

## Structure of the Cell Wall of *Staphylococcus aureus*, Strain Copenhagen. IX. Teichoic Acid and Phage Adsorption\*

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**ABSTRACT:** Selective degradation of the teichoic acid and the peptidoglycan polymers of the walls of *Staphylococcus aureus* (Copenhagen) by several defined techniques shows that 4-*O*- $\beta$ -(*N*-acetyl-D-glucosaminy) substitution of the D-ribitol units of the teichoic acid moiety is

essential to phage fixation and that, in order to be operative, these groups must possess a definite configuration which, in the native walls, is imparted by the binding of the teichoic acid to the supporting peptidoglycan.

The cell wall of *Staphylococcus aureus* strain Copenhagen is mainly composed of two polymers: a peptidoglycan which is the rigid matrix of the walls and a teichoic acid which is one of the immunological determinants in the staphylococcal cells. The structure of the peptidoglycan has been established by means of enzymatic degradations which have been previously described (Ghuysen and Strominger, 1963a,b; Tipper *et al.*, 1965, 1967; Ghuysen *et al.*, 1965a-c; Tipper and Strominger, 1966; Petit *et al.*, 1966; Muñoz *et al.*, 1966a,b; Jarvis and Strominger, 1967). The peptidoglycan contains linear strands of  $\beta$ -1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues. This glycan is linked through *N*-acetylmuramyl-L-alanine linkages to peptide subunits  $N^{\alpha}$ -(L-alanyl-D-isoglutaminyl)-L-lysyl-D-alanine. These peptide subunits are in turn cross-linked by pentaglycine bridges extending from the *N*-lysine residue of one peptide subunit to the C-terminal D-alanine of another. The teichoic acid (Baddiley, 1962; Sanderson *et al.*, 1962; Torii *et al.*, 1964) is a linear polymer of 4-*O*- $\beta$ - and 4-*O*- $\alpha$ -*N*-acetyl-D-glucosaminyl-D-ribitol units bridged by 1,5-phosphodiester linkages. Approximately one-half of the ribitol is esterified either at C-2 or C-3 by a D-alanine residue. According to the staphylococcal strain, the teichoic acid varies in its content of  $\alpha$ - or  $\beta$ -*N*-acetylglucosamine residues. In the case of *S. aureus* Copenhagen a proportion of 7 $\beta$ -linked for 1 $\alpha$ -linked residue was reported (Nathenson *et al.*, 1966).

The teichoic acid is covalently bound to the peptidoglycan (Strominger and Ghuysen, 1963; Ghuysen *et al.*, 1965a) through phosphodiester bonds, probably involving C-6 of some of the *N*-acetylmuramic acid residues (Liu and Gotschlich, 1967).

It has been shown that a given phage type of *S. aureus* is capable of inactivating a variety of staphylococcal phages to which it is not necessarily susceptible (Rountree, 1947). The general hypothesis is that the difference

in lytic activity results from the inability of the phage to penetrate or replicate in the particular staphylococcal strain. Heat-killed staphylococci or isolated cell walls have been shown to retain this ability to adsorb the homologous as well as the heterologous phages (Rountree, 1947; Hotchin *et al.*, 1952; Morse, 1962; Bradley, 1965) and the attachment of the phages through the tips of their tails was revealed by electron microscopy (Hotchin *et al.*, 1952; Bradley, 1965).

Reports dealing with the locus and the nature of the phage receptor sites in the walls has led to divergent conclusions (Morse, 1962; Rosato and Cameron, 1964; Wolin *et al.*, 1966; Matthew and Rosenblum, 1967). This communication describes the effect of specific hydrolyses of the cell walls in their glycan, peptide, or teichoic acid moieties on phage inactivation. The hydrolytic procedures used were those previously developed in the studies establishing the structure of the peptidoglycan.

