown to be temperature sensitive in mannitol fermentation. Cells were harvested from PA broth cultures of *S. aureus* strain S-800 during the logarithmic phase and mutagenized by suspension in 0.5 ml of saline containing 400 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) per ml and incubation for 10 min at 37 C. A colony that was surrounded by a yellow zone on a mannitol-salt agar when grown at 30 C for 2 days but not at 42 C was isolated. No detectable difference in production of toxins and enzymes and in virulence against rabbits was observed between the temperature-sensitive mutant in mannitol fermentation (designated as Mtl-ts hereafter) and its parent strain S-800. Growth of the mutant occurred at 42 C at a rate similar to that of the parent in the absence of added sugar or in the presence of glucose, but it was a little slower than that of the parent when mannitol was added. After 5 h of incubation, the pH of the medium was tested. Both the parent and the mutant caused the pH of the medium to drop to 6.8 in the absence of added sugar and to less than 5.8 in the presence of glucose. The pH of the mannitol-containing culture of the parent was less than 5.8, and that of the mutant was 6.2. From these results, the mutant YST-801 was considered to be temperature sensitive not in growth but in mannitol fermentation. However, this temperature sensitivity is not absolute because the pH of the mannitol-containing culture of the mutant was lower than that of the culture without sugar at 42 C. Hence, the use of a strictly defined method to examine mannitol fermentation at 30 and 42 C is required. This mutant was used throughout the following experiments as the parent, and the temperature

sensitivity of mannitol fermentation was examined at each step in this investigation to avoid confusion by contamination.

Characteristics of alpha-hemolysin hyperproducers. Cells were harvested from a PA broth culture of the Mtl-ts strain YST-801 at the logarithmic phase and mutagenized with NTG. The washed cell suspension was then, directly or after growth in PA broth, plated on blood agar plates containing washed rabbit erythrocytes. Colonies with larger hemolytic zones were picked and subjected to the hemolysin assay. Six independent mutants with higher hemolysin production were obtained. The frequency of mutation was 10^{-3} to 10^{-4} of the survivors. They were characterized as shown in Table 1. There was indeed a high coincidence between hyperproduction of alpha-hemolysin and that of DNase, protease, and coagulase. Thus, out of six hemolysin hyperproducers, five were DNase hyperproducers, five were protease hyperproducers, and four were coagulase hyperproducers. Colonies of all six hyperproducers were less yellow than the parent YST-801. All of these mutants showed a slight but reproducible decrease in salt fastness, judging by growth on nutrient agar containing 12.5% NaCl. This apparent pleiotropic alteration of alpha-hemolysin production and of other genetic characters may not be ascribed to elevated growth rate of the mutants as shown in Fig. 1, which revealed that the growth of the mutants, YST-809 and YST-810 as the representative, was a little slower than that of the parent YST-801. The other four mutants also gave similar growth results.

Characteristics of DNase hyperproducers. Cells of the Mtl-ts strain YST-801 were similarly mutagenized and plated on DNase agar plates, which were incubated overnight at 37 C. The DNase plates after overnight incubation served as the master plates for replication on nutrient agar plates and then were subjected to the DNase tests. The colonies which were surrounded with larger clear zones on the DNase plates were picked from the corresponding colonies on the nutrient agar and subjected to the test for DNase activity. Fourteen independent mutants with higher DNase production were obtained and characterized (Table 2). A high coincidence was shown between hyperproduction of DNase and that of alpha-hemolysin, protease, and coagulase. Thirteen of 14 DNase hyperproducers were also alpha-hemolysin hyperproducers, 11 were coagulase hyperproducers, and 7 were protease hyperproducers. Ten of 14 DNase hyperproducers produced colonies less yellow than those of the parent. All showed a slight but reproducible decrease in salt fastness. The growth of these DNase hyperproducers was also shown to be a little slower than that of the parent. Thus, this curious pleiotropic alteration of DNase hyperproduction and other genetic characters may not be ascribed to the elevated growth rate of the mutants.

Catabolite repression-like mechanism is not responsible for this pleiotropism. Glucose

TABLE 1. *Characteristics of alpha-hemolysin producers*[a]

Strain designation	Relative alpha-hemolysin activity	Relative DNase activity	Relative coagulase activity	Relative protease activity	Colonial pigmentation
YST-801	1	1	1	1.0	Yellow
YST-803	32	0.6	>8	2.4	Yellow to white
YST-807	2	4.5	2	1.0	White
YST-809	32	2.9	1	3.1	White
YST-811	8	3.7	2	2.5	White
YST-812	>64	1.4	2	1.4	Yellow to white
YST-813	16	1.7	1	2.8	Yellow to white

[a] Activities were expressed relative to those of YST-801 (see Materials and Methods).

