

## Capsular polysaccharide typing of domestic mastitis-causing *Staphylococcus aureus* strains and its potential exploration of bovine mastitis vaccine developmen. I. capsular polysaccharide typing, isolation and purification of the strains

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One hundred seven isolates of *Staphylococcus aureus* from bovine mastitis were investigated for colony morphology in serum-soft agar (SSA), autoagglutination in salt, and capsular serotype. Capsular polysaccharide (CP) was purified and quantified from the extracts of clinical isolates. Overall, 89 isolates (83.2%) were diffuse in the SSA, without any difference in the proportion of diffuse colony between type 5 and type 8 strains. Some strains exhibited compact colonies in the SSA and expressed CP as determined by an enzyme-linked immunosorbent assay, indicating that compact morphology does not exclude encapsulation. The majority of the strains (11/12) showed autoagglutination in the salt aggregation test. The serotype 336 accounted for 46.7% of the isolates followed by serotype 5 (12.1%) and serotype 8 (12.1%). Particularly, twenty-six (24.3%) isolates reacted with two serotypes; 7 for type 8/336 and 19 for type 5/336. Five isolates (4.7%) were nontypeable with monoclonal antibodies specific for CP serotype 5, 8, or 336. The CP concentration in culture supernatants varied with the serotypes, and the total amount of CP produced by cells grown in a liquid medium was much less than that produced by cells grown on a solid medium. The Western blotting indicated that the CP bands of *S. aureus* serotype 5 and 8 were ranged in the molecular mass of 58-84 kilodalton (kDa), with additional bands in the region of approximately  $\leq 48$  or  $\geq 84$  kDa.

**Key words:** bovine mastitis, *Staphylococcus aureus*, capsular polysaccharide, serotype

### Introduction

*Staphylococcus aureus* produces a variety of extracellular and cell-wall associated components which are involved in the pathogenesis in bovine, ovine, and caprine mastitis [29]. In cows, intramammary infection (IMI) due to *S. aureus* is generally subclinical. Nevertheless, IMI causes considerable economic losses, particularly milk losses which account for a 10 to 25% of total yield depending on the intensity of inflammation and the stage of lactation [2]. Moreover, the presence of *S. aureus* in raw milk is a public health problem. It has been demonstrated that *S. aureus* strains isolated from human infections produced capsular polysaccharides (CP) that belonged to 11 different serotypes on the basis of immunologic specificity. Both type 5 and type 8 are predominant, representing 70-80% of the isolates from all sources [3, 17, 29]. However, only a small amount of CPs was produced from the type 5 and 8 strains and the significance of these CPs in *S. aureus* virulence has been controversial [4, 38]. Monoclonal antibodies (MAB) reactive with the type 5 and 8 CP have been described, and the usefulness of MAB in characterizing *S. aureus* from clinical isolates has also been demonstrated [20, 32].

Highly encapsulated and mucoid strains, usually belonging to serotype 1 or 2, are rarely isolated from clinical specimens [3, 20, 32]. The strains are phage-non-typeable and clumping factor-negative. When cultivated *in vitro*, the strain exhibit the mucoid phenotype and produce diffuse colonies in serum-soft agar (SSA). They are more lethal for mice and more resistant to opsonophagocytic killing in the absence of anti-capsule antibody and complement than their non-encapsulated variants and other non-encapsulated strains [23, 26, 28]. The other serotypes are not mucoid when cultivated *in vitro* and are characterized as "micro-encapsulated strains" and their capsules are not apparent by the negative stains like India ink due to a small amount

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of capsule [37].

There was only one report on the capsular serotypes of *S. aureus* strains isolated from domestic animals in Korea [33]. The type 5 was predominant in both cows and dogs. In the previous study, the sample size of the isolates was not large enough to make a definitive conclusion for certain types. In this study, we determined the prevalence of CP types of *S. aureus* isolated from bovine mastitis milk in Korea. We also describes the methodology for the purification process of the CP from clinical isolates.

## Materials and Methods

### Bacteria and media

A total of 107 isolates collected from mastitic milk of cows were tested. The prototype strains used for the preparation of antisera to type-specific CP were isolate nos. 225 (CP type 5), 54 (CP type 8), 46 (CP type 336), and Wood 46 (ATCC 10832). All positive cultures were processed and identified by conventional methods [30] based on mainly colony morphology, Gram stain, tube coagulase, mannitol, coagulase, catalase, oxidase, and DNase. Columbia agar or broth (Difco) was used as a culture medium for all the experiments to minimize variation from acclimation [21].