### Material and Methods

**Cell Walls.** Cell wall preparations of *S. aureus* Copenhagen were obtained as previously described (Ghuysen and Strominger, 1963a). The procedure does not involve any heat treatment but includes treatment with trypsin at pH 7.0. The purified preparation contained, per mg dry weight, approximately 500 m $\mu$ equiv of peptidoglycan units (one disaccharide plus one peptide subunit plus one peptide bridge) and 480 m $\mu$ equiv of *N*-acetylglucosaminyl ribitol phosphate.

**Bacteriophages.** Three temperate phages (3C, 71, and 77) were selected to yield differences in type series, serological groups, morphology, and infectivity. Phages 3C and 71 belong to the group II of the typing series and phage 77 to the group III (Blair and Williams, 1964). Phages 3C belongs to the serological group A, phage 71 to group B, and phage 77 to group F (Blair and Williams, 1964). As described by Lapchine and Enjalbert (1965), the selected phages have long thin tails (1600 Å for phage 71, 2200 Å for phage 77, and 3000 Å for phage 3C), without contractile sheath. Phage 3C has an elongated head (830  $\times$  400 Å) while phages 77 and 71 have

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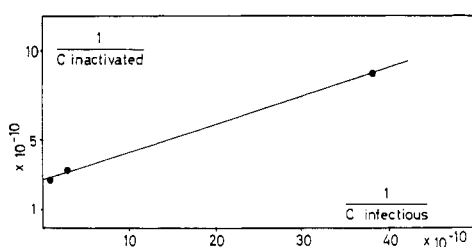


FIGURE 1: Isotherm of adsorption of phage 3C on cell walls of *S. aureus* Copenhagen (for conditions, see text).  $C_{\text{infectious}}$  = phage concentration, in plaque-forming units per milliliter, at the equilibrium, after adsorption on cell walls.  $C_{\text{inactivated}}$  = difference between the phage concentration (in plaque-forming units per milliliter) before adsorption on cell walls and the phage concentration after adsorption on cell walls ( $C_{\text{infectious}}$ ).

polyedric heads (550 Å). Phages 3C and 71, but not phage 77, are infectious for *S. aureus* strain Copenhagen.

**Bacteriophage Preparation.** Each phage was grown on the appropriate *S. aureus* propagating strain (PS 3C, PS 71, and PS 77, respectively). Growth medium (Bactotrypton Difco-yeast extract Difco-NaCl-glucose-water, 10:5:10:1:1000, w/w/w/w/v) was enriched with 400 µg/ml of calcium chloride (Blair and Williams, 1964). Cultures of the propagating strains grown overnight, on a shaker, at 37°, were added to fresh media (dilution 1:100) and inoculated with virus to give final dilutions of  $10^5$ – $10^6$  pfu (plaque-forming units)/ml. The mixtures were incubated, with shaking, at 37° for 6 hr (final titer  $10^9$ – $10^{10}$  pfu/ml). Bacterial cells and debris were removed by centrifugation at 6500g for 30 min. The supernatants were centrifuged at 105,000g for 1 hr and the pellets were gently resuspended in fresh growth medium. The purification was repeated four times to yield a final concentration of  $10^{11}$ – $10^{12}$  pfu/ml. These suspensions were the phage preparations used throughout the present work.

**Bacteriophage Inactivation. Standard Conditions.** INTACT CELL WALLS. Cell walls of *S. aureus* Copenhagen (final concentration 10 µg/ml) and phages (final concentration  $10^8$  pfu/ml) were mixed and incubated at 37° in 0.01 M Tris buffer (pH 7) supplemented with 0.005 M  $\text{CaCl}_2$ . The extent of phage inactivation was measured by titration on the propagating staphylococcal strains without prior centrifugation of the incubation mixtures.

DEGRADED CELL WALLS. Two sets of conditions were used. Under condition 1 the phages were incubated exactly as with intact walls. Condition 2 involved decreasing the phage concentration to  $10^4$ – $10^5$  pfu/ml and increasing the concentration of degraded walls to 100 µg/ml. When the inactivation tests were performed with soluble glycan or teichoic acid polymers, the wall products were added in concentrations, expressed in total amino sugar residues or in phosphorus, equivalent to those of intact cell wall suspensions.

