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Interaction of Bacteriophage N1 with Cell Walls of *Micrococcus lysodeikticus*[1]

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Bacteriophage N1 does not irreversibly adsorb to cell walls isolated from its host *Micrococcus lysodeikticus* strain 1 (ML-1). ML-1 walls do bind the virus in a specific but completely reversibly union. Electron microscopic examination of OsO_4-treated mixtures of phage and walls revealed phage bound to wall fragments by their tail tips, suggesting that reversible phage attachment to walls involves a "tail-first" adsorption of the virus. Treatment of ML-1 walls with fluorodinitrobenzene confers upon the walls the ability to inactivate N1 phage. The relationship between reversible phage attachment to walls and the mechanism of infection by N1 phage is discussed.

In a previous report (9), it was found that, although intact cells of *Micrococcus lysodeikticus* strain 1 (ML-1) irreversibly adsorbed N1 phage, this capacity was destroyed by any of several treatments of the cells including lysozyme digestion. The latter suggested that the cell wall was involved in phage adsorption. However, the initial step for the isolation of the bacterial cell wall, mechanical disruption, destroyed over 90% of the irreversible receptor activity present in intact cells. In the present study we examined the interaction of N1 phage with walls isolated from ML-1. The results demonstrate that, although isolated walls do not irreversibly adsorb N1 phage, the virus does bind to the walls in a specific, completely reversible, "tail-first" interaction. The relationship between reversible phage attachment to walls and the mechanism of infection by N1 phage is discussed.

MATERIALS AND METHODS

Bacteria. The indicator strain for phages N1 and N4, *M. lysodeikticus* strain 1(ML-1), was obtained from H. B. Naylor. ML-P, which is resistant to N1 (9) and is relatively resistant to lysozyme (4), was obtained from J. T. Park.

Bacteriophage. Phages N1 and N4 were obtained from the American Type Culture Collection, Rockville, Md. N1 was propagated and purified as previously described (9). N4 phage was propagated on ML-1 by scraping the agar-overlay from confluently lysed plates. The debris was removed by low-speed centrifugation and the phage were stored at 4 C over chloroform. N4 was not further purified.

Cultivation media and buffers. The composition of TM and TMA buffers and the peptone broth and the methods for cultivation of ML have been reported (9).

Cell walls. Lyophilized, stationary-phase ML cells were disrupted by shaking with styrene-divinyl copolymer beads for 30 min as previously described (9). After disruption, all manipulations were performed at 4 C. The beads were removed by filtration and the disrupted cells were concentrated by centrifugation (25,000 × *g*, 60 min). The resulting pellet was washed (generally eight times) with cold distilled water until the washes failed to show an absorbance peak at 260 nm when examined over the spectral range of 220 to 300 nm. After the third wash, the disrupted cells were centrifuged three times at 1,000 × *g* for 30 min to remove intact cells. During the washing procedure, a thin skim of yellow-pigmented material (presumably cell membranes) was layered on top of the cell wall pellets. This yellow material was carefully removed after each centrifugation by gently washing the cell wall pellets with water, and was discarded before resuspension of the walls. Purified walls were frozen, lyophilized, and stored in vacuo at 4 C over P_2O_5. From 1 g (dry weight) of cells, approximately 150 mg (dry weight) of cell walls were recovered. *Streptococcus faecalis* 9790 LOG walls were prepared as previously described (15). Cell walls of *Micrococcus roseus* R27, *Micrococcus radiodurans*, *Staphylococcus aureus* Copenhagen, and *Sarcina lutea* R262 were generously donated by J.-M. Ghuysen. *Bacillus subtilis* 168 walls were generously provided by F. E. Young.

Sodium decylsulfate (SDS) extraction of cell walls. Cell walls were occasionally extracted with 2% SDS as previously described (15). Walls of *B. subtilis* 168 and *S. faecalis* 9790 were routinely treated

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with SDS before use. This was necessary due to autolysis of the untreated walls of both organisms.

Phage attachment to cells and cell walls. Three assays were used to detect the interaction of phage with an absorbing substrate. The first was the dilution assay (9). One milliliter of cells (suspended in TM buffer and preincubated at 32 C for 30 min) was mixed with 1 ml of phage [0.2×10^5 to 10^5 (plaque-forming units) PFU/ml in TMA', similarly incubated in a tube (18×150 mm) at 32 C with rotary shaking (240 rev/min). After 45 min (unless otherwise specified), a portion of the adsorption mixture was diluted 100-fold in 3 ml of cold TMA containing 0.5 ml of chloroform. After thorough mixing, the number of infectious particles in the aqueous layer was determined. To determine the number of phage initially present, phage were incubated as described above, but in the absence of cells. In the second assay, the centrifugal assay, phage were incubated with cells or walls as in the dilution assay. After 45 min of incubation, 1 ml of the mixture was centrifuged, without dilution, at $10,000 \times g$ for 10 min at 2 C. A portion of the supernate was diluted in TMA and sampled for infectious virus as previously described (9). In many experiments, a single adsorption mixture was concomitantly assayed by both the dilution and centrifugal assays. In the third assay, the competition assay, ML-1 cells (freshly harvested from an exponentially growing culture, washed, resuspended in TM buffer, and sonically treated) were combined with cell walls in TM buffer to yield a final cell concentration of 2.2 (± 0.4) $\times 10^8$/ml. The mixture was preincubated for 30 min at 32 C. The remainder of the procedure is the same as that described for the dilution assay, except that the adsorption mixture was sampled at 2.5- or 5-min intervals. Appropriate controls (i.e., phage incubated without cells or walls, phage incubated with only cells, and phage incubated with only walls) were run.

