*REQUIREMENT OF GLUCOSYLATED TEICHOIC ACID FOR ADSORPTION OF PHAGE IN BACILLUS SUBTILIS 168**

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Communicated by I. C. Gunsalus, September 28, 1967

The cell wall of *Bacillus subtilis* 168 is composed of two heteropolymers, teichoic acid and peptidoglycan.[1] Teichoic acid is a polyglycerol phosphate with D-alanine or D-glucose on glycerol carbons 1 or 2 and a poly N-acyl hexosamine on the terminal phosphate.[2-4] This polymer is linked via a peptide bond from muramic acid on the poly hexosamine to a muramic acid residue of the peptidoglycan. A phage-resistant mutant, *Bacillus subtilis* 168/29, isolated by Dr. B. Reilly,[5] provided an opportunity to examine the alterations of the cell wall associated with phage resistance. This strain did not adsorb phages ϕ25, ϕ29, and SP10.[5] Subsequent experiments have demonstrated that resistance to five groups of phage is acquired by a single modification of teichoic acid. This communication describes the structural alterations and the enzymatic defects in phage-resistant strains of *B. subtilis*.

Materials and Methods.—Bacterial and phage strains: *Bacillus subtilis*, strains 168, 168/29,[6] and H, later reclassified as *Bacillus amyloliquefaciens*,[7] were used. Mutants of *B. subtilis* 168 resistant to phage were obtained spontaneously and by treatment of the parental strain with ultraviolet light[8] or with nitrosoguanidine.[9] Clones were selected which failed to adsorb phage and did not liberate phage after repeated subculture. A mutant of *B. subtilis* 168 resistant to SPO_2, Mu8u5u5 (SPO_2)/SPO_2 here designated as 70, was provided by Dr. Loubelle Boyce. Indicator strains were: *B. subtilis* 168 for phages ϕ1, ϕ25, SPO_2, and SP3; *B. subtilis* W23 for SP10, and *B. amyloliquefaciens* H for ϕ29.

Phages ϕ1, ϕ25, ϕ29,[10] and SP10[11] were kindly supplied by Drs. B. Reilly and J. Spizizen and SPO_2 by Drs. L-B. Boyce and R. Romig. Lysates were prepared according to Reilly[6] and radioactive phage by growth in a tryptophan-thymidine double auxotroph of *B. subtilis* 168[12] using minimal minus phosphate medium at pH 7[13] containing 10 μg per ml thymidine and either 2.5 μc H^3 thymidine or 10 μc P^{32} per ml. The phage were harvested as recommended by Reilly and Spizizen,[6] dialyzed for 25 hr against 3 changes of 100 vol of PM buffer at 4°C, filtered through a Millipore filter, and stored at 4°C.

Phage assay: Cell walls, prepared from 5-hr cultures of *B. subtilis*,[4] were incubated with phage at either 37°C or 4°C for 5, 15, or 30 min in PM or PAB. The walls were removed by centrifugation at 10 Krpm for 10 min at 4°C, and the supernatant solution was assayed for phage by plating an appropriate dilution in semisolid agar over TBAB; for SPO_2, Ivanovic's semisolid agar[14] was used over AK agar (Difco).

Electron microscopy: The phage and phage-wall complexes were stained with phosphotungstic acid[15] and examined in a Hitachi model 11 electron microscope.

Extract and membrane preparation: Wild-type *B. subtilis* strain 168, and the phage-resistant mutants, were grown overnight in pH 7.2 tryptose broth (gm/liter: Bacto-tryptose, 10; Bacto-beef extract, 3; and NaCl, 5). Minimal medium[4] was inoculated to optical density of 10–15 Klett units (filter no. 66), and incubated 4 hr at 37°C (35–55 Klett units). The cultures were harvested, washed once at 4°C with 50 mM Tris buffer, pH 7.0, and 1–3 gm of wet cells suspended in 10 ml of Tris buffer and disrupted in an ice-cooled bath with a sonic probe. The debris was removed by centrifugation at 11 Krpm for 12 min at 4°C. The activities of phosphoglucomutase (PGM) and UDPC pyrophosphorylase (UDPG-PPase) were assayed immediately.

