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Increased Cell Size and Shortened Peptidoglycan Interpeptide Bridge of NaCl-Stressed *Staphylococcus aureus* and Their Reversal by Glycine Betaine

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Staphylococcus aureus **cells grown in a defined medium under conditions of high ionic stress (2.5 M NaCl) were significantly larger than cells grown under unstressed conditions, even though the cells grew much more slowly under stressed conditions. Analysis of the structure of peptidoglycan from stressed cells showed a shorter interpeptide bridge than in peptidoglycan from unstressed cells. Glycine betaine inclusion in the high-NaCl medium resulted in cells with sizes and interpeptide bridges similar to those of cells grown under unstressed conditions.**

The gram-positive coccus *Staphylococcus aureus* is one of the most halotolerant eubacteria. The organism can grow in medium containing up to 3.5 M NaCl (28). During an investigation of the effects of growth in the presence of NaCl on the production of penicillin-binding protein 2a and autolytic activity (20), it was observed that cells grown in complex medium containing 0.85 M NaCl were larger than cells grown in complex medium alone (21). Similar observations of larger cells in complex medium containing NaCl than in complex medium alone have also been reported by others (13, 17).

When bacterial cells such as *Escherichia coli* are exposed to a sudden increase in external osmotic pressure, there is initially a fast efflux of water, resulting in decreased turgor pressure, plasmolysis, and cell shrinkage (1, 5, 18). Mitchell and Moyle (25) have shown a similar response for *S. aureus* cells transferred from dilute to concentrated NaCl solutions. Bacteria respond to a decrease in turgor by accumulating high concentrations of osmotically active, compatible solutes in order to restore turgor, either by transport from the medium or by biosynthesis (5). This allows the restoration of growth of the organism, and a new steady state is established.

There have been several recent studies of osmoregulation in *S. aureus* (11, 19, 24), and choline, glycine betaine, L-proline, and taurine have been shown to act as osmoprotectants. Because complex media contain the osmoprotectants L-proline and glycine betaine (10), cells of *S. aureus* osmotically stressed by growth in complex media containing high concentrations of NaCl accumulate proline and glycine betaine to high levels (19, 24). Thus, complex media are not well suited to study of the effects of NaCl on cell size because of potential modifying effects of osmoprotectants present in such media. In this paper, we report a careful examination of the effects of ionic and osmotic stress on the cell morphology and structure of peptidoglycan of *S. aureus* grown in defined medium in the presence and absence of the osmoprotectant glycine betaine.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. aureus* RN450 (NCTC 8325-4), a derivative of strain NCTC 8325, was used in this study. All experiments were carried out with cells grown in a defined medium that contained glycerol as the primary carbon source (31). The osmotic strength of this medium is approximately 0.20 osmol (kg of water)$^{-1}$ (11). The ionic and/or osmotic strength of the medium was adjusted by adding various amounts of NaCl or other solutes. Medium osmolality was determined with a vapor pressure osmometer (model 5100C; Wescor). Cells were grown with shaking (200 rpm) at 37°C in 300-ml nephelometer flasks containing 25 ml of medium. A 2% (vol/vol) inoculum from an overnight culture (12 to 18 h) in defined medium was the standard inoculum. When required, osmoprotectants were added to a final concentration of 1 mM.

Electron microscopy. To prepare samples for transmission electron microscopy (TEM), cultures were typically allowed to grow until the mid-exponential phase. Cells were fixed by adding glutaraldehyde to the culture to a final concentration of 2.5% (vol/vol). After 30 min, the cells were harvested by centrifugation (7,310 × *g*, 5 min, 4°C). The pelleted bacteria were mixed with 1.25% (wt/vol) water-agar, and the agar was allowed to solidify. The agar was then cut into approximately 1-mm^3 pieces and fixed for an additional 30 min in phosphate-buffered 2.5% (vol/vol) glutaraldehyde. The agar pieces were rinsed three times with phosphate buffer and postfixed in phosphate-buffered 1% (wt/vol) osmium tetroxide for 1 h. The agar pieces were then rinsed with water and fixed for 1 h in 1% (wt/vol) aqueous uranyl acetate. All fixations were carried out at room temperature. After dehydration in a graded series of ethyl alcohol and two changes in propylene oxide, the agar pieces containing bacterial cells were embedded in Epon 812. Thin sections stained with uranyl acetate and lead citrate were examined in a Zeiss 10-C TEM operating at 60 kV. For scanning electron microscopy (SEM) studies, cells were fixed with glutaraldehyde followed by osmium tetroxide, dehydrated in a graded series of acetone, and critical-point dried with liquid CO_2. The cells were coated and examined in a Cambridge model 240 SEM operating at 15 kV.