The terms phage inactivation or irreversible adsorption are used synonymously to indicate phage adsorption detected by the dilution assay. Reversible phage adsorption is used to indicate the binding of phage to an adsorbing substrate which was not detected by the dilution assay, but which was detected by both the centrifugal and competition assays.

Electron microscopy. To examine the interaction of N1 with ML-1 walls, 10^{11} N1 particles were mixed with 25 μg of SDS-extracted walls in 0.5 ml of TMA. After incubation of the mixture for 2 hr at 37 C, 0.5 ml of a 2% aqueous solution of OsO_4 was added. The suspension was held at room temperature for 15 min and centrifuged ($25,000 \times g$, 30 min, 2 C). The pellet was washed once with an equal volume of TMA, resuspended in 0.5 ml TMA, and examined in a Siemens Elmiskop 1A after staining with 1% phosphotungstic acid.

Chemical analyses. Cell walls (7 to 10 mg; held over P_2O_5 for a minimum of 2 weeks) were hydrolyzed under vacuum in 6 N HCl at 105 C for 16 hr. The HCl was then evaporated under vacuum for 24 hr. The amino acid content of the residue was determined on a Beckman-Spinco model 120 amino acid analyzer.

Total hexosamines were determined by the modified Morgan-Elson reaction (6), after hydrolysis in 3 N HCl for 3 hr at 95 C in sealed tubes and then reacetylation. Hydrolyzed and unhydrolyzed *N*-acetyl-D-glucosamine and *N*-acetylmuramic acid were used as standards.

Anthrone determinations were performed on unhydrolyzed walls by using the procedure of Toennies and Kolb (17). The 11-min reaction time was always employed. Anhydrous glucose was used as a standard and all values are reported as glucose equivalents of anthrone reacting material.

Glucose determinations were performed on cell walls hydrolyzed for 1 hr in 2 N H_2SO_4 and neutralized with NaOH, by using the Glucostat Reagent Kit (Worthington Biochemical Corp). The hydrolysis conditions liberated the maximal amount of glucose from the walls without causing detectable destruction of the glucose in a solution of known concentration (P. S. Lovett, Ph.D. Thesis, Temple University, 1968).

Determination of total free amino groups (6) was made on the supernatant fraction ($10,000 \times g$) of a 24-hr lysozyme digest (carried out at 37 C in 0.01 M sodium phosphate and 0.001 M magnesium chloride (*p*H 7.0) with 2 μg of lysozyme per mg of walls) of SDS-extracted ML-1 walls (turbidity reduction was 92%). ϵ-dinitrophenyl-L-lysine (Calbiochem) was used as a standard.

Treatment of cell walls with fluorodinitrobenzene. The procedure followed was that described by Ingram and Salton (8). SDS-extracted walls (10 mg) were suspended in 5 ml of distilled water containing 500 mg of $NaHCO_3$. Ten milliliters of a 5% (v/v) solution of fluoro-2,4-dinitrobenzene (FDNB) in absolute alcohol was added, and the suspension was incubated in the dark with constant stirring for 5 hr at room temperature. The walls were then washed four times with each of the following: distilled water, absolute alcohol, and anhydrous ether; the walls were concentrated between washes by centritugation at $10,000 \times g$ for 30 min at 2 C. The walls were resuspended in distilled water (2 ml) and air was bubbled through the suspension until the ether odor was gone. The preparation was frozen, lyophilized, and stored in vacuo at 4 C in a brown bottle covered with aluminum foil.

RESULTS

Interaction of N1 phage with ML-1 cell walls. As little as 20 μg of intact ML-1 cells per ml was sufficient to irreversibly adsorb 90% of the phage in 45 min at 32 C (9). Under the same conditions, concentrations of ML-1 cell walls as high as 3 mg/ml failed to inactivate a significant percentage of the phage (Table 1, experiment 1). Attempts to inactivate the phage with ML-1 cell walls by (i) supplementing adsorption mixtures with 1% peptone and 0.01 M $CaCl_2$, (ii) increasing the incubation time to 2 hr, (iii) pretreating the walls

TABLE 1. *Interaction of N1 phage with cell walls*[a]

