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Heterogeneity of Cell-Associated CP5 Expression on *Staphylococcus aureus* Strains Demonstrated by Flow Cytometry

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It was reported previously that two capsular polysaccharides, types 5 and 8 (CP5 and CP8), account for 70 to 80% of *Staphylococcus aureus* strains isolated from human and animal sources. The capsular material has been shown to play a part in virulence and in resistance to phagocytosis. With a view to investigating the role that CP plays in pathogenicity or protection, relative measurement of cell-associated CP is desirable. Flow cytometry, which permits the analysis of individual bacteria, was used to that end. Thirty isolates expressing CP5, of human (*n* = 7) and animal (cow, *n* = 11; goat, *n* = 3; swine, *n* = 3; hen, *n* = 3; and rabbit, *n* = 3) origin, were cultivated on either brain heart infusion agar (BHI) or modified medium 110 (mod 110) agar. Staphylococci were incubated with a mouse anti-CP5 monoclonal antibody (an immunoglobulin M, which does not react with staphylococcal protein A) and then stained with a fluorescein-labeled anti-murine antibody. The bacteria were washed, sonicated, and analyzed by flow cytometry. Except for three isolates, the expression of cell-bound CP5 was higher when bacteria were cultivated on mod 110 than when they were cultivated on BHI. We found a wide intraisolate phenotypic heterogeneity in surface-exposed CP5 in many strains, which appeared as mixtures of stained and unstained bacteria. Four main patterns could be distinguished on the basis of the distribution of the fluorescence of individual bacteria within the strain population as a function of growth medium. Great variations in both percentages of stained bacteria and fluorescence intensity were recorded among strains regardless of their origin. Flow cytometry analysis provided information on both the relative amounts and the distribution patterns of the surface expression of CP. This information is potentially useful for the evaluation of the part played by the capsule in the interaction of bacteria with host cells or for the study of the activities of antibodies to this target antigen, such as opsonization or prevention of adherence.

Staphylococcus aureus, which causes infections in humans (23, 24) and animals (1), can produce capsular polysaccharides (CP) that belong to 11 different serotypes (14, 25). Two of these serotypes, 5 and 8, have been shown to comprise 70 to 80% of isolates of human as well as animal origin (4, 19, 21, 25). These two predominant CP are thought to contribute to the virulence of *S. aureus* by interfering with phagocytosis. In in vitro phagocytosis assays, nonencapsulated strains were readily ingested and killed by polymorphonuclear leukocytes while CP type 5 (CP5) and CP8 encapsulated strains resisted phagocytosis (13, 14), although the requirement for CP5- or CP8-specific antibodies for efficient phagocytosis has been challenged (27). On the other hand, type-specific antibodies opsonized type 5 and type 8 strains, rendering them prone to phagocytic killing (13, 27). The two CP are known to be elaborated in vivo during *S. aureus* infections (3, 5), but it is also known that the amount produced in vitro depends on culture conditions (17, 20, 26) and that changes in nutritional environment modify virulence for mice (18, 28). It thus seems logical to pay attention to the amount of CP produced by the different clinical isolates, as well as to the influence of growth conditions.

We report here on the examination of *S. aureus* strains by flow cytometry, which proved suitable for the analysis of the amount of cell-associated CP5 on several strains of different origins. In addition to allowing the measurement of the effect of the culture medium on the amount of cell-associated CP5, flow cytometry, which permits the analysis of thousands of individual cells, revealed the heterogeneity of encapsulation of many strains, which appeared as mixtures of highly and poorly encapsulated bacteria. The results are an incentive to use flow cytometry to examine the characteristics of *S. aureus* strains used in in vitro studies or retrieved from infection foci.