Membranes were prepared by the method of Glaser and Burger[16] from cells grown 4 hr in minimal medium.

Enzymatic assays: UDPG-PPase was determined spectrophotometrically by coupling to TPN reduction in the presence of glucose-6-phosphate dehydrogenase.[17] PGM was assayed in a similar

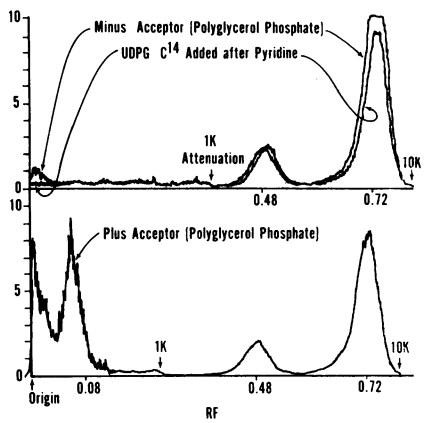

FIG. 1.—Assay of membrane-bound TAG transferase. A modification of the assay of Glaser and Burger[17] was utilized. Membranes were pre-incubated with one μmole of UDP-glucose, 20 μmoles of Tris-chloride (pH 8.0), 4 μmoles of $MgCl_2$ for 2 hr at 37°C. The reaction was diluted with 8 ml Tris chloride and the membranes reisolated by centrifugation at 17,000 rpm for 12 min. The membranes were incubated with the same amounts of Tris-chloride and $MgCl_2$ plus 400 mμmoles UDPG-UC^{14}, and 250 μg polyglycerol phosphate. After 2 hr at 37°C, an equal volume of pyridine was added, aliquots were chromatographed in ethanol-ammonium acetate pH 3.8, and the radioactive areas located in a strip counter. The radioactivity was quantitated in a Packard scintillation spectrophotometer. In calculation of specific activity it was assumed that the rate of glucosylation was linear during the 2-hr period of the reaction.

fashion, with glucose-1-phosphate as the substrate in Tris buffer, pH 8.5, and pyrophosphate omitted. Correction was made for the destruction of TPNH by crude extracts.

The intracellular pool of UDPG was measured in ethanol extracts of cells[18] with UDPG dehydrogenase.[17] The cell walls and teichoic acid were hydrolyzed for 3 hr at 100°C in 0.1 *N* HCl and were analyzed for glucose with glucostat (Worthington).

TAG transferase was assayed as described by Glaser and Burger,[16] using purified polyglycerol phosphate prepared from autolyzed cell walls of phage-resistant mutants by chromatography on agarose and hydroxyl apatite.[3] The transfer of glucose from UDPG-UC^{14} to acceptors of low *Rf* is dependent on the presence of polyglycerol phosphate (Fig. 1). The product was identified as glucosylated teichoic acid by cochromatography of the mutant teichoic acid (labeled with glucose-UC^{14} by transfer from UDPG-UC^{14} with TAG transferase) with P^{32}-labeled wild-type glucosylated teichoic acid.

Chemical analysis: Cell walls were hydrolyzed and analyzed for amino acids, amino sugars, and phosphorus as described previously.[2] Glycerol was assayed by gas liquid chromatography.[3] Carbohydrates were detected in cell walls and teichoic acid following hydrolysis in 0.1 *N* HCl at 100°C for 3 hr by chromatography on Kieselguhr G impregnated with sodium acetate using an ethyl acetate–isopropanol–water solvent (65:23.5:11.5) and an anisaldehyde spray.[19]

Results.—Phage-resistant mutants of B. subtilis: Reilly observed that mutants of *B. subtilis* 168 selected for resistance to phage ϕ25 are also resistant to phage ϕ29, despite marked differences in their morphology, host range, and antigenicity.[5] In

TABLE 1

ADSORPTION OF PHAGE TO *B. subtilis* CELL WALLS

Strain*	Mutant class	Growth on galactose	Phage (Titer/ml × 10^{-7}) ϕ1	ϕ29	ϕ25	SP10	SP3	SPO$_2$
			7.7	53	1.0	13.0	69	0.9
Initial Phage Titer:								
After Adsorption (30 min at 37°C)								
168	Wt	Stimulated	0.002	1.9	0.002	0.03	0.05	0.01
12	A	Stimulated	2.5	—	—	2.9	34	0.9
168/29	B	Nil	7.1	58	0.9	8.4	48	0.6
8	B	Nil	3.5	—	—	7.9	39	0.6
51	C	Lysis	2.8	—	—	7.5	28	0.6
70	C	Lysis	5.6	—	—	11.0	48	0.9

* Cell walls (1 mg/ml dry weight) were incubated with phage in 1 ml Pen Assay medium. The walls were separated by centrifugation at 10 Krpm for 10 min at 4°C, and the supernatant assayed for PFU.