### SSA technique

Appropriately diluted suspensions of each strain were added to staphylococcus medium no. 110 (S-110, Difco) with normal rabbit serum (1%, v/v) and incubated at 37°C up to 40 h, and then colonial morphology was evaluated. Streaming colonies were considered to be capsulated and compact growth was deemed to represent non-capsulate organism [11].

### Autoagglutination and salt aggregation test (SAT)

To simulate growth conditions in the udder, a medium containing bovine whey was used for the cultivation of *S. aureus*. Autoagglutination test was carried out as described by Watson *et al* [18]. SAT was performed as described elsewhere [25, 36].

### Preparation of rabbit anti-type specific serum

Rabbit antisera to each strain were prepared as previously described [12]. Serum samples were tested 5 days after the last injection by direct cell agglutination with the homologous strains as follows: the serum samples were diluted with an equal amount of phosphate buffered saline (PBS). A 0.25 µl of the diluted serum was mixed with 0.25 µl of bacterial suspension and the mixture was incubated for 4 h at 37°C. After determination of agglutination, the mixture was allowed to stand overnight at 4°C. When agglutination titers were 640 or more, the rabbit was exsanguinated. Pooled normal human serum was obtained by

venipuncture from healthy adult volunteers. Normal rabbit serum was obtained from the rabbit prior to immunization, and the serum samples were stored at -80°C until use.

### Serotyping for CP

MAbs for CP type 5, 8, and 336 used in this study were kindly gifted by Dr. A. J. Guidry (United States Department of Agriculture). CP serotyping of clinical isolates by the agglutination test were performed as previously described [17]. (This work was undertaken in the laboratory of United States Department of Agriculture). Briefly, bacteria grown on Columbia agar were harvested from plates by washing with 0.01 M Tris hydrochloride buffer (pH 7.2) and bacterial suspension was adjusted to a density of  $3 \times 10^9$  live bacteria. The suspension of  $10^9$  bacterial cells per ml were heated for 30 min at 100°C to kill the bacteria and then sonicated for 2 min. For the agglutination test, 25 µl of the staphylococcal suspension was mixed with 50 µl of MAb solution in PBS in the wells of the Costar 96-well microplates. The MAbs were also diluted in PBS for the titration. The reaction mixture was incubated at room temperature for 10 min with gentle shaking and the bacterial samples were then checked for the agglutination. Results were recorded as 3+ for strong positive and +/- for weak positive.

### Transmission electron microscopy (TEM)

Using transmission electron microscopy, *S. aureus* strains were examined by the methods described previously [32] with a little modifications. Columbia agar supplemented with milk whey was used to increase the expression of CP and the bacteria were incubated with antibodies to the homologous strain during the process. After washing three times for 10 min with PBS, the bacteria were observed with a transmission electron microscope (Hitachi H-7100 FA, Electron Microscope, Japan).

### Purification of type-specific CP

Type 5 and 8 CP were purified by the methods of Fournier *et al* [12, 13] and Karakawa *et al* [19]. Nucleic acids and proteins were partially removed by fractionation with 30% ethanol (v/v) at 4°C overnight. After centrifugation at  $25000 \times g$  for 30 min, the supernatant was precipitated in 80% (v/v) ethanol at 4°C overnight. The white, flocculent precipitate was suspended in approximately 100 ml of 0.05 M PBS (pH 7.2). The suspension was dialyzed against 0.05 M sodium acetate in 0.1 M NaCl (pH 7.2) for 48 h at 4°C and lyophilized. The dialysate was suspended in PBS (20 mg/ml) and digested overnight at 37°C with 100 mg/ml of DNase, 100 mg/ml of RNase, and 100 mg/ml of lysostaphin (Sigma), followed by overnight digestion at 37°C with 100 mg/ml of pronase. After dialysis and lyophilizing the extract was dissolved in PBS at 20 mg/ml and treated with 0.05 M sodium metaperiodate at room