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MATERIALS AND METHODS

MAb specific for CP5. Female BALB/c mice were immunized four times with 10^8 CFU of heat-inactivated *S. aureus* type 5 prototype strain Reynolds (12). Spleen cells of one of these mice were fused with P3-X63-Ag 8.653 mouse myeloma cells (15) according to the method of Kohler and Milstein (16). Hybridoma supernatants were screened by an enzyme-linked immunosorbent assay (ELISA) with purified CP5 conjugated to poly-L-lysine (molecular weight, 52,000) (Sigma Chemical Co., St. Louis, Mo.) by a modification of the method previously described (9). Positive hybridomas were cloned by limiting dilution. Clones secreting large amounts of antibody were selected and injected into pristane-primed BALB/c mice for production of ascites. Isotypes were determined from ascitic fluids by an ELISA with rabbit anti-mouse immunoglobulin G1 (IgG1), IgG2a, IgG2b, IgG3, IgM, or IgA conjugated with peroxidase (Bio-Rad Laboratories, Richmond, Calif.). Monoclonal antibodies (MAb) were precipitated from ascites by ammonium sulfate.

The specificity of MAb 120D3 (selected on the basis of strong ELISA signals and ease of production) was studied by slide agglutination with strains of CP5 (*n* = 8, including prototype strain Reynolds), CP8 (*n* = 8, including prototype strain Becker), and CP2, -4, -9, -10, and -11 (*n* = 1, prototype strain of each CP type) from our collection. Bacteria were grown on modified medium 110 (mod 110) agar plates at 37°C overnight. A loopful of bacteria was mixed with 200 μl of phosphate-buffered saline (PBS), and 20 μl of each suspension was mixed on a glass slide with 20 μl of the MAb diluted 1/10 in PBS. Agglutination was read after 2 min of gentle shaking. MAb 120D3, an IgM, did not react with purified protein A (Pharmacia, Uppsala, Sweden) as tested by ELISA with the protein A being used to coat wells of microtiter plates (data not shown).

Bacterial strains and growth conditions. A total of 31 strains of human (*n* = 8) and animal (cow, *n* = 11; goat, *n* = 3; swine, *n* = 3; hen, *n* = 3; and rabbit, *n* = 3) origin were used in this study. The strains were serotyped by ELISA (19); they were all CP5 except for the prototype CP8 strain Becker, of human origin (12), which was used as the negative control. The strains had been kept freeze-dried in the collection of our laboratory. Identification of *S. aureus* was based on colony morphology, Gram stain, hemolytic pattern, tube coagulase test, and clumping factor.

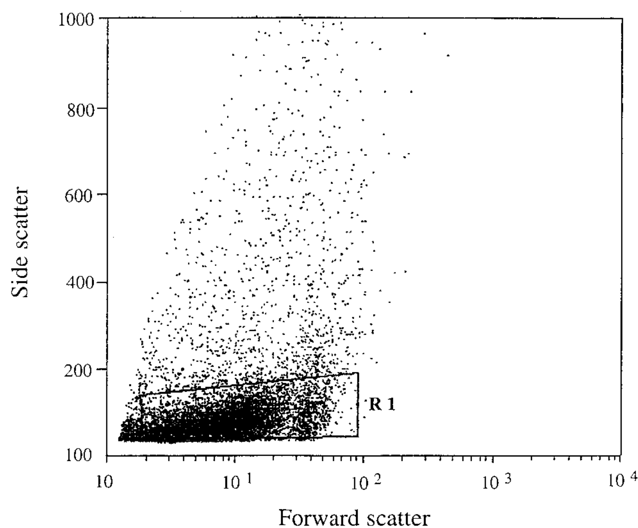

FIG. 1. Forward (low-angle)-scatter versus side (right-angle)-scatter dot plots of bacteria after processing and labeling for analysis by flow cytometry. The region R1 was defined in order to exclude aggregates of bacteria (high values of side scatter) and small debris (lowest values of side and forward scatter). Acquisition of data was restricted to R1.

