JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1988, p. 535–539
0095-1137/88/030535-05$02.00/0
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Vol. 26, No. 3

Production of Gamma-Hemolysin and Lack of Production of Alpha-Hemolysin by *Staphylococcus aureus* Strains Associated with Toxic Shock Syndrome

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Received 2 September 1987/Accepted 16 November 1987

The hemolytic activity of toxic shock syndrome isolates of *Staphylococcus aureus* is enhanced when agarose is substituted for agar in blood plates or when strains are grown in liquid culture in the presence of 20% (vol/vol) CO_2 in air. Hemolytic activity of a representative panel of toxic shock syndrome isolates was rigorously assessed both on blood agar and in liquid culture to unequivocally identify the predominant hemolysins produced. As determined by isoelectric focusing and Western immunoblotting, 15 of 15 TSS isolates produced gamma-lysin and 10 of 15 produced delta-lysin. None produced beta-lysin, and only 2 of 15 produced alpha-lysin. The low rate of alpha-lysin production was a most striking characteristic, since all strains were found to have the alpha-lysin gene by Southern blot hybridization.

Toxic shock syndrome (TSS) is a multisystem illness associated with phage group I strains of *Staphylococcus aureus*, most of which produce toxic shock syndrome toxin 1 (TSST-1) (2, 3, 20). Purified TSST-1 has been shown to produce many of the clinical symptoms of TSS in baboons and in rabbits (9, 18) and has been proposed to be a major factor in TSS (2, 9, 10, 20). However, not all the symptoms of TSS are reproducible in animal models, and strains of *S. aureus* that do not produce TSST-1 have been isolated from fatal cases of TSS (13), suggesting that other toxins may play a role in the disease.

In addition to TSST-1 production, a number of other phenotypic characteristics appear to typify TSS isolates. These include typability by phage group I phages, resistance to penicillin, cadmium, and arsenate, proteolysis of hemoglobin and casein, and susceptibility to bacteriocin produced by phage group II staphylococci (1, 11, 21). TSS isolates are less hemolytic than other clinical isolates when grown on sheep blood agar, although this difference is not as striking on rabbit or human blood agar. On the basis of hemolysin patterns on blood agar, Chow et al. (6) suggested that TSS isolates were more likely to produce delta-lysin than other staphylococcal hemolysins. Recently however, it was shown that the hemolytic activity of TSS isolates on blood plates was enhanced by substituting agarose for agar (5), which is a characteristic of gamma-lysin production (12, 14).

Little attention has been paid to the isolation and characterization of the hemolysins produced by TSS isolates in liquid culture. This may be due in part to reports of low hemolytic activity in culture supernatants. However, Carlson (5) recently showed that hemolytic activity in liquid culture is increased in the presence of 20% (vol/vol) CO_2. There is an obvious need to unequivocally identify the hemolysins produced by TSS isolates and to investigate whether the hemolysins are candidate virulence factors in TSS. Thus the hemolytic properties of a representative panel of TSS isolates were rigorously assessed both on blood agar and in liquid culture. Culture conditions were selected to enhance hemolysin production in liquid culture, thereby facilitating isolation and characterization of the principal hemolysins.

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MATERIALS AND METHODS

Bacterial strains. The TSS isolates used in this study were all from either confirmed or highly probable cases of TSS, whereas the non-TSS isolates were from a variety of clinical cases (Table 1).

Hemolytic activity on blood plates. Hemolysis on rabbit, sheep, and human blood agar was assessed by stab inoculating each strain on tryptic soy agar containing 3% (vol/vol) thrice-washed erythrocytes. Each strain was also stab inoculated onto similar blood plates on which 1% (wt/vol) agarose was substituted for agar; plates were incubated at 37°C for 24 h, followed by further incubation at 4°C for 16 h to determine whether beta-lysin, which is a hot-cold lysin, was produced.

