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Effects of Nonionic Detergents on Mycobacteriophage Bo 20 and Some Characterization of the Phage Adsorption to *Mycobacterium diernhoferi*

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ABSTRACT

The plaque formation of phage Bo 20 against *Mycobacterium diernhoferi* ATCC 19340 was markedly blocked by the presence of polyoxyethylene sorbitan long-chain acyl esters, Tweens 20, 40, 60, and 80 and polyoxyethylene monooleate, but not by two other nonionic detergents, sorbitan monooleate (Span 80) and glycerol monooleate. The viability of phage Bo 20 was reduced by incubating the phage particles with those nonionic detergents which showed the plaque formation blockage. The phage-adsorbing ability of *M. diernhoferi* cells pretreated with periodate, trypsin or heat was lower than that of untreated cells, whereas the ability of cells was not affected by the treatment with lipase or Tween 80. The adsorption of phage Bo 20 to *M. diernhoferi* cells was markedly inhibited by authentic D-galactose, D-galactosamine, D-melibiose, D,L-2, 6-diaminopimelic acid and L-lysine, respectively. These results suggest that an essential part in structures of positive nonionic detergents for inactivation of phage Bo 20 is the polyoxyethylene residue and fatty acid linked through an ester bond, and that the adsorption of the phage to the host cells may be initiated by the cell wall-associated polysaccharides and/or peptide chains.

There have been many studies on mycobacteriophages of various mycobacteria, reporting their morphological and other properties and also receptors for them[1,2,5-13]. Among mycobacteriophages reported, except for their sentitivities to nonpolar solvents[9], their biological and morphological properties are similar to each other. It is also known that the propagation of mycobacteriophages is inhibited by Tween 80 (polyoxyethylene sorbitan monooleate) which is routinely used in culture of mycobacteria[4,9,10]. However, little is known about the influence of Tween 80 on the mycobacteriophage-mycobacteria system.

This paper deals with the effects of Tween 80 on the adsorption of mycobacteriophage Bo 20 to *Mycobacterium diernhoferi* ATCC 19340 and the adsorption characteristics of the phage to the host cells.

MATERIALS AND METHODS

Phage and bacterial strains

Phage Bo 20 strain was obtained from Dr. I. Tarnok, Forschungsinstitut, Borstel, West Germany, and *M. diernhoferi* ATCC 19340 strain had been stored in our laboratory.

Nonionic detergents

Nonionic detergents having ester bond used in this study were: Tween 20 (polyoxyethylene sorbitan monolaurate), Tween 40 (polyoxyethylene sorbitan monopalmitate), Tween 60 (polyoxyethylene sorbitan monostearate), Tween 80 (polyoxyethylene sorbitan monooleate), Span 80 (sorbitan monooleate), polyoxyethylene monoole-

ate and glycerol monooleate.

Preparation of M. diernhoferi cells grown in heart infusion broth containing glycerol or Tween 80.

A half ml of *M. diernhoferi* cells (about 10^5/ml) grown on 1% Ogawa egg medium at 37°C for 3 days added and cultured in 50 ml heart infusion (HI) broth containing 0.4% Tween 80 or 4% glycerol at 37°C for 48 hr. Cells which had been passed twice through in each medium under the same conditions were harvested, washed with phosphate buffered saline (PBS) and stored at 4°C until use.

Treatment of M. diernhofer cells

Cells (10^8) grown in glycerol-HI broth at 37°C for 3 days were treated with 5% Tween 80 in PBS at 37°C for 1 hr, with 0.1 M sodium metaperiodate in ice bath for 1 hr in the dark, with 0.1 mg enzymes (lipase and trypsin) in the presence of 10 mM $CaCl_2$ and 1 mM $MgCl_2$ at 37°C for 1 hr or boiled for 15 min, respectively.