Two culture media were used: brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) and mod 110, prepared by the method of Yoshida and Ekstedt (28). Purity of strains was checked by culturing on sheep blood agar plates, and at least five colonies were used for broth cultures. After two 24-h subcultures in either BHI broth or mod 110 broth at 37°C, strains were grown overnight at 37°C on either BHI agar or mod 110 agar, respectively. Bacteria were harvested and suspended in PBS (pH 7) containing 0.1% Tween 80 (PBS-Tw). They were centrifuged (2,500 × *g*, 10 min, 4°C), washed once in PBS-Tw, and sonicated (150W ultrasonic disintegrator; MSE, Crawley, United Kingdom) to disrupt possible bacterial clumps under conditions such that viability was not altered (power setting 5, tune 4, 30 s). The concentration of bacteria was then adjusted to approximately 10^7 CFU/ml by spectrophotometric measurement with reference to standard curves.

Flow cytometry assay of MAb binding to viable bacteria. Bacteria (ca. 10^7/ml) were mixed in 200-μl portions with 200 μl of MAb 120D3 (ascitic fluid) diluted 1/10,000 in PBS-Tw and incubated at 37°C for 1 h. For each strain and growth medium there was a control tube incubated with PBS-Tw instead of diluted MAb. Bacteria were washed twice with PBS-Tw, mixed with 200 μl of fluorescein-labeled goat $F(ab')_2$ anti-mouse IgM (Jackson Immunoresearch Laboratories, West Grove, Pa.), and sonicated for 10 s. After 1 h of incubation at 37°C, bacteria were washed once, resuspended in 1 ml of PBS-Tw, and sonicated for 10 s. Ten thousand bacteria were acquired by flow cytometry (FACScan; Becton Dickinson Immunocytochemistry Systems, Mountain View, Calif.) and analyzed with LYSIS II software (Becton Dickinson). Small debris and bacterial aggregates were electronically excluded on the basis of light scatter signals. Antibody binding (fluorescence) was expressed as the percentage of cells fluorescing more intensely than those subjected to the fluorescein conjugate alone (i.e., no anti-CP5 MAb, which was the negative control) and as mean fluorescence intensity (MFI) of bacteria in the region of positively stained cells, i.e., excluding the region of negative controls (see M1 in Fig. 2).

RESULTS

Thirteen clones secreting CP5-specific antibody were obtained from the fusion. Five high-secreting clones were selected and used to produce ascites (one IgG2a, three IgM, and one IgA). The MAb 120D3, of the IgM isotype, agglutinated all CP5 strains and did not react with other CP strains except the CP4 prototype strain. This reaction was expected since the cross-reactivity between CP4 and CP5 has been described (14).

Light scatter properties of bacteria were used to define the region of individual bacteria retained for flow cytometry analysis. For the majority of bacterial cells, heterogeneity of forward-scatter values was more pronounced than the heterogeneity of side-scatter values (Fig. 1). Also, growth conditions (culture in mod 110) favoring the production of capsular material were associated with slightly higher forward-scatter values (not shown). For these two reasons, the acquisition window defined by region R1 was stretched along the forward-scatter axis in order not to exclude highly encapsulated bacteria. Dispersion along the side-scatter axis concerned fewer bacteria and mainly reflected aggregation, as the strong reduction in side-scatter values after sonication demonstrated (not shown).

Fluorescence-activated cell sorter analysis of the CP5-reactive MAb revealed fluorescence profiles against CP5 strains clearly distinguishable from those obtained with a simultaneously run CP8 strain (Becker), which produced negligible fluorescence (Fig. 2, pattern I). Only one strain (of bovine origin) of 30 did not differ from the negative control. Pilot experiments showed that heat treatment of bacteria at 65°C for 45 min reduced the MFI by 10 to 25%. That is why we chose to use untreated live bacteria. Nevertheless, the decrease caused by heating would not have altered the conclusions of this work, and the use of heat-killed bacteria can be contemplated.

