AN ABSTRACT OF THE THESIS OF

KAREN ROBERTS AUBORN for the M.S. in MICROBIOLOGY
(Name) (Degree) (Major)

Date thesis is presented October 30, 1962

Title LYSOGENY IN BACILLUS SUBTILIS

Abstract approved Redacted for privacy
(Major Professor)

Studies were directed toward stabilizing a phage-host system in Bacillus subtilis. The phage-host system was a lysogenic strain, W-23-S^r-L9, which perpetuated the bacteriophage, Sp-10. Its instability was characterized by a decrease in the number of spores carrying infective centers in new spore crops and variations in phage yields from several spore crops.

W-23-S^r-L9 was lysogenic by most criteria. Spontaneous phage production occurred after inactivation of free phage by heat treatment of spores or incubation of spores with antiserum. The strain was immune to the phage it produced.

Three clones derived from colonies of W-23-S^r-L9 that appeared from preliminary tests not to carry phage determinants were studied to determine differences that could explain variations in spore crops of W-23-S^r-L9. Spore suspensions of these clones were called PNL-A, PNL-B and PNL-C for "presumably non-lysogenic."

With PNL-A, phage was not detected in any medium used for growth of the strain although its parent, W-23-S^r-L9, produced phage in each medium tested. Ultraviolet light induced lysis of the strain in some media, but no phage could be demonstrated. The possible existence of defective phage was discussed.

PNL-B gave poor yields of phage in the test medium which was nutrient broth with yeast extract and glucose. Glucose decreased phage yields from PNL-B in all media tested although yields of phage from W-23-S^r-L9 were not decreased when glucose was added to nutrient broth or nutrient broth with yeast extract. High yields of phage were obtained from PNL-B in PA broth which is nutrient broth with $MnSO_4$, $MgSO_4$, $CaCl_2$ and NaCl. High yields were obtained if only $MnSO_4$ or $CaCl_2$ was added to nutrient broth. Although phage yields from W-23-S^r-L9 were poor in nutrient borth, addition of any one of the salts in PA broth stimulated high phage yields.

PNL-C produced no detectable phage in the test medium, but high yields were obtained in PA broth. Glucose decreased yields in any medium tested, and $MnSO_4$ was the only salt in PA broth that stimulated phage production. In the sporulation medium, a potato extract broth with peptone and yeast extract, lower phage yields were obtained from PNL-C than from W-23-S^r-L9. Addition of $MnSO_4$ increased the yield from PNL-C, and addition of sodium citrate reduced yields from PNL-C but not from its parent.

Proportions of spores carrying infective centers in spore suspensions of PNL-C were determined. Those suspensions whose spores were produced on potato agar or in potato broth with sodium citrate contained few infective centers compared to numbers of viable spores. Spore crops grown in potato broth or potato broth with $MnSO_4$ gave spore crops with many more infective centers. Growth from spore suspensions containing low proportions of infective centers gave little or no phage in the test medium and high yields in PA broth, and spore suspensions with high proportions of infective centers gave high phage yields in both media.

Differences between the phage derived from W-23-S^r-L9 and the PNL strains were not found in serological tests. However, a population of phage derived from the PNL strains always contained a lower percentage of virulent mutants than phage derived from W-23-S^r-L9.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LYSOGENY IN BACILLUS SUBTILIS

by

KAREN ROBERTS AUBORN

A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

June 1963

APPROVED:

[redacted]

Professor of Microbiology

In Charge of Major

[redacted]

Chairman of Department of Microbiology

[redacted]

Chairman of School Graduate Committee

[redacted]

Dean of Graduate School

Date thesis is presented October 30, 1962

Typed by Carol Baker

ACKNOWLEDGEMENTS

The author wishes to acknowledge:

Dr. Thorne for his guidance and help throughout this course of study;

faculty, staff, and fellow graduate students for their assistance;

the medium of oil paints for expression and diversion;

my husband and parents for love and understanding;

a teacher I once had for the maxim, "This too shall pass away," as it applies to research failures and thesis writing.

LYSOGENY IN BACILLUS SUBTILIS

INTRODUCTION

Lysogenic bacteria constitute a rather unique example of a host-virus relationship in which healthy cells carry indefinitely the potentiality of producing bacteriophage. The phages released by lysogenic bacteria are called temperate phages which differ from virulent phages in their ability to lysogenize. Transduction is usually mediated by temperate phage. An interest in the Bacillus subtilis lysogenizing systems resulted from studies on phage mediated genetic transfer. Using phage isolated from soil, Thorne (28, 29) demonstrated transduction in this species. The phage was capable of lysogenizing B. subtilis, and a lysogenic strain prepared in the laboratory served as the source of the transducing phage. His conclusions have been confirmed in other laboratories (12, 27). Since DNA-mediated transformation has been demonstrated in B. subtilis (24), comparative studies between the two genetic systems became possible (7, 25). In addition the occurrence of a type of sexual recombination may also exist in this species (15).

When individuals of the lysogenic strain of B. subtilis maintained at Oregon State University (the same strain used by C. B. Thorne for transduction studies) appeared to lose their lysogenic character, the maintainance of a stable lysogenic strain became

important. Earlier work was directed toward a possible physiological relationship between sporulation and phage production by lysogenic spore formers. However, it was found that phage production occurred in media conducive to sporulation as well as in media not conducive to sporulation; thus, such a relationship probably did not exist. It was during this study that the lysogenic strain appeared to be unstable; several spore suspensions prepared under similar experimental conditions contained different proportions of spores with infective centers. Difficulty in obtaining a stable lysogenic strain in this species had been reported by Romig and Brodetsky (22, p. 139). Since maintenance of a stable lysogenic strain as a source of transducing phage was a practical problem, studies involving the phage-host system were undertaken.

REVIEW OF LITERATURE

General view of lysogeny. In 1921 Bordet and Ciuca (30, p. 56) used the term "lysogenic" to describe bacteria which release bacteriophage giving rise to lysis of sensitive bacteria. A lysogenic bacterium is one which possesses and transmits the power to produce bacteriophage. The ability of a bacterium to carry the genetic material of a bacteriophage is now known to be dependent on the existence of a close homology between regions of the bacterial genome and the phage genome or prophage. The prophage is attached to a particular region of the bacterial chromosome and there it replicates as a normal chromosomal constituent with no expression of its viral nature (13, p. 468-469). For those lysogenic systems that have been investigated extensively, it has been shown that the majority of the individuals of a population do not produce phage. Phage is produced by a small proportion of the cells and is liberated by cell lysis (18, p. 272).

Pseudolysogenic or carrier strains are also phage producing strains. The phage producing ability is ascribed to a population equilibrium between resistant and sensitive cells in which the latter are constantly being infected by free phage. These cultures are easily freed from phage by exposure to antiphage serum. Lysogenic bacteria, on the other hand, produce bacteriophage after successive

reisolations or prolonged exposure to antiphage serum (1, p. 365).

Lysogeny appears to be widely spread in nature. It has been detected in many strains of various species and genera including Staphylococcus, Vibrio, Bacillus, Corynebacterium and is widely distributed among species of Enterobacteriaceae (1, p. 365-381). Its occurrence in the genera Mycobacterium and Streptococcus has been reported (10, p. 154-166).

Much of the present concept of lysogeny resulted from studies of lysogeny in Bacillus species. When den Dooren de Jong (18, p. 278) and Cowles (4) found that Bacillus spores heated to temperatures known to inactivate free phage gave rise to lysogenic clones, den Dooren de Jong concluded phage must exist in spores in some form other than free phage. Wollman and Wollman (18, p. 278) found no infectious particles released when lysogenic cells of B. megaterium were lysed with lysozyme. Their proposed gene theory (18, p. 278-279) was thus supported. They concluded that phage exists in two separate forms, the noninfectious form and the mature infectious particle. Lwoff and Gutmann (19, p. 318) named the noninfectious form probacteriophage, and from their experiments with isolated B. megaterium in microdrops (19, p. 312-330), they concluded that the replication of the probacteriophage was apparently coordinated with that of the bacterium.

Temperate and virulent phages. The temperate phages are

those phages which can establish a permanent union with their host cells. This establishment confers on the cell and its progeny the property of lysogeny (30, p. 56). The virulent phages are those unable to give lysogenic systems (18, p. 329).

Many mutations of the temperate phages have been described. Variations in host range, plaque type, plaque size, and the ability to lysogenize are among these. Plaques formed by temperate phages on an appropriate indicator strain are turbid because their centers are occupied by lysogenic cells. Plaques formed by certain mutants, i.e., mutation from temperate to virulent, are clear because such phages are unable to lysogenize (1, p. 369). Studies (14, p. 76-81) of the genetics of the characters of temperate phages have been performed with mutants of lambda coliphage. Ability to lysogenize was used as a marker in crosses between mutant strains.

