JOURNAL OF BACTERIOLOGY, July 1972, p. 220-230
Vol. 111, No. 1

Regulation of the Bacterial Cell Wall: Isolation and Characterization of Peptidoglycan Mutants of *Staphylococcus aureus*[1]

ANADI N. CHATTERJEE AND FRANK E. YOUNG

Department of Microbiology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

Received for publication 3 February 1972

Temperature-sensitive mutants of *Staphylococcus aureus* H which require 1.0 M NaCl for growth at 42 C can be divided into two major classes. Most of the mutants (class A) do not accumulate nucleotide precursors of cell wall biosynthesis in the absence of salt at the nonpermissive temperatures, whereas the class B mutants accumulate these precursors. The most extensively studied mutant RUS 1 (carrying *peg-1*) is defective in biosynthesis of peptidoglycan at the nonpermissive conditions as evidenced by: (i) reduced incorporation of cell wall precursors into peptidoglycan; (ii) accumulation of the nucleotide, uridine diphosphate (UDP) muramyl-L-alanyl-D-glutamic acid; (iii) reduced specific activity of UDP *N*-acetylmuramyl (MurNAc)-L-alanyl-D-glutamate:L-lysine ligase (EC 6.3.2.7); and (iv) an increased susceptibility to lysis with sodium dodecyl sulfate. Addition of 1.0 M NaCl reverses these defects with the exception of the specific activity of UDP-MurNAc-L-alanyl-D-glutamate:L-lysine ligase. Nevertheless, the structure of the cell wall is normal at the nonpermissive conditions if 1.0 M NaCl is present. An alteration in the binding of a fluorescent dye, 8-anilino-1-napthalene-4-sulfonic acid at the nonpermissive conditions in the absence of 1.0 M NaCl suggests that there may also be defects in the membrane in this strain.

The chemical structure and the biosynthetic pathways of bacterial cell wall are now well established (24); however, relatively little is known about the regulation of its synthesis. One approach to this problem would be the study of mutants defective in peptidoglycan biosynthesis. Since the peptidoglycan layer provides the strength and rigidity to the bacterial cell wall, one predictable phenotype of mutant strains defective in peptidoglycan would be development of osmotic fragility. In recent years, mutant strains requiring osmotic protection for growth have been described in *Bacillus subtilis* (29, 30, 43) and *Escherichia coli* (17, 18). Some of these mutants in *B. subtilis* were defective in teichoic acid synthesis (3; D. Brooks and F. E. Young, Bacteriol. Proc., p. 24, 1971). Others required the addition of growth factors like glutamine to preclude the need for osmotic stabilization, suggesting the primary lesion was not in peptidoglycan synthesis (31). To date, therefore, no well defined peptidoglycan mutants have been isolated in *B. subtilis* despite efforts in this and other laboratories. On the other hand, in *E. coli* peptidoglycan mutants have been isolated and preliminary mapping studies have been presented (18).

This paper describes the isolation and characterization of temperature-sensitive (ts) mutants of *Staphylococcus aureus* which become osmotically fragile when grown at the nonpermissive temperature with an associated inhibition of peptidoglycan synthesis. Thus, it is possible at least in *S. aureus*, to select mutants defective in either peptidoglycan or teichoic acid synthesis (4). A preliminary account of this work has already been published (Bacteriol. Proc., p. 49, 1971). Mutants of *S. aureus* with similar phenotype were described simultaneously and independently by Good and Pattee (10; C. M. Good, D. J. Tipper, and P. A. Pattee, Bacteriol. Proc., p. 50, 1971).

MATERIALS AND METHODS

Media and strains. The wild-type strain used in this study was the streptomycin-resistant strain *S.*

[1] This paper was presented in part at the 71st Annual Meeting of the American Society for Microbiology, Minneapolis, Minnesota, 2-7 May 1971.

aureus H (Str). Its isolation and maintenance have been described previously (4). The cells were grown in the complex PYK medium (5) containing 0.2% glucose. In some experiments a chemically defined medium was used. This minimal medium (MAG medium) contained the four cell wall amino acids (glutamic acid, glycine, alanine, and lysine), glucose, nicotinamide, thiamine, and inorganic salts buffered to *p*H 7.0 (6).

Isolation of mutant strains. *S. aureus* H (Str) was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described previously (4); about 60% of the cells were killed by the mutagen. Survivors were diluted into PYK medium and incubated at 30 C for 2 hr, and samples were plated on nutrient agar (NA) at 30 C. After 48 hr, colonies were replicated on NA with or without 1.0 M NaCl. After 16 hr at 42 C all colonies which required NaCl for growth were tested on PYK agar or Trypticase soy agar to confirm their requirement for NaCl. Of the 36 original isolates only 9 showed an absolute requirement for 1.0 M NaCl for growth at 42 C. Sucrose (1.2 M) could replace NaCl. All these mutant strains were coagulase-positive, streptomycin-resistant, and exhibited the parental phage type (phages 52A, 79, and 80). They will be designated as RUS for Rochester University Staphylococcus and the defect will be designated *peg* to denote abnormalities in biosynthesis of peptidoglycan.

Growth and viability studies. PYK medium (supplemented when necessary with 1.0 M NaCl) was used for growing both wild-type and mutant strains. In all metabolic experiments a 4% inoculum of a 13- to 15-hr culture was used. Cells were allowed to grow for at least two generations before a temperature shift (from 30 to 42 C) or addition of antibiotics and radioactive amino acids. All cultures were grown with shaking in New Brunswick G-76 (setting 5) or in G-25 (200 rev/min) gyrotory shakers. Growth was monitored by following the optical density (OD) at 585 nm. An OD of 0.44 was equivalent to 0.1 mg cells (dry weight)/ml. Viable cell numbers were determined by standard plating techniques on NA.