The two main results of this study were demonstrations of the influence of the growth medium on the expression of CP5 and of the heterogeneity of the expression of capsular material within the strain populations. The superiority of mod 110 over BHI for the production of CP5 was patent with 26 of the 30 CP5 strains tested. Apart from the strain that was negative by flow-cytometric examination, three strains (169.31, 126.60, and 520.24) bound the CP5-reactive MAb poorly, irrespective of the growth medium. The effect of the growth medium manifested itself both in the proportion of stained bacteria and in the intensity of fluorescence (MFI), whether the origin of the isolates was human beings (Table 1), ruminants (Table 2), or other animals (Table 3). The relative increase in CP5 surface

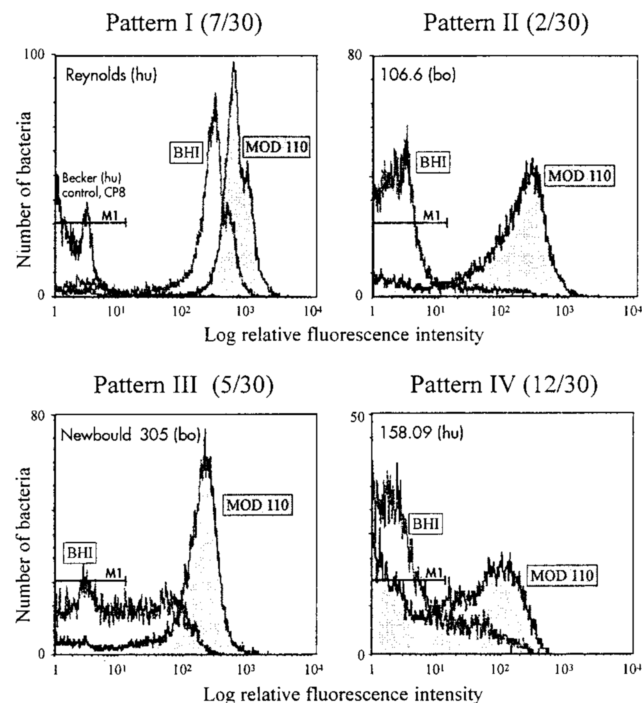

FIG. 2. Frequency histograms of fluorescence distribution of *S. aureus* cells labeled with the CP5-specific MAb 120D3. Four patterns of fluorescence profile were distinguished (see Results) on the basis of the heterogeneity of the distribution of individual bacteria as a function of cell-associated capsular material. Strains were grown on either BHI agar or mod 110 agar, labeled, and analyzed by flow cytometry. The area of unstained bacteria, defined with control samples (no MAb), is indicated by the M1 bar. A control strain (Becker, CP8) is shown with pattern I. hu, human; bo, bovine.

TABLE 1. Effect of culture medium on expression of CP5 by *S. aureus* strains of human origin

Strain	Fluorescence pattern	BHI		Mod 110		Relative increase[b]
		% CP5-positive bacteria	MFI[a]	% CP5-positive bacteria	MFI[a]	
Reynolds	I	88	323	95	739	2.5
101.32	IV	53	140	66	189	1.7
101.33	IV	19	14	22	48	4.0
157.32	NA[c]	90	209	68	193	0.7
158.09	IV	22	45	55	92	5.1
158.13	IV	58	190	79	271	1.9
158.17	I	86	237	83	511	2.1

[a] Arbitrary units.
[b] Derived as follows: (% bacteria × MFI)$_{mod\ 110}$/(% bacteria × MFI)$_{BHI}$.
[c] NA, not applicable, due to comparable expression of CP5 regardless of the culture medium or to poor reaction with MAb.

expression, which takes into account the percentage of labeled bacteria and the MFI, varied widely among the 26 strains, from 1.7 to 38.5. The absolute binding of the CP5-reactive MAb also varied widely, demonstrating that strains serotyped as CP5 by ELISA can express at their surface amounts of antibody-accessible CP differing by a factor of 20 (MFI) or more (percentage of bacteria × MFI).