**Enzymes.** The following enzymes were employed: pig epididymis exo- $\beta$ -N-acetylglucosaminidase (Sanderson *et al.*, 1962), *Streptomyces* F<sub>1</sub> endo-N-acetylmuramidase (Muñoz *et al.*, 1966a), *Myxobacter* AL I enzyme (Ensign and Wolfe, 1965, 1966; Tipper *et al.*, 1967), and the

lysostaphin endo-N-acetylglucosaminidase (Schindler and Schuhardt, 1964, 1965; Tipper and Strominger, 1966).

**Analytical Methods.** They were described in Ghuysen *et al.* (1966).

**Electron Microscopy.** Negatively stained samples were prepared by the phosphotungstic acid technique of Brenner and Horne (1959) and examined with an Hitachi Model HS6 electron microscope.

**Wall Degradation.** The various degradation procedures to which the cell walls were submitted, the chemical characterization of the alterations produced, and the isolation and characterization of the degraded products were fully described in previous papers of this series.

## Experimental Results

**1. Phage Inactivation by Intact Cell Walls.** Under the standard conditions (see Methods), the incubation times required to irreversibly inactivate 95–99% of the selected phages were 15 min for phage 3C and 77, and 60 min for phage 71. Examination of the pellets obtained after centrifugation of the suspension mixtures revealed the classical attachment of the virions to the cell walls by the tail tips (Hotchin *et al.*, 1952; Bradley, 1965). The irreversibility of the inactivation was proved by dilution tests using the method described by Adams (1959). The suspensions of cell walls (10 µg/ml) and inactivated phages (initial concentration  $10^8$  pfu/ml) were diluted 100-fold in the nutrient growth medium (see Methods) supplemented either with calcium chloride (final concentration 0.005 M) or with sodium chloride (final concentration 0.1 M). After 2-hr incubation, at 37°, in either media, phage titration showed a liberation of infectious phages not exceeding 6% of the initial titer. Kinetics of phage inactivation were measured under the standard conditions but using a walls concentration equal to 1 µg/ml and phage concentrations ranging from  $1.5 \times 10^9$  to  $1.5 \times 10^{10}$  pfu/ml. Plotting the reciprocal of the concentration of inactivated phages as measured at the equilibrium (from 30 min to 2 hr according to the phage concentration) *vs.* the reciprocal of the concentration of infectious phages gave straight lines (Figure 1), thus demonstrating that phage inactivation by isolated staphylococcal walls is an adsorption phenomenon mediated through specific receptor sites (Krueger, 1931). Extrapolation to the ordinate of the adsorption isotherm of phage 3C indicates that, at saturation, one cell wall (estimated dry weight  $1.7 \times 10^{-13}$  g) can fix about 630 virions.

**2. Effects of Cell Wall Degradations on Phage Adsorption.** DEGRADATION OF THE TEICHOIC ACID PORTION OF THE WALLS. No enzyme is known which hydrolyzes phosphodiester bonds within the teichoic acid or at the junction between teichoic acid and peptidoglycan. Treatment of the walls with a cold 10% trichloroacetic acid solution induces the appearance of soluble phosphomonoester groups, leaving at completion of the process, an insoluble peptidoglycan material which is essentially free of teichoic acid. (Strominger and Ghuysen, 1963; Ghuysen *et al.*, 1965a). Under these mild conditions, trichloroacetic acid is only slightly hydrolytic for the wall peptidoglycan, as proved by end-group analyses;

cleavage of covalent linkages occurs to such a low extent that the peptidoglycan network does not undergo solubilization. Cell walls were suspended in 10% trichloroacetic acid and maintained at 4° with occasional stirring. After various lengths of time, from 16 hr to 15 days, samples were withdrawn and the residual walls were thoroughly washed. The phage adsorption abilities of the treated cell walls were estimated under condition 1; with phages 71 and 77, however, the incubation times were prolonged up to 1 and 2 hr, respectively. As shown in Figure 2, the removal of the teichoic acid (expressed in organic phosphate) resulted in a parallel decrease in the ability of the walls to inactivate phage.