Defective lysogeny. Certain mutants of various phages have been described which cannot give rise to normal infectious phage and can be propagated only in the prophage state. Bacteria carrying such mutant but inducible prophages generally retain their sensitivity to ultraviolet light and lyse after irradiation, but they do not release active phage (30, p. 62).

In the Bacillus species, a lysogenic B. megaterium was isolated (23) which produced very little phage compared to the parent

strain. Irradiation of the strain with ultraviolet light resulted in lysis without release of phage. Siminovitch, after estimation of DNA during the latent period, postulated that release of phage was interrupted by a defect in DNA synthesis. The hypothesis was that ultraviolet light induced only the development of the protein part of the phage.

Multiple lysogeny. It is known that some lysogenic bacteria perpetuate a number of different prophages. Some Staphylococci produce five types of phage (18, p. 311). When homologous phage enters a bacterium for which it is lysogenic, the phage may be diluted out or establish itself as a prophage giving rise to doubly lysogenic strains. In some instances it can displace a pre-existing prophage (30, p. 59).

Immunity. Jacob and Wollman (13, p. 471-472) defined immunity as the property of a lysogenic bacterium to survive infection by a phage homologous to the prophage it perpetuates. This immunity is established against all mutants except those of a special class.

The establishment of lysogeny and concomitant immunity is controlled by a short segment of the linkage group of a temperate phage (30, p. 59). Experiments leading to the above conclusion were done with the Escherichia coli (λ) system.

An understanding of the mechanism of immunity has come from studies of zygotic induction in E. coli (14, p. 301). When a lysogenic

Hfr (male) cell is crossed with a F^- (female) recipient, the prophage, λ, is induced after entering the recipient. The development of λ results in destruction of the bacterial cell (14, p. 93). There would be in the cytoplasm of lysogenic bacteria an immunity factor which prevents the vegetative multiplication of the prophage and homologous superinfecting phages. This theory, as proposed by Jacob, for immunity in lysogenic bacteria (14, p. 304) is comparable to that for inducible enzyme systems. A regulator gene directs the synthesis of a repressor. This repressor in turn probably acts on a specific structure (operator) of the phage genome preventing the expression of early functions needed for the initiation of phage production. Lysogenization and vegetative replication appear to be mutually exclusive. Lysogenization would be characterized by the prevalance of the repressor mechanism and vegetative reproduction by the productive chain of synthetic reactions. The different types of mutations which affect the capacity of a temperate phage to lysogenize, its sensitivity to immunity, or its inducibility are readily explained by the repressor model. The work leading to the proposed theories of immunity has been done only with the E. coli (λ) system.

Although the loss of immunity has been used as a criterion for loss of lysogeny (3, 6), little work has been done in Bacillus species on the phenomenon of immunity. McCloy (21) found a lysogenic

B. cereus strain not to be completely immune even to the temperate phage. The colonial appearance of the strain was altered after lysogenization in a way suggesting that the alteration was due to incomplete immunity to the temperate phage produced during growth.

Induction. The phenomenon of induction of bacteriophage from lysogenic cultures evolved from studies on lysogenic B. megaterium. When Lwoff and Gutmann (19) observed that in some microclones of lysogenic B. megaterium high proportions of bacteria lysed and liberated phage while in other microclones only small proportions produced phage, this suggested to them that phage production was controlled by external factors. Sudden and considerable increases during certain periods in development of cultures caused Lwoff (18, p. 284) to suggest that phage production was controlled by some changes in the medium resulting from bacterial metabolism. Lwoff et al. (20) found that bacteria lysed and liberated bacteriophage after ultraviolet irradiation in yeast medium but not in synthetic medium. Huybers (11, p. 234-252) found that lysogenic B. megaterium produced phage after ultraviolet irradiation only when manganese was present after irradiation. Cobalt and zinc inhibited phage production, but the inhibition was relieved by manganese. Lwoff and Siminovitch (16) found that certain reducing agents would induce phage development in lysogenic B. megaterium only in yeast extract

medium. Lwoff (17) found his inducing agents to be effective in media containing Mn^{++}, Cu^{++}, and Fe^{++}. Addition of a chelating agent, oxime, caused the medium to no longer promote phage development. Although he was not studying induction of phage, Friedman (8) found that some of the phages of B. megaterium were inactivated by monovalent ions in the absence of divalent ions. Divalent ions, Ca^{++}, Mn^{++}, Mg^{++}, or Zn^{++}, would protect against inactivation by Na^{+} or K^{+}.

If Jacob's proposed repressor theory (14, p. 320-303) is assumed, the effect of inducing agents could be visualized as resulting from an arrest, or a decrease in rate, of the synthesis of a labile repressor. Initiation of vegetative replication would take place when the level of the repressor inside the cell fell below a certain threshold. Another hypothesis (30, p. 60-61) to explain induction is as follows: Competition exists between the small prophage and the relatively huge nucleus for intermediates involved in their synthesis. Under normal conditions, the prophage would be restricted by the need of essential intermediates by predominant chromosomal regions. In this system an inducing agent could alter the functional capacity of the active regions along the chromosome and release the phage from competitive inhibitions.

Loss of lysogeny. Lwoff (18, p. 312) said in his review on lysogeny:

> "References are often found in papers around 1925-1930 to lysogenic strains which spontaneously lost their lysogeny and then spontaneously regained it. It is known today that some temperate phages are exceedingly fragile. They are easily adsorbed on porcelain filters and readily inactivated by monovalent cations in the absence of divalent cations. Moreover, some of them gave tiny plaques on indicator strains and only in certain batches of broth or peptone. . . . It should be said immediately that no example is known of a nonlysogenic bacterium becoming spontaneously lysogenic . . . . It seems highly probable that the losses and gains of lysogeny reported around 1925-1930 were due to clinical failure to recognize the presence of phage."

A loss of lysogeny has been reported in species related to Bacillus subtilis; den Dooren de Jong (18, p. 312-313) reported loss of lysogeny in B. megaterium and B. undilatus. His strains lost their original immunity towards the homologous phage. In their studies Ehrlich and Watson (6) found that growth in a nutrient medium containing glucose would render lysogenic B. megaterium cells no longer immune to its phage. Their work was confirmed by DeCarlo et al. (5). Clarke (3) cured B. megaterium of its lysogenic condition by subculturing his lysogenic strain in a synthetic medium containing citrate. Lysogenicity was lost after 61 subcultures. The loss resulted in reversion of the culture to sensitivity to its original bacteriophage. Absence of Ca^{++} was believed to be the cause of the loss. Expanding on Clarke's experiments, Lwoff (18, p. 313) also working

with B. megaterium, obtained nonlysogenic strains after 25-34 subcultures whether or not a calcium binding substance was present. Wollman and Wollman (31) found that lysogenic B. megaterium failed to produce phage in a medium free of calcium but did not lose its lysogenic character after 21 transfers. Romig and Brodetsky (22, p. 139) reported a rapid loss of lysogeny in B. subtilis. Individuals lost their ability to produce bacteriophage and became sensitive to the original bacteriophage.

It has been found that heavy irradiation of E. coli (λ) with ultraviolet light cures bacteria of their lysogenic character. Among the survivors very few produced phage. Defectively lysogenic and nonlysogenic cells were differentiated by their sensitivity to λ phage (14, p. 60). "Curing" of lysogenicity in Staphylococcus by fumagillin was reported by Taylor and Koft (26) as evidenced by the loss of phage producing ability; however, immunity to the phage was not lost.

Transduction. Certain temperate phages (and occasionally their mutant virulent phages) are able to carry a piece of genetic material from a donor bacterium to a recipient bacterium. Among the recipient cells, some have acquired new genetic properties originating in the donor bacteria. Among the characters which can be transduced from donor to recipient bacteria is the lysogenic character itself (1, p. 377-379). A great variety of genetic characters

can be transduced such as nutritional characters, utilization of sugars, resistance to inhibitors, motility, surface antigens, etc. Transduction and lysogenization are two distinct phenomena. Only certain types of temperate phages have the ability to transduce. On the other hand, transduction can occur without concomitant lysogenization of the transduced bacterium (14, p. 23).

Transduction was first observed in Salmonella by Zinder and Lederberg (32). Transduction has been studied in a great many Salmonella strains, in Shigella, and in Escherichia coli (14, p. 24). Its occurrence in Streptococcus is being studied at Oregon State University (2).