Isolation and characterization of nucleotide-bound peptidoglycan precursors. A logarithmic-phase culture of a peptidoglycan-defective mutant RUS 1 (carrying *peg-1*) growing in PYK medium was shifted from 30 to 42 C (at OD 1.20) and incubated for an additional 90 min at the nonpermissive temperature. The cells were then harvested by centrifugation, the cell pellet was extracted with cold 5% trichloroacetic acid, and the nucleotides were isolated on a Dowex-1 column (36) except that the charcoal step was omitted and the extract was first partially purified over a Sephadex G-25 column (5). A single peak of bound *N*-acetyl hexosamine was eluted from the Dowex-1 column, was lyophilized and was resuspended in distilled water. The ultraviolet (UV) spectrum of this fraction was similar to uridine nucleotides (at *p*H 7.0 the ratios were: 250 nm/260 nm = 0.72; 280 nm/260 nm = 0.40). After hydrolysis with 0.02 N HCl for 5 min at 100 C, the fraction was chromatographed in solvent A (isobutyric acid–0.5 M NH_4OH; 5:3, v/v) on Whatman no. 1 paper with uridine mono- and diphosphate (UMP and UDP) as standards. The hydrolysate gave a single UV-absorbing spot which co-chromatographed with UDP. The unhydrolyzed material gave a single UV spot in two solvent systems: solvent A and solvent B (95% ethanol–1 M ammonium acetate, *p*H 3.8; 7:5, v/v). The ratio of bound *N*-acetyl hexosamine to phosphate to uridine (E_{max} at 262 nm = 9,800) was 1.00: 2.11:1.15. Identification and quantitative analysis of the components was done on a Technicon amino acid analyzer after hydrolysis with 6 N HCl for 11 hr at 102 C. Results were computed from a standard containing the wall amino acids and amino sugars that had been hydrolyzed by an identical procedure.

Protein and peptidoglycan synthesis. Logarithmic-phase cultures growing in PYK broth at 30 C were divided into four equal parts. Two of these were incubated at 30 C and the other two shifted to 42 C. One flask at each temperature was supplemented with 1.0 M NaCl. After 2 min, ^{3}H-L-lysine (0.1 μCi/ml) or ^{14}C-glycine (0.05 μCi/ml) was added to each flask. Samples from these flasks were pipetted at intervals into cold 10% trichloroacetic acid (final concentration 5%), and incorporation into protein and peptidoglycan fractions was determined after Park-Hancock fractionation (25). Essentially all the radioactivity in the protein or peptidoglycan fractions could be recovered as lysine or glycine after acid hydrolysis (6 N HCl, 105 C, 15 hr). In other experiments, the cells growing at 30 C in PYK medium were harvested and washed in phosphate buffer (*p*H 7.0, 0.05 M), resuspended in the same buffer, and added to the MAG medium which had been prewarmed to 30 or 42 C. Thus, the incorporation of radioactive amino acids into cell walls and proteins was determined in both a complex and a chemically defined medium.

Fluorometry. Intensity of fluorescence of cell suspensions in the presence of the fluorescent dye 8-anilino-1-naphthalene-4-sulfonic acid (ANS) was measured in an Aminco-Bowman spectrophotometer (American Instrument Co.). A standard solution was prepared by mixing 3.0 ml of bovine serum albumin (BSA; 0.2 mg/ml) in tris(hydroxymethyl)aminomethane (Tris) buffer (0.05 M, *p*H 7.2) with 0.1 ml of ANS (1.0 mM) solution. The excitation and emission wave lengths were set at 380 and 475 nm, respectively. Fused quartz cells were used for all measurements with the slit arrangement set for maximum sensitivity. The spectrofluorometer was standardized by adjusting the relative fluorescent intensity (RFI) of the standard BSA solution to 90 by the sensitivity setting. A 0.1-ml sample of ANS (1.0 mM) was added to 3.0 ml of cells suspended in Tris buffer (*p*H 7.2, 0.05 M), and 0.1 ml of Tris buffer was added to another 3.0-ml sample of cells. The RFI of each cell suspension was corrected by the blank value from a sample containing no ANS.

Preparation and assay of UDP-MurNAc-L-alanyl-D-glutamate:L-lysine ligase (EC 6.3.2.7; L-lysine adding enzyme). The L-lysine adding enzyme was partially purified from cell-free extracts by a published method (13). Cell-free extracts were made either by mechanical disintegration in a Braun homogenizer (6) or enzymatically by lysing the cells with lysostaphin (33). The assay was based on the

formation of UDP-*N*-acetylmuramyl-L-alanyl-D-isoglutamyl-L-lysine (UDP-MurNAc-tripeptide) when the precursors UDP-MurNAc-L-alanyl-D-isoglutamic acid (UDP-MurNAc-dipeptide) and L-lysine were incubated with the enzyme extract together with adenosine triphosphate (ATP) and magnesium chloride. The precursor UDP-MurNAc-dipeptide was isolated from *S. aureus* H cells which were incubated in MAG medium in the absence of lysine (39). The control consisted of the incubation mixture minus the UDP-MurNAc-dipeptide. The reaction was stopped by heating to 100 C for 2 min. The whole reaction mixture was then spotted on Whatman no. 3MM filter paper, and chromatographed in solvent A for 15 hr which separated lysine (R_F = 0.67) from UDP-MurNAc-tripeptide (R_F = 0.12). The marker compound UDP-MurNAc-tripeptide was isolated and purified from the wild-type cells by treating them with D-cycloserine (40). After drying, the area corresponding to the UDP-MurNAc-tripeptide was cut in small strips and the radioactivity determined in a scintillation counter. Quantitative assay of the amount of product formed was based on the analysis of an identical chromatogram containing lysine of known specific activity. Under the conditions used, 1 nmole of product had about 800 counts/min.

Assay of radioisotopes. Both aqueous solutions and filter papers were counted with a toluene base scintillation fluid (36). For aqueous samples a detergent, Triton X-100, was added to the scintillation fluid. All radioactivity was measured in a Beckman LS 230 scintillation counter with correction for quenching by an internal standard.

Other methods. Bound *N*-acetyl amino sugars, as a measure of nucleotide-bound peptidoglycan precursors, were assayed by the Morgan-Elson reaction (27) after hydrolysis with 0.01 N HCl for 4 min at 100 C. UDP-*N*-acetylglucosamine (UDP-GlcNAc) was used as standard. Protein was determined by the method of Lowry et al. (16) and phosphate by the method of Chen et al. (7). Cell walls were isolated by mechanical disintegration of cells with glass beads as previously described (4). All quantitative analysis was done on a Technicon amino acid analyzer. The starting *p*H was 3.25, and the column was run at 60 C; an internal standard of norleucine was included in every run. Cell walls were hydrolyzed for 15 hr at 105 C, and the nucleotide-bound peptidoglycan precursors for 11 hr at 102 C. All hydrolyses were done in 5 N HCl. The results were calculated from a standard mixture containing the cell wall amino sugars and amino acids that were hydrolyzed and analyzed by an identical procedure.

RESULTS

Growth and viability studies. All the nine ts mutants grew normally at 30 C; however, at 42 C they exhibited osmotic fragility and loss of viability after about one generation. This effect was reversed by NaCl. The minimal concentration of NaCl which could stabilize the mutant was 0.4 M and the optimal concentration was 1.0 M. Sucrose (1.6 M), but not glycerol (up to 2 M), could replace NaCl. All this evidence was in general agreement that the primary role of NaCl was of osmotic protection at the nonpermissive temperature.