Hemolytic activity in liquid culture. Selected TSS strains were grown in 25 ml of Todd-Hewitt broth (Difco Laboratories) in 250-ml Erlenmeyer flasks in the presence of 30% (vol/vol) CO_2 in air at 37°C for 24 h with shaking (150 rpm). The strains were also tested for hemolysin production in Todd-Hewitt broth and in Bernheimer medium (4) without added CO_2. Hemolysin production was optimal in Todd-Hewitt broth, and each strain used in this study was subsequently grown in Todd-Hewitt broth under conditions found to be optimum for hemolysin production (i.e., 125 ml of Todd-Hewitt broth in 250-ml Erlenmeyer flasks at 37°C at 150 rpm for 24 h). Each culture supernatant was titrated for hemolytic activity against rabbit, sheep, and human erythrocytes in microdilution trays (Sarstedt). Doubling dilutions of culture supernatant (25 μl) were made in either phosphate-buffered saline (0.067 M phosphate and 0.077 M sodium chloride, pH 7) for testing against rabbit and human erythrocytes or in Tris-buffered saline (0.01 M Tris, 0.9% [wt/vol] NaCl, pH 7) containing 1 mM $MgCl_2$ for testing against sheep erythrocytes. Rabbit and human erythrocytes were washed three times in phosphate-buffered saline and then suspended in phosphate-buffered saline to give a final concentration of 1% (vol/vol). Sheep erythrocytes were washed and suspended in Tris-buffered saline. An equal volume (25

TABLE 1. Source of bacterial strains screened for hemolysin production

Strain	Supplier[a]	Isolation site	TSST-1[b]
TSS isolates			
8384	PHLS	Vagina	+
8566	PHLS	Vagina	+
82/803	PHLS	Vagina	+
8606	PHLS	Vagina	+
81/8	PHLS	Vagina	+
8723	PHLS	Vagina	+
8904	PHLS	Vagina	+
4221	PHLS	Vagina	+
FRI1169	P & G	Vagina	+
Todd555	P & G	Vagina	+
TSS55	P & G	Vagina	+
PG23	P & G	Vagina	+
465	PHLS	Vagina	+
9886	PHLS	Vagina	+
2303	PHLS	Vagina	+
Non-TSS isolates			
685	FDVH	Urine	+
613	FDVH	Wound	−
601	FDVH	Throat	−
755	FDVH	Thigh	−
664	FDVH	Wound	−
679	FDVH	Throat	+
693	FDVH	Postgraft	−
616	FDVH	Wound	+
731	FDVH	Sputum	−
678	FDVH	Nasal	−

[a] PHLS, Public Health Laboratory Service, Colindale, London, England; P & G, Procter and Gamble Co., Cincinnati, Ohio; FDVH, Federated Dublin Voluntary Hospitals.

[b] TSST-1 was measured by Western immunoblotting.

μl) of a 1% (vol/vol) suspension of washed, packed erythrocytes was added to each well, and then the trays were incubated at 37°C for 30 min, followed by further incubation at 4°C for 1 h. The hemolytic titer (hemolytic units per milliliter) was expressed as the reciprocal of the highest dilution which showed 50% hemolysis as assessed visually. Certain assays were conducted in the presence of 50 μg of egg yolk lecithin (Sigma Chemical Co.) per ml to inhibit delta-lysin (15). Hemolytic activity was also measured after heating culture supernatants at 60°C for 30 min.

Neutralization tests with specific antisera. Culture supernatants (2 μl) of TSS isolates were each mixed with anti-alpha, anti-beta, anti-delta, or anti-gamma serum (2 μl), spotted onto rabbit blood agarose plates, and incubated at 37°C for 16 h. Culture supernatants from *S. aureus* W46, BB, NCTC 10345, and Smith 5R, known to produce alpha-, beta-, delta-, and gamma-lysin, respectively, were used as controls.

Analytical isoelectric focusing. Culture supernatants were concentrated 10-fold by using a CX-10 immersible membrane (Millipore Corp.) under vacuum. After dialysis against 1% (wt/vol) glycine, the concentrates were examined by analytical isoelectric focusing (IEF) with polyacrylamide gels (1 mm) with a pH gradient of 3.5 to 9.5 (LKB Instruments, Inc.). The gels were run according to the manufacturer's instructions at 1,500 V, 50 mA, and a constant power supply of 30 W for 1.5 h. Standards for pH gradient determination were amyloglucosidase (pI 3.5), soybean trypsin inhibitor (pI 4.55), beta-lactoglobulin A (pI 5.2), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), horse myoglobin (pI 6.85), lentil lectin (pI 8.45), and trypsinogen (pI 9.3) (Pharmacia Fine Chemicals). Samples were run in duplicate sets such that one set was stained in an acid extract of Coomassie blue R-250 (Sigma) (B. Billcliffe and J. P. Arbuthnott, Application note, LKB, 1974), and the other was overlaid with agar containing 3% (vol/vol) thrice-washed rabbit erythrocytes; overlays were incubated in a moist box at 37°C for 16 h.