Assay for adsorption of phage Bo 20 to variously treated M. diernhoferi cells

A half ml of the phage suspension (1×10^6PFU/ml) was incubated with or without 0.5 ml of variously treated cells (1×10^8/ml) at 37°C for up to 5hr. At various time intervals, the mixture was centrifuged at 3,000 × *g* for 20 min, and PFU in the supernatant fluids was assayed.

Assay for inhibition of phage Bo 20 adsorption to M. diernhoferi cells by saccharides and amino acids

The inhibition of phage adsorption by authentic saccharides and amino acids was tested as described previously[14,16]. Briefly, 0.5 ml of the bacterial suspension (1×10^8/ml) and 0.5 ml of the compounds (0 — 600 mM) were incubated with 0.5 ml of the phage suspension (1×10^5PFU/ml) at 37°C for 2 hr. The mixture was then centrifuged at 3,000 × *g* for 20 min and PFU in the supernatant fluids was assayed. After treatment, cells were washed three times with PBS by centrifugation at 3,000 × *g* for 20 min.

One-step growth test

One-step growth of phage Bo 20 was tested by the method of Jones and David[8]. Briefly, 0.1 ml of the phage particles at a concentration of 1×10^5 plaque-forming units (PFU) per ml suspended in PBS was added to 1 ml of *M. diernhoferi* cells (6×10^5/ml) suspended in 4% glycerol-HI broth with or without 0.1% Tween 80, and then incubated at 37°C for 24 hr. At various time intervals, the incubation mixture was harvested, treated with chloroform (one drop) and centrifuged at 5,000 × *g* for 30 min. PFU in the supernatant fluids was assayed by plating the 10-fold diluted samples on 0.8% glycerol-HI soft agar plates containing 10^7 of *M. diernhoferi* cells as indicator cells.

Assay for action of nonionic detergents against plaque-formation and viability of phage Bo 20

One-fifth ml of phage suspension (1×10^3PFU/ml) was mixed with 3 ml of 0.8% glycerol-HI soft agar containing 0.2% detergents and host cells (10^7), overlaid on 1.5% glycerol-HI agar plates immediately and incubated at 37°C for 48 hr. To test the viability, 0.5 ml of phage suspension (1×10^8PFU/ml) was incubated with an equal volume of 0.4% detergent solution or with PBS (control) at 37°C for up to 5 hr. At various time intervals, the mixture was withdrawn, serially diluted 10-fold until the detergent remaining is negligible in the mixture, and PFU was assayed.

RESULTS

One-step growth of phage Bo 20 in M. diernhoferi cells

As shown in Fig. 1, the number of PFU increased with incubation time up to 8 hr when the phage suspension was incubated with *M. diernhoferi* cells in glycerol-HI broth. From the result, it was found that phage Bo 20 had a latent period of about 60 min and a burst size of approximate to 80. In contrast, the number of PFU in glycerol-HI cultures containing 0.1% Tween 80 reduced logarithmically and was lost completely after 3 hr of incubation.

Plaque formation of phage Bo 20 in glycerol-HI soft agar plate containing various nonionic detergents

When 0.2 ml of the phage suspension (1×10^3PFU/ml) was mixed with 0.8% glycerol-HI soft agar containing host cells (10^7) and 0.2% Tweens (20, 40, 60 or 80), Span 80, polyoxyethylene monooleate or glycerol monooleate, the formation of phage plaques was not observed on plates containing these Tweens or polyoxyethylene monooleate. In contrast, Span 80 and glycerol monooleate permitted the plaque forma-