Of these nine mutants, only one, *peg-1* (which we shall use to designate peptidoglycan mutants) accumulated nucleotide-bound peptidoglycan precursors when grown at 42 C. The present study deals primarily with this mutant. The effects of shifting an actively growing culture of *peg-1* from 30 to 42 C is shown in Fig. 1. Although cultures supplemented with 1.0 M NaCl continued to grow and divide normally (as judged by OD and viability), the cultures without NaCl stopped growing (as judged by OD) after about 1 hr and eventually started to lyse. The viability increased for the first 30 min (about one generation) and then decreased exponentially for the next 60 min (Fig. 1). It is important to note that this killing could be reversed in part by diluting and by plating in media supplemented with 1.0 M NaCl. Addition of chloramphenicol (CAP; 80 μg/ml) at the time of temperature shift prevented in part the dramatic

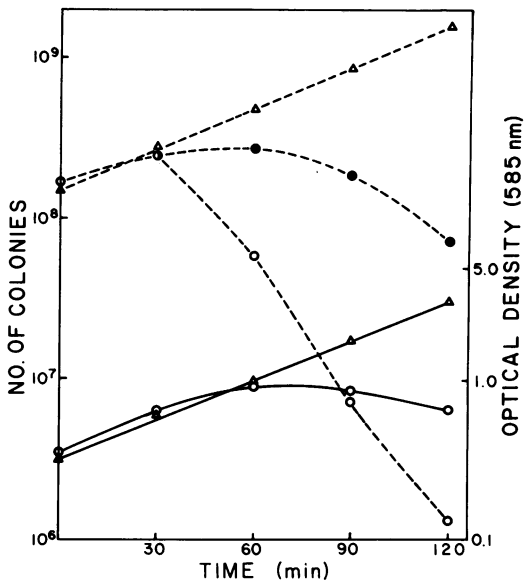

FIG. 1. *Growth and viability of peg-1 at 42 C. A logarithmic-phase culture of peg-1 in PYK broth at 30 C was split in two halves and one was supplemented with 1.0 M NaCl. The flasks were then shifted to 42 C, and incubation was continued. Growth was monitored by OD at 585 nm and viability by plating suitable dilutions in 0.85% NaCl on nutrient agar. Symbols:* ○, *control culture;* △, *culture supplemented with 1.0 M NaCl;* ●, *control culture diluted and plated in media containing 1.0 M NaCl. Broken lines denote cell titer; solid lines denote OD.*

loss of viability under nonpermissive conditions in the absence of 1 M NaCl (Table 1). At 42 C growth of the wild type was inhibited by CAP; however, no decrease in viability occurred if the CAP was diluted out within 2 hr. This would suggest unbalanced growth at the nonpermissive temperature as a major factor in the loss of viability of the mutant strain.

Evidence of osmotic damage to mutant cells grown at 42 C was shown by treatment with sodium dodecyl sulfate (SDS). Bacterial cells with a damaged peptidoglycan layer are lysed preferentially by SDS (19). Table 2 shows that a culture of *peg-1* grown at 42 C was lysed by SDS. It should be noted that this osmotic fragility was comparable to cells grown at 30 C in the presence of penicillin, a known inhibitor of peptidoglycan synthesis (Table 2). No lysis could be seen with *peg-1* cells grown at 30 or at 42 C when the medium was supplemented with 1.0 M NaCl. Table 2 also shows that the wild-type cells grown either at 30 or at 42 C were resistant to lysis by SDS. Although CAP appeared to overcome the loss in viability at the nonpermissive temperature (Table 1), the cell walls were still defective as evidenced by lysis with SDS (Table 2).

Accumulation of peptidoglycan precursors. *peg-1* accumulated nucleotide-bound peptidoglycan precursors when grown at the nonpermissive temperature. The rate of this accumulation was determined by shifting log-phase cultures of *S. aureus* from 30 to 42 C. At suitable intervals, crushed ice was added to the culture, the cells were harvested by centrifugation and extracted with trichloroacetic acid, the extract was partially purified by chromatography on Sephadex G-25 column (as described in Materials and Methods), and the content of C-1 bound *N*-acetyl hexosamines (as a measure of nucleotide-bound peptidoglycan precursors) was determined. An identical procedure was carried out with the parent strain treated with penicillin G. As shown in Fig. 2, the rate and extent of accumulation of bound *N*-acetyl hexosamines were strictly comparable in both cases. There was no accumulation when *peg-1* was grown at 30 C. Accumulation of peptidoglycan precursors (measured as C-1 bound *N*-acetyl hexosamines) under various conditions by the wild type and mutant strain is shown in Table 3. For the parent strain (growing at 30 or 42 C) amount of bound *N*-acetyl hexosamines per g of cells

TABLE 1. *Effect of chloramphenicol (CAP) on the viability of peg-1 cells growing at 42 C*[a]

Culture	Conditions	Viable cell titer at			
		0 min	30 min	60 min	120 min
peg-1	42 C	6.0×10^8	9.1×10^8	1.0×10^8	1.3×10^6
peg-1	42 C plus CAP at 0 min	5.9×10^8	6.1×10^8	4.0×10^8	1.1×10^8

[a] A log-phase culture of *peg-1* growing at 30 C in PYK broth was split into two, and both flasks were shifted to 42 C (0 min) with continued shaking. CAP (80 μg/ml) was added to one flask at 0 min. Viable cell titer was determined by dilution in 0.85% NaCl and plating on nutrient agar.

TABLE 2. *Sodium dodecyl sulfate (SDS)-induced lysis of wild-type and peg-1 cells*

Strain	Growth conditions[a]	OD at 585 nm		ΔOD (OD H_2O − OD SDS)
		In H_2O	In SDS	
Wild type	30 C	0.450	0.466	−0.016
	42 C	0.470	0.488	−0.018
peg-1	30 C	0.425	0.428	−0.003
	30 C + penicillin G (10 μg/ml)	0.410	0.166	+0.244
	42 C	0.395	0.110	+0.285
	42 C + 5% NaCl	0.460	0.449	+0.012
	42 C + chloramphenicol (80 μg/ml)	0.415	0.162	+0.253

[a] A log-phase culture of wild-type strain growing in PYK broth at 30 C was split in two and one was shifted to 42 C. A similar culture of *peg-1* growing at 30 C was split into five portions; two of these continued to be shaken at 30 C while the other three were shifted to 42 C. Additions of NaCl or antibiotics were made at time of shift. All cultures were incubated further for 90 min. Two 10-ml samples of each culture were then centrifuged. One was suspended in 10 ml of water and the other in 10 ml of SDS (0.02 M), and OD was determined after incubation of the cell suspensions at 37 C for 5 min.