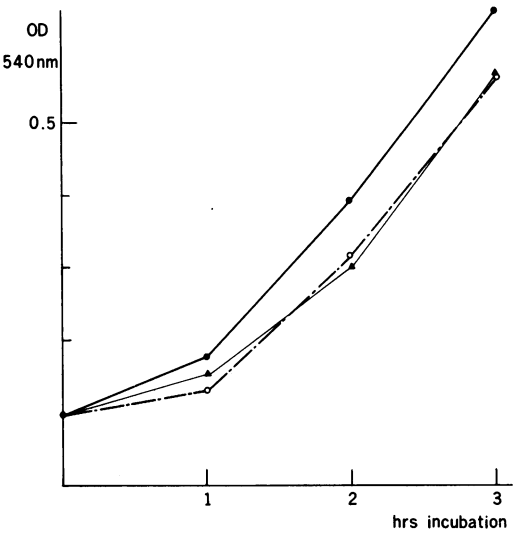

FIG. 1. *Growth kinetics of the parent YST-801 and the mutants YST-809 and YST-810. Overnight Penassay broth cultures of these strains were centrifuged, and the cells were resuspended in 5 ml of Penassay broth to give a reading of 0.1 at 540 nm with a Bausch & Lomb photometer. Growth was read at the intervals indicated. No marked difference in the number of viable cells was observed between the inocula of the parent and the mutants. Symbols:* ●, *YST-801;* ▲, *YST-809;* ○, *YST-810.*

might have inhibited exoenzyme or exotoxin production by a mechanism similar to catabolite repression. The growth medium used was PA broth containing glucose at 0.1%. Some of these alpha-hemolysin and/or DNase hyperproducers were shown to be less effective than the parent in either incorporation (permeation) or accumulation of α-methyl glucoside, and hence of glucose itself (unpublished data). Thus, the decrease in the intracellular concentration of glucose might have been the cause of simultaneous hyperproduction of the several exotoxins. This possibility was, however, excluded by the experiments shown in Table 3. Hyperproduction of the mutants was observed irrespective of the presence or absence of glucose, the change of the pH and also the addition of cyclic AMP.

Pleiotropic reversion of hyperproducers to hypoproducers. Several revertants in either alpha-hemolysin or DNase production were isolated from representative strains of alpha-hemolysin hyperproducers and DNase hyperproducers by mutagenesis with ethyl methane sulfonate (Tables 4 and 5). Many alpha-hemolysin revertants were shown to have lower activities of DNase and protease. Many DNase hypoproducing revertants were also hypoproducers of alpha-hemolysin and protease. As for the coagulase activities, pleiotropic reversion to hypoproduction could not be observed; in fact, there seemed to be no reversion at all.

DISCUSSION

Since the report by Voureka (7) that "noxious substances" induce pleiotropic loss of multiple genetic characters including toxin production in *S. aureus*, many examples of pleiotropic alterations in this microorganism have been reported (1-5, 8). It is, however, curious that the combinations of genetic characters lost or recovered together were different from report to report. One of the reasons for this may be the difference in the mechanisms underlying these phenomena. For example, acquisition of fibrinolysin production and loss of beta-hemolysin production were shown to occur simultaneously because of phage conversion (8). The apparent pleiotropism may in some cases be ascribed to multiple functions or catalytic activities of a single enzyme (4). Still other investigators assumed a common regulatory mechanism in synthesis or release of these exoenzymes (1, 5). Although they asumed a common regulatory mechanism, their experimental results always dealt with absolute loss or recovery rather than quantitative alteration. Furthermore, saprophytic staphylococci are often encountered as contaminants in the laboratory and could be very similar to the mutants with simultaneous loss of toxin production described here. We started our investigation after considering these

TABLE 2. *Characteristics of DNA hyperproducers*

Strain designation	Relative DNase activity	Relative alpha-hemolysin activity	Relative coagulase activity	Relative protease activity	Colonial pigmentation
YST-801	1	1	1	1	Yellow
YST-815	3.4	16	1	3.0	Yellow to white
YST-816	5.7	>64	1	3.1	Yellow to white
YST-817	5.8	32	2	2.9	Yellow to white
YST-818	2.8	16	2	2.6	White
YST-819	3.8	2	4	1.5	White
YST-820	2.8	4	4	1.3	Yellow
YST-821	2.4	4	4	1.3	Yellow
YST-822	4.6	16	8	0.9	Yellow
YST-823	2.1	16	4	1.0	Yellow
YST-824	2.8	32	2	1.0	White
YST-825	1.4	2	2	1.0	Yellow to white
YST-826	2.8	8	2	0.5	White
YST-827	4.1	2	4	1.0	White
YST-828	2.1	1	1	1.0	Yellow to white

TABLE 3. *Comparison of alpha-hemolysin production and the change in pH under various growth conditions*[a]

Growth medium	YST-801	YST-811	YST-812	YST-822
Nutrient broth				
Final pH	7.4	7.4	7.4	7.2
Hemolysin	1	16	8	8
Nutrient broth + 0.2% glucose				
Final pH	4.4	4.4	4.4	4.4
Hemolysin	1	4	4	4
Nutrient broth + 0.1 M phosphate buffer				
Final pH	7.6	7.6	7.6	7.6
Hemolysin	1	8	8	4
Nutrient broth + 0.2% glucose + 0.1 M phosphate buffer				
Final pH	6.8	6.8	6.8	6.8
Hemolysin	1	16	4	16
Penassay broth				
Final pH	7.0	7.0	7.0	7.0
Hemolysin	1	32	8	16