Cell area measurement. All cell sections for area measurements were recorded with a video printer at a magnification of ×10,000. The video printer was connected to the TEM via a video camera. Each field to be recorded was selected randomly, making sure that all sections used in area measurements came from one field to prevent recording the same cell more than once. The cell sections to be measured were numbered to avoid repeated measurement of the same cell. Cell areas were determined by tracing the cell outline with a digitizer and image analysis program, which computed the cell area. A total of 50 cells from each group were measured.

Preparation and analysis of *S. aureus* peptidoglycan. *S. aureus* cultures were grown to the mid-exponential phase (optical density at 580 nm of 0.4) in defined medium with or without 2.5 M NaCl, in either the presence or absence of osmoprotectants. The cells were quickly chilled and harvested by centrifugation (16,000 × *g* at 4°C). The cells were resuspended in boiling 4% (wt/vol) sodium dodecyl sulfate and boiling was continued for 30 min. The pellets were washed thoroughly with water to remove sodium dodecyl sulfate and further treated with various enzymes as described previously (6) to obtain purified cell walls. Preparation and separation of the muropeptides from purified peptidoglycan were performed as described by de Jonge et al. (6).

Statistical analysis. All statistical analyses were performed with the SPSSX

TABLE 1. Growth of *S. aureus* in defined medium under various conditions[a]

Addition to defined medium	Mean generation time (h)	Time to stationary phase (h)	Maximum cell density (A_{580})
None	1	11	2.5
Proline	1	11	2.5
Proline betaine	1	11	2.5
Glycine betaine	1	11	2.5
Choline	1	11	2.5
Taurine	1	11	2.5
NaCl	3.75	50	0.6
NaCl + proline	2.5	22	0.85
NaCl + proline betaine	2	22	0.95
NaCl + glycine betaine	1.5	21	1.3
NaCl + choline	2.5	22	0.9
NaCl + taurine	3	45	0.8

[a] Mean generation time, time to stationary phase, and maximum cell density were determined from standard semilogarithmic plots of growth curves. The concentrations of the additions to defined medium were 2.5 M (NaCl) and 1 mM (all others).

statistical package (29). Data were analyzed by analysis of variance. Statistical significance was accepted at the $P < 0.01$ level of probability.

RESULTS

Growth of *S. aureus* in high-NaCl medium in the presence and absence of osmoprotectants. The growth of *S. aureus* in defined medium containing 2.5 M NaCl with no added osmoprotectants was drastically retarded compared with that in low-osmotic-strength defined medium (Table 1). Glycine betaine, L-proline, proline betaine, and choline stimulated the growth rate and stationary-phase population size, with glycine betaine being the most effective osmoprotectant. Taurine was a much weaker osmoprotectant. These results are consistent with previous findings (11, 19, 24).

Electron microscopic observations. Exponential-phase cells of *S. aureus* grown in defined medium, defined medium plus 2.5 M NaCl, and defined medium plus 2.5 M NaCl and 1 mM glycine betaine are shown in Fig. 1. Addition of 2.5 M NaCl to the growth medium resulted in a significant increase in cell size (Fig. 1B) compared with cells grown in defined medium without NaCl (Fig. 1A). Addition of the osmoprotectant glycine betaine reversed the effect of NaCl (Fig. 1C). The mean and standard deviation of cell areas after each treatment are shown in Table 2. The frequency distribution curves of the cell areas for each growth condition are shown in Fig. 2. A statistically significant difference was found in the mean cell areas between the control (0.43 μm^2) and NaCl-grown (0.58 μm^2) cells. A similar difference was also found between the NaCl-grown cells and the cells grown in the presence of NaCl and glycine betaine. The mean cell area of the latter group (0.41 μm^2) was very close to that of the control group (0.43 μm^2). The increase in cell size due to growth with high NaCl and its reduction by glycine betaine were also observed with stationary-phase cells (Table 2). Proline is a less potent osmoprotectant than glycine betaine in *S. aureus*, and it decreased the size of cells growing in the presence of 2.5 M NaCl less than glycine betaine did (Table 2).