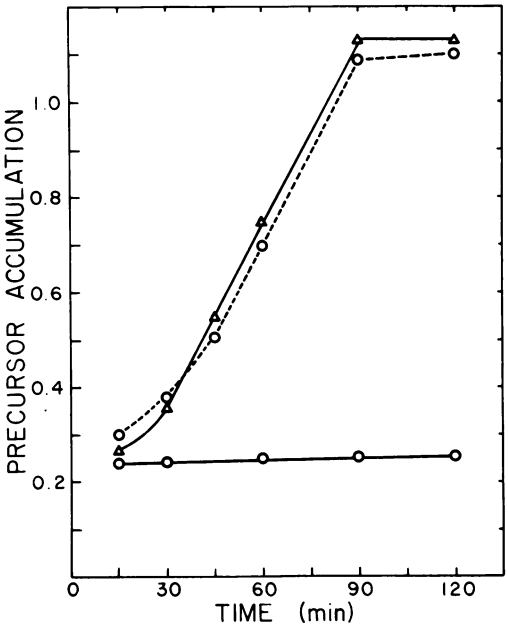

FIG. 2. *Kinetics of accumulation of bound N-acetyl hexosamine. Log-phase culture of wild type growing in PYK broth at 37 C was treated with penicillin G (10 μg/ml) and the shaking was continued at 37 C. A similar culture of peg-1 growing at 30 C was split into two halves. One of these was shifted to 42 C and the other continued to be incubated at 30 C. At the indicated times, 20-ml samples of each culture were poured into crushed ice, the cells were harvested by centrifugation, and the bound N-acetyl hexosamines accumulated in the cell pellets were determined as in Materials and Methods. Symbols: O—O, peg-1 incubated at 30 C; O- - -O, peg-1 incubated at 42 C; Δ—Δ, wild type plus penicillin G (10 μg/ml). Precursor accumulation expressed as μmoles of bound N-acetyl hexosamine/200 ml of culture.*

was approximately 2 μmoles. This was increased 10-fold by treatment with penicillin. The accumulation pattern with the mutant strain grown at 30 C was very similar to that of the wild type (Table 3). However, *peg-1* cells incubated at 42 C accumulated about 20 μmoles of bound *N*-acetyl hexosamines per g of cells. This value is comparable to that induced with penicillin. Addition of penicillin to *peg-1* growing at 42 C did not enhance the amount of accumulation (A. N. Chatterjee, *unpublished data*). It should be noted that the wild phenotype was restored when 1.0 M NaCl was added to a culture of *peg-1* incubated at 42 C.

UDP-MurNAc-dipeptide was identified as the major precursor that was accumulated by *S. aureus* (carrying *peg-1*) growing at 42 C (Table 4). It co-chromatographed in two solvents (A and B) with the authentic UDP-MurNAc-dipeptide which was isolated from *S. aureus* H cells incubated in MAG medium in the absence of lysine (39). UDP-MurNAc and UDP-MurNAc-L-ala (these were not resolved on the Dowex-1 column) also accumulated to a small extent (about 10% of the total). The major precursor accumulated by incubating *peg-1* with penicillin at 30 C was identified as the normal peptidoglycan precursor, UDP-MurNAc-pentapeptide (Table 4).

Fluorescence studies. The fluorescent dye ANS has been used as a probe to detect changes on bacterial cell surfaces (12). This dye has a weak fluorescence in aqueous solutions but fluoresces strongly when adsorbed to proteins (9). In the present study a differential effect of ANS was observed with *peg-1* grown at 30 and 42 C. Thus, as shown in Table 5, there was a marked increase in the RFI of *peg-1* cells grown at 42 C, but there was no such difference with wild-type cells. Table 5 shows that the mutant phenotype was reversed when *peg-1* was grown at 42 C in the presence of 1.0 M NaCl. The last two lines in Table 5 give the RFI of autoclaved cell suspensions which

TABLE 3. *Accumulation of nucleotide-bound N-acetyl hexosamines under different growth conditions*[a]

Strain	Growth conditions	Micromoles of bound *N*-acetyl hexosamines/g of cells (dry wt)
Wild type	30 C	1.8
	42 C	2.2
	30 C + penicillin G (10 μg/ml)	21.8
peg-1	30 C	2.0
	42 C	21.8
	30 C + penicillin G (10 μg/ml)	19.8
	42 C + NaCl (1.0 M)	1.8

[a] Log-phase cultures of wild type growing at 30 C in PYK broth were divided into three equal portions and one of these was shifted to 42 C while the other two continued to be shaken at 30 C. A similar log-phase culture of *peg-1* growing at 30 C was divided into four equal portions; two of these were shifted to 42 C while the other two continued to be shaken at 30 C. Penicillin G and NaCl were added at the moment of shift. After an additional 90 min of incubation at 30 or 42 C, the cells were centrifuged and extracted with cold 10% trichloroacetic acid, and the bound *N*-acetyl hexosamines were determined as in Materials and Methods.

TABLE 4. *Characterization of cell wall precursors of S. aureus*[a]

Strain and growth conditions	Probable structure of major precursor accumulated	Composition[c] (molar ratios)			
		Muramic acid	Alanine	Glutamic acid	Lysine
Wild type + penicillin G (10 μg/ml) at 37 C	UDP-*N*-acetyl-muramyl-L-alanyl-D-isoglutamyl-L-lysyl-D-alanyl-D-alanine[b]	1.00	2.98	1.15	0.97
Wild type + D-cycloserine (75 μg/ml) at 37 C	UDP-*N*-acetyl-muramyl-L-alanyl-D-isoglutamyl-L-lysine[b]	1.00	0.98	1.08	0.97
peg-1 + penicillin G (10 μg/ml) at 30 C	UDP-*N*-acetyl-muramyl-L-alanyl-D-isoglutamyl-L-lysyl-D-alanyl-D-alanine[b]	1.00	3.11	1.12	0.98
peg-1 at 42 C	UDP-*N*-acetyl-muramyl-L-alanyl-D-isoglutamic acid[b]	1.00	0.99	1.11	<0.05

[a] For details of isolation and characterization see Materials and Methods.
[b] Small amounts of UDP-*N*-acetyl-muramic acid and UDP-*N*-acetyl-muramyl-L-alanine also accumulate under these conditions.
[c] Normalized to muramic acid = 1.00.

would represent the maximum values for these cell suspensions as such treatment is known to break down completely the permeability barrier.

