ISOLATION OF BACTERIOPHAGES FROM PASTEURELLA MULTOCIDA

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ABSTRACT

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PASTEURELLA MULTOCIDA

By

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A modification of the Fisk technique was employed to screen 35 strains of *Pasteurella multocida* for lysogeny. In this technique each potential lysogenic culture was spotted on a lawn of each indicator strain. Cultures were also examined for lysogeny before and after treatment with ultraviolet light (UV) and mitomycin C.

Those combinations of producer vs. indicator strain which showed signs of lytic activity in the above tests were mixed together in soft agar in an attempt to demonstrate plaque formation. Four combinations showed signs of plaque formation and one of them was selected for further study.

The bacteriolytic agent was isolated from the selected producer strain and electron micrographs were taken. Tests were also performed to determine whether or not divalent cations were required as lysis cofactors.

It was found that mitomycin C induced a higher percentage of lysogeny among the 35 strains than did UV treatment. It was also demonstrated that the bacteriolygic agent was a bacteriophage rather than a bacteriocin. The electron micrographs provided the first visual evidence of a temperate bacteriophage isolated from a lysogenic strain of *P. multocida*.

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INTRODUCTION

For many years *Pasteurella multocida* has been recognized as an important animal pathogen (20). A considerable number of human infections due to this species have been reported in recent years (19). The following animal diseases are associated with *P. multocida*: hemorrhagic septicemia, enzootic swine pneumonia, fowl cholera, and snuffles in rabbits. Human infections by *P. multocida* are most often local infections following cat or dog bites, and less frequently infections involving the respiratory or central nervous system (43).

Many serologic varieties are included in the species *P. multocida*. Over the years there have been numerous attempts to group the members of this species on the basis of their serologic characteristics (20). One method devised by Carter separated strains on the basis of differences in capsular substances (18). Five serotypes, viz., A, B, C, D and E, were identified by this procedure. Namioka and Murata devised an agglutination procedure for the identification of somatic or O antigens (65). They identified 11 different O groups. They then combined Carter's identification of the capsular or K antigen with their identification of the O antigens to fully designate strains in a manner similar to that used for the designation of serotypes of *Escherichia coli*, e.g., the well known bovine hemorrhagic septicemia strains were designated 6:B, 6 representing the O group and B standing for the capsular antigen. Although these procedures are effective they are rather onerous and there is a need for a simpler typing procedure.

Since it has been known for many years that lysogeny exists among strains of *P. multocida*, the development and application of a phage typing procedure would seem to be a possibility. Such a typing method would probably be less onerous than the procedures in current use.

In this study 35 strains of *P. multocida* were examined for lysogeny using a modification of the Fisk method (31). The strains were then exposed to ultraviolet light (UV) and mitomycin C to induce the lysogenic strains to produce bacteriophage. One bacteriophage was selected and partially purified from those demonstrated. This agent was examined with the electron microscope. Tests were performed to determine whether or not divalent cations were required as lysis cofactors. In the course of this study an effective method for isolating temperate bacteriophages from lysogenic strains of *P. multocida* was developed.

REVIEW OF THE LITERATURE

Historical

The discovery of bacterial viruses probably dates back to 1915, when F. W. Twort isolated a filterable agent which produced a "glassy transformation" of micrococcal colonies during their growth on an agar surface (77). Twort believed that this bacterial lysis was a result of an acute infectious disease of the bacteria. He succeeded in transferring the active principle from vitreous to normal colonies, showed that it was filterable, that the number of lesions was inversely proportional to the dilution, and that it multiplied and reproduced. Twort's observations were published in 1915 (80). Twort made some speculations on the possible nature of the infectious agent. He stated that it could be either of the following: (a) an ultravirus, (b) a small parasite reproducing at the expense of the bacterium, (c) a phase in the life cycle of the micrococcus, (d) an autocatalytic enzyme, or (e) a primitive form of life.

Two years later, d'Herelle published his observations on a filterable bacteriolytic substance that he isolated from feces. D'Herelle (23) named the agent bacteriophage, meaning "eater of bacteria". D'Herelle (2) pointed out the ability of bacteriophages to lyse growing bacterial cultures; he also presented evidence that the lysis was accompanied by production of more bacteriophage and that the lytic agent was transmissible in series from culture to culture of susceptible bacteria.