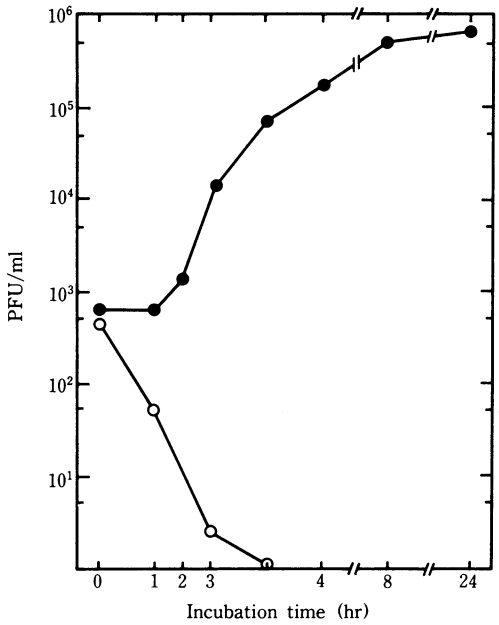

Fig. 1. One-step growth of phage Bo 20 in *M. diernhoferi* ATCC 19340. One-fifth ml of the phage suspension (1 × 10^5PFU/ml) was added to the host cells suspended in 4% glycerol-HI broth (6 × 10^5) containing with (○) or without (●) 0.1% Tween 80, and then incubated at 37°C for 24 hr. At indicated intervals, the incubation mixture was harvested, treated with chloroform and centrifuged at 5,000 × *g* for 30 min to remove the cell debris. PFU in supernatant fluids serially diluted 10-fold with PBS was assayed.

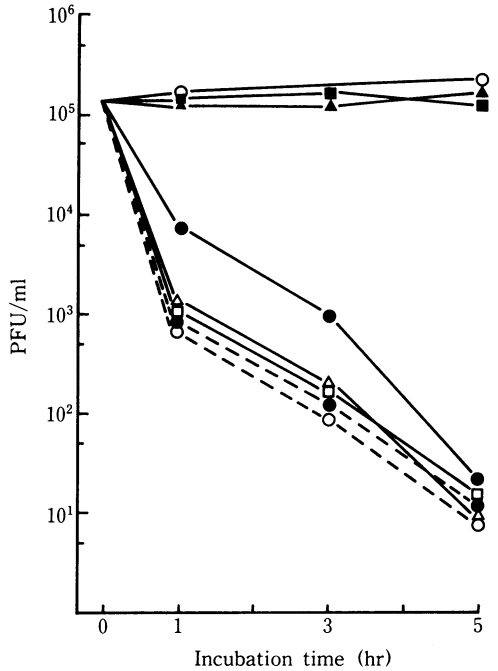

Fig. 2. Effects of various nonionic detergents on viability of phage Bo 20. A half ml of the phage suspension (1 × 10^8PFU/ml) was incubated with 0.5 ml of 0.4% detergents or with PBS at 37°C for up to 5 hr. at indicated time, the mixture was withdrawn, serially diluted 10-fold and PFU in each sample was assayed. Phage alone (○), + Tween 20 (--○--), + Tween 40 (□), + Tween 60 (△) + Tween 80 (--●--), + polyoxyethylene monooleate (●), + Span 80(▲), + glycerol monooleate (■).

tion (data not shown).

Direct effects of various nonionic detergents on viability of phage Bo 20

When the phage particles were treated with 0.2% nonionic detergents tested and PFU was assayed, the number of PFU was markedly reduced by Tweens (20, 40, 60 or 80) and polyoxyethylene monooleate, but not by Span 80 and glycerol monooleate (Fig. 2).

Adsorption of phage Bo 20 to Tween 80-treated M. diernhoferi cells, cells grown in glycerol-HI broth, in HI-broth containing Tween 80 and on Ogawa egg medium

As shown in Fig. 3, any difference in the phage-adsorbing ability was not observed among *M. diernhoferi* cells grown in glycerol HI broth, HI broth containing Tween 80 and on Ogawa egg medium. Phage particles were also adsorbed to cells (grown in glycerol-HI broth) treated with Tween 80 as well as to untreated cells.

Adsorption of phage Bo 20 to variously treated M. diernhoferi cells

When the phage suspension was incubated with the lipase-treated or the intact cells, the number of PFU was strongly reduced, whereas it was less adsorbed to the periodate-treated, trypsin-treated or the heat-treated cells (Fig. 4).