temperature in the dark room for 45 min to remove teichoic acid [24]. The reaction was stopped by adding 25% (v/v) ethylene glycol. The sample was then dialyzed against 0.05 M sodium acetate-0.1 M NaCl (pH 6.0) and lyophilized. The dialysate was applied to a DEAE-Sephacel column (2.6 by 45 cm), at a flow rate of 10 ml/min which was developed with a 800 ml gradient of 0.1 M to 0.5 M NaCl in 0.05 M sodium acetate (pH 6.0). Fractions that were positive with anti-type specific serum were pooled, desalted, and lyophilized. Pooled fractions were then applied on a Sephacryl S-300 column (Pharmacia) (1.5 by 90 cm, 0.5 ml/min) with 0.05 M sodium acetate buffer (pH 6.0) for elution. Protein content was determined in test tubes using protein assay reagent kit (Micro BCA, Pierce, USA).

### CP preparation for serotyping and its detection in culture supernatant

Cells were suspended in PBS containing lysostaphin (100 µg/ml), and then incubated at 37°C overnight. The suspension was autoclaved at 121°C for 60 min, to release CP from the cell. After centrifugation at 25,000 × g for 20 min, the supernatant was harvested and stored at -20°C prior to type CP assay. Type-specific CPs were detected in the supernatants of autoclaved bacteria by an inhibition enzyme-linked immunosorbent assay (ELISA), as described previously [28] except that anti-mouse peroxidase-conjugated whole immunoglobulin G (Sigma) was used. After addition of the enzyme substrate (O-phenylenediamine) and incubation at 37°C for 1 h, the optical density at 492 nm was read with a Titertek Multiscan MCC 340 microplate reader (Flow Laboratories SA, Puteaux, France).

### SDS-PAGE and western blotting

CP was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the discontinuing system of Laemmli [22]. CP was separated in 10% and 5% (w/v) acrylamide as resolving and stacking gels, respectively. After electrophoresis, the gel was subjected to the western blotting as described previously [7]. Polysaccharide was transferred to nitrocellulose sheets in a Hoefer Trans-Blot cell with plate electrodes, following the manufacturer's instructions. Transfer was carried out at a constant voltage of 70 V for 1.5 h in running buffer containing 25 mM Tris/HCl and 192 mM glycine (pH 8.3). Thereafter, the sheets were placed in a sealed plastic box with blocking buffer containing 10% skim milk and incubated at room temperature for 10 min. The nitrocellulose membrane was rinsed in PBS-0.05% Tween 20 (PBST) three times for 10 min and then incubated with rabbit antiserum diluted 1 : 500 in blocking buffer containing 2.5% skim milk in PBS, at 25°C for 2 h. The membrane was then washed three times with PBST for 10 min each and finally were incubated at 25°C for 1 h with

peroxidase-conjugated goat anti-rabbit IgG antibodies (Sigma) diluted 1 : 500 in PBS. The membrane was washed in PBST and developed in a freshly prepared substrate solution containing 4 CN peroxidase substrate and 30% H<sub>2</sub>O<sub>2</sub> peroxidase solution (Kirkegaard & Perry Laboratories, KPL, Maryland). The reaction was stopped by washing the membrane in water for 5 min.

## Results

### Colony morphology in SSA

Of a total of 107 isolates, *S. aureus* bacterial colonies from 89 isolates (83.2%) were diffuse in SSA (Table 1). There were no differences in the proportion of diffuse colony morphology between type 5 (92.3%, or 12/13) and type 8 (84.6% or 11/13) strains. The bacteria from five isolates (4.7%) were nontypeable by the SSA assay. The diffuse colony morphology in SSA, as considered by a criterion of encapsulation, seemed to be a characteristic of most *S. aureus* strains from mastitic milk either freshly isolated or cultivated under suitable conditions. Five strains of each capsular type were tested on CP expression by ELISA with polyclonal antibodies (Table 2). Regardless of the colony morphology, the strains of type 8 and type 336 expressed CP as determined by ELISA inhibition. However, the

**Table 1.** Colonial morphology of *S. aureus* from 107 isolates in serum soft agar

Capsular type	Morphology in SSA			Total
	Diffuse	Compact	Indeterminate	
5	12	1	0	13
8	11	1	1	13
336	45	1	4	50
5 and 336	15	0	4	19
8 and 336	5	0	2	7
NT *	1	1	3	5
total	89	4	14	107

\* nontypeable.