The increase in cell size due to the presence of NaCl in the growth medium and its reduction by glycine betaine were also observed when cells were examined by SEM (Fig. 3), confirming the TEM data.

The influence of a range of concentrations of NaCl (0, 0.5, 1.0, 1.5, and 2.0 M) on cell size was investigated. Increases in cell size were noted at concentrations of 1.0 M NaCl (medium osmolarlity, about 2.0 osmol [kg of water]$^{-1}$) and above. In order to attempt to determine whether the increase in cell size was due solely to the osmotic stress provided by high concentrations of NaCl, the effects of various nonionic osmolytes on

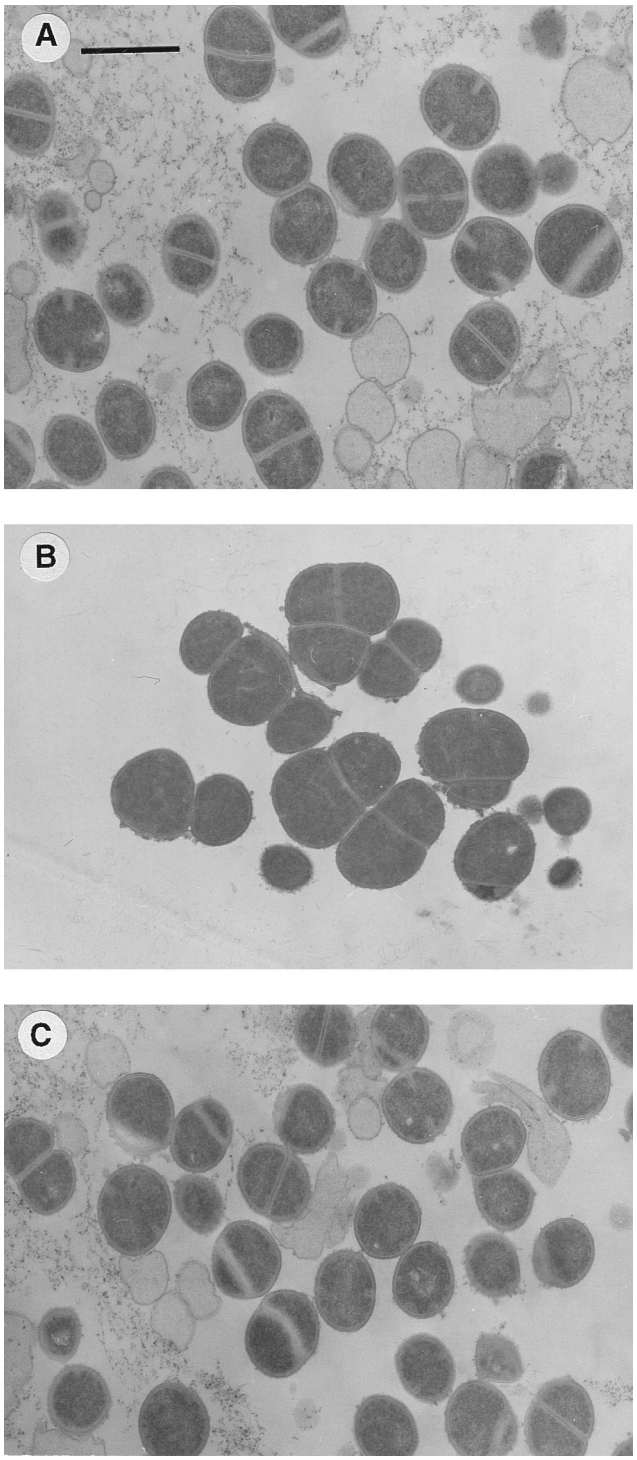

FIG. 1. Electron micrographs of thin sections of exponential-phase cultures of *S. aureus* grown in (A) defined medium, (B) defined medium containing 2.5 M NaCl, and (C) defined medium containing 2.5 M NaCl and 1 mM glycine betaine. All micrographs are at the same magnification. Bar, 1 μm.

