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Synthesis of Microcapsule by *Staphylococcus aureus* Is Not Responsive to Environmental Phosphate Concentrations

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The polysaccharide microcapsule of *Staphylococcus aureus* has been reported to be differentially expressed depending on growth conditions, with phosphate concentration being the critical environmental component. This study evaluated the effect of growth of a serotype 8 strain of *S. aureus* in phosphate-replete and phosphate-limiting media on microcapsule production. The presence of the cell wall polymers microcapsule and teichoic acid was measured by both gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry. Production of microcapsule was unaffected by changes in the environmental phosphate concentration. There was, additionally, no evidence for a shift from teichoic acid to teichuronic acid synthesis.

Eleven capsular serotypes for *Staphylococcus aureus* have been described (13, 23). Capsule production by this bacterium falls into two distinct categories. Types 1 and 2 capsules, exemplified by strains M and Smith, consist of a prominent polysaccharide capsule which gives rise to mucoid colony formation. Production of the mucoid capsule has been associated with increased resistance to phagocytosis and enhanced virulence (19). However, few clinical isolates of *S. aureus* actually produce this prominent capsule. The mucoid strains of *S. aureus*, as well as nonmucoid isolates, produce a second type of capsule (27). This polysaccharide capsule is not readily apparent by colony morphology or by typical capsule-staining procedures. It has, therefore, been designated a microcapsule. A serotyping scheme based on the microcapsule has been developed, and the majority of human clinical isolates, approximately 80%, fall into serotypes 5 and 8 (1). Furthermore, preliminary studies indicate that these two serotypes predominate in animal mastitis isolates (20). The chemical compositions and structures of the various serotypes of microcapsules have been elucidated (7, 16–18). The genes encoding the type 1 capsule from strain M, the type 5 microcapsule from strains Newman and Reynolds, and the type 8 microcapsule from strain Becker have been cloned, and their DNA sequences have been determined (14, 22). Antibodies directed against the microcapsule have been shown to be opsonic and to be protective, in certain animals, of infection (6, 12, 21, 25).

Synthesis of microcapsules appears to be a regulated event. It has been reported that the degree of expression of the microcapsules is dependent on the composition of the growth medium, the stage of growth, and whether the organisms are cultured on solid or liquid medium (6, 15, 21, 24, 26). Microcapsule expression has been reported to be enhanced by growth in Columbia broth or agar, and this phenomenon has been attributed to the low-phosphate nature of this medium (5, 6, 15, 21). Giving further credence to this observation is the chemical composition of the microcapsule. The microcapsule contains acidic sugars which are usually associated, in other gram-positive bacteria, with teichuronic acids. In organisms such as *Bacillus subtilis*, growth under conditions of limiting phosphate is associated with a shift in synthesis of cell wall-associated teichoic acid to production of the acidic sugar-containing teichuronic acid (3, 4, 11, 28). The chemical similarity between teichuronic acid and microcapsules of *S. aureus*, as well as the enhanced expression of the microcapsule in Columbia medium, has led to the suggestion that microcapsule synthesis may be induced by limitation of environmental phosphate. To investigate this possibility, the expression of microcapsule in a semidefined phosphate-limited growth medium was assessed by gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS).