**Table 2.** Expression of CP from type 5 and 8 strains of *S. aureus* isolates

Capsular type	Colony morphology	No. of strains	ELISA inhibition by supernatant *
5	Diffuse	4	++
	Compact	1	-
8	Diffuse	4	++
	Compact	1	++
336	Diffuse	3	++
	Compact	1	++
	Indeterminate	1	++

\* supernatants containing type 5 or 8 CP inhibited the fixation of polyclonal antibodies on plates coated with purified homologous CP. ELISA inhibition: ++, 100%; -, no inhibition.

**Table 3.** Autoagglutination assay using *S. aureus* grown in agar with and without supplemented with bovine milk whey

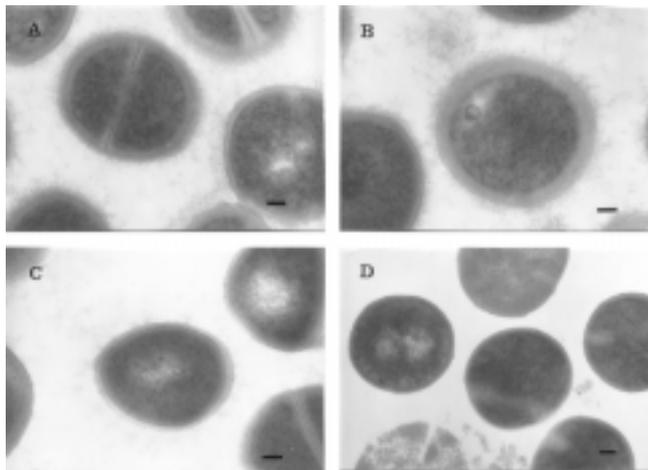
Molarity of NaOH	<i>S. aureus</i> grown in	
	Agar	Agar with whey
0.005	2/12	12/12*
0.003	0/12	12/12
0.001	0/12	11/12
0.05	0/12	9/12
0.02	0/12	6/12
0.01	0/12	4/12
0.1	0/12	0/12

\* no. of positive strains / no. of tested strains.

strain of type 5 with compact colony morphology did not express CP.

### Cell hydrophobicity

Increasing the concentrations of sodium hydroxide progressively inhibited autoagglutination by the bacteria (Table 3). At the concentrations of < 0.001 M sodium hydroxide, most of staphylococci (91.7%) grown in the agar supplemented with whey were autoagglutinable whereas at the concentrations > 0.02 M sodium hydroxide, the process was inhibited remarkably by 50%. All isolates but one aggregated in SATs at the concentration of 1 M ammonium sulfate. Autoaggregating strains were subcultured 15 times on agar medium containing whey without any loss of surface hydrophobicity. The presence of capsular material on the surface of 12 randomly selected *S. aureus* could additionally be demonstrated by electron microscopic studies, whereas no capsular material could be observed for the non-mucoid wood 46 strain (Fig. 1).

**Fig. 1.** Transmission electron micrographs of *S. aureus* capsules after reaction with homologous antibody. (A) isolate no. 225 (serotype 5), (B) isolate no. 54 (serotype 8), (C) isolate no. 73 (serotype 336), and (D) unencapsulated strain Wood 46. Bars represent 0.01  $\mu\text{m}$ .**Table 4.** Serotyping of 107 clinical *S. aureus* isolates

No. (%) of isolates						
Type 5	Type 8	Type 336	Type 5 and 336	Type 8 and 336	NT *	Total
13(12.1)	13(12.1)	50(46.7)	19(17.8)	7(6.5)	5(4.7)	107(100)

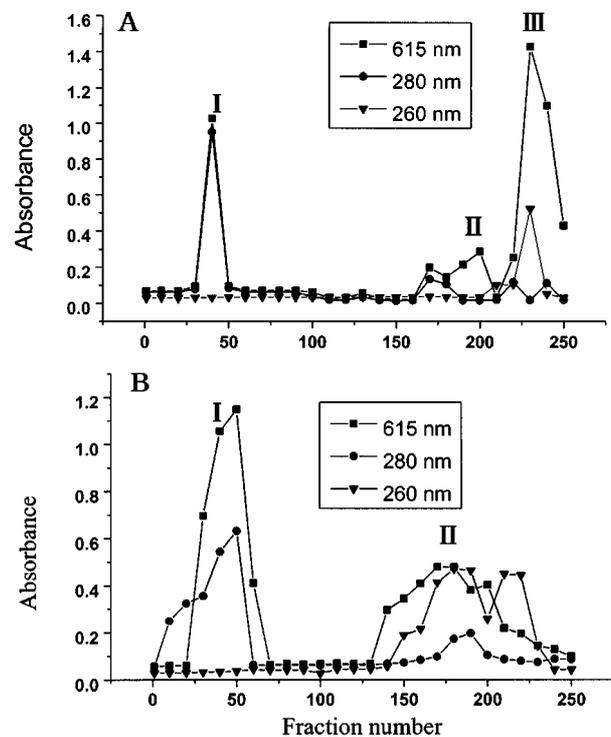
\* nontypeable.