TABLE 2. Cell areas of cells grown under various osmotic conditions[a]

Growth phase and addition to defined medium	No. of cells measured	Mean avg cell area (mm^2) ± SD
Exponential-phase cultures		
None	50	0.43 ± 0.09
NaCl	50	0.58 ± 0.12
NaCl + glycine betaine	50	0.41 ± 0.09
Stationary-phase cultures		
None	50	0.38 ± 0.05
NaCl	50	0.56 ± 0.16
NaCl + glycine betaine	50	0.36 ± 0.06
NaCl + L-proline	50	0.50 ± 0.01

[a] The concentrations of the additions to defined medium were 2.5 M (NaCl) and 1 mM (all others). Addition of 1 mM glycine betaine to the defined medium had no effect on cell area (data not shown).

cell size were examined. Media were adjusted to an osmotic strength of 2.0 osmol (kg of water)$^{-1}$ with NaCl, KCl, glycerol, sorbitol, and sucrose. It was not practical to use the nonionic osmolytes at higher concentrations because of solubility and medium viscosity considerations. Glycerol lowers medium water activity but does not provide an osmotic stress because it freely permeates bacterial cells and equilibrates across the membrane (25). Cells grown in the presence of sucrose or sorbitol were not enlarged but appeared to be somewhat smaller than control cells, in contrast to NaCl- and KCl-grown cells, which were larger, as expected. The size of glycerol-grown cells was not changed. These observations suggest that high ionic strength, which includes an osmotic component, and not an osmotic effect alone, is responsible for the increase in cell size. The size of cells grown in the presence of 2 M NaCl, KCl, or $NaNO_3$ was increased with each of the solutes, indicating that the effect is not specific to Na^+ or Cl^- ions.

Muropeptide analysis. To determine whether the changes in morphology were associated with alterations in cell wall structure, the muropeptide composition of peptidoglycan was analyzed by reversed-phase high-performance liquid chromatography (HPLC). In cells grown in the absence of NaCl, the major components of peptidoglycan were pentaglycine muropeptides (peaks 5, 11, and 15 to 21). As shown in Fig. 4, the presence of 2.5 M NaCl had a profound effect on the muropeptide composition. The amount of pentaglycine-substituted species was dramatically reduced, and increased amounts of muropeptides with fewer than five glycine residues (serving as interpeptide bridges) were observed (peaks 1 and 4, arrow II; for structures, see de Jonge et al. [6]). Also, the degree of cross-linkage was slightly reduced. Glycine betaine (1 mM) inclusion in the high-NaCl medium resulted in a muropeptide profile similar to that of cells grown under unstressed conditions (Fig. 4C).