Western immunoblotting. The presence of alpha-, beta-, delta-, and gamma-lysin in concentrated supernatants was determined by Western immunoblotting (19) with specific antisera. Sera were used at a 1/100 dilution, and the antigen-antibody complexes were detected with a protein A-peroxidase conjugate (Sigma). Blots were developed with 4-chloro-1-naphthol as the substrate.

Southern blotting. Chromosomal DNA prepared from 12 TSS isolates and pDU1212 (17) was cleaved with *Cla*I (Boehringer Mannheim Biochemicals) and probed with a 700-base-pair gene probe internal to the alpha-lysin gene (17).

Preparative IEF. The TSS isolate PG23 was grown in Todd-Hewitt broth (Difco) for 24 h at 37°C with shaking (150 rpm). The culture supernatant was concentrated 10-fold by ultrafiltration with a PM-10 membrane (Amicon Corp.). Preparative IEF in a 110-ml column (LKB 8100-1) was performed in the pH range 3 to 10 with 1% ampholine. The column was stabilized in a 5 to 55% (wt/vol) sucrose gradient according to the manufacturer's instructions. Fractions of 1 ml were collected and assayed for hemolysin production as described above. Hemolysin-positive fractions were analyzed on sodium dodecyl sulfate-polyacrylamide gels (16) and stained with Coomassie blue R-250 (Sigma). The presence of alpha-, beta-, delta-, and gamma-lysin was confirmed by Western immunoblotting with specific antiserum.

Antiserum. Anti-alpha, anti-beta, and anti-delta sera were raised in rabbits against purified hemolysins and were supplied by C. Adlam, Wellcome Biotechnology, Beckenham, Kent, England. Anti-gamma rabbit antiserum was provided by T. H. Birkbeck, Department of Microbiology, Glasgow University, Glasgow, Scotland.

RESULTS

Hemolytic activity on blood plates. TSS isolates showed increased zones of hemolysis when agarose was substituted for agar in blood plates (Fig. 1). Agarose enhancement was observed regardless of whether rabbit, sheep, or human erythrocytes were used. However, non-TSS isolates in general exhibited larger zones of hemolysis on blood agar than the TSS isolates, and the agarose effect was not as striking.

Hemolytic activity in liquid culture. Two media were tested for hemolysin production in liquid culture, namely, Todd-Hewitt broth and Bernheimer medium. Neither medium supported hemolysin production in either shake (150 rpm) or static cultures grown in 25-ml volumes of medium in 250-ml Erlenmeyer flasks. However, good yields were obtained in 25-ml shake cultures grown in an atmosphere of 30% (vol/vol) CO_2 in air, as previously reported (5). Good yields were also obtained with Todd-Hewitt broth when in the absence of added CO_2 the volume of medium was increased to either 100 or 125 ml (Table 2). This modification greatly simplified the procedure for hemolysin production and was used for all subsequent work. It should be noted that Bernheimer medium, which is widely used for the production of staphylococcal hemolysins, was less effective than Todd-Hewitt broth in supporting hemolysin production by TSS isolates. Hemolysin production by TSS isolates in