Expt	Cells or cell walls	Dilution assay of infectious phage		Centrifugal assay of infectious phage in supernatant		Infectious phage in resuspended pellet
		Titer	Per cent of control	Titer	Per cent of control	
	μg/ml					
Expt 1						
Control	0	2.64×10^{-4}	100	—[b]		—
ML-1 walls	5,000	0.92×10^{-4}	34	—		—
ML-1 walls	3,000	2.24×10^{-4}	85	—		—
ML-1 cells	1,000	0.04×10^{-4}	2	—		—
Expt 2						
Control	0	2.99×10^{-4}	100	2.88×10^{-4}	100	0.29×10^{-4}
Micrococcus roseus walls	200	4.00×10^{-4}	134	2.48×10^{-4}	86	0.42×10^{-4}
M. radiodurans walls	200	3.10×10^{-4}	104	2.84×10^{-4}	99	0.60×10^{-4}
ML-1 walls	100	3.20×10^{-4}	107	0.34×10^{-4}	12	2.88×10^{-4}
ML-1 cells	100	0.34×10^{-4}	11	0.14×10^{-4}	5	0.08×10^{-4}
Expt 3						
Control	0	1.24×10^{-4}	100	1.22×10^{-4}	100	—
Streptococcus faecalis walls	200	1.24×10^{-4}	100	1.18×10^{-4}	97	—
Staphylococcus aureus walls	200	1.14×10^{-4}	92	1.24×10^{-4}	102	—
Bacillus subtilis walls	200	1.32×10^{-4}	106	1.26×10^{-4}	103	—
Sarcina lutea walls	200	1.28×10^{-4}	104	0.09×10^{-4}	7	—
ML-1 walls	100	1.26×10^{-4}	102	0.12×10^{-4}	10	—
ML-1 cells	100	0.02×10^{-4}	2	0.04×10^{-4}	3	—
Expt 4 (at 4 C)						
Control	0	1.70×10^{-4}	100	1.72×10^{-4}	100	0.20×10^{-4}
S. faecalis walls	100	1.72×10^{-4}	101	1.78×10^{-4}	103	0.30×10^{-4}
ML-1 walls	100	1.84×10^{-4}	108	0.08×10^{-4}	5	1.62×10^{-4}
ML-1 cells	100	0.56×10^{-4}	32	0.04×10^{-4}	2	0.08×10^{-4}
Expt 5						
Control	0	4.22×10^{-4}	100	4.22×10^{-4}	100	—
ML-1 walls	500	4.26×10^{-4}	101	0.24×10^{-4}	6	—
ML-P walls	500	4.34×10^{-4}	103	4.28×10^{-4}	102	—
ML-1 cells	500	0.18×10^{-4}	4.3	0.11×10^{-4}	3	—
ML-P cells	500	3.92×10^{-4}	93	4.02×10^{-4}	95	—
Expt 6 (N4 phage)						
Control	0	0.63×10^{-4}	100	0.62×10^{-4}	100	—
S. faecalis walls	210	0.83×10^{-4}	132	0.60×10^{-4}	97	—
ML-1 walls	500	0.67×10^{-4}	106	0.07×10^{-4}	11	—
ML-1 cells	100	0	0	0.01×10^{-4}	2	—

[a] The figures shown in column 7 were obtained by resuspending the thoroughly drained cell or cell wall pellet, obtained from the centrifugal assay, to its original volume (1 ml) in fresh TMA buffer. A sample of the resulting suspension was diluted 100-fold and a portion of this dilution was plated with indicator bacteria.

[b] Dash indicates procedure was not performed.

with 2% SDS or trypsin (1 mg/ml), (iv) eliminating the 30-min preincubation of the walls before the addition of phage, or (v) diluting adsorption mixtures into broth or buffer not containing chloroform were unsuccessful. These observations suggested that either the virus had no affinity to adsorb to isolated cell walls or the phage did attach to the walls in a weak, perhaps reversible, association which was not detected by the dilution assay. To distinguish between these possibilities, the centrifugal assay was employed. This assay is based on the technique used by Puck and col-

leagues to examine the reversible binding of T1 phage to *E. coli* (5, 11, 12). Cell walls of ML-1 and 6 other gram-positive bacteria were tested for receptor activity by both the dilution and centrifugal assays concomitantly. None of the cell wall preparations significantly inactivated the phage when tested by the dilution assay (Table 1, experiments 2–5). However, cell walls of ML-1 and those of closely related bacterium, *S. lutea*, appeared to adsorb the virus when tested by the centrifugal assay. Virus which sedimented with ML-1 cell walls in the centrfugal assay could be virtually entirely recovered as infectious particles by plating a dilution of the resuspended cell wall pellet (Table 1, experiments 2 and 4). In experiments where N4 phage was substituted for N1, similar results were obtained (Table 1, experiment 6).

The reversible receptor activity of ML-1 cell walls, as determined by the centrifugal assay, was quantitively similar to the irreversible receptor activity of the ML-1 cells, as determined by either the centrifugal or the dilution assay (Fig. 1). The phage-adsorbing efficiency [PAE; reciprocal of the dry weight of cells or cell walls which adsorbs 50% of the phage particles in 45 min at 32 C (9)] of different preparations of cells or walls ranged from 0.10 to 0.25. Phage attachment to cells determined by either the dilution or centrifugal assay gave similar results (Fig. 1B).