Thorne (28, 29) first reported transduction in Bacillus subtilis. After isolation of a temperate phage from soil, he lysogenized wild type W-23. The phage was prepared by harvesting it after growth of the lysogenic strain. With this phage he transduced prototrophy to auxotrophic mutants of W-23. The same phage transduced another strain, W-168, but would not propagate on the strain. Lysogeny could not be detected in W-168. Using another lysogenic system in this species Takahashi (27) independently reported transduction of streptomycin resistance, prototrophy, and sporogenesis. Ivánovics and Csiszár (12) isolated a series of temperate phages for this species and found different frequencies of transduction among the phages.

METHODS AND MATERIALS

Bacterial strains. Bacillus subtilis, W-23-S^r, is a streptomycin-resistant mutant of the wild type strain, W-23 (prototrophic). Although the organism is resistant to streptomycin, this characteristic was not studied in this investigation. B. subtilis, W-23-S^r-M4 is a histidine-requiring mutant isolated by C. B. Thorne following ultraviolet irradiation of W-23-S^r spores (29, p. 106). B. subtilis, W-168 (indole$^-$) is the strain used as a recipient by several investigators in studies on transformation (7, 24). This strain, as well as several auxotrophic mutants of W-23-S^r, was used also as a recipient in transduction studies by C. B. Thorne (29). B. subtilis, W-23-S^r-L9 was obtained by lysogenizing W-23-S^r with phage Sp-10 described below (29, p. 110). L9 is an arbitrary designation indicating that it was the ninth isolate among several such lysogenic cultures. The strain of B. licheniformis was ATCC 9945A. Spores of each culture were stored in soil and brought from Fort Detrick to Oregon State University by C. B. Thorne.

Phage. The phage, Sp-10, used to lysogenize W-23-S^r giving rise to the phage producing strain, W-23-S^r-L9, was isolated from soil (29, p. 107). It was derived from a single plaque produced by phage which had been propagated on W-23-S^r when spores of this

bacterium were used as an indicator in assaying soil filtrates. Sp-10, because it is temperate for W-23-S^r, gives a turbid plaque morphology on this indicator. The phage is also temperate for the Bacillus licheniformis strain which will also serve as an indicator. A few plaques in a lawn with the appropriate dilution of Sp-10 and indicator are clear because some of the phage particles (virulent mutants) have lost the ability to lysogenize. A clear plaque was picked and the phage was propagated on W-23-S^r. Another phage mutant whose plaque morphology is distinct from that of either Sp-10 or its virulent mutant was isolated and designated VL. It produces a very clear plaque which contains a distinct colony in the center. The lysogenic colony originating in the center of such a plaque was streaked onto nutrient agar to isolate colonies from free phage, and a single colony was transferred to potato agar and allowed to sporulate. Spores arising from such colonies yielded phage which gave rise to plaques of only this type. Photographs of Sp-10 and its mutants are shown in Figure 1. Sp-10 was the transducing phage used by C. B. Thorne (29).

Media. Nutrient broth, NB, contained Difco nutrient broth, 8 g/liter. NBY medium was NB with 0.3 percent Difco yeast extract. Phage assay, PA, broth was NB with the following salts: NaCl, $MgSO_4$, $MnSO_4$, and $CaCl_2$. The concentrations are shown

in Table 1. The pH of PA medium was adjusted to 5.9 - 6.0 with HCl. When the concentration of one salt was varied, or when only one salt was used in the medium, the pH and the concentrations of all other constituents were held constant. If glucose was desired in any of the above medium, sterile 20 percent glucose was added to give a final concentration of 0.1 percent. NBY with 0.1 percent glucose was designated NBYG. Distilled water was routinely used in all media.

Potato extract media was prepared as follows: Diced potatoes, 100 g, were boiled 5 minutes in 500 ml of water, and the material was filtered through a Whatmann No. 1 paper on a Büchner funnel. One g Difco yeast extract and 10 g of N-Z Case peptone were added. The pH was adjusted to 7.2 with NaOH or HCl, and the solution was diluted to 1 liter. If solid medium was desired, 20 g agar were added.

The formula for minimal medium is shown in Table 2. Casamino acids, 3 g/liter, were added to the medium in some cases to allow colonies to develop faster. Since Casamino acids are free of tryptophan and indole, minimal medium with Casamino acids was used to select for W-168 transductants. To simplify procedures, stock solutions were stored containing ingredients at 10 times their final concentrations used in the medium. Solution A contained KH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, and sodium citrate. Solution B

contained $MgSO_4$, $FeCl_3$, and $MnSO_4$. A third solution contained only glutamic acid. It was necessary to store the glutamic acid and solution A at refrigerator temperatures. Sterile 20 percent glucose was added to give 0.5 percent glucose in the medium. If solid medium was desired, sterile agar (30 g/liter) was added to an equal quantity of double strength sterile minimal medium. This method prevented darkening of the medium when it was stored with agar.

Sterile medium was stored in prescription bottles until ready for use. Sterilization consisted of autoclaving for 20 minutes at 121° C.

Cultural conditions. When shaken cultures were desired, cotton-plugged flasks or bottles were incubated on a Brunswick Gyrotory shaker held at 37° C and running at a speed of 160 revolutions per minute. Slants, plates or bottles containing agar were held the desired time in a 37°C incubator. If screw caps were used in lieu of cotton plugs, they were loosened to allow circulation of air.

Propagation of Spores. Spores were produced either in potato broth on a shaker or on potato agar. In all instances, sterile distilled water was the wash medium or diluent for spores. Centrifugation was done in a Serval model SS-34 centrifuge at 8,000 revolutions per minute. If potato agar was used, small volumes of water

were added to the growth on slants or in Roux bottles. The growth was then removed with a sterile pipet and washed. If potato broth was used, spores were removed from the medium by centrifugation. With either method spores were grown 4 days at 37°C and washed twice by resuspending in water and centrifuging. The heat treatment was 30 minutes at 65°C to inactivate remaining cells and 60 minutes to inactivate free phage in the case of lysogenic cultures. Viable counts were made on nutrient agar.

Assay of phage. Cells were removed by centrifugation at 5, 000 revolutions per minute in a Serval SS-34 for 15 minutes and filtration through Millipore DA filters (0. 65μ pore size). For quantitative estimations of phage at high titers the use of supernatant fluid after centrifugation gave results comparable to those obtained with filtered samples. The agar overlay technique was used for phage titration. The base layer was about 25 ml of PA agar, and soft PA agar was the overlay medium. Difco peptone, 0. 5 percent was the diluent for phage preparations. One-tenth ml of the appropriate dilution of phage was added to 3 ml of soft agar containing 1×10^8 spores of indicator strain in 16 mm culture tubes and the mixture was poured onto the base agar. The indicator was W-23-S^r unless stated differently. Plates of agar base were prepared the same day they were used. Unglazed porcelain covers were used to eliminate smearing

of plaques caused by moisture. Plaques were counted after 16-25 hours of incubation at 37°C, and phage titers were expressed as pfu (plaque-forming units) per ml.

Phage propagation. When lysogenic strains were used for phage production, about 1×10^8 spores of the lysogenic strain were added to 250 ml Erlenmeyer flasks containing 50 ml of medium. Phage was harvested from shaken flasks which had been incubated at 37°C for 14-16 hours. Cells were removed by centrifugation and filtration if desired. Sometimes phage was propagated on the appropriate organisms either in shaken flasks or on PA agar with an overlay of soft agar. In shaken flasks indicator spores (1×10^8) and phage (a definite multiplicity or single plaque) were incubated 14-16 hours at 37°C before phage was harvested. When the soft agar method was used, 1 ml of a phage suspension was added to 3 ml of soft PA agar with 10^8 indicator spores. The mixture was poured onto a petri plate of hard PA agar and the plate was incubated 16 hours at 37°C. To harvest phage from the lysate, the plate was flooded with 5 ml peptone diluent, and the soft agar was macerated with a sterile glass spreader and transferred to a centrifuge tube. The supernatant fluid was collected after centrifugation.

Isolation of PNL strains. A spore suspension of W-23-S^r-L9, was spread on nutrient agar to give isolated colonies, and colonies

were picked at random onto phage assay agar with a lawn of W-23-S^r spores (10^8) in an overlay of soft PA agar. Plates were incubated overnight at 37°C. Those colonies not causing lysis of the indicator strain were called PNL (presumably nonlysogenic). Three colonies designated PNL-A, PNL-B, and PNL-C were sporulated on potato agar, and their spore suspensions were used for further study.