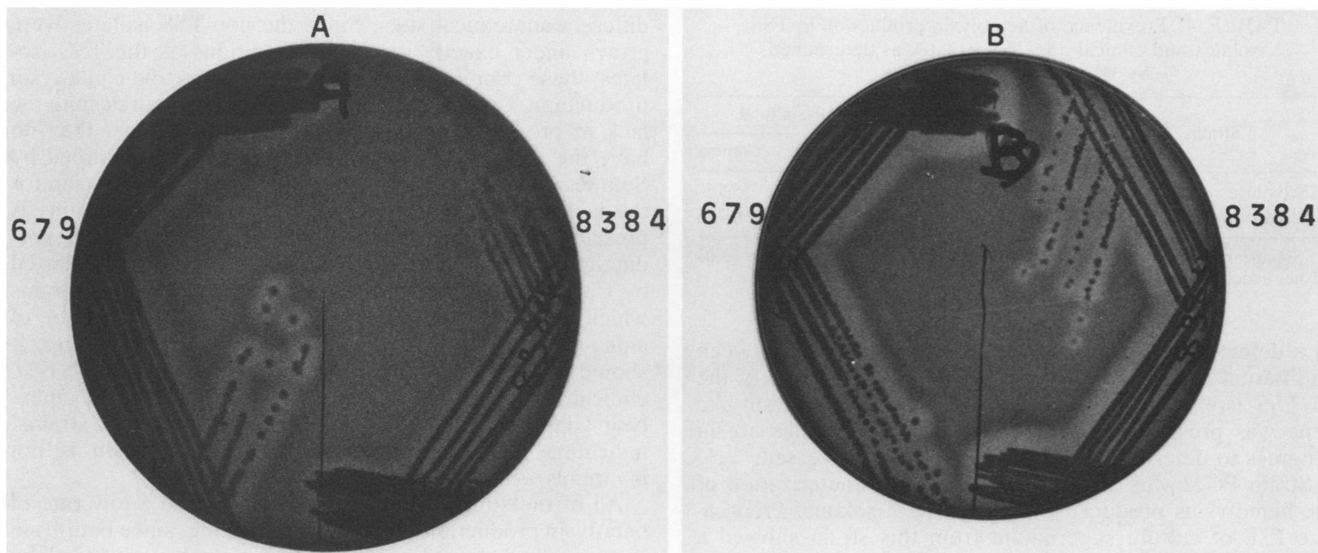

FIG. 1. Hemolytic activity of 8384, a TSS isolate, and 679, a clinical non-TSS isolate, on rabbit blood agar (A) and rabbit blood agarose (B).

Todd-Hewitt broth was fairly significant, and activity was greatest against rabbit erythrocytes (Table 3).

Identification of hemolysins in TSS isolates. A number of observations suggested that the prime hemolysin produced by TSS isolates was gamma-lysin. The hemolytic activity of culture supernatants was heat sensitive, being destroyed by heating at 60°C for 30 min, and was not inhibited by lecithin (50 μg/ml). Both of these findings excluded delta-lysin as the predominant hemolysin, since delta-lysin is thermostable and is also inhibited by lecithin at the concentration used. Also, had delta-lysin been present as the main hemolysin a different pattern of hemolytic activity versus rabbit, sheep, and human erythrocytes (Table 3) would have been expected, since delta-lysin is relatively nonspecific against these erythrocytes. Hemolytic activity of culture supernatants versus sheep erythrocytes was not enhanced by further incubation at 4°C after preliminary incubation at 37°C. This indicates that beta-lysin which exhibits hot-cold hemolysis was also not the predominant hemolysin. Analytical IEF combined with the use of blood overlays showed that concentrated supernatants of representative TSS isolates (PG23, FRI1169, Todd555, TSS55) gave zones of hemolysis close to the cathode in the pH range 9 to 9.5. Zones of hemolysis corresponding to alpha-lysin (pI 8.5) were not detected. In addition, hemolytic activity of representative culture supernatants spot tested on rabbit blood agarose was not neutralized by specific antiserum raised against alpha-, beta-, or delta-lysin, even though these sera did neutralize the supernatants from control strains. Anti-gamma-lysin neutralized the hemolytic activity of the TSS isolates and of the control strain Smith 5R. All of the above findings are consistent with the production of gamma-lysin as the main hemolysin in culture supernatants of TSS isolates. Concentrated culture supernatants were analyzed by Western immunoblotting to unequivocally identify the hemolysin produced by the TSS isolates (Table 4). Gamma-lysin was detected in all samples from 15 TSS isolates, and delta-lysin was detected in 10 of 15 isolates. Surprisingly, alpha-lysin was present in only two of the concentrates, and beta-lysin was not detected. Non-TSS isolates were screened for comparison. These also showed high rates of gamma- and delta-lysin production but differed markedly in that 9 of 10 of them produced alpha-lysin.