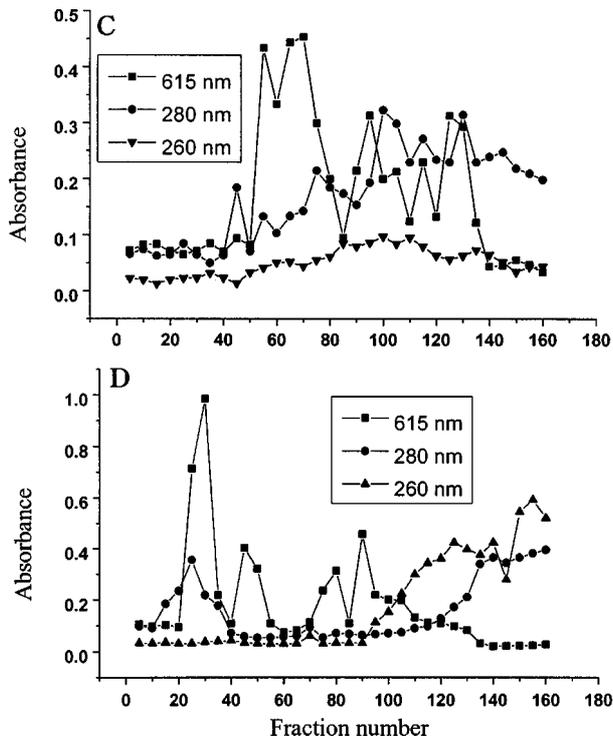
### Serotyping of *S. aureus* with MAb

Characterization of MAb was described elsewhere [9, 10]. Among 107 *S. aureus* strains, serotype 336 was the most prevalent (50 isolates), followed by serotype 5 (13 isolates) and serotype 8 (13 isolates) (Table 4). Particularly, 26 isolates (24.3%) contained two serotypes; 7 for CP type 8/336 and 19 for CP type 5/336, and 5 (4.7%) were nontypeable with MAbs specific for serotype 5, 8, or 336 CP, ie, probably encapsulated with other than 11 prototype capsules.

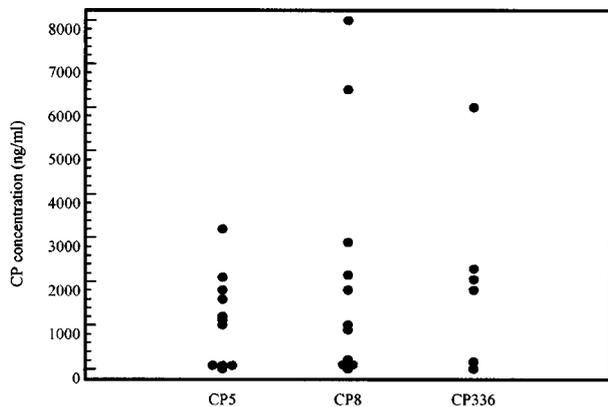
### Purification and concentration of CPs

The type-specific CP released into the supernatant by autoclaving or lysostaphin treatment of cells was absorbed onto DEAE-cellulose. The type 5 CP was eluted as a shoulder under the last peak, but not distinct in type 8 CP

**Fig. 2.** Ion-exchange chromatography of the autoclaved extract of *S. aureus* expressing CP type 5 (A) or CP type 8 (B). Extracts (500 mg) in 40 ml of 0.05 M sodium acetate buffer containing 0.1 M NaCl (pH 6.0) was applied to a column of DEAE-Sephacel equilibrated in the same buffer. Bound material was eluted with a linear gradient of 0.1 to 0.5 M NaCl. 10 ml fractions were collected.