PCR-based serotype determination. Approximately 80% of *S. aureus* clinical isolates belong to serotype 5 or 8. The operons encoding these microcapsules have been cloned and sequenced (22). Analysis of the sequence has indicated that 4 determinants located in the central region of each operon, *capH* to *capK*, are sequence distinct, whereas the 12 determinants which flank these 4 are essentially identical in the two serotypes. The two serotypes can thus be distinguished based upon the four-gene unique regions. PCR primers have been designed to allow exact annealing to sequences flanking the unique sequences and thus to allow amplification of the serotype-unique sequences. The primer sequences are as follows: cap585, ATACTTGGAGGAAATGACGATG; cap583, CAC TCATCTAATCGACGTCCT. PCR was performed under the following conditions. Initial denaturation of the template was done at 94°C for 3 min, followed by 35 cycles of amplification including denaturation of the template at 94°C for 30 s, primer annealing at 59°C for 45 s and then at 67°C for 30 s, and chain extension at 72°C for 5 min. A final extension at 72°C for 4 min was included. The resulting PCR products were digested with the restriction endonucleases *Sma*I (American Allied Biochemical) and *Tth*111I (Promega) to determine if the organism was serotype 5 or serotype 8. Results of such amplifications with strains 8325-4 (serotype 5), Becker (serotype 8), and DAW (a 1978 methicillin-resistant clinical isolate) are illustrated in Fig. 1. The expected PCR products are DNA fragments of 4,145 bp (serotype 5) and 4,327 bp (serotype 8). Furthermore, the serotype 5 fragment has a single *Tth*111I site which, after digestion, yields fragments of 2,921 and 1,225 bp. The serotype 8 fragment lacks a *Tth*111I cleavage site. The

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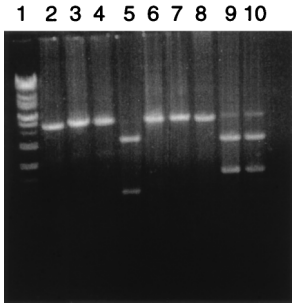

FIG. 1. PCR-based serotyping of strain DAW. *capH*- to *capK*-containing DNA fragments from serotype 5 strain 8325-4 (lanes 2, 5, and 8), serotype 8 strain Becker (lanes 4, 7, and 10), and the methicillin-resistant clinical isolate DAW (lanes 3, 6, and 9) were electrophoresed in a 0.8% agarose gel. The PCR fragments were digested with the restriction endonuclease *Tth*111I (lanes 5 to 7) or *Sma*I (lanes 8 to 10) or were not treated with a restriction endonuclease (lanes 2 to 4). Lane 1 contains the Supermarker (Bioworks) DNA size standard.

serotype 8 fragment possesses a single *Sma*I site, and cleavage with this endonuclease produces fragments of 2,740 and 1,587 bp. The serotype 5 fragment lacks a *Sma*I cleavage site. The PCR amplifications gave the expected patterns for the type 5 (8325-4) and type 8 (Becker) strains, and the DAW clinical isolate yielded a serotype 8-specific pattern.

Carbohydrate analysis of strain DAW. Carbohydrate analysis was carried out on *S. aureus* DAW cells grown in phosphate-replete (10.5 mM total phosphate) and phosphate-deficient (0.023 mM total phosphate) semidefined media (28). *B. subtilis* W23 cells were grown in the same media to serve as controls for environmental phosphate levels. Under phosphate-limiting conditions, this organism shifts from synthesis of a ribitol-containing teichoic acid to a teichuronic acid (11, 28). Neutral and aminosugar profiles were determined by the alditol acetate method, as described previously (8, 9). Analysis of neutral and acidic sugars by LC-MS was performed by the method of Fox et al. (11). The major sugars observed for *S. aureus* DAW by GC-MS analysis were anhydroribitol (derived from ribitol by dehydration on hydrolysis), ribose, fucosamine, glucose, muramic acid, and glucosamine (Fig. 2). Glucosamine and glucose were found in high concentrations. Although these sugars can be present in teichoic acid, they are also found in other cellular constituents and thus were not quantified. For example, glucosamine (along with muramic acid) is present in peptidoglycan.

Fucosamine and anhydroribitol served as markers for the serotype 8 microcapsule and teichoic acid, respectively, for *S. aureus*. Residual ribitol (as well as ribose derived from RNA) produces ribitol pentaacetate on conversion to alditol acetates for GC-MS analysis. Thus the "ribitol-ribose" peak does not accurately represent teichoic acid content. Anhydroribitol and fucosamine were present at high concentrations under all growth conditions for *S. aureus*. Fucosamine was produced equally well under conditions of phosphate excess and limitation (0.2 and 0.3% [dry weight], respectively). This behavior is inconsistent with microcapsule expression being induced by phosphate limitation.