Southern blotting with an alpha-lysin gene probe was done to determine whether the absence of alpha-lysin production by TSS isolates was due to the loss of the alpha-lysin gene or

TABLE 2. Hemolytic activity of strain Todd555 grown in different volumes of Todd-Hewitt broth

Vol of broth in 250-ml culture flask (ml)	Hemolytic activity (HU[a]/ml) vs erythrocytes	
	Rabbit	Human
25		
50	128	8
75	128	32
100	256	64
125	256	64
150	128	16

[a] HU, Hemolytic units.

TABLE 3. Hemolytic titers of TSS isolates

Strain	Hemolytic activity (HU[a]/ml) vs erythrocytes		
	Rabbit	Sheep	Human
8384	32	16	4
8566	16	8	2
82/803	16	8	2
8606	64	32	8
81/8	128	16	8
8723	64	8	16
8904	64	32	8
4221	2	2	0
FRI1169	32	64	4
Todd555	128	128	32
TSS55	64	32	8
PG23	64	64	8
465	256	64	8
9886	256	64	32
2303	32	16	8

[a] HU, Hemolytic units.

TABLE 4. Prevalence of hemolysin production in TSS isolates and clinical non-TSS isolates as determined by Western immunoblotting

Strains	No. positive for hemolysin/total			
	Alpha	Beta	Delta	Gamma
TSS isolates	2/15[a]	0/15	10/15	15/15
Clinical non-TSS isolates	9/10	3/10	10/10	10/10

[a] Significant difference from the clinical non-TSS isolates with $P < 0.001$ (Fisher exact test, two tailed).

to a defect in expression. Hybridization occurred between an internal alpha-lysin gene probe and DNA from all of the 12 TSS isolates tested, indicating that an alpha-lysin-like gene was present in these strains (Fig. 2). Studies are in progress to determine why this gene is not expressed.

Strain PG23 was selected for further characterization of the hemolysins produced by typical TSS isolates. Preparative IEF of culture supernatant from this strain showed a single peak of hemolytic activity in the pH region 9 to 9.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblotting confirmed that fractions from this region contained both gamma- and delta-lysin. No alpha- or beta-lysin was detected in any fraction from any region of the column.

DISCUSSION

TSS strains of *S. aureus* have previously been reported to be poor producers of hemolysins when compared with other clinical isolates (1, 6, 11, 21). Contrary to these reports, we have shown that TSS isolates are hemolytic when grown under the appropriate conditions, i.e., substituting agarose for agar in blood plates and growing liquid cultures in the presence of CO_2. We also found that hemolysin production was not dependent upon an atmosphere of 30% (vol/vol) CO_2 but rather on a critical surface area/volume ratio. In view of these findings it is not surprising that other workers missed hemolysin production by TSS isolates. Our findings confirm the work of Carlson (5), who also recently reported that TSS isolates of *S. aureus* show enhanced hemolysis on agarose.

Hemolytic activity of TSS strains on agarose but not on agar strongly suggests the production of gamma-lysin, since this is the only staphylococcal hemolysin which is inhibited by agar. Using a number of different criteria, we have confirmed that TSS isolates produce predominantly gamma-lysin and that some strains also produce delta-lysin. It is interesting that although delta-lysin was detected by Western immunoblotting, the hemolytic activity of culture supernatants did not indicate that delta-lysin was present, suggesting that the latter is produced in negligible quantities or is perhaps masked by gamma-lysin. Production of gamma- and delta-lysins was not confined to TSS strains, since clinical strains also produced these hemolysins. However, the most striking difference was that, unlike other clinical isolates, TSS strains did not produce alpha-lysin. Alpha-lysin is associated with pathogenic staphylococci and is considered to be an important virulence factor (17). Therefore, it is surprising that TSS isolates have such a low rate of alpha-lysin production. Even with the very sensitive technique of Western immunoblotting, which is capable of detecting nanogram quantities of protein, only 2 of 15 TSS isolates were positive for alpha-lysin production, whereas 9 of 10 of the other clinical isolates produced alpha-lysin. These strains were nonendemic and were isolated from a variety of different anatomical sites. Since the non-TSS isolates were grown under exactly the same conditions as the TSS isolates, these conditions are capable of supporting alpha-lysin production. The most interesting finding was that despite the lack of production of alpha-lysin by TSS strains, they do have the alpha-lysin gene, which was clearly identified by Southern blotting with an alpha gene probe. The reason for the lack of expression of this gene in TSST-1$^+$ strains is presently under investigation. Decreased alpha-lysin production in *S. aureus* septicemia strains was recently reported by Christensson and Hedstrom (7); they noted that strains which produced TSST-1 had a particularly low rate of alpha-lysin production, which coincides with our findings. It should be noted, however, that in this study three TSST-1$^+$ clinical non-TSS strains also produced quantities of alpha-lysin similar to those in other TSST-1$^-$ clinical strains, indicating that lack of production of alpha-lysin is not invariably associated with TSST-1 production.