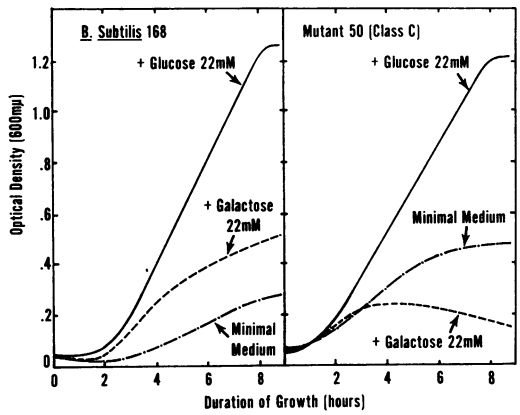

FIG. 2.—Growth of *B. subtilis* strains 168 and 50 (Class C) on galactose. Growth measured in minimal medium containing 0.02% casein hydrolysate, 5 mM $MgSO_4$, and 250 mM L-tryptophan at 37°C was compared with growth in the same medium supplemented with 22 mM glucose or galactose.

subsequent studies these mutants were also found to be resistant to phages SPO_2 and SP10. In liquid medium the mutants are resistant to phages $\phi 1$ and SP3, but in semisolid or solid medium they are infected at a reduced efficiency. One exception, mutant 70, is resistant in all media. As shown in Table 1, phage resistance results from the inability of the viruses to adsorb to the cell walls of the mutants. Three classes of resistant mutants can be recognized by growth in medium containing galactose. Class A strains, like the wild type, are stimulated, Class B mutants are unaffected, and Class C strains lyse in minimal galactose medium, (Fig. 2). *B. subtilis* strain 168/29, which served as the impetus for this study, is morphologically atypical. On minimal glucose agar it forms smooth glistening colonies about one half the diameter of the parent strain. In other respects, it resembles Class B mutants. Twenty-five other mutants form wild-type colonies in minimal glucose.

Phage resistance can be transferred by PBS-1 transduction or by DNA-mediated transformation, either nonselectively with the morphological mutant, *B. subtilis* 168/29,[20] or by cotransformation with other auxotrophic markers.

Adsorption and release of phage by cell walls: Two types of response were found on adsorption of phage by wild-type cell walls. Phages $\phi 29$ and SPO_2 were adsorbed and were released on autolysis of the cell walls when the pH was adjusted to the optimum for the autolytic enzyme, N-acyl-muramyl-L-alanine amidase;[4] phages $\phi 25$ and SP10 were adsorbed but were not released again as infective agents (Table 2). The following experiments indicate that inactivation is due to alteration of the tail, and not to ejection of DNA; (1) neither biologically active nor radioactive DNA was released into the supernatant medium after adsorption of radioactive phage $\phi 25$ to cell walls, and (2) electron microscopy demonstrated that the phage adsorbed to the cell walls had full heads and contracted distorted tails.

TABLE 2

RELEASE OF ADSORBED PHAGE FROM WILD-TYPE WALLS BY AUTOLYSIS

Phage	Released (%)
$\phi 29$	30
SPO_2	10
SP10	0.005
$\phi 25$	<0.001

Cell walls from strain 168 (wt) were incubated 10 min at 37°C in 10 mM potassium phosphate buffer pH 7.0 containing 5 mM $MgSO_4$ and 10^8 phage/ml. After one wash by differential centrifugation the walls were resuspended in 20 mM potassium phosphate buffer pH 8.2 and incubated at 37°C to permit autolysis. After 90 min the mixture was centrifuged 10 min, 10 Krpm at 4°C, and the supernatant assayed for phage.