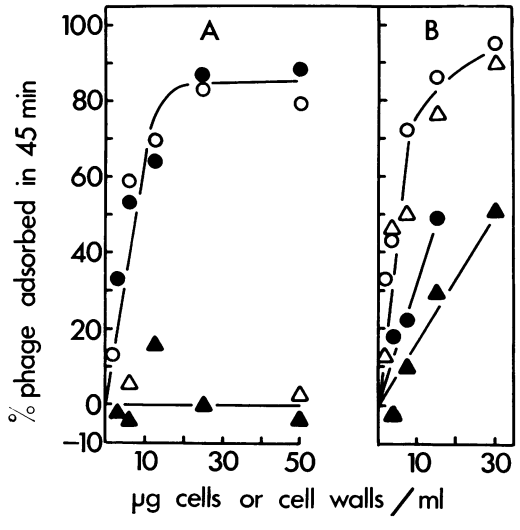

FIG. 1. *Reversible and irreversible adsorption of bacteriophage N1 to heated and nonheated ML-1 cell walls and cells. Lyophilized ML-1 cell walls (A) and ML-1 cells (B) were suspended in TM buffer at respective concentrations of 1 and 1.2 mg/ml and were sonically treated for 2.5 min. Both suspensions were diluted 10-fold in buffer, and a portion of each was held at 100 C for 60 min. The heated (solid symbols ●, ▲) and nonheated (open symbols ○, △) cell walls and cells were serially diluted in TM buffer. Selected dilutions were assayed for receptor activity by the centrifugal assay (circles ●, ○) and the dilution assay (triangles ▲, △).*

Holding ML-1 cell walls at 100 C for 60 min had no effect on the reversible receptor activity (Fig. 1A). Similar treatment of the cells reduced the receptor activity 60 to 75%, as determined by both the dilution and centrifugal assays (Fig. 1B). The reversible receptor activity of isolated cell walls was unaffected by extraction of the walls with 2% SDS or by heating the walls (100 C, 15 min) and incubating them with trypsin for 3 hr at 37 C. In contrast, over 95% of the reversible receptor activity of the walls (400 μg/ml) was lost during incubation with 0.5 μg of lysozyme per ml for 3 hr at 37 C (Lovett, Ph.D. Thesis). Such treatment reduced the initial turbidity by 80%.

In addition to the reversible binding of N1 phage to ML-1 cell walls as demonstrated by the centrifugal assay, the cell walls were capable of "competitively inhibiting" the rate of irreversible phage attachment to intact cells. The results of a typical experiment are shown in Fig. 2. In this experiment, the adsorption velocity constant of irreversible phage adsorption (determined by the dilution assay) to ML-1 cells (1.2×10^8/ml) or ML-1 cells mixed with *S. faecalis* cell walls (650 μg/ml) was 1.03×10^{-9}/min. In contrast, the rate of phage adsorption (K) to ML-1 cells mixed with ML-1 cell walls (127 μg/ml) was reduced over 85% ($K = 0.13 \times 10^{-9}$/min). At cell wall concentrations below 20 μg/ml, the degree of competition was proportional to the amount of

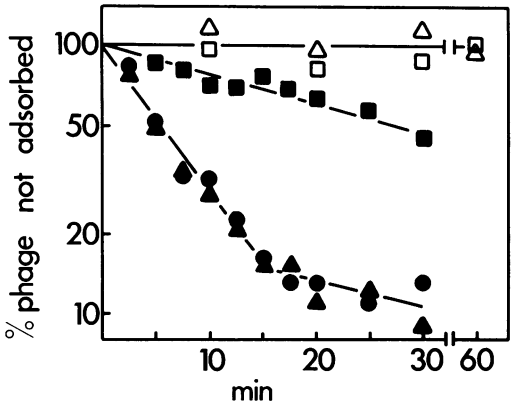

FIG. 2. *Competition of irreversible phage adsorption to cells by ML-1 cell walls. The procedure for the competition assay is described in the text. The composition (per ml) of adsorption mixtures (excluding phage) is ML-1 cell walls (127 μg), □; S. faecalis cell walls (650 μg), △; ML-1 cells (1.2×10^8), ●; ML-1 cells (1.2×10^8) + ML-1 cell walls (127 μg), ■; ML-1 cells (1.2×10^8) + S. faecalis cell walls (650 μg), ▲.*

added cell walls (Fig. 3). Cell wall concentrations of greater than 100 μg/ml exerted the maximal competitive effect, reducing the rate of irreversible phage attachment to cells approximately 90%. The residue remaining after trichloroacetic acid extraction (5%, 95 C, 15 min) of ML-1 cell walls possessed a similar amount of competitive activity as untreated cell walls (Fig. 3). The trichloroacetic acid residue represented 40% of the dry weight of the wall and contained only 20% of the anthrone-reacting material present in untreated ML-1 walls. Attempts to demonstrate competitive activity by using the soluble fraction of ML-1 cell walls after lysozyme digestion were unsuccessful (Lovett, Ph.D. Thesis).

Electron microscopic examination of adsorption mixtures containing N1 phage and SDS-extracted ML-1 cell walls (after fixation with 1% OsO_4) revealed that over 90% of the virus particles observed were associated with the walls in a "tail-first" orientation (Fig. 4A and B). In adsorption mixtures not fixed with OsO_4, less than 15% of the phage present were seen in a "tail-first" association with the walls. In control experiments in which *S. faecalis* cell walls were substituted for those of ML-1, the phage showed no tendency to bind to the walls before (*not shown*) or after (Fig. 4C) fixation with OsO_4.