In an attempt to describe the frequency histograms of fluorescence distribution of the strains involved in this study, we distinguished four main fluorescence patterns (Fig. 2). Pattern I (7 of 30 strains) corresponds to homogeneous distributions of bacterial cells, whatever the growth medium. Pattern II (2 of 30 strains) also shows homogeneous distributions, but with an all-or-none response to the growth medium. Pattern III (5 of 30 strains) describes strains with a heterogeneous distribution when grown on BHI agar, with many unstained cells, and a homogeneous distribution of stained cells when grown on mod 110 agar. Finally, pattern IV (12 of 30 strains) groups many strains which are heterogeneous under both growth conditions, comprising many unstained cells even when grown on mod 110 agar.

TABLE 2. Effect of culture medium on expression of CP5 by *S. aureus* strains isolated from cases of mastitis of cows or goats

Animal and strain	Fluorescence pattern	BHI		Mod 110		Relative increase[b]
		% CP5-positive bacteria	MFI[a]	% CP5-positive bacteria	MFI[a]	
Cow						
N 305	III	39	66	88	218	7.5
102.51	IV	36	10	69	49	9.4
106.6	II	8	64	86	229	38.5
116.30	IV	4	64	49	86	16.5
133.10	IV	30	16	75	66	10.3
135.48	II	2	93	81	40	17.4
159.06	IV	24	21	78	109	16.9
159.13	III	20	131	87	340	11.3
159.14	IV	5	78	21	184	9.9
169.31	NA[c]	2	15	2	16	1.1
400.22	IV	37	54	75	97	3.6
Goat						
126.60	NA[c]	31	33	24	47	1.1
133.18	III	56	52	91	616	19.3
144.16	III	24	128	80	349	9.1

[a] Arbitrary units.
[b] Derived as follows: (% bacteria × MFI)$_{mod\ 110}$/(% bacteria × MFI)$_{BHI}$.
[c] Not applicable. See Table 1.

TABLE 3. Effect of culture medium on expression of CP5 by *S. aureus* strains isolated from rabbits, hens, or swine

Animal and strain	Fluorescence pattern	BHI		Mod 110		Relative increase[b]
		% CP5-positive bacteria	MFI[a]	% CP5-positive bacteria	MFI[a]	
Rabbit						
452.14	I	88	172	93	302	1.9
452.15	I	85	175	95	439	2.8
526.14	I	95	240	94	544	2.2
Hen						
522.08	IV	8	35	65	96	22.3
522.15	IV	16	45	71	97	9.6
522.24	III	64	161	95	219	2.0
Swine						
520.23	I	90	142	96	330	2.5
520.24	NA[c]	35	24	20	36	0.9
526.18	I	83	110	90	466	4.6

[a] Arbitrary units.
[b] Derived as follows: (% bacteria × MFI)$_{mod\ 110}$/(% bacteria × MFI)$_{BHI}$.
[c] Not applicable. See Table 1.

DISCUSSION

The feasibility of measuring antibody interactions with surface-exposed epitopes on live bacteria by flow cytometry was established previously (2, 7). We used a MAb directed to an epitope of CP5 which does not cross-react with CP8. Being an IgM, this MAb did not interact with the staphylococcal protein A, as checked by ELISA with whole staphylococci or with purified protein A in preliminary experiments. This permitted its use for specifically staining whole, intact *S. aureus* cells. Epitope accessibility is a critical determinant of antibody reactivity with corresponding epitopes on antigens borne on the surfaces of bacteria. As the capsule is the outermost bacterial structure, its accessibility to antibodies is certain, even though the accessibility of all the copies of the epitope recognized by the MAb is not assured, particularly as the thickness of the capsule increases. Consequently, the intensity of fluorescence is probably not an accurate quantitative indirect measure of the cell-associated CP5. Nevertheless, our results show that the widely varying MFI permitted us to distinguish strains (by the MFI) or bacterial cells within strains (by the profile of fluorescence intensity). In particular, the method was suitable for demonstrating the effect of the growth medium on the surface expression of capsular material.