TABLE 3

VARIABLE COMPONENTS OF CELL WALLS FROM PHAGE-RESISTANT MUTANTS

Wall components	Walls Prepared from Strains 168 (wt)*	12(A)	8(B)	51(C)
Glutamate†	1.0	1.0	1.0	1.0
Glucose	2.4	<0.01	<0.01	<0.01
Alkaline (total)	2.1	4.0	3.1	2.3
Alkaline-labile	0.8	2.9	2.0	0.7
GalNAc	0.5	0.3	<0.1	<0.1
Phosphorus	3.4	4.3	3.0	2.8

* The letters in parentheses refer to the wild type, or mutant class (see Table 1).
† Molar ratios referred glutamate = 1.

Structural requirements of the cell wall for adsorption of phage: Several lines of evidence linked the presence of glucose in teichoic acid to the ability of phage to adsorb to cell walls. First, the primary alteration in the cell wall is in teichoic acid, whereas the molar ratios of the components of the peptidoglycan are normal. Chemical analysis demonstrated that three modifications could occur in teichoic acid, but only one of these, the deletion of glucose, was essential for phage resistance (Table 3). The amount of alkaline-labile D-alanine varies. For instance, mutants 8 and 12 have D-alanine substituted on approximately 65 per cent of the glycerol equivalents. Lower levels of GalNAc, an amino sugar which occurs only in teichoic acid, are consistently observed in Class B and Class C mutants. Secondly, Reilly noted a similar, simultaneous resistance to phages ϕ25, ϕ29, and SP10[5, 20] in *B. amyloliquefaciens* which does not contain GalNAc in the cell wall.[21] The omission of glucose from teichoic acid is the only chemical change in walls of phage-resistant mutants of this strain. Thirdly, transformation from phage sensitivity to phage resistance is accompanied by the deletion of glucose from teichoic acid.

Macromolecular glycosylated teichoic acid obtained either by hydrolysis of the cell wall with lysozyme or by the endogenous autolytic enzyme does not inactivate any of the phages. Phage ϕ25 was chosen to investigate the structural requirements for adsorption because it was irreversibly inactivated by adsorption to cell walls. Figure 3 shows the progressive decrease in adsorption of phage ϕ25 during autolysis of the cell wall. The shoulder corresponds to hydrolysis of approximately 60 per cent of the amide bonds between N-acyl muramic acid and L-alanine.

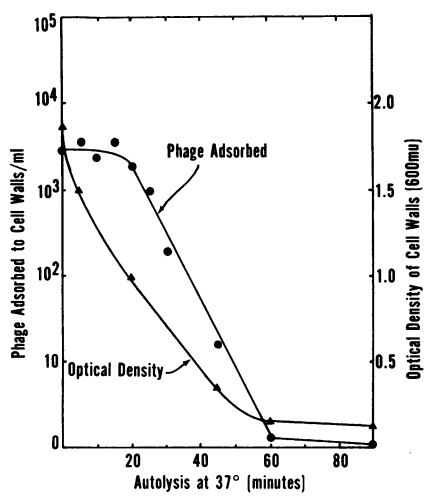

FIG. 3.—Adsorption of phage ϕ25 as a function of autolysis of walls of *B. subtilis* 168 (wt). Cell walls were suspended at a mg/ml in 20 mM $(NH_4)_2CO_3$ and incubated at 37°C. Aliquots of 0.2 ml were transferred to 0.8 ml of 50 mM potassium phosphate buffer, pH 6.8, containing 5 mM $MgSO_4$ and 2×10^7 phage ϕ25 at 4°C. After 15 min the mixtures were centrifuged at 10 Krpm for 10 min at 4°C and the supernatant liquid assayed for PFU.

TABLE 4

ENZYME ACTIVITIES IN STRAIN 168 (WT) AND MUTANTS*

Mutant	Class	PGM	UDPG-PPase (units)	TAG transferase
168	Wt	13.7	4.4	2.14
1c	A	4.5	2.2	0.06
12	A	10.5	2.6	0.02
47	A	8.0	4.0	0.01
9	B	14.3	4.0	0.37
61	B	11.2	5.3	1.52
62	B	8.4	3.6	0.62
168/29	B	6.8	4.0	1.30
38	C	<1	5.0	0.81
50	C	<1	4.0	1.07
51	C	<1	4.6	0.70
70	C	<1	5.2	1.38

* Assays, phosphoglucomutase (PGM), UDPG pyrophosphorylase (UDPG-PPase), and UDPG: polyglycerol teichoic acid glucosyl transferase (TAG transferase), see *Materials and Methods*. One unit equals conversion of one n mole/min/mg protein.