Reversible binding of N1 phage to cetyltrimethylammonium bromide (CTB)-treated cells. ML-1 cells incubated with 1% CTB lost over 95% of their irreversible receptor activity (9). However, as determined by the centrifugal assay, such cells retained a significant amount of reversible receptor activity (Fig. 5). The reversible receptor activity of CTB-treated cells (PAE = 0.02) represented a loss of activity compared with the control cells (similarly incubated, omitting the detergent; PAE = 0.2). However, as the activity of the latter is virtually entirely irreversible, the comparison is not really valid.

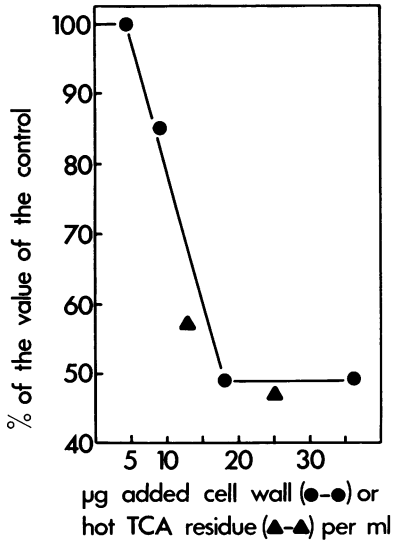

FIG. 3. *Dependence of the competition assay on the concentration of added ML-1 cell walls. Competition assays were performed as described in the text. To obtain the hot trichloroacetic acid residue, ML-1 cell walls were extracted with 5% trichloroacetic acid at 95 C for 15 min. The insoluble residue was washed six times with water and lyophilized.*

As indicated above, reversible phage attachment appears to be a function of the wall. Therefore, the reversible phage attachment to CTB-treated cells should be expressed, not in terms of the cellular dry weight, but rather in terms of the dry weight of the cell wall substance present. Estimates of the proportion of the total mass of ML which is cell wall have ranged from 20 to 35% (14). In Fig. 6, the results of reversible phage attachment to CTB-treated cells (from Fig. 5) are replotted, assuming that the contribution of the cell wall to the total mass of the cell is either 20 or 35% along with typical data for the reversible binding of N1 phage to cell walls prepared by mechanical disruption. The concentration curve based on the 20% assumption is virtually superimposable on that for reversible binding to cell walls.

The possibility that the reversibility of the interaction of phage with walls was an artifact created by a lytic enzyme located either in the phage or in the cell wall was examined. The function of such an enzyme might result in the hydrolysis of portions of the cell wall after irreversible phage attachment, thereby releasing infectious virus. Based on the following, this suggestion seems unlikely. First, walls extracted with 2% SDS or heated at 100 C for 1 hr bound phage only reversibly. Second, walls (400 μg/ml) incubated with phage (10^{10}/ml) at 37 C for 24 hr lost no more turbidity (20% of the initial value) and released no more hexosamine (200 μmoles per g) than was observed with control walls incubated without phage. The latter findings are consistent with the report that, at a multiplicity of 1,000 phage per cell, N1 did not lyse ML-1 from without (7).

Chemical analyses of cell walls. It was previously shown that, of several ML strains examined, only ML-P cells did not reversibly or irreversibly adsorb N1 phage (9). Chemical analysis of the walls of ML-P and ML-1 (Table 2) revealed no major qualitative differences between the strains. The only significant quantitative difference detected was a lower glucose concentration in the walls of ML-P. ML-P walls were

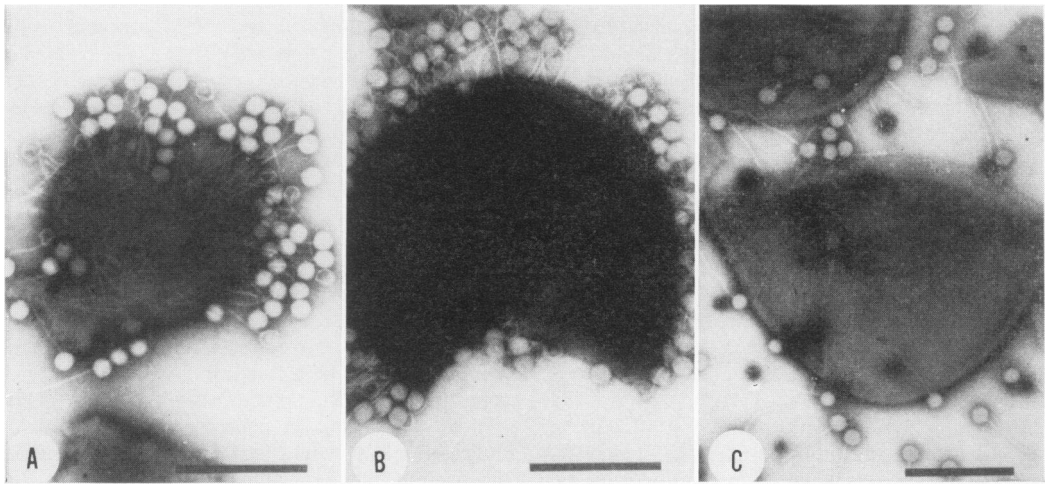

FIG. 4. *Mixtures of N1 phage and SDS-treated cell walls after fixation with OsO_4. Each bar represents 0.5 μm. A, ML-1 walls; B, ML-1 walls; C, S. faecalis walls.*