Protein and peptidoglycan synthesis. The synthesis of protein and peptidoglycan in *peg-1* was measured after allowing the cells to incorporate radioactive lysine or glycine. As shown in Fig. 3, protein synthesis continued when the temperature of the culture was shifted from 30 to 42 C. The rate of protein synthesis was not affected by the presence of 1.0 M NaCl. In striking contrast to this, the rate of peptidoglycan synthesis was markedly inhibited after 30 min at the nonpermissive temperature, and there was virtually complete inhibition within 60 min. Peptidoglycan synthesis at 42 C continued normally when the medium was supplemented with 1.0 M NaCl. The rate of protein and peptidoglycan synthesis in the parent strain at 42 C was not affected by NaCl and was comparable to *peg-1* when the latter was supplemented with 1.0 M NaCl. Essentially similar results were obtained when peptidoglycan synthesis was measured in the chemically defined MAG medium. The radioactive amino acids lysine and glycine could be used interchangeably. The data in Fig. 3 are with ^{14}C-glycine because there are five glycine per one lysine residue in the repeating unit of peptidoglycan.

Activity of L-lysine adding enzyme (UDP-MurNAc-L-alanyl-D-glutamate: L-lysine ligase; EC 6.3.2.7). The development of osmotic fragility and failure to synthesize peptidoglycan at the nonpermissive temperature could be due to a specific lesion(s) in the biosynthetic enzymes. Since *peg-1* accumulated the precursor UDP-MurNAc-L-alanyl-D-isoglutamic acid when grown at 42 C, we studied the activity of UDP - MurNAc - L - alanyl - D-glutamate: L-lysine ligase (EC 6.3.2.7), the enzyme which adds lysine to the incomplete precursor UDP-MurNAc-dipeptide (13). As shown in Table 6, the enzyme preparation from the wild-type strain could actively synthesize UDP-MurNAc-tripeptide from UDP-MurNAc-dipeptide and lysine. The rate was linear for 30 min and the synthesis was abso-

TABLE 5. *Penetration of 8-anilino-1-naphthalene-4-sulfonic acid (ANS) into wild-type and peg-1 cells*[a]

Strain	Growth conditions	RFI of cell suspensions
Wild type	30 C	2.3
	42 C	2.1
peg-1	30 C	1.8
	42 C	5.9
	42 C + 1.0 M NaCl	1.9
Wild type	30 C + autoclaved[b]	16.6
peg-1	30 C + autoclaved[b]	15.8

[a] A log-phase culture of wild type growing at 30 C in PYK medium was divided in two and one was shifted to 42 C. A similar culture of *peg-1* growing at 30 C was divided in three equal portions and two of these were shifted to 42 C. NaCl was added to one flask at the time of shift. After a further 90 min of incubation at 30 or 42 C, the cells were harvested and washed and the relative fluorescent intensity (RFI) of the suspensions was determined as in Materials and Methods.
[b] Washed cell suspensions in Tris buffer autoclaved at 120 C for 10 min.

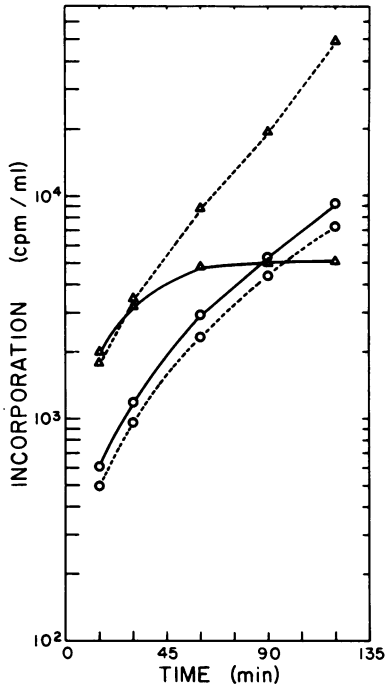

FIG. 3. *Synthesis of protein and peptidoglycan by peg-1 at 42 C. A log-phase culture of peg-1 growing in PYK broth at 30 C was split into two halves and one was supplemented with 1.0 M NaCl. Both flasks were shifted to 42 C and shaking continued.* ^{14}C*-glycine (0.05 μCi/ml) was added to each flask 2 min after the temperature shift. At the indicated intervals, 1.0 ml of each culture was pipetted into 1 ml of 10% trichloroacetic acid, and incorporation into protein and peptidoglycan fractions was determined (see Materials and Methods). Symbols: O, incorporation in protein; Δ, incorporation in peptidoglycan. Solid lines denote control culture; broken lines denote culture supplemented with 1.0 M NaCl.*

lutely dependent on added UDP-MurNAc-dipeptide. In marked contrast, the enzyme preparation from cultures of *peg-1* grown at 42 C had no detectable activity. Unexpectedly, the enzyme from the *peg-1* grown at 30 C had an extremely low activity (less than 5% of the wild type). Addition of NaCl to the reaction mixture, growing the mutant strain in media containing 1.0 M NaCl, or lowering the temperature of incubation to 30 C failed to restore activity. The activity of the extract from the wild type was not inhibited by the addition of extract from *peg-1* grown at 30 or 42 C, demonstrating that an inhibitor of this enzyme was not present in the extracts of this mutant. In other experiments, the ammonium sulfate fractionation step was omitted and cell-free extracts of *peg-1* (grown at 30 C and 42 C) were assayed directly for enzyme activity. All such assays were uniformly negative though significant activity could be readily detected in similar extracts of the wild type.

Analysis of cell walls. Three independent batches of cell walls were prepared from the parent strain grown at 37 C and of the mutant strain grown at 30 or at 42 C. These walls were analyzed quantitatively in the amino acid analyzer after hydrolysis with HCl. As shown in Table 7, cell walls of the mutant strain grown at 30 and 42 C had the same quantitative and qualitative composition, which was identical to that of the wild-type wall as published previously (36). In addition, the phage-binding capacity of the mutant cell walls (both 30 and 42 C) was similar to the parent strain (4), suggesting a normal cell wall peptidoglycan (35) and teichoic acid (4). Thus, despite the apparent absence of the L-lysine adding enzyme, the chemical composition and biological properties of the cell wall of *peg-1* (grown at 30 or 42 C) were similar to the parent strain.