Enzymatic defects in phage-resistant cells: The transfer of glucose from glucose-6-phosphate to glucosylated teichoic acid requires three enzymes: phosphoglucomutase (PGM), UDPG:pyrophosphorylase (UDPG-PPase), and UDPG:polyglycerolteichoic acid glucosyl transferase (TAG transferase). The primary block for Class A mutants is in TAG transferase (Table 4). The activity of other enzymes may be decreased. Class C mutants lack PGM. In contrast, Class B mutants usually contain wild-type levels of enzyme, and thus present an intriguing problem. The slight depression of enzymatic activity in some mutants does not seem adequate to explain the complete absence of glucose from the cell walls. Therefore, additional experiments were performed to determine the availability of UDPG and the efficiency of the acceptor. Under the conditions employed, neither UDPG nor UDPG dehydrogenase could be detected in the parent strain or in the mutants. The acceptor capacity of nonglucosylated teichoic acid from three Class B mutants tested was equal to the nonglucosylated teichoic acid from a type A mutant. Thus, although the inability to utilize galactose as a carbon source and the decreased levels of GalNAc in the cell walls suggest that the Class B mutants are limiting in UDPG, enzymatic analyses have not confirmed this supposition.

Discussion.—Phages which infect *B. subtilis* have been isolated in many laboratories (for review, see refs. 5 and 22). Many of these phages have been examined and classified on the basis of antigenicity, host range, physical properties of DNA, and morphology.[5] The phages utilized in this study are representative of five distinct groups. Three of these groups differ markedly in tail structure. For instance, Davison has demonstrated that SP8 (a phage which resembles ϕ25 in antigenicity, morphology, and physical properties of DNA[5]) has a tail which measures 1500 Å in length and terminates in a plate (850 Å in diameter), bearing a number of specialized structures.[23] The 2500-Å tail of SP3 terminates in a fiber bundle attached to a 360-Å tip.[24] Phage ϕ29 is a small virus with 315-Å hexagonal head Its 325-Å tail contains 12 tail appendages, and has a distal diameter of 60 Å. Despite marked differences in morphology, these phages and phages SP10, ϕ1, and SPO_2 require a glucosylated teichoic acid in the cell wall for adsorption.

The exact role of the glucosylated teichoic acid in adsorption of phage to cell walls is not known. Could the glucose on the glycerol teichoic acid serve as the

primary binding site for phage or does the deletion of glucose result in an alteration of the geography of the cell wall that masks or distorts the primary binding site? Two observations indicate that the arrangement of molecules of teichoic acid on the surface of the cell is critical: (1) hydrolysis of 60 per cent of the amide bonds between N-acyl muramic acid and L-alanine (the first amino acid in the peptide) results in diminution of adsorption of phage $\phi 25$ on the cell wall (2) none of the viruses studied to date are inactivated by macromolecular teichoic acid. Because phages $\phi 1$ and SP3 can infect most of the resistant mutants in semisolid or on solid agar, but not in liquid medium, glucosylated teichoic acid cannot be the sole determinant for adsorption of the viruses. It is possible that the vigorous shaking in liquid cultures retards adsorption to a weaker site. The glucose is, however, required for adsorption of the viruses to isolated cell walls. Recently, Glaser, Ionesco, and Schaeffer[9] have demonstrated a strict requirement for glucosylated teichoic acid in the inactivation of the phage-like killer particle μ by *B. subtilis* W23. Unlike the present study, glucosylated macromolecular teichoic acid inactivates this particle to the same extent as glucosylated teichoic acid in the cell wall.