Inhibition of phage adsorption to M. diernhoferi cells by saccharides and amino acids

As shown in Fig. 5, D-galactose, D-galactosamine, D-melibiose (D-galactopyranosyl-D-glucofuranose), D, L-2, 6-diaminopimelic acid (DAPA) and L-lysine markedly inhibited the adsorption of phage particles to the host cells, and their inhibiting effects increased with an increase in concentrations of them. L-Rhamnose and L-glutamic acid also showed slight inhibition. However, D-glucose, D-mannose, L-alanine and

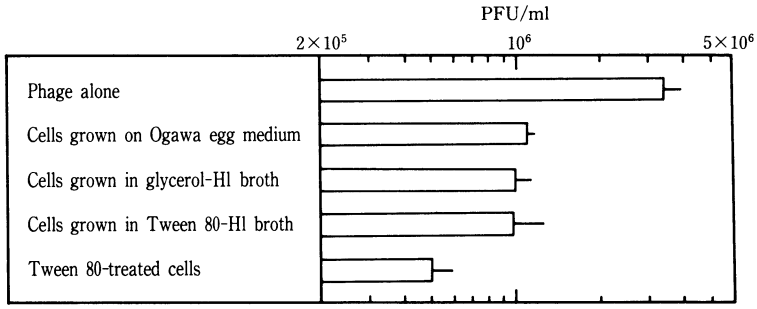

Fig. 3. Adsorption of phage Bo 20 to Tween 80-treated cells, cells grown in glycerol-HI broth, in Tween 80-HI broth and on Ogawa egg medium. A half ml of the phage suspension (1 × 10^6PFU/ml) was incubated with 0.5 ml of Tween 80-treated *M. diernhoferi* cells and cells grown in 4% glycerol-HI broth, in 0.4% Tween 80-HI broth or on Ogawa egg medium at 37°C for 2 hr, respectively. The mixture was centrifuged and PFU in the supernatant was assayed.

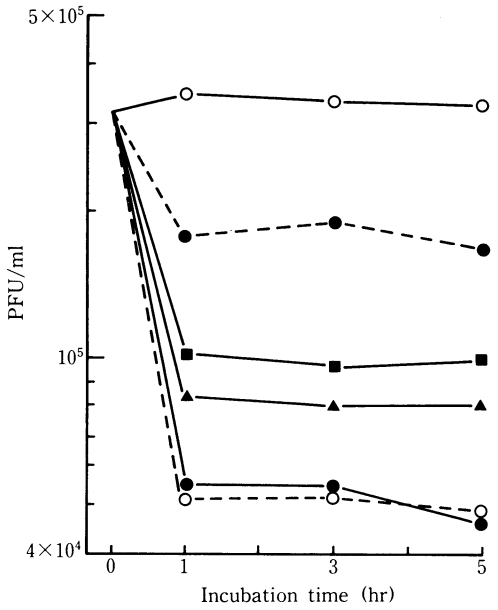

Fig. 4. Adsorption of phage Bo 20 to variously treated *M. diernhoferi* cells. A half ml of the phage suspension (1 × 10^6PFU/ml) was incubated with 0.5 ml of variously treated cell suspensions (1 × 10^8/ml) at 37°C for up to 5 hr. At indicated time, the mixture was withdrawn, centrifuged and PFU in the supernatant was assayed. Phage alone (○), phage + intact cells (●), phage + lipase-treated cells (--○--), phage + trypsin-treated cells (■), phage periodate-treated cells (--●--), phage + heat-treated cells (▲).

L-arginine did not exhibit such inhibition.