DISCUSSION

Functionally, the bacterial cell wall is composed of two different kinds of heteropolymers. One of these, the peptidoglycan, is ubiquitous in nature and responsible for the strength and rigidity of the cell. The second group, which comprises a large variety with specialized structure and function, could be regarded as accessory or special polymers. By using phage resistance as a tool, we have in the past been able to isolate and study mutants in teichoic acid (an accessory polymer), both in *S. aureus* (4, 36) and *B. subtilis* (3, 42). Mutants of *B. subtilis* defective in glucosylation of teichoic acid or in synthesis of polymerized teichoic acid were isolated readily. However, this approach has failed so far for isolation of peptidoglycan mutants, presumably because this layer is less frequently exposed. Since mutations leading to a defective peptidoglycan would probably be a lethal event, we have sought to isolate ts mutants which would grow normally under permissive conditions but would make a defective peptidoglycan and be killed at the nonpermissive temperature. The results presented in this paper indicate that *S. aureus* (*peg-1*) is a ts mutant defective in peptidoglycan synthesis at the nonpermissive temperature. Thus the mutant cells failed to grow after one generation and eventually started to lyse when shifted from 30 to 42 C. Viability dropped more drastically than turbidity (Fig. 1). At first glance, failure of the

TABLE 6. *Activity of UDP-MurNAc-L-alanyl-D-glutamate: L-lysine ligase (ADP) (EC 6.3.2.7) (L-lysine adding enzyme)*

Enzyme preparation[a]	Reaction mixture	Amount of UDP-MurNAc-tripeptide (nmoles/mg protein) formed after:		
		15 min	30 min	60 min
Wild type grown at 37 C	Complete	21	39	49
	Minus UDP-MurNAc-dipeptide	<1	<1	<1
peg-1 grown at 30 C	Complete	1.2	1.8	1.8
	Minus UDP MurNAc-dipeptide	<1	<1	<1
peg-1 grown at 42 C	Complete	<1	<1	<1

[a] The complete incubation mixture contained 20 μliters of ATP (0.01 M), 5 μliters of $MgCl_2$ (0.1 M), 5 μliters of Tris buffer (pH 8.5, 0.5 M), 20 μliters of ^{3}H-L-lysine (0.001 M 18,000 counts/min), 20 μliters of UDP-MurNAc-dipeptide (0.001 M), and enzyme in a total volume of 100 μliters. Incubation was at 37 C. Incorporation of ^{3}H-lysine in UDP-MurNAc-dipeptide was measured after separating the product by paper chromatography (Materials and Methods). Cell-free extract in each case was prepared with lysostaphin.

TABLE 7. *Cell wall composition of wild type and peg-1 grown at the permissive and nonpermissive temperatures*

Wall component analyzed	Wild type grown at 37 C		*peg-1* grown at 30 C		*peg-1* grown at 42 C	
	μmoles/mg of wall	Molar ratio[a]	μmoles/mg of wall	Molar ratio[a]	μmoles/mg of wall	Molar ratio[a]
Muramic acid	0.52	0.94	0.48	0.90	0.53	0.96
Glucosamine	0.96	1.81	0.93	1.75	0.98	1.80
Glutamic acid	0.53	1.00	0.53	1.00	0.55	1.00
Glycine	2.33	4.40	2.22	4.19	2.22	4.04
Alanine	1.31	2.47	1.26	2.50	1.28	2.33
Lysine	0.58	1.09	0.56	1.06	0.52	1.06
Phosphate	0.94	1.77	0.96	1.81	0.96	1.75

[a] Molar ratio expressed with glutamic acid = 1.00.

cells to lyse actively at 42 C is disturbing, but we have found a very similar picture when the culture is treated with a specific inhibitor of peptidoglycan synthesis, e.g., penicillin G or D-cycloserine. Presumably, the observed lysis in other systems where peptidoglycan synthesis is similarly inhibited is primarily due to the action of autolytic enzymes (28). In fact, the difficulty in isolating peptidoglycan-deficient mutants in *B. subtilis* may be related to the more active autolysin(s) in this organism. The killing of cells at 42 C could be partially reversed when the cells were diluted and plated in the presence of 1 M NaCl. A similar recovery of osmotically damaged cells was reported by Schuhardt and Klesius (34). Direct osmotic damage was shown by exposing the 42 C grown cells to SDS (Table 2). Thus, while the wild-type cells grown at 42 C or the mutant cells grown at 30 C are resistant to SDS, the mutant cells grown at 42 C are lysed on exposure to SDS. A similar sensitivity to SDS was observed by Good and Pattee (10). The enhanced binding of the fluorescent dye (ANS) at 42 C by *peg-1* indicates an altered permeability of mutant cells at the nonpermissive temperature. Increased binding of fluorescent dyes has been observed when the surface structures of bacteria were damaged or modified by antibiotics (23), heat or cold shock (1, 32), or by parasitization with *Bdellovibrio bacteriovirus* (8). A specific alteration in membrane proteins was demonstrated in mutants of *E. coli* with defective deoxyribonucleic acid synthesis. These strains showed an increased uptake of ANS at the nonpermissive temperature (12, 37). The strongest evidence that *peg-1* is a peptidoglycan mutant comes from the studies on in vivo peptidoglycan synthesis and precursor accumulation at 42 C. In *S. aureus* carrying *peg-1*, peptidoglycan synthesis is inhibited specifically at 42 C (Fig. 3). There is a time lag of about 30 to 40 min before this inhibition becomes significant. Complete inhibi-

tion occurs after about 1 hr of incubation at 42 C. Such a time lag in the onset of inhibition is also seen in its growth characteristics (OD and viability) and probably is a measure of the half life of the temperature-sensitive protein at the elevated temperature.

S. aureus peg-1 accumulates nucleotide-bound hexosamines when grown at 42 C. Accumulation of such compounds in *S. aureus* by penicillin (followed later on by other antibiotics) and the kinetics of such accumulation (38) was one of the key points for the hypothesis that this antibiotic is a specific inhibitor of peptidoglycan synthesis (26). The verification of this hypothesis occurred much later in both in vivo and in vitro studies (15, 41). Figure 2 shows that such compounds begin to accumulate within 15 min of the temperature shift to 42 C. Furthermore, the kinetics and also the extent of accumulation closely parallel that induced by penicillin. In the present study we have tried to compare the phenotypic effects of penicillin on the wild type to that of a temperature shift on *peg-1*, and in every case we have found the two effects strictly comparable. Similarly, Good et al. reported that phenocopies of their osmotically fragile mutants could be made by treating the parent strain with D-cycloserine (10).