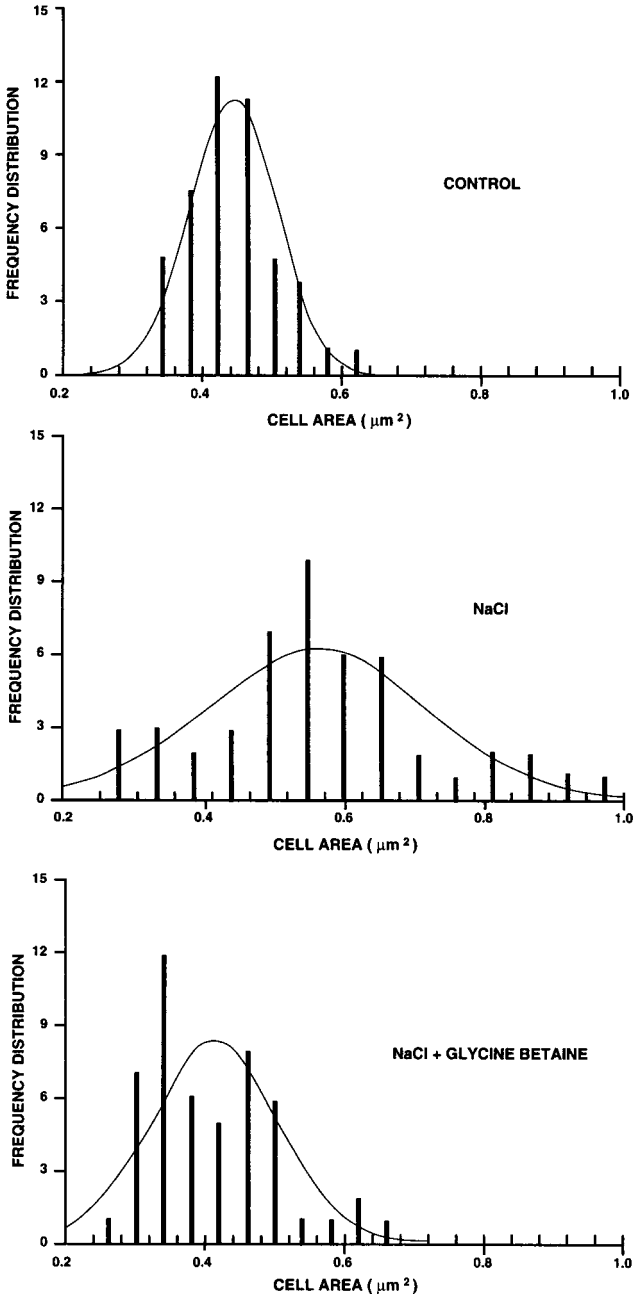

FIG. 2. Frequency distribution of areas of exponential-phase *S. aureus* cells grown in defined medium (control), defined medium containing 2.5 M NaCl, and defined medium containing 2.5 M NaCl and 1 mM glycine betaine. Fifty cell areas from each group are divided among a maximum of 21 size classes. The continuous curve represents the computer-generated Gaussian distribution.

DISCUSSION

The most striking findings of this study were the increased size of cells grown in the presence of high NaCl and the reversal of this effect by the osmoprotectant glycine betaine. Proline is known to be a less potent osmoprotectant than glycine betaine (11, 24) and had less effect on cell size than glycine betaine. Through studies of the effects of various ionic and nonionic osmolytes on cell size, it appears that the increase in cell size is due to high ionic strength rather than high osmotic strength. Ionic solutes provide the stresses of high osmotic strength and high concentrations of ions.

In *E. coli*, it is well established that slowly growing cells are significantly smaller than rapidly growing cells (26). In our experiments, cells growing in high-NaCl medium grew much more slowly than those growing in medium without added NaCl, yet the slowly growing cells were larger than the more rapidly growing cells. In this connection, Cayley et al. (2) have proposed that osmotic stress controls the growth rate of *E. coli* by a fundamentally different mechanism than that occurring when the carbon source is used to vary the growth rate. The enlargement of cells is not due to an artifact introduced by