DISCUSSION

In the experiments presented here, we investigated the effects of nonionic detergents on mycobacteriophage Bo 20 and some characterization of the phage adsorption to *M. diernhoferi* ATCC 19340. From the one-step growth test, the phage showed a latent period of 60 min and a burst size of approximate 80. The plaque formation was markedly blocked by the presence of polyoxyethylene sorbitan long-chain acyl esters (Tweens 20, 40, 60 and 80) and polyoxyethylene monooleate, but not by sorbitan monooleate (Span 80) and glycerol monooleate. The viability of phage Bo 20 was also reduced by incubating the phage particles with those nonionic detergents which showed the plaque formation blockage. Our findings seem to suggest that an essential part in structures of positive nonionic detergents for inactivation of phage Bo 20 is the polyoxyethylene residue linked through the ester bond to the carboxyl group of long-chain fatty acid.

It has been reported that the lipid portion of lipopolysaccharide (LPS) from mycobacterial cell wall is indispensable for the adsorption of mycobacteriophages such as D 29, GS-7 and ϕ 630 to their host cells[6,8,9,13]. If the lipid portion of LPS from the cell wall of *M. diernhoferi* cell is essential for adsorption of phage Bo 20, the phage-adsorbing ability of cells will be lost by the treatment with Tween 80 possessing an extractable action of some lipids from the bacterial

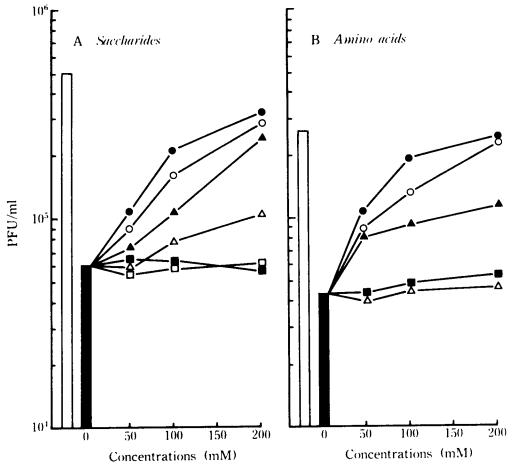

Fig. 5. Inhibition of phage Bo 20 adsorption to *M. diernhoferi* cells by saccharides and amino acids. A half ml of the bacterial suspension (1×10^8/ml) and 0.5 ml of authentic saccharide (A) or amino acid (B) at final concentration of 50 to 200 mM were combined and incubated with 0.5 ml of the phage suspension (1×10^5PFU/ml) at 37°C for 2 hr. After incubation, the mixture was withdrawn, centrifuged and PFU in the supernatant was assayed.
A. Saccharides: phage alone (□), phage + cells (■), phage + cells + D-melibiose (●), phage + cells + D-galactosamine (○), phage + cells + D-galactose (▲), phage + cells + L-rhamnose (△), phage + cells + D-glucose (■), phage + cells + D-mannose (□).
B. Amino acids: phage alone (□), phage + cells (■), phage + cells + D, L-DAPA (●), phage + cells + lysine (○), phage + cells + L-glutamic acid (▲), phage + cells + L-alanine (△), phage + cells + L-arginine (■).

cells[10] or with lipase. In the present study, the phage particles were strongly adsorbed to the Tween 80-treated and the lipase-treated cells. In contrast, the adsorption of phage Bo 20 to *M. diernhoferi* cells treated with periodate, trypsin or with heat was markedly reduced. We further examined the phage adsorption-inhibiting ability of authentic sugars and amino acids. The phage-adsorption inhibition test by using authentic compounds had been done by many investigators to determine the determinant group of receptors for various bacteriophages[3,14-17]. D-galactose, D-galactosamine, D-melibiose, D, L-DAPA and L-lysine markedly inhibited the adsorption of phage Bo 20 to the host cells, and L-rhamnose and L-glutamic acid also showed a slight inhibition. However, D-glucose, D-mannose, L-alanine and L-arginine did not exhibit such inhibition. Our findings may indicate that the cell wall-associated saccharides such as galactose and galactosamine and the cell wall-associated peptides consisting of DAPA, lysine and/or glutamic acid are required for initial binding of phage Bo 20 to *M. diernhoferi* cells. Although, mycolic acid, which is species specific and main component of the lipid portion in LPS of mycobacterial cell wall, is thought to be an essential element of receptors for mycobacteriophage GS-7[6], the present data suggest the possibility that galactose, galactosamine, DAPA and/or lysine occupy a determinant part of binding sites for phage Bo 20 of *M. diernhoferi* ATCC 19340.