Phage antiserum Lyophilized rabbit antiserum against Sp-10 phage was obtained from Dr. C. B. Thorne. Antiserum against the phage from PNL-C was prepared in rabbits by intravenous injections of the phage. The phage had been collected by high speed centrifugation and suspended in a solution containing 0.15 M NaCl, 0.5 percent gelatin and 0.05 M K_2HPO_4 at pH 7.0. Each of three rabbits was injected with 0.5 ml of suspension containing 3×10^{10} pfu/ml. Injections were repeated after 2, 5, 7, 9, and 13 days. One ml of suspension was injected on the last day. Sera were collected and pooled a week after the last injection.

Resistance to phage attack. A lawn containing 1×10^8 spores of the strain under question was prepared in soft PA agar overlay on hard PA agar. A drop of the appropriate dilution of phage was added with a sterile rod or pipet. The lawn was examined for lysis after incubation at 37°C for at least 16 hours. If a more precise test was desired, the phage was titrated against the strain in

question using the method described for phage assay.

Induction of phage by ultraviolet irradiation. Only cultures in the logarithmic phase of growth were irradiated. These were 5 hour shaken cultures grown from a 10 percent inoculum of an overnight culture. The source of ultraviolet rays was two 15 watt General Electric Germicidal lamps held 16 inches above cultures. Five ml samples were shaken under the lamps in open petri dishes. After irradiation, the entire sample was added to 50 ml medium (the same medium used for irradiation) and incubated under shaking conditions. The percent transmission was read on a Beckman model B Spectrophotometer.

Transduction. Transduction procedures described by C. B. Thorne (29, p. 106-107) were used. Recipient strains were five-hour shaken cultures in NBY grown from a 10 percent inoculum of an overnight culture. Phage, free of bacteria, was harvested from 16 hour cultures of lysogenic strains. Only W-23-S^r-M4 (histidine$^-$) and W-168 (indole$^-$) were used as recipients. Incubation of phage and recipient occurred in 25 ml cotton-plugged serum bottles which were shaken at 37°C for 30 minutes. Total volume of each transducing mixture was 1.0 ml. W-23-S^r-M4 transductants were selected on minimal medium while W-168 transductants were selected on minimal medium supplemented with Casamino acids.

Controls included tests of auxotrophs for revertants and phage for sterility.

RESULTS AND DISCUSSION

When spores of the phage-producing strain, W-23-S^r-L9, are inoculated into the appropriate medium and incubated on a shaker at 37°C, phage production occurs. In early studies NBYG medium was used routinely for phage production. In this medium a culture of W-23-S^r-L9 inoculated with 10^6 spores per ml and grown for 16 hours contained about 1×10^9 plaque forming units per ml of supernatant fluid.

From earlier results obtained by Thorne (29, p. 110), it was concluded that strain L9 was stable with respect to maintenance of lysogeny. This conclusion was based on the fact that when the culture was carried through several cycles of growth and sporulation in potato extract broth, the numbers of viable spores and infective centers in the spore suspensions were equal within experimental error. This was true even though each successive spore crop was heated 60 minutes at 65°C to inactivate any free phage present in the suspensions. The use of spore suspensions prepared in this manner had resulted in high yields of phage in NBYG medium.

When attempts were made to repeat these experiments with new spore crops, the numbers of infective centers in the spore suspensions tested did not agree with the numbers of viable spores. The numbers of infective centers equaled only 0.5 times the numbers of spores. Individual colonies were tested for liberation of phage that lysed the indicator.

With two separate spore suspensions, one produced in potato broth and one produced on potato agar, only 56 percent and 50 percent of the colonies tested caused lysis on a lawn of indicator. Colonies not giving rise to lytic areas on lawns of indicator were called "presumably nonlysogenic" or PNL.

Later on difficulty was encountered in reproducing spore suspensions of W-23-S^r-L9 that gave high yields of phage. Some spore suspensions gave high yields while other preparations produced under apparently the same experimental conditions, gave poor yields. For these reasons the phage-host system was investigated in more detail.

Effect of antiserum on "curing" of lysogeny. The possibility existed that W-23-S^r-L9 was not truly lysogenic, and the phage it produced was perpetuated in the strain in some other manner. A B. cereus strain is known to perpetuate a virulent phage which is viable after heat treatment of spores (9). If true lysogeny did not exist, the phage could perhaps be adsorbed onto or into the spores in some manner causing the phage to be heat stable. Antiphage serum might eliminate phage adsorbed onto spores. Incubation with antiserum known to inactivate Sp-10 phage did not cause the spores to lose their phage producing ability. The results are shown in Table 3. The existence of true lysogeny in W-23-S^r-L9 was thus

supported.

Immunity of PNL clones. Since lysogenic bacteria by definition lose their immunity to homologous phage when lysogeny is lost, the PNL clones of L9 were tested to determine their susceptibility to Sp-10 phage. W-23-S^r-L9 is immune to both Sp-10 and its virulent mutant. However, at very high multiplications of superinfection of L9 with the virulent mutant, lysis occurs. The cause of this phenomenon is thought to be adsorption or so many phage particles to cells that lysis occurs without phage multiplicities. In early spot tests PNL strains displayed some resistance to Sp-10, more than the normal susceptible strain, W-23-S^r, but less than the parent strain, W-23-S^r-L9. When Sp-10 and a virulent mutant were titrated against spore suspensions of PNL strains, these strains behaved differently from either W-23-S^r or L9. Results are shown in Table 4. An intermediate resistance pattern appeared to exist with the degree of immunity varying among the PNL strains. Two PNL strains appeared to produce their own phage as lawns showed lytic activity without addition of phage. One of the strains, PNL-C, formed about 10^3 plaques per 10^8 spores in its own lawn.

Activity against another indicator. It seemed possible that L9 might be lysogenic for more than one phage, and that the properties of the PNL strains derived from L9 might be explained by the loss of

one or more of the prophages. If the PNL strains were lysogenic for phage existing in the wild type of W-23-S^r, this organism would not serve as a suitable indicator because it would be immune to its own phage. Since the B. licheniformis ATCC 9945A strain will also serve as an indicator for Sp-10 phage, it was selected as a test indicator for phages produced by the PNL strains. Stock spore suspensions and filtrates from 16 hour NBYG broth cultures were tested for their activities on a lawn of B. licheniformis. Results obtained are shown in Tables 5 and 6. All spore suspensions, including the B. subtilis indicator, causes lysis of the B. lichenformis strain. However, the filtrate of only the L9 strain caused a lytic area on the lawn of indicator; therefore, the cause of lysis when spore suspensions were used was probably due to another bacterial inhibitor rather than phage originally present in W-23-S^r. The cause of the lytic action when spores were used was not persued. A fallacy in the above experiments with filtrates was found: NBYG broth was not a good medium for phage production by PNL strains.

Phage production by PNL strains. Tests were made for phage production by the PNL strains in media other than NBYG. When PA broth was substituted for NBYG, high titers of phage were obtained from PNL-B and PNL-C as evidenced by titration of filtrates against the B. subtilis indicator. PNL-C produced no detectable

phage in NBYG but produced 10^9-10^{10} phage particles in PA broth. PNL-B produced low yields in NBYG medium and high yields in PA. These strains were studied further since they appeared to have different requirements for phage reduction than the parent strain, L9.

Irradiation of PNL-A. Since phage production by PNL-A could not be demonstrated in any medium tested, attempts were made to induce phage production by ultraviolet light. Irradiation of PNL-A in NBYG caused no detectable lysis of the culture, and no phage was detected when supernatant fluids were assayed against W-23-S^r. When the irradiation was done in PA broth, lysis of the culture occurred as evidenced by an increase in percent transmission. Results of an irradiation treatment in PA broth are given in Table 7. When supernatant fluids were titrated for phage against W-23-S^r (and tested qualitatively on B. licheniformis ATCC 9945A and B. subtilis, W-168), no phage was detected. An explanation for the behavior of PNL-A could be the presence of a defective prophage since most defective phages are inducible, i.e., cells will lyse but will not give rise to infective particles after induction. A possible way to demonstrate the presence of defective phage would be to show the production of recombinant phage after infection with phage. For example, if wild type phage appeared after incubation of the strain believed to be carrying a defective prophage with mutant phage,

but not after incubation of the mutant phage with indicator, one would have evidence for existence of defective phage. The virulent mutant isolated was not suitable since a few temperate phages (presumably revertants) occurred after incubation with the indicator as well as with PNL-A. Incubation of PNL-A and W-23-S^r with the VL phage did not show any recombinants. Cultures were incubated for 16 hours after infection with a multiplicity of 1, and supernatant fluids were titrated against W-23-S^r.