Of the nine ts mutants isolated, only *peg-1* accumulated nucleotide-bound peptidoglycan precursors when grown at 42 C. The other properties of these eight mutants were very similar to those described for *peg-1*. Mutants accumulating different cell wall precursors have been described both in *S. aureus* (10) and *E. coli* (18). Good and Tipper (*personal communication*) have obtained similar variation in nucleotide accumulation with a larger series of mutants.

The major precursor accumulated by *peg-1* was identified as UDP-MurNAc-dipeptide (Table 5). A similar precursor was isolated from ts peptidoglycan mutants in *E. coli*, and this was shown to be due to the inactivation of the *meso*-diaminopimelic acid synthesizing enzyme at 42 C (18). In the case of *peg-1* the defective locus (or loci) could be the L-lysine adding enzyme. It could also be at some later stage in the biosynthetic process. It should be emphasized that in most studies with antibiotics there are several intervening steps between the enzyme(s) inhibited and the precursor that is accumulated. The activity of the L-lysine adding enzyme in a cell-free extract could be readily demonstrated in the parent strain (Table 6) at levels similar to that reported by Ito and Strominger (14). To our surprise, the enzyme isolated from the mutant strain even under permissive conditions was virtually inactive (Table 6). We have repeated the assay more than a dozen times with different batches of cells grown at different temperatures in media supplemented with or without NaCl but the results were uniformly negative. This suggests that the enzyme in *peg-1* is already damaged at 30 C so that, although it can function in vivo, any effort to extract it results in an almost complete loss of activity. In the only work with partially purified L-lysine adding enzyme from *S. aureus* (14), there was no specific mention of its being a relatively unstable enzyme. Since our experiments with the dye ANS indicated that the membrane might be damaged in the mutant strain, the intracellular localization of the L-lysine adding enzyme in the wild type was studied. Virtually all of the activity could be recovered in a supernatant fraction, suggesting a cytoplasmic localization of the enzyme (A. N. Chatterjee, *unpublished data*). The L-lysine adding enzyme must be able to function adequately in vivo in the mutant strain at 30 C as shown by its ability to synthesize normal amounts of UDP-MurNAc-pentapeptide when treated with penicillin G (Tables 3 and 4). Also, the mutant strain is able to grow and synthesize peptidoglycan at a normal rate at 30 C (Fig. 1 and 2). The cell wall composition of the mutant strain grown at 30 and at 42 C is very similar to that of wild type (Table 7), which is confirmed by the ability of these walls to bind phages, indicating a normal content of phage receptor sites. As the phage receptor site in *S. aureus* is located both in the teichoic acid (4) and the peptidoglycan (35), the rate of phage binding by isolated walls is a rapid and sensitive assay for detecting any abnormalities in cell wall composition. We are now analyzing these walls in detail for peptide and polysaccharide chain lengths and N- and C-terminal groups.

The ability of NaCl to reverse the mutant phenotype at 42 C is similar to the osmotic remedial auxotrophic mutants reported in yeast (2, 11), *Neurospora* (20) and *E. coli* (12). NaCl was chosen as the osmotic stabilizer because of its known ability to protect protoplasts of *S. aureus* (21, 22) and also because it does not inhibit growth of the wild type at concentrations needed for osmotic stabilization. The lowest concentration of sucrose that could replace NaCl was 0.8 M. Since sucrose inhibited growth in liquid media at these concentrations it could only be used on plates. Other stabilizing agents like raffinose and fi-

coll, diethylene glycol, KCl, and LiCl inhibited growth of the wild type. The use of $MgCl_2$ or $CaCl_2$ at high concentrations was precluded by their tendency to remove phosphate from the medium. Glycerol was the least inhibitory at high concentrations. This could not replace NaCl (up to a concentration of 2.0 M), which correlates with the known inability of glycerol to stabilize *S. aureus* protoplasts (22). The phenotypic reversions of osmotic remedial mutants reported in the literature can be affected by various inorganic and organic solutes including glycerol (11). Although the exact mechanism of osmotic correction of these defects is unknown, it is generally believed that these are due to suppression of a missense mutation. Studies are in progress to determine more precisely the role of NaCl in the repair of this biosynthetic defect.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants A1-10141 and A1-10093 from the National Institute of Allergy and Infectious Diseases and by grant GB-24379 from the National Science Foundation.