In order to preclude the possibility that the loss of activity might result from minor modifications within the peptidoglycan, a suspension of intact cell walls was treated with an *exo-β-N-acetylglucosaminidase* (Sanderson *et al.*, 1962). This enzyme can only liberate the *β*-linked *N*-acetylglucosamine residues which substitute some of the *D*-ribitol 5-phosphate units of the teichoic acid and has no hydrolytic action on any other linkages present in the walls. Walls, thus, remain insoluble.

The action of this enzyme upon both the teichoic acid and the peptidoglycan have been studied. When acting on teichoic acid extracted from the walls by trichloroacetic acid, thus free of any contaminating peptidoglycan, the *exo-β-N-acetylglucosaminidase* liberates those *N*-acetylglucosamine residues which are *β*-glycosidically linked to the ribitol phosphate polymer, *i.e.*, about 0.85 residue/organic phosphate (Sanderson *et al.*, 1962). When acting on glycan chains, it liberates those *N*-acetylglucosamine residues located at the nonreducing ends of the chains and which are *β* linked to the adjacent *N*-acetylmuramic acid residue in the sequence. No further degradation of the glycan chains can occur since the enzyme has no *exo-N*-acetylmuramidase activity. Thus the disaccharide *GlcNAc-MurNAc*<sup>1</sup> is hydrolyzed into its two hexosamine residues (Ghuysen and Strominger, 1963; Tipper *et al.*, 1965) but the tetrasaccharide *GlcNAc-MurNAc-GlcNAc-MurNAc* is split into free *GlcNAc* residue and the trisaccharide *MurNAc-GlcNAc-MurNAc* (Leyh-Bouille *et al.*, 1966). The intact glycan moiety of the *S. aureus* peptidoglycan is known to be a polydisperse system with an average chain length of about 12–16 disaccharide units. The former figure results from the estimation of the formaldehydogenic end groups originating from the *N*-acetylhexosaminitol terminals of the intact glycan moiety which has been reduced by *NaBH<sub>4</sub>* (Tipper *et al.*, 1967). The latter figure is based on the estimation of the free *N*-acetylglucosamine residues liberated from the walls after complete hydrolysis of the linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine by means of an *endo-N*-acetylmuramidase (Ghuysen and Strominger, 1963a). The liberation of free *N*-acetylglucosamine under these conditions (actual data 32 *μ*moles/mg of walls or per about 500 *μ*equiv of disaccharide units) shows that *N*-acetylglucosamine residues are located at the reduc-

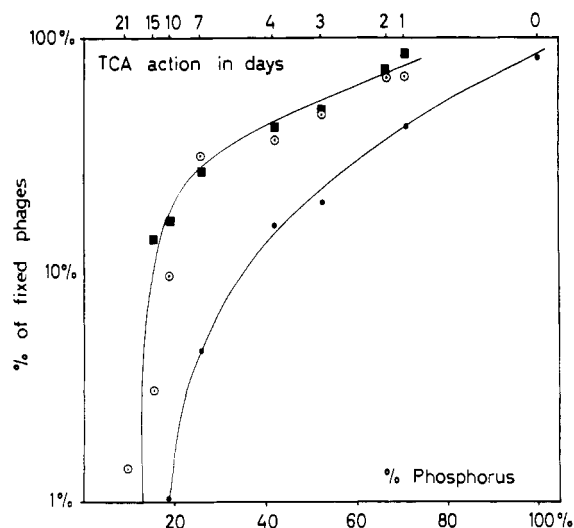


FIGURE 2: Trichloroacetic acid treatment (4°) of cell walls of *S. aureus* Copenhagen. Hydrolysis of teichoic acid and loss of phage inactivation ability. Upper abscissa: hydrolysis time in days. Lower abscissa: organic phosphorus in treated walls expressed in per cent of the content of intact walls. Ordinate: phage inactivation of treated walls expressed in per cent of the phage concentration before the adsorption. (○) Phage 3C, (■) phage 71, and (●) phage 77.