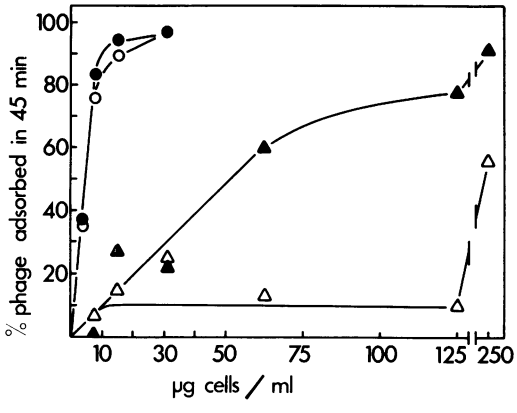

FIG. 5. *Reversible and irreversible binding of N1 phage to ML-1 cells treated with 1% CTB. An overnight culture (incubated 18 hr at 32 C) of ML-1 cells was washed, sonically treated, and incubated with 1% CTB for 90 min as previously described (9). During the incubation, the turbidity of the cells was reduced 22%. After treatment, the cells were washed and serially diluted in TM buffer, and samples of selected dilutions were assayed for receptor activity by the dilution assay (open symbols) and the centrifugal assay (solid symbols). Cells incubated in the absence of the detergent are indicated by circles. The detergent treated cells are designated by triangles.*

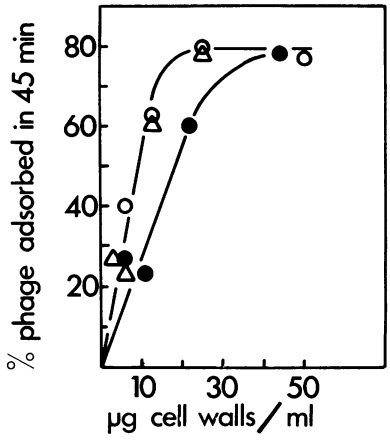

FIG. 6. *Reversible phage adsorption to CTB-treated cells plotted as a function of the cell wall concentration. The results of the binding of phage to CTB-treated cells as detected by the centrifugal assay (from Fig. 5) have been replotted in terms of the weight of the cell wall substance present. It is assumed that 20% (△) or 35% (●) of the dry weight of the cell is cell wall. The binding of the phage by ML-1 cell walls (prepared by mechanical disruption), as determined by the centrifugal assay, is shown for comparison (○).*

reported to contain a higher concentration of *O*-acetyl groups than were found in the lysozyme-sensitive parent of this mutant (2, 4). This parameter was not examined in the present study. However, treatment of ML-P walls with alkali (2), which presumably releases *O*-acetyl groups, had no effect on the inability of the walls to bind N1.

Interaction of N1 phage with dinitrophenylated (DNP) walls. Treatment of ML-1 walls with FDNB confers upon the walls the ability to inactivate N1 phage (Table 3). Similarly treated ML-P walls did not inactivate the phage. The PAE of phage inactivation by DNP ML-1 walls (PAE = 0.18, Fig. 7) was unaffected by holding the walls at 100 C for 15 min. The kinetics of phage inactivation by DNP ML-1 walls (Fig. 8)

showed a biphasic inactivation curve, similar to that seen with irreversible adsorption of N1 by ML-1 cells (9).

DNP ML-1 walls were sensitive to lysis by lysozyme, although the rate of lysis of the DNP walls was slower than that of untreated walls (*unpublished data*). The soluble fraction obtained after digestion of 200 μg of DNP ML-1 walls with lysozyme (turbidity reduction, over 90%) did not inactivate N1 phage.

The reduction in the ability of DNP ML-1 walls to inactivate N1 during progressive dissolution of the walls by lysozyme was examined (Fig. 9). During the initial stage of lysozyme digestion (until the turbidity was reduced 20%) little effect on the phage inactivating property was observed. At the stage corresponding to 20 to 40% reduction in turbidity, a rapid loss of the phage inactivating property was seen, with complete loss of activity occurring after 40% reduction of turbidity.

Based on the data cited above, phage inactivation by DNP walls is similar to reversible phage attachment to untreated ML-1 walls. The only difference found is that DNP walls inactivate the phage. In many instances we have unsuccessfully attempted to recover infectious phage after inactivation by DNP ML-1 walls by digestion of the walls with lysozyme. Preliminary electron microscopic examination (*not shown*) of mixtures of DNP-ML-1 walls and N1 phage (after over 90% phage inactivation) revealed that, without OsO_4 fixation, over 90% of the phage were bound to the walls in a "tail-first" orientation. Under the same conditions, untreated walls bound 10 to 15% of the phage.