LITERATURE CITED

1. Allwood, M. C., and A. D. Russell. 1967. Mechanism of thermal injury in *Staphylococcus aureus*. I. Relationship between viability and leakage. Appl. Microbiol. **15**:1266-1269.
2. Bassel, J., and H. C. Douglas. 1968. Osmotic remedial response in a galactose-negative mutant of *Saccharomyces cerevisiae*. J. Bacteriol. **95**:1103-1110.
3. Boylan, R. J., N. H. Mendelson, D. Brooks, and F. E. Young. 1972. Regulation of the bacterial cell wall: analysis of a mutant of *Bacillus subtilis* defective in biosynthesis of teichoic acid. J. Bacteriol. **110**:281-290.
4. Chatterjee, A. N. 1969. Use of bacteriophage mutants to study the nature of the bacteriophage receptor site of *Staphylococcus aureus*. J. Bacteriol. **98**:519-527.
5. Chatterjee, A. N., D. Mirelman, H. J. Singer, and J. T. Park. 1969. Properties of a novel pleiotropic bacteriophage-resistant mutant of *Staphylococcus aureus* H. J. Bacteriol. **100**:846-853.
6. Chatterjee, A. N., and J. T. Park. 1964. Biosynthesis of cell wall mucopeptide by a particulate fraction from *Staphylococcus aureus*. Proc. Nat. Acad. Sci. U.S.A. **51**:9-16.
7. Chen, P. S., T. Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. Anal. Chem. **28**:1756-1758.
8. Crothers, S. F., and J. Robinson. 1971. Changes in the permeability of *Escherichia coli* during parasitization by *Bdellovibrio bacteriovorus*. Can. J. Microbiol. **17**: 689-697.
9. Edelman, G. M., and W. O. McClure. 1968. Fluorescent probes and the conformation of proteins. Accounts Chem. Res. **1**:65-70.
10. Good, C. M., and P. A. Pattee. 1970. Temperature-sensitive osmotically fragile mutants of *Staphylococcus aureus*. J. Bacteriol. **104**:1401-1403.
11. Hawthorne, D. C., and J. Friis. 1964. Osmotic remedial mutants. A new classification for nutritional mutants in yeast. Genetics **50**:829-839.
12. Hirota, Y., J. Mordoh, and F. Jacob. 1970. On the process of cellular division in *Escherichia coli*. III. Thermosensitive mutants of *Escherichia coli* altered in the process of DNA initiation. J. Mol. Biol. **53**:369-387.
13. Ito, E., and J. L. Strominger. 1962. Enzymatic synthesis of the peptide in bacterial uridine nucleotides. I. Enzymatic addition of L-alanine, D-glutamic acid and L-lysine. J. Biol. Chem. **237**:2689-2695.
14. Ito, E., and J. L. Strominger. 1964. Enzymatic synthesis of the peptide in bacterial uridine nucleotides. III. Purification and properties of L-lysing adding enzyme. J. Biol. Chem. **239**:210-214.
15. Izaki, K., M. Matsuhashi, and J. L. Strominger. 1966. Glycopeptide transpeptidase and D-alanine carboxypeptidase: penicillin-sensitive enzymatic reactions. Proc. Nat. Acad. Sci. U.S.A. **55**:656-663.
16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
17. Mangiarotti, G., D. Apirion, and D. Schlessinger. 1966. Selection of sucrose-dependent *Escherichia coli* to obtain envelope mutants and fragile cultures. Science **153**:892-894.
18. Matsuzawa, H., M. Matsuhashi, A. Oka, and Y. Sugino. 1969. Genetic and biochemical studies on cell wall peptidoglycan synthesis in *Escherichia coli* K 12. Biochem. Biophys. Res. Commun., **36**:682-689.
19. Metcalf, R. H., and R. H. Deibal. 1969. Differential lytic response of enterococci associated with addition order of lysozyme and anions. J. Bacteriol. **99**:674-680.
20. Metzenberg, R. L. 1968. Repair of multiple defects of a regulatory mutant of *Neurospora* by high osmotic pressure and by reversion. Arch. Biochem. Biophys. **125**:532-541.
21. Mitchell, P., and J. Moyle. 1957. Autolytic release and osmotic properties of "protoplasts" from *Staphylococcus aureus*. J. Gen. Microbiol. **16**:184-194.
22. Mitchell, P., and J. Moyle. 1959. Permeability of the envelopes of *Staphylococcus aureus* to some salts, amino acids, and non-electrolytes. J. Gen. Microbiol. **20**:434-441.
23. Newton, B. A. 1954. Site of action of polymyxin on *Pseudomonas aeruginosa*: antagonism by cations. J. Gen. Microbiol. **10**:491-499.
24. Osborn, M. J. 1969. Structure and biosynthesis of the bacterial cell wall. Annu. Rev. Biochem. **38**:501-538.
25. Park, J. T., and R. Hancock. 1960. A fractionation procedure for studies of the synthesis of cell wall mucopeptide and of other polymers in cells of *Staphylococcus aureus*. J. Gen. Microbiol. **22**:249-258.
26. Park, J. T., and J. L. Strominger. 1957. Mode of action of penicillin. Science **125**:99-101.
27. Reissig, J. L., J. L. Strominger, and L. F. Leloir. 1955. A modified colorimetric method for the estimation of N-acetyl amino sugars. J. Biol. Chem. **217**:959-966.
28. Rogers, H. J. 1967. Killing of staphylococci by penicillins. Nature (London) **213**:31-33.
29. Rogers, H. J., M. McConnell, and I. D. J. Burdett. 1968. Cell wall or membrane mutants of *Bacillus subtilis* and *Bacillus licheniformis* with grossly deformed morphology. Nature (London) **219**:285-288.
30. Rogers, H. J., M. McConnell, and I. D. J. Burdett. 1970. The isolation and characterization of mutants of *Bacillus subtilis* and *Bacillus licheniformis* with disturbed morphology and cell division. J. Gen. Microbiol. **61**:155-171.
31. Rogers, H. J., and M. McConnell. 1970. The role of L-glutamine in the phenotypic change of a *rod* mutant derived from *Bacillus subtilis* 168. J. Gen. Microbiol. **61**:173-181.
32. Sato, M., and H. Takahashi. 1969. Cold shock of bacteria. II. Magnesium-mediated recovery from cold shock and existence of two critical temperature zones

in various bacteria. J. Gen. Appl. Microbiol. **15**:217-229.
33. Schindler, C. A., and V. T. Schuhardt. 1965. Purification and properties of lysostaphin—a lytic agent for *Staphylococcus aureus*. Biochim. Biophys. Acta **97**: 242-250.
34. Schuhardt, V. T., and P. H. Klesius. 1968. Osmotic fragility and viability of lysostaphin-induced staphylococcal spheroplasts. J. Bacteriol. **96**:734-737.
35. Shaw, D. R. D., and A. N. Chatterjee. 1971. *O*-Acetyl groups as a component of the bacteriophage receptor on *Staphylococcus aureus* cell walls. J. Bacteriol. **108**: 584-585.
36. Shaw, D. R. D., D. Mirelman, A. N. Chatterjee, and J. T. Park. 1970. Ribitol teichoic acid synthesis in bacteriophage-resistant mutants of *Staphylococcus aureus* H. J. Biol. Chem. **245**:5101-5106.
37. Siccardi, A. G., B. M. Shapiro, Y. Hirota, and F. Jacob. 1971. On the process of cellular division in *Escherichia coli*. IV. Altered protein composition and turnover of the membranes of thermosensitive mutants defective in chromosomal replication. J. Mol. Biol. **56**:475-490.
38. Strominger, J. L. 1962. Biosynthesis of bacterial cell walls. Fed. Proc. **21**:134-143.
39. Strominger, J. L., and R. H. Threnn. 1959. Accumulation of a uridine nucleotide in *Staphylococcus aureus* as the consequence of lysine deprivation. Biochim. Biophys. Acta **36**:83-92.
40. Strominger, J. L., R. H. Threnn, and S. S. Scott. 1959. Oxamycin, a competitive antagonist of the incorporation of D-alanine into a uridine nucleotide in *Staphylococcus aureus*. J. Amer. Chem. Soc. **81**:3803-3804.
41. Wise, E. M., and J. T. Park. 1965. Penicillin: its basic site of action as an inhibitor of a peptide cross-linking reaction in cell wall mucopeptide synthesis. Proc. Nat. Acad. Sci. U.S.A. **54**:75-81.
42. Young, F. E. 1967. Requirement of glucosylated teichoic acid for absorption of phage in *Bacillus subtilis* 168. Proc. Nat. Acad. Sci. U.S.A. **58**:2377-2384.
43. Young, F. E., P. Haywood, and M. Pollock. 1970. Isolation of L-forms of *Bacillus subtilis* which grow in liquid medium. J. Bacteriol. **102**:867-870.