Effect of glucose on phage yields. The effect of glucose was tested on the PNL-strains since it is present in NBYG but not in PA medium. The results are given in Table 8. In NBYG medium L9 produced phage as usual. PNL-B produced a small amount, and PNL-C produced no detectable phage. When glucose was omitted, phage yields from PNL-B and PNL-C were increased. Glucose decreased phage yields from these strains in PA and NB. Also, it decreased the yield from L9 in PA but not in NBY or NB. No phage was detected in any instance from PNL-A. When glycerol was added to NBY, yields from PNL-B and PNL-C were again decreased.

Effects of salts on phage yields. Since PA differs from nutrient broth only in its salts contents, the salts present in PA were tested individually for their effects on phage yields. The results are

given in Table 9. The addition of $MnSO_4$ resulted in higher yields from all spore suspensions yielding phage. $CaCl_2$ increased yields from L9 and PNL-B. The effect of $CaCl_2$ on PNL-C was somewhat erratic as a consistent level of phage could not be obtained in replicate flasks. $MgSO_4$ increased the yields from L9 but not from PNL-B or PNL-C. Likewise, the addition of NaCl resulted in a high yield from L9 but not from the PNL strains. When all salts were added, as in PA medium, maximum yields were obtained with PNL-B and PNL-C; and the yield from L9 was the same as when NaCl alone was added. PNL-A produced no detectable phage in any of the media.

Different levels of $CaCl_2$ and $MnSO_4$ in NB were tested with PNL-B as shown in Tables 10 and 11. One could conclude from these experiments that very small additions of $MnSO_4$ or $CaCl_2$ would increase phage yields from PNL-B; therefore, it is conceivable that different batches of nutrient broth or contamination of glassware with these salts could alter results. The concentration of $CaCl_2$ normally used in PA broth appeared to be adequate, but the concentration of $MnSO_4$ should possibly be increased to 0.0015 M to give optimum yields of phage.

Antisera cross reactions. Once phage was produced by PNL strains, attempts were made to detect any serological differences between this phage and the phage of L9. As shown in Table 12,

Sp-10 antiserum inactivated the phage produced by PNL-C or PNL-B. Table 13 gives the results obtained when antiserum prepared against PNL-C phage was tested against Sp-10. Sp-10 was inactivated by the antiserum. Table 14 gives results of testing both antisera for their activity against the VL mutant. The VL mutant was tested since it was derived from a single lysogenic colony, and its serological specificity was not known. No serological differences were distinguished between the phages derived from L9 and PNL-C.

Phage yields in potato broth. L9 had been shown to produce phage in a medium used for sporulation, potato broth, as well as in a medium not conducive to sporulation, NBYG. Results from a comparative study are compiled in Table 15. Conclusions from this study were: W-23-S^r and L9 show similar sporulation characteristics. Phage yields from L9 are comparable in both media although sporulation is much better in potato broth than in NBYG. Hence, no correlation between sporulation and phage production exists. The reason for decreases in phage titers is not known but is probably a result of the lability of the phage under these conditions.

Table 16 shows results obtained when phage yields from PNL-C and L9 were compared in potato broth with $MnSO_4$ or sodium citrate added. PNL-C gave a considerably lower phage yield in potato broth than did L9. When $MnSO_4$ was added to potato broth, the

yield from PNL-C was increased at least 100 times. When sodium citrate was added to potato broth (possibly making manganese unavailable), the yields from L9 were still high; but those from PNL-C were very low.

Effects of sporulation media on phage yields. The finding that phage yields from PNL-C in potato broth could be altered by the addition of $MnSO_4$ or sodium citrate suggested that the amounts of phage produced before sporulation might be reflected in variations in the proportions of spores carrying infective centers. Results of an experiment to test this are shown in Table 17. Spores were prepared under the various conditions, i.e., in potato broth with and without sodium citrate or $MnSO_4$ and on potato agar; and the numbers of infective centers and viable spores were determined. The proportion of spores carrying infective centers was very low when the sporulation medium for PNL-C was potato agar or potato broth with sodium citrate added. Spores produced on potato agar gave rise to only 1 infective center in 10^5 spores, and spores produced in potato broth with sodium citrate had 1 infective center per 10^4 spores. In both potato broth and potato broth with added $MnSO_4$, 1 spore out of 3 had an infective center.

In Table 18 phage yields in NBYG and PA broth from PNL-C spores produced under the various conditions described above are given.

Only those spore suspensions with high proportions of infective centers gave high yields of phage in NBYG. Spore suspensions containing a very low proportion of infective centers, however, produced high yields in PA broth.

It seems possible, on the basis of these results, to explain some of the difficulties experienced with respect to reproducibility of results with various spore crops when NBYG was used routinely for phage production. It is probable that various batches of potato medium varied with respect to manganese content. Also, storage of potato agar could lead to batches of media having lower moisture content thus reducing the chance of reinfection which apparently must occur in order to obtain spore crops with high proportions of infective centers.

It appears that true lysogeny, if it exists at all in this system, is unstable under the conditions of our tests; and the production of spores carrying infective centers depends on phage production and reinfection in the sporulation medium. The difference in phage yields in NBYG medium and PA medium, particularly when spore suspensions with low proportions of infective centers are used, is not completely understood. It appears that glucose and manganese and perhaps other salts are important factors contributing to this effect.

A possible explanation for the peculiar behavior of this phage-host system is that true lysogeny, as it is known to exist in other genera, occurs in L9, and that another system of incorporating phage particles into spores also occurs. The latter system might readily release its phage when the spores germinate and grow, but induction of the prophage in the truly lysogenic system might require the presence of cofactors such as manganese.

Another phenomenon which is not understood is the variation in the percentage of virulent mutants occurring in a population of phage. This percentage varied among L9 spore crops from 1 percent in one suspension to 60 percent in another when the phage was produced in NBYG. When PA broth was used, virulent mutants in the phage yields from the two spore crops above increased to 10 percent and 80 percent. Other values were obtained with other L9 spore crops. It was of particular interest that the PNL strains gave phage yields with only a few virulent mutants. PNL-B gave a yield with 0.1 percent virulent mutants, and PNL-C gave a yield with only 0.01 percent virulent mutants. These percentages were determined for phage produced in PA broth.

A possible use of the PNL strains might be in transduction studies because the phage they produce contains a lower proportion of virulent mutants. Virulent phage lyses many of the recipient

cells resulting in a reduced frequency of transduction. W-168 (indole$^-$) and W-23-S^r-M4 (histidine$^-$) were transduced to prototrophy by PNL-C phage, but detailed studies and comparison with transduction by L9 phage were not carried out.

SUMMARY

Studies were directed toward stabilizing a phage-host system in Bacillus subtilis. The strain of B. subtilis, W-23-S^r-L9, was a lysogenic strain perpetuating the bacteriophage, Sp-10. Its importance was in propagation of the phage for several transduction studies (7, 29). Reasons for believing the strain was unstable were:

1. After isolation of W-23-S^r-L9 (29, p. 111) all spores in a series of spore crops were believed to carry infective centers; however, a decrease in the number of spores carrying infective centers in new spore crops occurred.
2. Some spore suspensions gave high yields of phage in the test medium, NBYG, which is nutrient broth with yeast extract and glucose, while other preparations produced under apparently the same conditions gave poor yields.

The strain was lysogenic by criteria given in the literature, i.e., spontaneous phage production after inactivation of free phage and immunity to homologous phage. Phage was produced upon growth of the culture after spores were either heated to inactivate free phage or incubated with antiserum to inactivate free phage. The strain was immune to the phage it produced.

The instability problem was approached by an investigation of

three clones of W-23-S^r-L9, picked because their original colonies did not appear to carry infective centers. Studies to determine why these clones were different from their parent were undertaken. These differences would perhaps explain variations in spore crops of W-23-S^r-L9. Spore suspensions of these clones were called PNL-A, PNL-B, and PNL-C for "presumably nonlysogenic."

With PNL-A, phage was not detected in any medium used for growth of the strain although its parent W-23-S^r-L9 produced phage in each of the media tested. Ultraviolet light induced lysis of the strain in some media, but no phage could be demonstrated. The possible existence of a defective phage was discussed.