[a] Five milliliters of the growth media indicated was inoculated with washed cells harvested from overnight PA broth cultures to give a reading of 0.1 at 540 nm with a Bausch & Lomb photometer. After the culture was shaken overnight at 37 C, the pH was measured with a test paper (bromothymol blue or bromocresol green), and alpha-hemolysin activities were assayed. The alpha-hemolysin activities were expressed relative to that of nutrient broth for YST-801 culture.

TABLE 4. *Characterization of revertants with respect to alpha-hemolysin production*[a]

Strain designation	Relative alpha-hemolysin activity	Relative DNase activity	Relative Protease activity	Relative coagulase acivity
YST-801	1	1	1	1
YST-809	16	1.45	1.94	2
YST-848	1	0.82	0.48	16
YST-849	1	0.93	0.48	16
YST-850	1	0.87	0.33	16
YST-851	1	0.85	0.48	16
YST-852	4	1.01	0.48	16
YST-853	1	0.81	0.18	16
YST-854	1	1.08	0.18	8
YST-855	1	1.06	0.18	8
YST-856	1	1.13	0.48	16
YST-857	1	1.32	0.33	16

[a] Strains YST-848 to -857 were independently isolated revertants with respect to alpha-hemolysin activity.

TABLE 5. *Characterization of revertants with regard to DNase production*[a]

Strain designation	Relative DNase activity	Relative alpha-hemolysin activity	Relative protease activity	Relative coagulase activity
YST-801	1	1	1	1
YST-822	2.02	32	1.60	8
YST-841	1.18	1	1.19	16
YST-842	1.14	2	0.92	16
YST-843	1.05	1	1.19	16
YST-844	1.27	2	1.15	8
YST-845	1.36	2	1.32	8
YST-846	0.72	2	1.48	32
YST-847	1.08	4	1.42	32

[a] Strains YST-841 to -847 were independently isolated revertants with respect to DNase production.

criticisms and observed a marked coincidence between hyperproduction of alpha-hemolysin, DNase, protease, coagulase, and pigment and slight increase in salt sensitivity.

Because the growth rate of these hyperproducers was almost the same as or slower than that of the parent, the cause of toxin hyperproduction may not be an elevated growth rate of the mutants. Glucose, which was contained in the growth medium (PA broth), was suspected of inhibiting production of some exoenzymes by a mechanism similar to that of catabolite repression. Some of the mutants were shown to be less effective than the parent in incorporation or accumulation of α-methyl glucoside and hence of glucose itself (unpublished data). Thus, the decrease of intracellular concentration of glucose have been considered the cause of toxin hyperproduction of the mutants. However, this possibility was excluded by the finding that hyperproduction was observed irrespective of the presence or absence of glucose. The addition of cyclic AMP was also ineffective in decreasing toxin production by the mutants to the level of the parent.

From alpha-hemolysin or DNase hyperproducers, several revertants with respect to the activities of alpha-hemolysin or DNase were isolated by mutagenesis with ethyl methane sulfonate. Many of them had simultaneously gained lower activities of alpha-hemolysin, DNase, and protease. As for coagulase activity, simultaneous reversion to hypoproduction was not observed. Thus, it could be concluded that these multiple characters are often simultaneously and even quantitatively altered, probably by a single event. Therefore, simultaneous mutations at multiple sites, although they occur very often in NTG mutagenesis, and phage conversion are very unlikely.

There is also the possibility that these characters are regulated by a common regulatory mechanism, including involvement of plasmid, for synthesis or release of these exoenzymes (1, 5). For example, if the structural genes for these multiple characters are under regulation of a single repressor or if there is an active release mechanism common to all of these exoenzymes, their activities could apparently be pleiotropically altered. A genetic study is now under progress to clarify the real mechanism underlying these observations.

However, there are two experimental results that require further consideration. First, there are many exceptions to complete pleiotropic alteration, and second, the activities are not strictly coordinated; i.e., although hyperproducers of a certain toxin are often simultaneously hyperproducers of another toxin, the ratio of the activities of these toxins is not the same in each mutant. This could be ascribed to the assay method, which is not sufficiently accurate except for DNase. This could also be the consequence of secondary involvement of a protease in this pleiotropism. The released protease hydrolyzes the toxins whose production is changed together with the protease, and thus could lead to exceptions in pleiotropism and absence of strict coordination. This could be excluded by studying the toxin production in the presence of antiprotease substances. Seemingly inverse effects of reversion on the activity of coagulase could also be explained by assuming an involvement of fibrinolysin in this pleiotropism. This enzyme may also give rise to an inverse effect on

the activity of coagulase by lysing the coagula produced by coagulase.

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