**Fig. 3.** Gel filtration patterns (C, CP5; D, CP8) of the fraction identified in ion-exchange chromatography on Sephacryl S-300. The column was eluted with 0.05 M sodium acetate buffer (pH 6.0) at a flow rate of 0.5 ml/min. The sample was applied in 1.5 ml of buffer, and 1 ml of each fraction was collected.

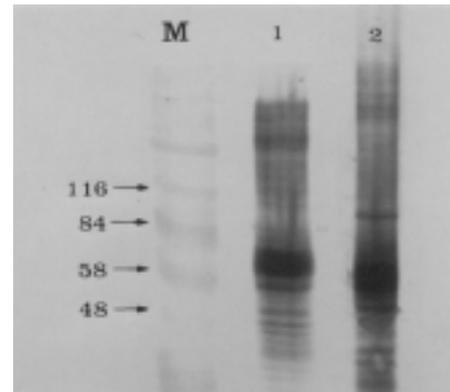


**Fig 4.** Distribution of CP concentration by serotypes of randomly selected 30 *S. aureus* isolates.

(Fig. 2). The type-specific CP containing fractions was further fractionated on DEAE-cellulose with a linear gradient of NaCl, resulting in two or three major peaks. Most of the protein was eluted in the first peak. The CP was finally purified by gel filtration (Fig. 3). A amount of 1.2  $\beta^2$  purified CP was recovered from 2-liter cultures of CP8 and 0.7 $\beta^2$  from the same amount of cultures of CP5. The protein content in the finally obtained CP was 1.2% in CP8 and 2.3% in CP5, respectively. To determine CP

**Table 5.** Capsular polysaccharide (CP) expression for *S. aureus* serotype 8 by culture medium

Strain	Medium	Amount of CP8 (mg/10 <sup>10</sup> CFU) at trial no.	
		1	2
no. 54	Agar	3.7	5.2
	Broth	0.024	0.029



**Fig 5.** Western blot of purified *S. aureus* CP with rabbit antibodies against serotype 5 and 8. Lanes: 1, clinical isolate no. 225 (serotype 5); 2, clinical isolate no. 54 (serotype 8). Lane M indicates the positions of molecular mass markers (kDa).

concentration in the culture supernatants, 30 isolates were randomly selected and tested on the concentration of CP (Fig. 4). It varied from 2 to 3200 ng/ml for serotype 5 CP, from 3 to 8000 ng/ml for serotype 8 CP, and 4 to 6000 ng/ml for serotype 336 CP. Some strains produced negligible amount of CP. Cells grown in liquid medium produced less amount of CPs than cells grown on agar medium (Table 5). Agar-grown *S. aureus* produced 3.7-5.2  $\mu\text{g}$  of CP8 per 10<sup>10</sup> CFU, whereas broth-grown cells produced only 0.024-0.033  $\mu\text{g}$  of CP8 per 10<sup>10</sup> CFU, indicating that agar-grown bacteria produced about 150-fold higher amount of CP8 per milligram of biomass than broth-grown bacteria.

**Electrophoretic and Western blot profiles**

The extracted CP preparations were subjected to the SDS-PAGE electrophoresis and the western blot. The CP bands of *S. aureus* serotype 5 or 8, which was reacted with antibodies raised against their homologous strains, were blotted in the molecular mass range of 48-84 kDa (Fig. 5). In addition, a common densely packed band in the region of approximately 58 kDa appeared in the CP preparations and a few additional bands of 48~84 kDa appeared.

**Discussion**

Although capsule production by staphylococci was first recognized in 1930 by Gilbert [14], the prevalence of encapsulation among *S. aureus* strains has been appreciated recently. Highly encapsulated staphylococci were not found

in the present study. Most of the isolates (83.2%) showed diffuse colony, and there were no differences in the proportion of diffuse colony between type 5 and type 8 strains. Notably, this study showed that some of clinically isolated strains exhibited compact colonies in SSA, but they expressed CP as detected by ELISA, indicating that even compact morphology may have a characteristic of encapsulation. This result is consistent with others [6, 34], describing that the expression of capsule is greatly influenced by the environmental and bacterial growth conditions, such as culture medium and growth phase of the organism. A conversion from compact to diffuse morphology has also been observed when human *S. aureus* isolates grown on routine medium were cultured on S-110 [39]. Similar results were obtained from bovine mastitic strains [27, 31].