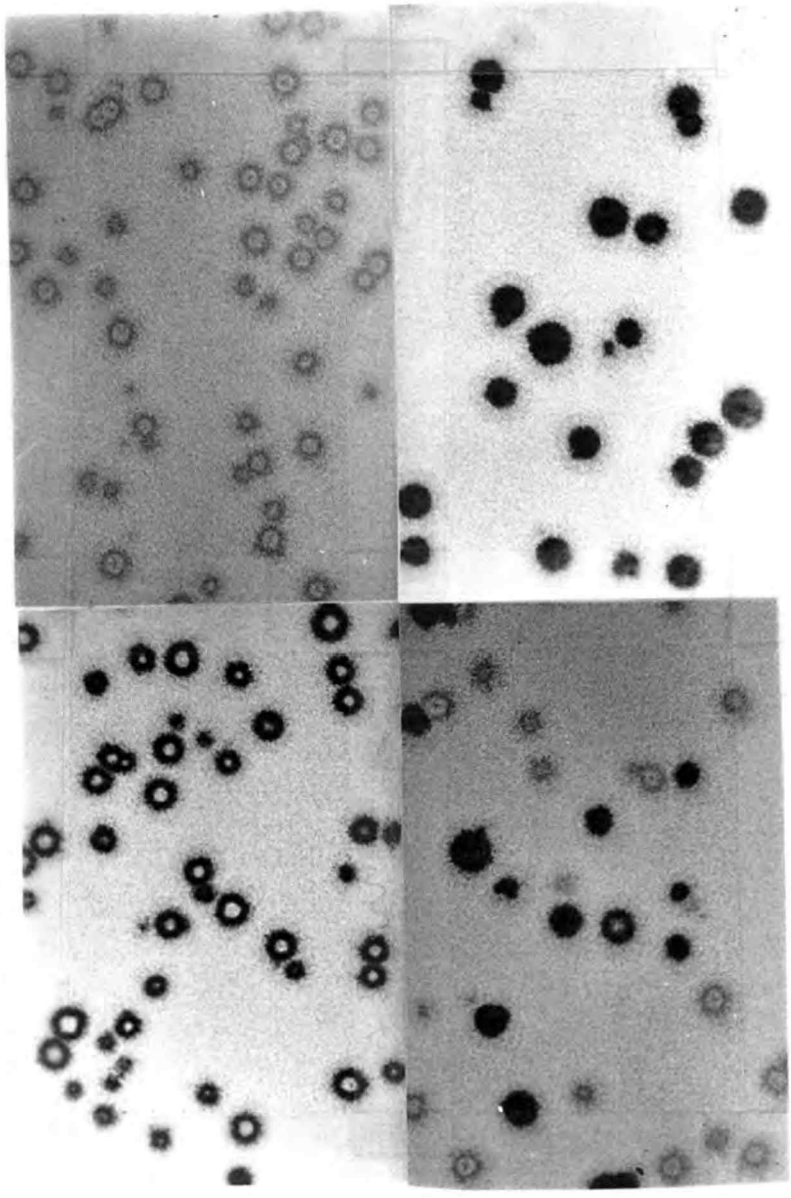
Different results were obtained with PNL-B. This strain gave poor yields of phage in the medium, NBYG, used routinely for phage production from W-23-S^r-L9. Glucose, which was present in the test medium, decreased phage yields from PNL-B in all media tested, but glucose did not decrease phage yields from W-23-S^r-L9 in nutrient broth or nutrient broth with yeast extract although it did in another medium, PA broth, which is nutrient broth with $MnSO_4$, $CaCl_2$, NaCl, and $MgSO_4$. PNL-B produced high yields of phage in PA broth or in nutrient broth with the addition of only $MnSO_4$ or $CaCl_2$. Although the phage yields from W-23-S^r-L9 were poor in nutrient broth, this strain produced high yields of phage in PA broth or in nutrient broth with the addition of any one of the salts in PA broth.

PNL-C produced no detectable phage in NBYG, but high yields were obtained in PA broth. Glucose decreased phage yields in any medium tested, and $MnSO_4$ was the only salt in PA broth which stimulated phage production. In the sporulation medium, potato extract broth with peptone and yeast extract, yields of phage from PNL-C were lower than those from W-23-S^r-L9. However, addition of $MnSO_4$ increased the yield from PNL-C in this medium, and addition of sodium citrate reduced yields from PNL-C but not from its parent.

Proportions of spores carrying infective centers in spore suspensions of PNL-C were determined. Those suspensions made from growth on potato agar or in potato broth with sodium citrate contained very few infective centers compared to the numbers of viable spores. However, spore suspensions grown in potato broth or potato broth with $MnSO_4$ gave spore crops with many more infective centers compared to viable spores. When the spore suspensions containing low proportions of infective centers were used as inocula, little or no phage was produced in NBYG but high yields were obtained in PA broth. The spore suspensions with high proportions of infective centers gave high phage yields in both media. The behavior of PNL-C appeared to be dependent upon whether phage production and reinfection occurred in the sporulation media.

Differences between the phage from PNL strains and W-23-S^r-L9 were not found in serological tests. However, populations of phage derived from the PNL strains always contained a lower percentage of virulent mutants than phage derived from W-23-S^r-L9. For this reason phage from PNL strains might be useful in transduction studies.

Figure 1. Plaque formation by Sp-10 phage and mutants on W-23-S^r.



Upper Left. Plaques formed by Sp-10.
Upper Right. Plaques formed by an Sp-10 virulent mutant.
Lower Left. Plaques formed by an Sp-10 VL mutant.
Lower Right. Plaques formed by Sp-10, its virulent mutant, and its VL mutant.

All pictures are enlarged 4 times.

Table 1. Composition of Phage Assay Medium (PA)

Difco nutrient broth	8 g/liter
agar	7 or 15 g/liter
NaCl	0.086 M
$MgSO_4$	0.0008 M
$MnSO_4$	0.0003 M
$CaCl_2$	0.001 M
pH 6.0	

Table 2. Composition of Synthetic Medium

$(NH_4)_2SO_4$	0.015 M
KH_2PO_4	0.04 M
K_2HPO_4	0.08 M
$MgSO_4$	0.0008 M
$FeCl_3$	0.00015 M
$MnSO_4$	0.0000015 M
Sodium Citrate	0.003 M
Glucose	5 g/liter
L-Glutamic Acid	1 g/liter

pH 7.0

Table 3. Activity of Sp-10 Phage Antiserum on W-23-S^r-L9 Spores

addition to 10^8 spores	pfu/ml	
	30 min. incubation	60 min. incubation
NS	1.0×10^8	8.5×10^7
AS	1.0×10^8	7.5×10^7

The spores, 0.1 ml, and serum, 0.9 ml, were incubated statically at 37°C. Spores were titrated against W-23-S^r. "NS" denotes normal serum, and "AS" denotes antiserum.

Table 4. Activity of Sp-10 Phage Against Presumably Nonlysogenic Strains

phage added	Plaques formed on the various indicators				
	W-23-S^r	W-23-S^r-L9	PNL-A	PNL-B	PNL-C
no phage	0	*0	0	*0	8×10^2
Sp-10 (1×10^7 pfu)	TNTC	*0	TNTC	*0	TNTC
Sp-10 (1×10^5 pfu)	TNTC	*0	TNTC	*0	TNTC
Sp-10 (1×10^3 pfu)	1×10^3	*0	137	*0	8×10^2
Sp-10-Virulent Mutant (1×10^8 pfu)	confluent lysis	lysis without distinct plaques	confluent lysis	confluent lysis	confluent lysis
Sp-10-Virulent Mutant (1×10^6 pfu)	confluent lysis	*0	confluent lysis	lysis without distinct plaques	confluent lysis
Sp-10-Virulent Mutant (1×10^4 pfu)	TNTC	*0	7×10^2	*0	8×10^2
Sp-10-Virulent Mutant (1×10^2 pfu)	82	*0	18	*0	8×10^2

*Lawns showed some lysis without added phage.

TNTC indicates plaques were too numerous to count.

Table 5. Activity of Spore Suspensions of PNL Strains on a Lawn of B. licheniformis

spore suspension (1×10^9 spores/ml)	lysis on W-23-S^r	lysis on B. licheniformis
W-23-S^r	-	+ Dilutions of 10^2 and 10^4 were not active
W-23-S^r-L9	+ Dilutions of 10^2 and 10^4 still active	+ Dilutions of 10^2 and 10^4 were still active
PNL-A	-	+ Dilutions of 10^2 and 10^4 were not active
PNL-B	+ Dilutions of 10^2 and 10^4 still active	+ 10^2 dilution was active; 10^4 dilution was not active
PNL-C	+ Dilutions of 10^2 and 10^4 not active	+ Dilutions of 10^2 and 10^4 were not active

Spore suspensions were added by drops from sterile rods to a lawn containing 10^8 spores of B. licheniformis. "+" denotes lysis on the B. licheniformis lawn, and "-" denotes no lysis.