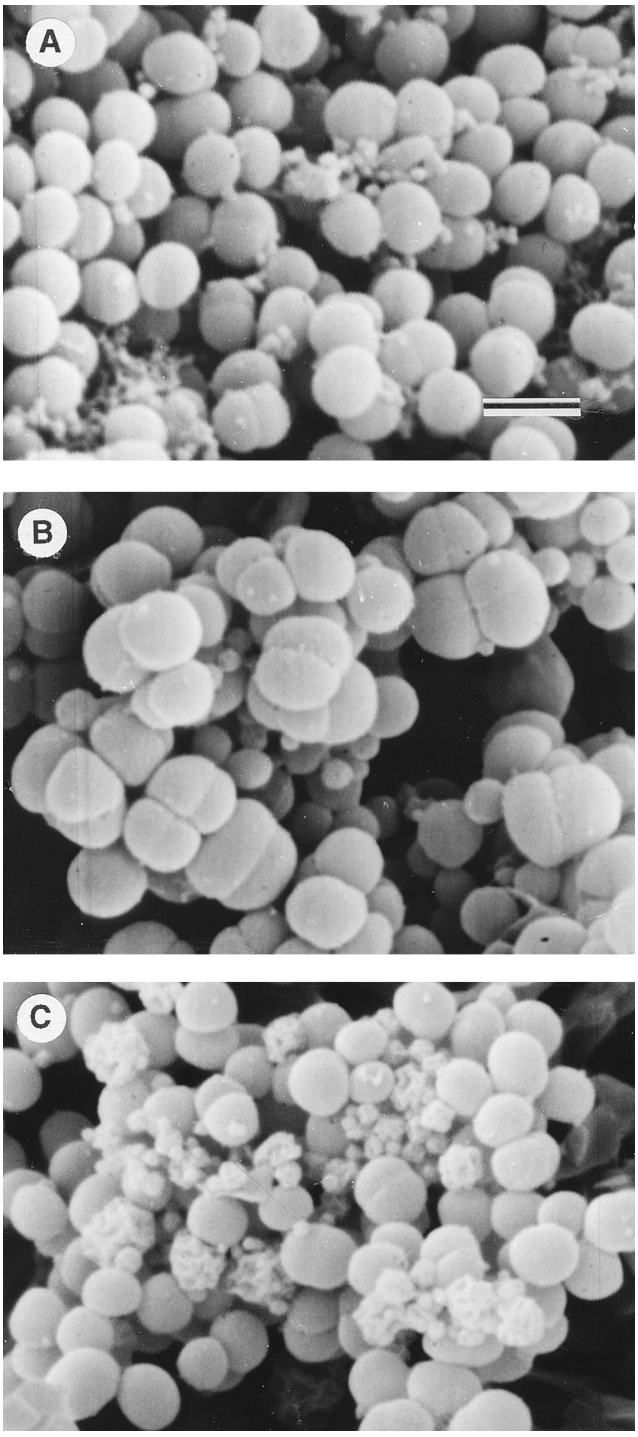

FIG. 3. SEMs of exponential-phase cultures of *S. aureus* grown in (A) defined medium, (B) defined medium containing 2.5 M NaCl, and (C) defined medium containing 2.5 M NaCl and 1 mM glycine betaine. All micrographs are at the same magnification. Bar, 1 μm.

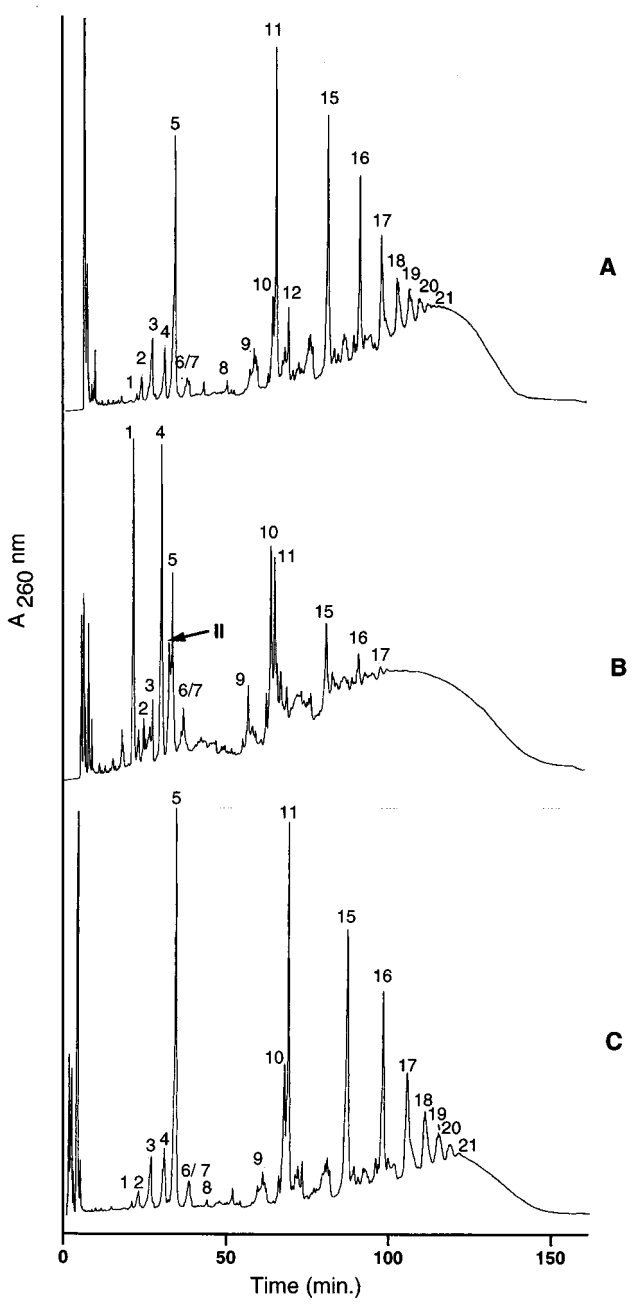

FIG. 4. Muropeptide profile of *S. aureus* RN450 grown in (A) defined medium, (B) defined medium containing 2.5 M NaCl, and (C) defined medium containing 2.5 M NaCl and 1 mM glycine betaine. The elution profiles were obtained as described before (6). Cells were grown, and cell walls were prepared. Peptidoglycan was obtained after teichoic acid was removed. Enzymatic hydrolysis with a muramidase, borohydride reduction, and separation of muropeptides were performed by reversed-phase HPLC. Peak assignments were made on the basis of retention times of a standard cell wall preparation. For chemical structures, see de Jonge et al. (6).