Because bacterial capsule lies as a discrete layer external to the rigid cell wall, it is lost rapidly in artificial media during culture. Lee *et al* [24] reported that staphylococci grown on surfaces, both in vitro and in vivo, produce large quantities of cell-associated CP8 than those grown in liquid cultures. There is a report that capsule production may be enhanced when cultivated in a medium with limiting phosphate level [8]. Our study clearly showed that the expression of CP in *S. aureus* was influenced depending on the pre-treatment conditions applied for *S. aureus*. The bacteria were cultured in the low phosphate-containing modified Columbia media supplemented with milk whey to pretend to be udder environment. The incubation of the bacteria with antibodies to the homologous strain to preserve the integrity of CP before TEM processes resulted in a good microscopic observation. Cells grown in liquid medium produced 150-fold less CP8 production than did cells grown on agar medium. Because staphylococci may release soluble capsular antigens during growth in broth cultures, it is essential to use both culture supernatants and CPs bound to the bacterial cells.

Unlike other studies [1, 3, 17, 29], serotypes 5 and 8 accounted for only 24.2% of all the isolates, and the majority of *S. aureus* isolates (>51%) were not typeable by either serotype 5 or 8 antisera. Sompolinsky *et al* [32] reported that 10% (34/348) of the bovine isolates were nontypeable. There is another report that 59% of the isolates from the United States were nontypeable [15]. Recently, Guidry *et al* [16] suggested that nontypeable isolates could be typed when adding newly developed serotyping antiserum 336 to the previous typing scheme. In our study, 50 of 55 nontypeable strains were typed as serotype 336 (90.9%). Further, multiple serotypes existed within herds; 7 for CP type 8/336 and 19 for CP type 5/336. One important observation in our study is that the relatively high frequency of serotype 336 may represent clones prevalent in the farm investigated, indicating that the distribution of serotypes may be different in geogra-

phic locations and clinical sources, as described previously [3, 32]. Eventually, these data may suggest that a vaccine containing *S. aureus* serotypes 5 and 8 would have limited potential for comprehensively preventing *S. aureus* mastitis in Korea.

Many bacterial pathogens associated with mucosal surfaces have been shown to bind to specific epithelial cell surface receptors, the binding being defined as lectin-ligand interaction [5]. Studies on *S. aureus* suggested that these interactions may be involved in the adhesion of these pathogens to epithelial cell surfaces and tissue matrix [35]. In a recent study, the strains freshly isolated from bovine mastitis often possess a high surface hydrophobicity, as measured by the tendency to autoagglutinate in saline and buffer with a physiological salt concentration [18]. In our study, the majority (91.7%) of the fresh isolates from bovine mastitis expressed the pattern of autoagglutination. Similarly, the high proportion of strains expressed a pronounced hydrophobicity. There was a correlation between the expression of capsule by the organism and the autoagglutination in the presence of low concentrations of sodium hydroxide. Although the addition of milk whey in some strains was not effective in promoting autoagglutination, a significant proportion of capsulated cells existed. The relationship between hydrophobicity and autoagglutination is still controversial. *S. aureus* Cowan I strain producing high amounts of protein A showed high surface hydrophobicity, but not autoagglutination. Thus, further studies should be performed to determine the correlation.

The capsule was isolated by ethanol precipitations and enzyme digestions, followed by chromatography. Removal of teichoic acid was achieved by subjecting the polysaccharide preparation to oxidation with sodium metaperiodate. Even though capsular antigens were recovered from both bacterial extracts and culture supernatants of the organism, the yield was very low, compared to 0.5-2.0 mg/liter of culture by Fournier *et al* [12]. Quantifying the percentage composition of CP was difficult because of the poor yield. However, the partial purity of our sample was deduced by the low protein content in the final batches and western blotting with polyclonal antibodies. In both serotypes, the major densely packed bands were blotted in the narrow molecular mass range of 48-84 kDa, with a few additional bands which are considered as being associated with peptidoglycan. This procedure is quite laborious and unsuitable for large scale purification. Therefore, a simple efficient method of purification of *S. aureus* need to be developed to reduce CP loss during the purification process. Further studies, preferably using CP antibodies, should be attempted to elucidate the protective effect in a mouse model of staphylococcal infection.

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