Staphylococci cultured under conditions of phosphate excess

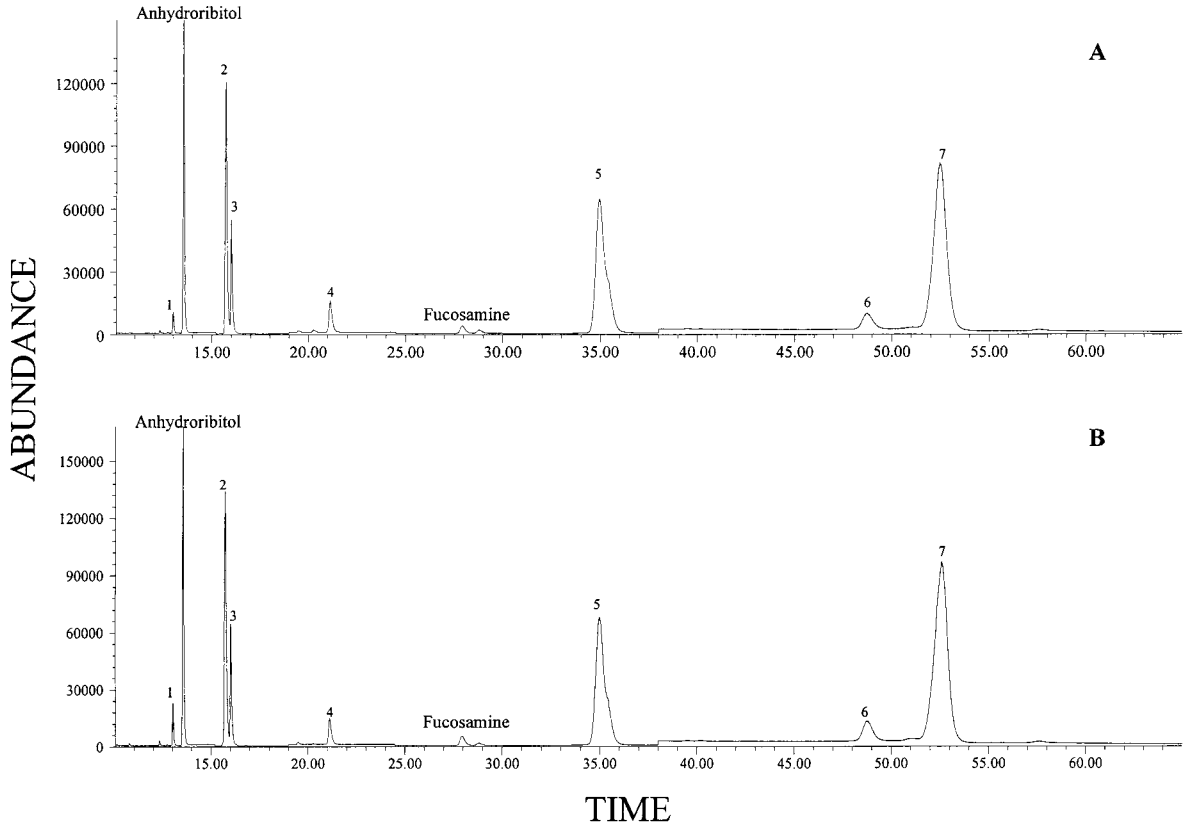

FIG. 2. Carbohydrate profiles of *S. aureus* determined by GC-MS. (A) Cells grown in phosphate-rich medium. (B) Cells grown in phosphate-limited medium. Note that the levels of anhydroribitol and fucosamine (markers for teichoic acid and capsule, respectively) are unchanged between the two growth conditions. Other peaks: 1, glycerol; 2, ribose plus ribitol; 3, arabinitol (internal standard); 4, glucose; 5, muramic acid; 6, methylglucamine (internal standard); 7, glucosamine.