TEM, as similar results were obtained when cells were observed by SEM after critical-point drying (Fig. 3). Glycine betaine (1 mM) increases the growth rate but decreases cell size. Meury (23) has reported that high osmolarity (NaCl and KCl were used as the stressing agents) causes *E. coli* to lengthen and attributed this to an inhibition of cell division. Glycine betaine stimulated DNA synthesis and restored cell division in *E. coli* in high-osmolarity medium. Slowing cell division in a coccus presumably would lead to cell enlargement if growth continued. Possibly glycine betaine also restores the rate of cell division in *S. aureus* growing under NaCl stress.

Peptidoglycan analysis of cells grown in the presence of 2.5 M NaCl showed a remarkable difference in muropeptide com-

position compared with that of cells grown in defined medium alone. The stressed cells had a shorter peptidoglycan interpeptide bridge than unstressed cells. A likely explanation for the relative abundance of muropeptides with fewer than five glycine residues in peptidoglycan is a disturbance in the glycine addition process. Glycine addition occurs sequentially at the lipid-linked stage of peptidoglycan precursor synthesis (16, 30). Since muropeptides with glycine substitutions ranging from zero to five were all present in peptidoglycan and no accumulation of a specific muropeptide was observed, high NaCl concentrations may affect the glycine addition process in general, possibly by a disturbance of membrane integrity. It is well established that NaCl-grown *S. aureus* cells have increased levels of the negatively charged membrane phospholipids phosphatidylglycerol and cardiolipin (14, 17). The muropeptide analyses suggest that in stressed cells, the peptidoglycan was somewhat more loosely cross-linked than the peptidoglycan of unstressed cells.

Recently, some peptidoglycan mutants with shortened interpeptide bridges were described, all of which had a decreased autolysis rate (7–9, 12, 22). The peptidoglycan of cells grown in the presence of 2.5 M NaCl in the absence of osmoprotectants may be similarly less susceptible to peptidoglycan hydrolase activity. In this connection, more empty cells were noted in cultures grown under unstressed conditions (Fig. 1A) or stressed conditions plus glycine betaine (Fig. 1C) than under stressed conditions without an added osmoprotectant (Fig. 1B). This may be a reflection of the autolytic activity of these cells. In the paper of Henze et al. (12), electron micrographs of *femA* and *femB* mutants of *S. aureus* are shown. The cells displayed a pseudomulticellular appearance, with suppressed separation of daughter cells. The *femA* mutant had more aberrant cross walls than the *femB* mutant. Examination of the cells grown in the presence of NaCl without osmoprotectants shows a similar pseudomulticellular appearance with retarded cell separation, more closely resembling the *femB* than the *femA* mutant.

One of the characteristics of osmotically stressed bacteria, including *S. aureus*, is that they have a decreased volume of cytoplasmic water (3, 4). The origin of the osmoprotective activities of glycine betaine and proline has been studied by Cayley et al. (3). In *E. coli*, accumulation of these osmoprotectants was accompanied by a significant increase in the volume of cytoplasmic water per unit dry weight, with glycine betaine causing a larger increase than proline. The alteration in the peptidoglycan composition caused by adding glycine betaine to cells growing in the presence of 2.5 M NaCl may be an indirect effect, a result of more global effects of glycine betaine, such as increasing the volume of cytoplasmic water. It is unlikely that glycine betaine is a source of glycine, as all available evidence suggests that accumulated glycine betaine is not further metabolized (11, 15).

In summary, we have shown that high NaCl caused an increase in the size of *S. aureus* and that normal cell size is restored by addition of glycine betaine. The increase in the cell size under stressed conditions is correlated with shorter interpeptide bridges, less cross-linked peptidoglycan, and retarded cell separation. Further work will be necessary to elucidate the mechanism of the increase in cell size caused by NaCl and its reversal by glycine betaine.

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