All of the strains tested in this survey had a low rate of beta-lysin production. This is not surprising, since beta-lysin is generally believed to be more frequently produced by strains of animal origin. Also, the expression of beta-lysin can be negatively controlled by two different types of converting phages. One type is a double converting phage; upon lysogenization the organism acquires the ability to express staphylokinase but loses the ability to express beta-lysin. The second type of phage converts to loss of beta-lysin only upon lysogenization (8). It is possible that TSS isolates harbor such phages.

In summary, we have shown that contrary to previous reports TSS isolates of *S. aureus* can be as hemolytic as other clinical strains. Characteristically they produce both gamma- and delta-lysin and not just delta-lysin, as is generally believed. They can be distinguished from other clinical isolates by their lack of alpha-lysin production, although

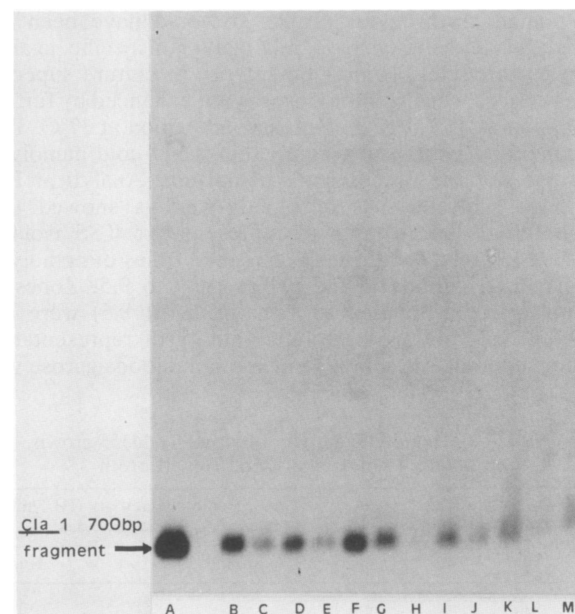

FIG. 2. Southern blot hybridization of chromosomal DNA from TSST-1$^+$ strains with a 700-base-pair alpha-toxin gene fragment. Track A contains pDU1212 DNA; tracks B through M contain whole cell DNA from 12 TSS isolates. The intensity of the bands varies from track to track because of variable DNA concentrations of the samples.

they do have the alpha-lysin gene. It has already been established that TSST-1 is an important virulence factor of TSS strains (9, 10, 18). However, TSST-1 does not reproduce all the symptoms of TSS in animal models. It is likely therefore that other toxins may be involved in the pathogenesis of TSS. It is not unreasonable to speculate that gammalysin could be one such toxin. We hope that further work in our laboratory will serve to evaluate whether gamma-lysin plays a role in the pathogenesis of TSS.

ACKNOWLEDGMENTS

This work was supported by the Procter and Gamble Co., Cincinnati, Ohio.

We thank Karen Kohrman and Roger Stone for helpful discussions at the initiation of this work. In particular we acknowledge Roger Stone's contribution in early experiments involving IEF blood overlays. We also thank Jerry Kirkland for sending us strains and Mary O'Reilly for supplying the alpha-toxin gene probe and for help with the Southern blotting experiment.

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