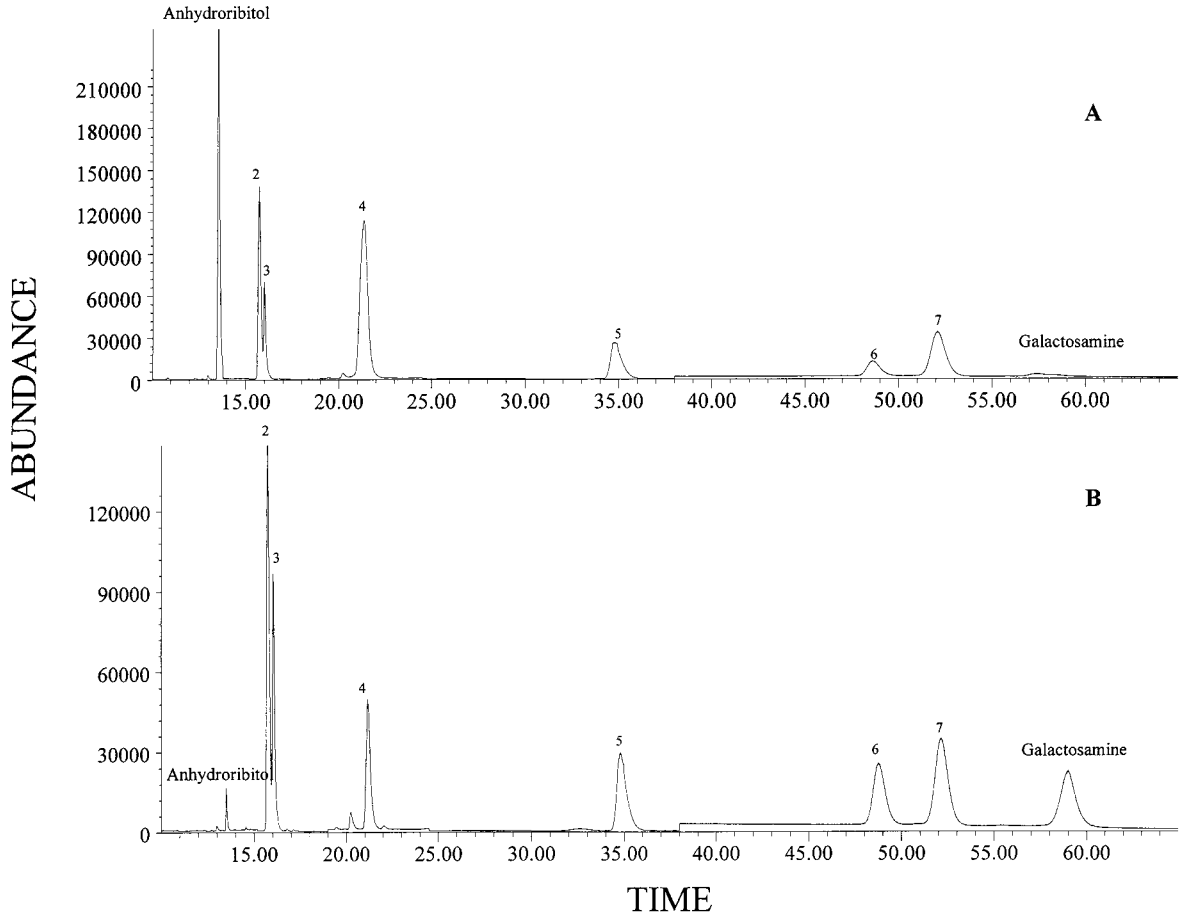

FIG. 3. Carbohydrate profiles of *B. subtilis* determined by GC-MS. (A) Cells grown in phosphate-rich medium. (B) Cells grown in phosphate-limited medium. Note that the level of anhydroribitol (marker for teichoic acid) is markedly reduced under phosphate-limited growth conditions. Other peaks: 2, ribose plus ribitol; 3, arabinitol (internal standard); 4, glucose; 5, muramic acid; 6, methylglucamine (internal standard); 7, glucosamine.