The extent of dinitrophenylation of the DNP ML-1 walls used in the above experiments was examined. DNP ML-1 walls were incubated at 37 C with lysozyme (1 μg of lysozyme per mg of walls) in 0.2 M ammonium acetate buffer (*p*H 6.6) for 24 hr in the dark. The turbidity was reduced by 93%. The supernatant fraction (10,000 × *g*, 60 min) was frozen, lyophilized, and resuspended in distilled water (2.5 μg/ml). Portions of this solution were taken through the FDNB reaction procedure (6) omitting the FDNB reagent. After acidification with 2 N HCl, the absorbancy at 420 nm was compared with ε-DNP-L-lysine (standard). The value for DNP groups in the walls was 344 μmoles/g. However, a solution of ε-DNP-L-lysine taken through the same procedure, including incubation with lysozyme and lyophilization, lost 25% of its absorbancy at 420 nm. The value for walls (344

TABLE 2. *Chemical composition of ML-1 and ML-P cell walls*[a]

Compounds	Untreated cell walls				Sodium decylsulfate-treated cell walls			
	ML-1		ML-P		ML-1		ML-P	
	Amt	Ratio[b]	Amt	Ratio[b]	Amt	Ratio[b]	Amt	Ratio[b]
	μmoles/g		μmoles/g		μmoles/g		μmoles/g	
Amino acids								
Aspartic acid	76	0.11	116	0.22	Trace[c]		Trace	
Threonine	33	0.05	Trace		Trace		Trace	
Serine	33	0.05	Trace		Trace		Trace	
Glutamic acid	689	1.00	531	1.00	616	1.00	593	1.00
Glycine	708	1.03	591	1.11	602	0.98	589	0.99
Alanine	1,353	1.96	1162	2.19	1,164	1.89	1,212	2.04
Valine	50	0.07	Trace		—[d]		—	
Methione	51	0.07	41	0.08	—		—	
Isoleucine	26	0.04	Trace		—		—	
Leucine	63	0.09	Trace		—		—	
Lysine	649	0.94	631	1.19	706	1.15	644	1.09
Arginine	45	0.07	12	0.02	—		—	
Total hexosamine	ND[e]		ND		1,391	2.26	972	1.64
Anthrone-positive material	547	0.79	ND		566	0.92	322	0.54
Glucose	ND		ND		206	0.33	115	0.19

[a] One preparation of cell walls for each ML strain was used for all analyses. ML-1 and ML-P walls were isolated from cells grown 24 hr at 32 C (stationary phase).

[b] Assuming glutamic acid is equivalent to 1.

[c] Concentration of less than 10 μmoles/g.

[d] Analysis not performed.

[e] None detected.

TABLE 3. *Inactivation of N1 phage by DNP ML-1 walls*

Walls[a]	Infectious virus	
	Dilution assay	Centrifugal assay
None	2.58×10^{-4}	2.46×10^{-4}
ML-1	2.46×10^{-4}	0.15×10^{-4}
DNP ML-1	0.02×10^{-4}	0.02×10^{-4}
ML-P	2.56×10^{-4}	2.60×10^{-4}
DNP ML-P	2.62×10^{-4}	2.56×10^{-4}

[a] All walls were at a concentration of 100 μg/ml.

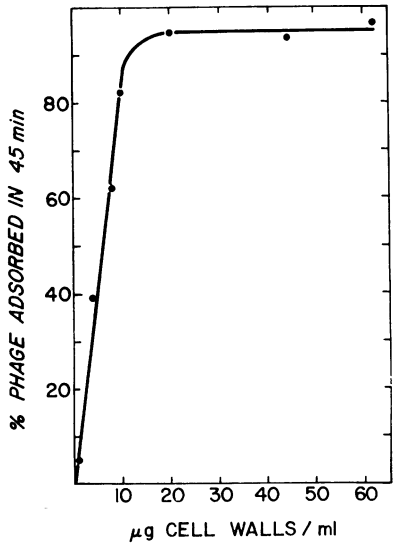

FIG. 7. *Inactivation of N1 phage by DNP ML-1 walls measured by the dilution assay.*

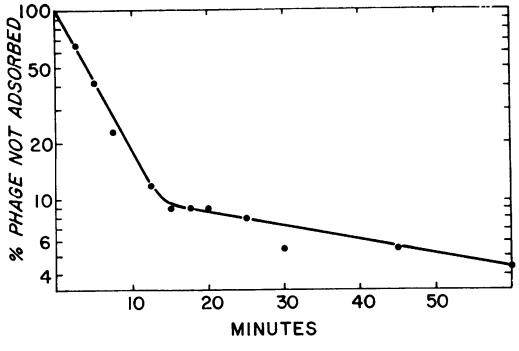

FIG. 8. *Kinetics of N1 phage inactivation by DNP ML-1 walls measured by the dilution assay. The wall concentration was 34 μg/ml.*

μmoles/g) when corrected for this loss was 459 μmoles/g. By comparison, the value for the FDNB reactive groups in SDS-extracted ML-1 walls was 567 μmoles/g. Therefore, we estimate