ing ends of the glycan chains while *N*-acetylmuramic acid residues are located at the nonreducing ends. *Exo-β-N-acetylglucosaminidase* is therefore devoid of any detectable activity on intact glycan strands. Furthermore, it has also been observed that the walls of *Micrococcus lysodeikticus* which do not contain teichoic acid, do not yield any detectable traces of free *N*-acetylglucosamine after treatment with the *exo-β-N-acetylglucosaminidase*. Whereas, treatment of intact walls of *S. aureus*, which contain teichoic acid, with the same enzyme resulted in the liberation of 300 *μ*moles of free *N*-acetylglucosamine residues/mg of walls. Since 620 *μ*moles of free *GlcNAc*/mg of cell walls are liberated when soluble teichoic acid, obtained by disruption of the cell walls with *Myxobacter* peptidase (*vide infra*), is used as substrate, this indicates that, with intact cell walls, 48.7% of the *GlcNAc* is liberated by the enzyme.

Again, the phage fixation abilities of intact and enzyme-treated walls were estimated under condition 1 (30 min for phage 3C; 1 hr for phages 71 and 77). As shown in Table I, there is a significant loss of the activity as a result of a partial elimination of the *N*-acetylglucosamine residues from the teichoic acid portion of the walls. Cell walls exposed to enzyme, then quickly centrifuged, and washed in the normal manner, exhibited the identical phage-inactivating ability as untreated walls. Therefore, the loss of inactivating ability after enzyme digestion cannot be attributed to some nonspecific phenomenon, such as the coating of the receptor sites by adsorbed protein from the enzyme preparation.

DEGRADATION OF THE PEPTIDOGLYCAN PORTION OF THE WALLS. From the foregoing, one might expect to obtain the phage receptor sites in a soluble form by isolating the intact teichoic acid portion of the walls. Walls were solubilized with the help of the *Streptomyces F<sub>1</sub>* endo-

<sup>1</sup> Abbreviations used that are not listed in *Biochemistry* 5, 1445 (1966), are: *GlcNAc*, *N*-acetylglucosamine; *MurNAc*, *N*-acetylmuramic acid.

TABLE I: Phage Concentrations (pfu/ml), Expressed in Per Cent of the Initial Titers, after Incubation with Intact and Degraded Walls of *S. aureus*. Phage Inactivation Tests Were Taken to Equilibrium in All Cases.

Phage	Cell Walls Treated with							<i>Myxobacter</i> Enzyme and Lysostaphin Endo- <i>N</i> - acetylgluco- saminidase
	Intact Cell Walls		Exo- $\beta$ - <i>N</i> - acetylgluco- saminidase	F <sub>1</sub> Endo- <i>N</i> - acetylglu- cosamini- dase	<i>Myxobacter</i> Enzyme			
	<i>a</i>	<i>c</i>			<i>a</i>	<i>b</i>	<i>c</i>	
3C	0.2	2.0	22.8	100	100	1.8	95.4	100
71	0.7	5.0	69.3	100	100	11.6	92.7	94.6
77	2.1	3.0	30.0	100	100	5.2	98.1	90.5

<sup>a</sup> Standard conditions (for intact cell walls) or conditions 1 (for degraded cell walls). <sup>b</sup> Condition 2; see Methods. <sup>c</sup> Phage concentration after further incubation at 37° for 2 hr of a 100-fold dilution of the suspension in the growth medium supplemented with CaCl<sub>2</sub> or with NaCl (see text). With intact walls, the fixation is virtually irreversible. With walls treated with *Myxobacter* AL I enzyme, the inactivation is reversible.