produced large amounts of ribitol (0.42% [dry weight]), as determined by LC-MS analysis. Ribitol production was essentially unchanged in cells grown under phosphate-limiting conditions (0.49%). Glucuronic acid (a marker for teichuronic acid) has been reported to be synthesized by certain strains of *S. aureus*. Trace levels of a peak at the retention time for glucuronic acid was observed under excess phosphate conditions. There was only a slight increase in the amount of glucuronic acid under phosphate-limiting conditions (total levels of 0.06%). Because there was no decrease in teichoic acid production and no significant synthesis of glucuronic acid, there is no evidence for a shift from teichoic acid to teichuronic acid synthesis. Similarly, no reduction in the teichoic acid marker was detected when staphylococcal strains 8325-4 (serotype 5) and Becker (serotype 8) were cultured under the various phosphate concentrations utilized in this study (10). Therefore, the results obtained with DAW appear to be more universally applicable to *S. aureus*. These findings are in contrast to the results obtained by Ellwood and Tempest but are consistent with the conclusions of Dobson and Archibald (2, 4).

The behavior of *B. subtilis* cells in phosphate-limiting medium was in agreement with a shift from teichoic acid to teichuronic acid synthesis. *B. subtilis* W23 synthesizes a ribitol-containing teichoic acid in the presence of excess phosphate and a glucuronic acid-containing teichuronic acid under phosphate limitation. The amount of anhydroribitol was seen to decrease during growth at the lower phosphate concentration (0.6 versus 0.04% [dry weight]) (Fig. 3). Galactosamine has been previously reported to be a major component of teichuronic acid but also has been reported to be a component of a "secondary" teichoic acid present in small amounts compared to the primary teichoic acids (3). Galactosamine was present at trace levels in cells grown in 10.5 mM phosphate but at high levels (0.9% [dry weight]) in cells grown in medium with 0.023 mM phosphate supplementation.

By LC-MS analyses, ribitol was readily detected in *B. subtilis* W23 cells grown under conditions of phosphate excess (0.59% on a dry weight basis) while the peak for glucuronic acid was too low to quantify. For cells grown under phosphate-limiting conditions, ribitol production was decreased 20-fold (0.03%) and glucuronic acid became a major component of chromatograms (0.31%).

The microcapsule of *S. aureus* is chemically similar to the teichuronic acid of *B. subtilis*. The wall-attached polymer contains uronic acid sugars and was reported to be expressed at elevated levels under conditions of phosphate limitation. The presence of the microcapsule does not confer a mucoid phenotype on the cells, again consistent with a teichuronic acid. However, the classical model of teichoic acid-teichuronic acid expression would suggest that teichoic acid markers (anhydroribitol and/or ribitol) should disappear from cells grown under

low-phosphate conditions whereas a teichuronic acid marker (e.g., glucuronic acid) should increase significantly under low-phosphate conditions. For *S. aureus* DAW, anhydroribitol and ribitol were present at similar levels under all growth conditions, suggesting continuing production of a teichoic acid. Almost no glucuronic acid was observed under any of the growth conditions tested. These results are inconsistent with a switch from teichoic acid to teichuronic acid synthesis. The level of fucosamine, a component of the type 8 capsule, did not increase under phosphate limitation. These results are consistent with production of a microcapsule under conditions of both phosphate excess and limitation. Thus the reported differences in the amount of microcapsule expressed in vitro must be due to environmental cues unrelated to phosphate availability. Consistent with this is the observation that microcapsule production can be enhanced by growth under conditions unrelated to environmental phosphate concentrations, such as in the presence of milk whey (26).

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