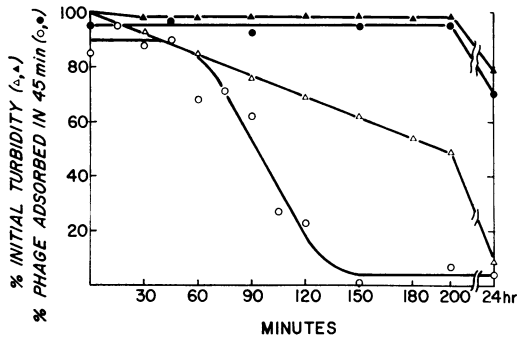

FIG. 9. *Loss of the phage-inactivating capacity of DNP ML-1 walls during lysozyme digestion. DNP ML-1 walls (600 μg/ml) were incubated in TM at 37 C with 1 μg/ml of lysozyme (open symbols) or heat-inactivated (100 C, 15 min) lysozyme (closed symbols). At intervals, samples were withdrawn and held at 100 C for 10 min. Phage inactivation was measured by the dilution assay at a final wall concentration of 10 μg/ml. Turbidity measurements were made at 675 nm in a Spectronic 20 colorimeter adapted with a digital readout.*

that over 80% of the free amino groups in the DNP ML-1 walls were blocked.

DISCUSSION

Although isolated ML-1 cell walls were incapable of inactivating N1 phage, a reversible but relatively specific binding of the virus to walls was demonstrated. This reversible interaction appears to involve the association of the viruses' tail tips with the walls, as demonstrated by electron microscopy. Such an orientation is consistent with the present concept of the initial event in phage infection of a bacterium (16). The requirement of fixation of the virus-wall complex with OsO_4 to see the interaction was not unexpected, since the complex has been shown to be entirely reversible. Presumably, the fixation process "cements" the reversible union between phage and wall into a stable structure.

Attempting to correlate the various data presented in this and the preceding study (9) into a working hypothesis which describes the events resulting in the irreversible binding of N1 phage to ML-1 cells, we make the following proposal. The initial attachment between phage and cells is believed to involve a completely reversible binding between components in the tail tip of the virus and the cell wall. Subsequent to the initial reversible binding process which recognizes the surface of the host, at least one other cellular component must be involved. The identity and function of the latter are obscure. Since the "other cell component" must be physically located close to the cell surface to be involved in phage attach-

ment, we suggest that either the cytoplasmic membrane or a structure located between the cell wall and membrane, such as the intracellular teichoic acid (14), may complete the necessary structural configuration which results in the irreversible attachment of the phage.

The above proposal will clearly require further investigation. However, evidence from other phage-bacterium systems suggests the participation of structures other than the cell wall in phage attachment. By examination of thin sections through electron microscopy, Bayer (1) has shown that members of the T series of phage attach virtually exclusively to areas of the cell envelope of *E. coli* B which retain connections with the cytoplasmic membrane after plasmolysis. It was suggested that the wall-membrane connections may represent passageways through which phage pass their nucleic acid to the cytoplasm of the host. The possibility that these connections also participate in the receptor sites for certain phage cannot be excluded. In another study, a phage receptor was isolated from spheroplasts of a group D streptococcus (18). After a rigorous purification, the receptor was found to contain both protein and carbohydrate. The investigators suggested that the origin of the protein was the cytoplasmic membrane. The activity of the purified receptor was sensitive to a number of agents, including proteolytic enzymes and periodate. In an investigation of the receptor sites for several phage active on *B. subtilis* 168, Young reported that 30% of the ϕ29 phage particles which bound to isolated cell walls were released as infectious particles during autolysis (19). Whether these results can be interpreted as a reversible type of phage attachment is not clear since the majority of phage bound to walls were not recovered.

The significance of phage inactivation by DNP ML-1 walls is obscure. It is possible that dinitrophenylation of ML-1 walls confers upon the walls a structural configuration present in intact cells. For instance, in intact cells certain of the free amino groups normally found in isolated cell walls may be blocked by some nonwall component of the cell. Alternatively, phage inactivation by DNP walls could be relatively nonspecific. Phage that reversibly bind to walls may simply be rendered sensitive to irreversible damage by the DNP molecules in the wall. At present there is insufficient data to explain this phenomenon.

Two previous studies of the bacteriophage receptor activity of ML walls have been published. Salton (13) reported that N4 phage was inactivated by ML walls. However, in the tryptone broth suspending medium used by Salton, 200 μg of ML walls inactivated only 50% of the phage after 90 min at 37 C. We have found that less than 10 μg of ML-1 cells is sufficient to inactivate 50% of the phage after 45 min at 32 C. If it is assumed that N1 and N4 are inactivated at a comparable rate (which is suggested by data in Table 1) then Salton's results can be explained by assuming that his walls were contaminated with approximately 2% cells. Brumfitt (3) stated that N4 phage was adsorbed by ML cells and walls with equal efficiency. It was implied that these results were also obtained with N1 phage. Since no description of the assay method was given, it is impossible to determine whether Brumfitt was studying reversible or irreversible phage attachment. Likewise, no description of the cell, wall, and phage concentrations was given. Therefore, an evaluation of Brumfitt's results cannot be made.

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