Culture conditions are known to influence the production of CP5 and CP8 in *S. aureus*, but most of the previous studies involved only one (17, 26) or two (20) strains. In the present study, 30 CP5 strains were examined by a method which proved to be straightforward, provided the equipment and reagent are available. The results confirmed that mod 110, a high-salt and high-carbohydrate culture medium, is more favorable than BHI for production of CP5, whatever the origin of the strains. However, the flow-cytometric analysis of MAb binding does not give the same kind of information on the bacteria as does the ELISA method, which involves an extraction of CP from bacterial cells, and it also has limitations. In addition to the limitation discussed above (in relation to accessibility), flow cytometry is also less sensitive than ELISA. The strain serotyped as CP5 by ELISA but considered unstained by flow cytometry illustrates this drawback. Possibly this is inherent in the method, since we encountered the same kind of false-negative result with *Streptococcus agalactiae* variants labeled

with a MAb directed to the surface X protein (22). On the other hand, flow cytometry analysis can provide information that ELISA cannot, e.g., on the expression of CP5 by thousands of individual cells. The resulting frequency histograms of fluorescence intensity showed that many strains were mixtures of unstained and stained bacteria (patterns III and IV), or in other words, that amounts of surface-exposed CP5 varied widely within bacterial populations. Flow cytometric analyses have revealed evidence of antigenic variation among bacterial populations assumed to be homogeneous, including *Neisseria gonorrhoeae* (2), *Escherichia coli* and *Salmonella minnesota* (7), *Streptococcus agalactiae* (22), and *Brucella* species (6) but not, to our knowledge, *S. aureus*. We did not attempt to separate highly stained from poorly stained bacteria by cloning or sorting, so we do not know whether expression of CP5 is a stable characteristic of a variant. Another factor which could contribute to the heterogeneity of CP5 expression is the stage of growth of the bacteria, since older cultures can produce more CP5 (20) and our cultures were asynchronous.

Another interesting aspect of the flow cytometry technique is the possible relationship between the amount of MAb-associated CP5 and the functional activity of CP5-reactive antibodies. In particular, staphylococcal CP5 and CP8 have been implicated in resistance to phagocytosis by neutrophils, and antibodies directed to CP are considered opsonic (13, 14, 27). It seems possible that the most heavily encapsulated cells of a heterogeneous bacterial population would be more resistant to phagocytosis than the least encapsulated cells, while the latter, on the other hand, could be more efficient in adherence as a result of the unmasking of adhesins, as has been postulated for populations of *S. agalactiae* composed of bacteria expressing various amounts of type III capsular material (10). Therefore, it is conceivable that the presence of phenotypic variants within bacterial strains would increase the ability of the strains to adapt rapidly to different environments and thus would increase their pathogenic potential (11).

The phenotypic variability demonstrated here could be accompanied by variations in virulence. It could also be of consequence for the evaluation of the opsonic efficacy of CP5-specific antibodies, since bacteria producing few CP could be more prone to phagocytosis without the help of antibodies directed to CP. As a case in point, a recent demonstration of the protective efficacy of antibodies to *S. aureus* CP has been partly ascribed to the precautions taken for favoring the full encapsulation of the challenge strains (8). We propose that another consideration should be the homogeneity of expression of CP at the strain level.

In conclusion, our study confirmed the effect of the nutritional environment on the expression of cell-associated CP5 by a substantial number of *S. aureus* strains of diverse origins. It also revealed large variations in the amount of CP5 in bacterial populations assumed to be homogeneous. Flow cytometry analysis appears to be well suited for further investigation of this phenomenon in relation to pathogenesis and immunoprophylaxis.

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