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REFERENCES

1. **Bowman, B.U., Jr., Newman, H.A.I., Mritz, J.M. and Koehler, R.M.** 1973. Properties of mycobacteriophage DS 6A. II. Lipid composition. Am. Rev. Resp. Dis. **107**: 42—49.
2. **Bradley, D.E.** 1967. Ultrastructure of bacteriophages and bacteriocins. Bacteriol. Rev. **31**: 230—314.
3. **Dawes, J.** 1975. Characterization of the bacteriophage T4 receptor site. Nature (London) **256**: 127—128.
4. **Dubos, R.J. and Davis, B.D.** 1946. Factors affecting the growth of tubercle bacilli in liquid media. J. Exp. Med. **83**: 409—423.
5. **Gadagkar, R.R. and Gopinathan, K.P.** 1978. Inhibition of DNA injection from mycobacteriophage I 3 by Tween-80. Virology **91**: 487—488.
6. **Imaeda, T. and Blas, F.S.** 1969. Adsorption of mycobacteriophage on cell-wall components. J. Gen. Virol. **5**: 493—498.
7. **Jones, W.D., Jr.** 1973. Studies on the bacteriophage of a naturally lysogenic *Mycobacterium fortuitum*. Am. Rev. Resp. Dis. **108**: 1438—1441.
8. **Jones, W.D., Jr. and David, H.L.** 1971. Inhibition by rifampin of mycobacteriophage D 29 replication in its drug resistant host, *Mycobacterium smegmatis* ATCC 607. Am. Rev.

Resp. Dis. **103**: 618—624.

9. **Jones, W.D., Jr. and Greenberg, J.** 1977. Host modification and restriction with a mycobacteriophage isolated from pseudolysogenic *Mycobacterium chelonei*. J. Gen. Microbiol. **99**: 389—395.
10. **Karnik, S.S. and Gopinathan, K.P.** 1980. Possible involvement of a calcium-stimulated ATP-hydrolyzing activity associated with mycobacteriophage I 3 in the DNA injection process. J. Virol. **33**: 967-975.
11. **Kozloff, L.M., Raj, C.V., Rao, R.N., Chapman, V.A. and DeLong, S.** 1972. Structure of a transducing mycobacteriophage. J. Virol. **9**: 390—393.
12. **Takeya, K. and Amako, K.** 1964. The structure of mycobacteriophages. Virology **24**: 461—466.
13. **Tokunaga, T., Kataoka, T. and Suga, K.** 1970. Phage inactivation by an ethanol-ether extract of *Mycobacterium smegmatis*. Am. Rev. Resp. Dis. **101**: 309—313.
14. **Watanabe, T.** 1976. Role of lipopolysaccharide in adsorption of coliphage T4D to *Escherichia coli* B. Can. J. Microbiol. **22**: 745—751.
15. **Watanabe, T. and Saito, H.** 1978. Inactivation of coliphage T2H by basic amino acids. Microbiol. Immunol. **22**: 167-172.
16. **Watanabe, T. and Shiomi, T.** 1975. Inhibiting materials for gamma phage adsorption to the cell wall of *Bacillus anthracis*, strain Pasteur No. 1-H. Jpn. J. Microbiol. **19**: 115—121.
17. **Yokokura, T.** 1971. Phage receptor material in *Lactobacillus casei* cell wall. I. Effect of L-rhamnose on phage adsorption to the cell wall. Jpn. J. Microbiol. **15**: 457—463.