Table 6. Activity of Filtrates Prepared from NBYG Broth Cultures on a Lawn of B. licheniformis

Filtrate	lysis on B. licheniformis
W-23-S^r	-
W-23-S^r-L9	+ Dilutions of 10^2 and 10^4 still active
PNL-A	-
PNL-B	-
PNL-C	-

Filtrates free of bacteria prepared from 16-hour cultures were added to a lawn containing 10^8 B. licheniformis spores. Drops were added with sterile rods. "+" denotes lysis of B. licheniformis, and "-" denotes no lysis.

Table 7. Effect of ultraviolet irradiation on PNL-A in PA broth

Irradiation time	W-23-S^r		PNL-A	
	Percent transmission	pfu/ml	Percent transmission	pfu/ml
0	3.3	0	4.0	0
15 sec	4.2	0	6.0	0
30 sec	7.9	0	8.0	0
45 sec	11.5	0	20.0	0
60 sec	18.4	0	58.0	0

Percent transmission was read at 660 mμ after cultures were incubated on a shaker for 7 hours. Supernatant fluids were assayed against W-23-S^r after the 7 hours incubation.

Table 8. Effect of Glucose on Phage Yields

medium	pfu/ml W-23-S^r-L9	PNL-A	PNL-B	PNL-C
NBY	3.1×10^8	0	4.0×10^8	5.5×10^4
	4.0×10^8	0	6.0×10^7	8.8×10^4
	4.3×10^8	0	1.4×10^8	2.2×10^4
NBY + glucose	2.5×10^9	0	3.6×10^4	0
	6.2×10^8	0	3.9×10^4	0
	6.5×10^8	0	2.6×10^4	0
NBY + 1 percent glycerol	1.3×10^8	0	4.5×10^4	0
	1.0×10^8	0	4.7×10^4	0
	1.2×10^8	0	4.4×10^4	0
NB	1.0×10^5	0	1.4×10^6	4.0×10^1
	1.5×10^5	0	7.0×10^5	1.0×10^2
	3.0×10^5	0	6.0×10^5	3.0×10^1
NB + glucose	1.5×10^5	0	2.5×10^4	0
	1.5×10^5	0	2.4×10^4	0
	1.0×10^5	0	2.4×10^4	0
PA	6.0×10^9	0	3.3×10^{10}	5.3×10^{10}
	6.0×10^9	0	3.3×10^{10}	3.9×10^{10}
	3.3×10^9	0	5.3×10^{10}	3.6×10^{10}
PA + glucose	1.5×10^7	0	1.7×10^8	1.7×10^9
	1.0×10^7	0	1.7×10^8	2.2×10^9
	2.0×10^7	0	1.5×10^9	6.2×10^9
			2.9×10^9	
			2.4×10^9	

Values are given for replicate flasks.

Table 9. Effects of Salts in PA Medium on Phage Yields

medium	pfu/ml W-23-S^r-L9	PNL-A	PNL-B	PNL-C
NB	1.5×10^5	0	7.0×10^5	4.0×10^1
	3.0×10^5	0	1.4×10^6	1.0×10^2
	1.0×10^5	0	6.0×10^5	3.0×10^1
NB + $MnSO_4$ (0.0003 M)	5.9×10^6	0	1.2×10^{10}	2.2×10^9
	2.2×10^6	0	6.6×10^9	1.1×10^9
	1.9×10^7	0	2.6×10^9	1.1×10^9
NB + $CaCl_2$ (0.001 M)	5.8×10^9	0	1.9×10^8	3.8×10^5
	2.7×10^9	0	2.2×10^9	6.0×10^3
	1.2×10^9	0	7.1×10^9	7.0×10^3
				6.8×10^6
				1.8×10^2
				1.4×10^2
NB + $MgSO_4$ (0.0008 M)	2.3×10^7	0	6.2×10^4	4.2×10^2
	7.6×10^7	0	1.2×10^5	4.4×10^2
	1.1×10^8	0	2.0×10^5	4.8×10^2
NB + NaCl (0.086 M)	7.7×10^9	0	1.5×10^4	4.4×10^2
	3.4×10^9	0	2.0×10^4	3.0×10^1
	1.4×10^9	0	3.0×10^4	6.0×10^1
NB + all salts (PA broth)	6.0×10^9	0	6.4×10^{10}	3.9×10^{10}
	6.0×10^9	0	4.2×10^{10}	5.3×10^{10}
	3.3×10^9	0	5.0×10^{10}	3.6×10^{10}

Values are given for replicate flasks.

Table 10. Effects of Different Concentrations of $MnSO_4$ on Phage Production by PNL-B in Nutrient Broth

$MnSO_4$ moles/liter	pfu/ml
none	1.4×10^6 7.0×10^5 6.0×10^5
0.000003	3.9×10^9 3.0×10^8 2.7×10^8
0.00006	2.7×10^8 1.8×10^8 1.4×10^9
0.0003	4.9×10^9 3.4×10^9 6.6×10^9
0.0015	2.3×10^{10} 1.0×10^{10} 1.6×10^{11}
0.0030	3.1×10^5 1.4×10^6 2.2×10^5

Values are given for replicate flasks

*Concentration is the same as that used in PA broth

Table 11. Effect of Different Concentrations of $CaCl_2$ in Nutrient Broth on Phage Production by PNL-B

$CaCl_2$ moles/liter	pfu/ml
none	1.4×10^6
	7.0×10^5
	6.0×10^5
0.0001	3.2×10^7
	2.5×10^7
	1.7×10^7
0.0008	3.0×10^8
	1.8×10^8
	3.7×10^8
0.0013 *	1.9×10^8
	1.3×10^7
	2.6×10^{10}
0.0078	1.3×10^7
	2.0×10^7
	3.5×10^8
0.0135	less than 10^5
	"
	"

Values are given for replicate flasks.

*Concentration is the same as that used in PA broth.

Table 12. Activity of Sp-10 phage antiserum against the phage of PNL strains

	pfu/ml after 30 min incubation
PNL-B filtrate + NS	1×10^{10} pfu/ml
PNL-B filtrate + AS	0
PNL-C filtrate + NS	4.2×10^{9} pfu/ml
PNL-C filtrate + AS	0
Sp-10 filtrate + NS	2.1×10^{9} pfu/ml
Sp-10 filtrate + AS	0

Phage from PNL strains was produced in PA broth. Equal volumes of filtrate and serum, totaling 1 ml, were incubated statically at 37°C. "NS" denotes normal serum, and "AS" denotes antiserum.

Table 13. Activity of PNL-C phage antiserum against Sp-10 Phage

	pfu/ml	
	30 min. incubation	60 min. incubation
PNL-C phage + PS	3×10^9	4×10^9
PNL-C phage + AS	0	0
Sp-10 phage + PS	1×10^8	1×10^8
Sp-10 phage + AS	0	0

Phage from PNL-C was produced in PA broth, and L9 phage (Sp-10) was produced in NBYG. Equal volumes of phage and serum totaling 1 ml were incubated statically. "PS" denotes preimmune serum, and "AS" denotes antiserum.

Table 14. Activity of Sp-10 Phage and PNL-C Phage Antisera Against the VL Mutant

Addition to VL phage	pfu/ml after 30 min. incubation
none	2.0×10^8
NS	2.4×10^8
Sp-10 AS	0
PNL-C phage AS	0

VL phage was produced in NBY broth. Volumes totaling 1 ml were incubated statically at 37°C. Mixtures contained 0.5 ml phage suspension and 0.5 ml serum or peptone diluent. NS is an abbreviation for normal serum, and AS stands for antiserum.

Table 15. Phage Production and Sporulation by W-23-S^r-L9 in Potato Broth and NBYG

strain	time	medium	spores/ml	pfu/ml*
W-23-S^r	16 hours	NBYG	2.4×10^2	
			1.1×10^2	
		potato broth	4.7×10^1	
			2.5×10^2	
	3 days	NBYG	2.9×10^4	
			5.0×10^3	
		potato broth	6.9×10^8	
			2.0×10^7	
	6 days	NBYG	1.8×10^5	
			5.5×10^6	
		potato broth	6.9×10^8	
			2.0×10^7	
W-23-S^r-L9	16 hours	NBYG	1.2×10^3	2.0×10^9
			2.9×10^3	1.6×10^9
		potato broth	3.4×10^3	2.5×10^9
			7.7×10^2	6.1×10^9
	3 days	NBYG	2.0×10^3	2.0×10^3
			3.1×10^4	9.0×10^6
		potato broth	2.9×10^8	5.0×10^5
			1.2×10^8	8.0×10^3
	6 days	NBYG	4.2×10^5	3.1×10^4
			4.3×10^5	3.8×10^4
		potato broth	6.0×10^8	1.0×10^3
			1.9×10^8	1.5×10^3

Values are given for duplicate flasks.