*N*-acetylmuramidase (in 0.02 M citrate buffer, pH 5.5). This enzyme hydrolyzes the glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine (Muñoz *et al.*, 1966a), thus degrading the wall glycan into disaccharide units. The entire soluble wall material was desalted by gel filtration on Sephadex G-25 and freeze dried. Under either incubation conditions 1 or 2, no phage inactivation by these F<sub>1</sub>-degraded walls was detected (Table I).

Walls were also solubilized with the help of the *Myxobacter* AL I enzyme which hydrolyzes both peptide linkages within the pentaglycine bridges and amidic *N*-acetylmuramyl-L-alanine linkages at the junction between glycan and peptide (Tipper *et al.* 1967). A preparation of the intact glycan chains, some being attached to the intact teichoic acid polymer, was obtained according to the technique of Tipper *et al.* (1967). Under incubation condition 2, a phage inactivation occurred; data indicated (Table I), however, that disruption of the wall peptide had reduced the inactivating efficiency by a factor of 10<sup>4</sup>–10<sup>5</sup>. Moreover dilution tests showed that, under these conditions, the adsorption was reversible (Table I). The above preparation of intact glycan and glycan-teichoic acid complexes was further degraded (in 0.01 M phosphate buffer, pH 7.5) with the lysostaphin endo-*N*-acetylglucosaminidase which rapidly hydrolyzes the glycosidic linkages between *N*-acetylglucosamine and *N*-acetylmuramic acid (Tipper *et al.*, 1967). As expected, the resulting desalted product exhibited, only a trace of phage fixation activities.

## Discussion

Direct evidence for the exact location of the teichoic acid polymer in walls of *S. aureus* is lacking; but it is probable that it is external to the peptidoglycan network.

The  $\beta$ -*N*-acetylglucosaminyl residues of the teichoic acid of *S. aureus* have been shown by haptenic inhibition studies to be the determinants of an immunological specificity responsible for cell wall agglutination (Nathenson *et al.*, 1966) and hence must be accessible to the exterior.

Furthermore careful electron microscopic studies of *Bacillus megaterium* KM, by Nermut (1967), clearly showed that the rigid peptidoglycan layer, 100 Å thick, is surrounded by a 120-Å thick plastic layer of teichoic acid "bristles."

The evidence now presented indicates that the 4-*O*- $\beta$ -*N*-acetylglucosaminyl-D-ribitol units of the teichoic acid, or at least some of them, are also specifically involved in phage fixation. This conclusion is in agreement with studies of Wolin *et al.* (1966) who showed that variations in phage sensitivity among *S. aureus* mutants parallel chemical changes in the teichoic acid complexes. Similarly, Young (1967) recently showed that, in *Bacillus subtilis*, glycosylation of the teichoic acid skeleton is required for phage adsorption to occur.

From the data presented here, however, and also from observations of Young (1967), it is obvious that phage fixation requires a definite configuration of the active groups of the teichoic acid polymer since any disruption of the supporting peptidoglycan network drastically reduces or completely abolishes the ability of the teichoic acid to interact with phage. Morse (1962) and Matthew and Rosenblum (1967) had previously observed that either preparations of cell wall peptidoglycan or teichoic acid alone were incapable of performing phage inactivation. The exact locus of the receptor site thus remains unidentified and it may well be that phages anchor across both teichoic acid and peptidoglycan layers, since the involvement of both these structures is clearly shown.

In contrast to these studies, it was proposed by Rosato

and Cameron (1964), that the receptor sites for phage 77 are located within the peptidoglycan. According to more recent reports from the same laboratory (Cameron and Osborne, 1967), phage 77 can be inactivated by the peptidoglycan precursor *N*-acetylglucosaminyl-*N*-acetylmuramyl peptide phospholipid. At present, there is no obvious explanation for these discrepancies.

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