Class B mutants remain an enigma. The growth of these mutants is not affected by galactose, they contain reduced levels of GalNAc in the cell wall, and they do not have marked defects in the enzymes known to be involved in glucosylation of teichoic acid. Furthermore, the polyglycerol phosphate (nonglucosylated teichoic acid) in the cell wall of Class B mutants is an excellent acceptor for glucose from UDPG. At least three possible explanations exist: (*a*) a block in an unknown enzyme(s) which is essential for the synthesis of glucosylated teichoic acid; (*b*) a false feedback inhibition or alteration of the binding (K_m) of enzyme(s) in this pathway; and (*c*) alteration of the structural protein of the membrane which affects enzymatic function. One of the enzymes involved in the glucosylation of teichoic acid, TAG transferase, is associated with the membrane. This enzyme is reaggregated under conditions which promote the formation of membranes from disaggregated membranes.[25] Conceivably, the enzymes involved in biosynthesis of specific heteropolymers in the cell wall may be associated with specific subunits of the cytoplasmic membrane in a fashion analogous to that suggested for mitochondrial membranes.[26] Genetic studies have demonstrated that phage-resistant mutants map in a cluster on the chromosome between the histidine A_1 and arginine C loci.[27] Furthermore, recent elegant studies by Woodward and Munkres[28] have demonstrated that mutations in the structural protein of mitochondria can result in abnormalities in the associative properties of the coenzyme nucleotides or malate dehydrogenase. Thus, deficiency in respiration can result from mutations which lead to abnormal protein–protein interactions between structural protein and enzymes as well as mutations which produce alterations of the catalytic properties of the enzymes involved in respiration. Class B mutants may provide a bacterial system in which the association between enzymes in synthesis of glucosylated teichoic acid and membrane subunits can be investigated.

Summary—Glucosylation of polyglycerol teichoic acid is essential for adsorption of six unrelated phages to the cell wall of *B. subtilis* 168. Phages $\phi 25$, $\phi 29$, SPO_2, and SP10 do not infect cells with nonglucosylated teichoic acid in liquid, semisolid, or solid media. Phages $\phi 1$ and SP3 cannot infect cells with nonglucosylated teichoic acid in liquid medium, but can parasitize most of the phage-resistant mutants on

semisolid agar or solid agar. The phage-resistant mutants have been separated into three classes. Class A mutants are deficient in UDP glucose:polyglycerol teichoic acid glucosyl transferase, contain N-acyl galactosamine in the cell wall, and can grow on galactose as a carbon source. Class B mutants do not have marked deficiencies in the enzymes known to be involved in the glucosylation of teichoic acid, have less N-acyl galactosamine in the cell wall, and cannot utilize galactose as a carbon source. Class C mutants are deficient in phosphoglucomutase, have markedly reduced levels of N-acyl galactosamine in the cell wall, and lyse when grown on galactose as the sole carbon source. These classes of phage-resistant mutants can be utilized as genetic markers in DNA-mediated transformation and PBS-1 transduction in a fashion analogous to other traits in *B. subtilis* 168.

The author is deeply indebted to Dr. B. Reilly for his interest and advice during the course of this investigation. He also wishes to acknowledge the participation of Mr. V. Maino and Miss H. Stieglitz in the early phase of this study.

Abbreviations: PM buffer, 50 mM potassium phosphate buffer pH 6.8 containing 5 mM $MgSO_4$ and 10 mM NaCl; PAB, Pen Assay broth; DNA, deoxyribonucleic acid; TPN, triphosphopyridine nucleotide; GlcNAc, N-acyl glucosamine; GalNAc, N-acyl galactosamine; PGM, phosphoglucomutase; UDPG, uridine-5′-diphosphoglucose; UDPG-PPase, uridine-5′-diphosphoglucose pyrophosphorylase; TAG transferase, UDPG:polyglycerol teichoic acid glucosyl transferase; PFU, plaque-forming units.

* Supported in part by grants GM-12956-02 from National Institute of General Medicine and E 288 C from the American Cancer Society. A portion of this study was reported in *Bacteriol. Proc.* (1967), p. 26.

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[1] Peptidoglycan denotes the polymer composed of N-acyl glucosamine, N-acyl muramic acid, alanine, diaminopimelic acid, and glutamic acid in *B. subtilis* 168 in accordance with the terminology of Anderson, J. S., P. M. Meadow, M. A. Haskin, and J. L. Strominger, *Arch. Biochem. Biophys.*, **116**, 487 (1966).

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