*pfu were determined in culture supernatant fluids.

Table 16. Phage Yields in Potato Broth from W-23-S^rL9 and PNL-C

Addition to medium	pfu/ml W-23-S^r-L9	PNL-C
none	3.4×10^9	1.1×10^7
	4.6×10^9	6.1×10^6
	1.6×10^9	4.8×10^6
sodium citrate (0.05 M)	1.1×10^9	2.2×10^3
	1.3×10^9	2.6×10^3
	3.6×10^9	3.1×10^3
$MnSO_4$ (0.0003 M)	not done	1.9×10^9
		6.3×10^8
		2.0×10^9

Values are given for replicate flasks.

Table 17. Infective Centers in Various Spore Suspensions of PNL-C

culture	sporulation medium	washed spore suspension	
		spores/ml	pfu/ml
W-23-S^r-L9	potato agar	4.0×10^8	2.0×10^8
PNL-C	potato agar	8.0×10^8	1.2×10^4
"	potato broth	8.6×10^8	2.9×10^8
"	potato broth + $MnSO_4$	1.0×10^9	2.9×10^8
"	potato broth + citrate	4.2×10^9	4.4×10^5

Spore suspensions had been heated 60 minutes at 65° C to inactivate any free phage particles.

Table 18. Phage Yields in PA and NBYG from PNL-C Spores Produced in Different Sporulation media

sporulation medium	experiment	phage yields (pfu/ml) NBYG	PA
potato agar		0	5.3×10^{10}
		0	3.9×10^{10}
		0	3.6×10^{10}
potato broth	1	8.9×10^{8}	2.5×10^{10}
		8.6×10^{8}	2.0×10^{10}
		9.7×10^{8}	2.2×10^{10}
"	2	8.0×10^{8}	2.8×10^{10}
		8.2×10^{8}	2.9×10^{10}
		7.2×10^{7}	1.5×10^{10}
"	3	2.0×10^{8}	1.6×10^{10}
		1.5×10^{7}	1.5×10^{10}
		5.9×10^{7}	1.6×10^{10}
potato broth + $MnSO_4$ (0.0003 M)	1	1.4×10^{9}	2.5×10^{9}
		1.4×10^{9}	2.4×10^{9}
		1.3×10^{8}	2.1×10^{9}
"	2	5.3×10^{8}	1.4×10^{9}
		7.6×10^{8}	5.0×10^{8}
		7.7×10^{9}	8.2×10^{10}
	3	1.0×10^{9}	1.1×10^{10}
		1.2×10^{9}	1.2×10^{9}
		1.5×10^{9}	8.0×10^{9}
potato broth + sodium citrate (0.05M)	1	8.4×10^{4}	1.4×10^{10}
		4.2×10^{4}	4.3×10^{10}
		3.1×10^{2}	2.4×10^{10}
	2	6.4×10^{2}	2.0×10^{10}
		6.5×10^{2}	2.9×10^{10}
		7.6×10^{2}	3.2×10^{10}
	3	2.3×10^{3}	3.9×10^{10}
		2.0×10^{3}	3.9×10^{10}
		1.9×10^{3}	3.3×10^{10}

Values are given for replicate flasks.

BIBLIOGRAPHY

1. Adams, Mark H. Bacteriophage. New York, Interscience Publishers, 1959. 592 p.

2. Allen, Lois Kendren. Studies on transduction in lactic Streptococcus organisms. M.S. Thesis. Corvallis, Oregon State University, 1962. 88 numb. leaves.

3. Clarke, Norman A. Studies on the host-virus relationship in a lysogenic strain of Bacillus megaterium. II. The growth of Bacillus megaterium in a synthetic medium. Journal of Bacteriology 63:187-192. 1952.

4. Cowles, Philip B. The recovery of bacteriophage from filtrates derived from heated spore suspensions. Journal of Bacteriology 22:119-123. 1931.

5. DeCarlo, M. R., W.B. Sarles and S.G. Knight. Lysogenicity of Bacillus megatherium. Journal of Bacteriology 65:53-55. 1953.

6. Ehrlich, H. L. and D. W. Watson. Lysogenesis of Bacillus megaterium. Journal of Bacteriology 58:627-632. 1949.

7. Ephrati-Elizur, Erela and Maurice S. Fox. Comparison of transduction versus transformation of linked markers in Bacillus subtilis. Nature 192:433-434. 1961.

8. Friedman, Murray. The bacteriophages of Bacillus megaterium. II. Stability in mono- and divalent ions. Journal of Bacteriology 66:501-502. 1953.

9. Goldberg, Ivan D. and Krishnamarty Gollakota. Bacteriophage in Bacillus cereus. In: Spores. Minneapolis, Burgess, 1961, p. 276-289.

10. Groman, Neal B. Phage-host relationships in some genera of medical significance. Annual Review of Microbiology 15: 153-176. 1961.

11. Huybers, Kenneth. Cationic reversion of induced phage development in Bacillus megaterium. Annales de l'Institut Pasteur 84:242-253. 1953.

12. Iványovics, G. and K. Csiszár. Some characteristics of subtilis phages with transducing activity. Naturwissenschaften 49: 309-310. 1962.

13. Jacob, Francois and Elie L. Wollman. Genetic aspects of lysogeny. In: The chemical basis of heredity. Baltimore, John Hopkins University Press, 1957, p. 468-499.

14. _____. Sexuality and the genetics of bacteria. New York, Academic Press, 1961. 334 p.

15. Kohiyama, M. and Y. Ikeda. Anomalous genetic interactions observed in Bacillus subtilis. Nature 184:208-209. 1959.

16. Lwoff, A. and L. Siminovitch. Induction of prophage development by reducing substances. Annales de l'Institut Pasteur 82:676-689. 1952. (Abstracted in Chemical Abstracts 46: 11308e. 1952.)

17. Lwoff, André. Significance of bivalent cations in inducing the development of prophage through the agency of reducing agents. Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences 234:366-368. 1952. (Abstracted in Chemical Abstracts 46:6697b. 1952)

18. _____. Lysogeny. Bacteriological Reviews 17:269-337. 1953.

19. Lwoff, André and Antionette Gutmann. Investigations on a lysogenic Bacillus megaterium. In: Papers on bacterial viruses. New York, Little, Brown, 1960, p. 312-331.

20. Lwoff, André, Louis Siminovitch and Neils Kjeldgaard. Induction of bacteriophage lysis of an entire population of lysogenic bacteria. In: Papers on bacterial viruses. New York, Little, Brown, 1960, p. 332-333.

21. McCloy, E. W. Lysogenicity and immunity to Bacillus phage W. Journal of General Microbiology 18:198-220. 1958.

22. Romig, W. R. and A. M. Brodetsky. Isolation and preliminary characterization of bacteriophages for Bacillus subtilis. Journal of Bacteriology 83:135-141. 1961.

23. Siminovitch, Louis. Relation between abortive development of prophage in Bacillus megatherium 91 and the synthesis of desoxyribonucleic acid. Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences 233:1694-1696. 1951. (Abstracted in Chemical Abstracts 46:8713i. 1952.)

24. Spizizen, John. Transformation of biochemically deficient strains of Bacillus subtilis by deoxribonucleate. Proceedings of the National Academy of Sciences 44:1072-1078. 1958.

25. Takagi, J. and Y. Ikeda. Transformation of genetic traits by the DNA prepared from a lysogenic phage, S-1. Biochemical and Biophysical Research Communications 7:482-485. 1962.

26. Taylor, W. J. and B. W. Koft. "Curing" of lysogenicity in Staphylococcus by fumagillin. Bacteriological Proceedings. 1961, p. 165.

27. Takahashi, I. Genetic transduction of Bacillus subtilis. Biochemical and Biophysical Research Communications 3:171-175. 1961.

28. Thorne, Curtis B. Transduction in Bacillus subtilis. Federation Proceedings 20:254. 1961.

29. _____. Transduction in Bacillus subtilis. Journal of Bacteriology 83:106-111. 1962.

30. Whitfield, J. F. Lysogeny. British Medical Bulletin 18:56-63. 1962.

31. Wollman, E. and Mme. E. Wollman. Behavior of lysogenic Bacillus megatherium and its bacteriophage in decalcified media. Comptes Rendus des Séances de la Société de Biologie 121:302-303. 1936. (Abstracted in Chemical Abstracts 30:3013. 1936.)

32. Zinder, N. and J. Lederberg. Genetic exchange in Salmonella. Journal of Bacteriology 64:679-699. 1952.