In 1926, d'Herelle (24) summarized his earlier findings on the multiplication of bacterial viruses:

> "The first act of bacteriophagy consists in the approach of the bacteriophage corpuscle toward the bacteria, then in the fixation of the corpuscle to the latter...The bacteriophage corpuscle penetrates into the interior of the bacterial cell. When, as a result of its faculty of multiplication, the bacteriophage corpuscle which has penetrated into the bacterium forms a colony of a number of elements, the bacterium ruptures suddenly, liberating into the medium young corpuscles which are then already to continue the action."

In 1929, Burnet offered some convincing support in favor of d'Herelle's conception that the infecting bacteriophage (later called phage) particle multiplies within the bacterium, and that its progeny are liberated upon lysis of the host cell (13). He showed that 20 to 100 viruses suddenly appeared some 20 minutes after a bacterial suspension was infected with a single phage particle.

Although Burnet accepted d'Herelle's basic view of the bacteriophage as a virus, he disposed of the idea propagated by d'Herelle that the phage should be considered as a single, highly variable species. Instead, Burnet showed that bacteriophages differ so widely in their physical and physiological properties that a great variety of agents must be included in this classification. Studies of the immunology of bacteriophages showed that different phage types carry entirely different antigens and that serological cross-reaction, rather than range of sensitive bacterial species, was the best criterion for establishing the relatedness of bacteriophage strains. Burnet also investigated the role of the bacterial surface in the fixation, or adsorption, of the bacteriophage particle to its host cell. To explain why each phage type is only active on a rather restricted and characteristic number of bacterial strains, he proposed that the initial contact between infecting virus

and bacterial surface is a stereospecific process between complementary structures on virus and cell, analogous to an antigen-antibody reaction and that phage resistant variants which appear in bacterial cultures after exposure to phage owe their resistance to a hereditary alteration in the cell surfaces. This cell surface alteration no longer permits the specific attachment of the virus particle to its bacterial receptor sites.

In addition to these experiments, Burnet (13) devised the progenitor of the modern single-burst experiment. This experiment adduced convincing proof of d'Herelle's contention that phage particles first accumulate inside the infected bacterium and are then suddenly released by the cell.

Schlesinger studied the properties of virus particles by chemical and physiochemical methods, and provided the first insights as to what bacteriophages really are. Using indirect means, such as the sedimentation velocity of a phage particle in centrifugal fields or the capacity of the bacterial cell for phage adsorption, Schlesinger (74) was able to determine the maximum linear dimension and mass of various phage particles. Schlesinger (73) also studied the adsorption mechanism of the virus to its host cell and found that the kinetics of this process imply that Brownian movement brings virus particles into random collisions with the bacterial surface. The small size of a bacterial virus renders it invisible under ordinary microscopes; however, Schlesinger was able to directly estimate the total number of phage particles in a purified virus preparation by counting the number of bright points produced in a dark-field microscope. Schlesinger (75) made an even greater contribution when he managed to purify a substantial amount of phage by differential centrifugation and graded filtration of crude phage suspensions. After purifying the phage he found by direct chemical analysis that the

virus consists mainly of protein and of deoxyribonucleic acid (DNA) in roughly equal proportions.

Schlesinger's work was extended by Delbrück (25), who showed that the physiological state of bacteria affects the rate of adsorption, and by Garen and Puck (33), who demonstrated that under certain conditions a reversible union between phage and bacterium can take place. It was also pointed out that the rate of phage adsorption reaches a maximum at high bacterial concentrations and is highly temperature dependent. Ellis and Delbrück (30) described the single burst experiment which made it possible to study phage growth in individual infected bacteria, rather than in mass culture. Subsequent electron-optical observations by Anderson (5) revealed that the organ of adsorption is the phage tail, in particular, that tail fibers are the structures which undergo the stereospecific fixation reaction with the phage receptors on the bacterial surface.

Bacteriophage Multiplication

Some important studies on the kinetics of intracellular phage multiplication were conducted by Doermann (29). He lysed phage-infected bacteria at various times during the latent period and assayed the infectivity of the material released by premature lysis. It was found that the infectivity associated with the original parental virus is lost at the outset of the reproductive process, since no infective particles are found in any of the bacteria lysed within ten minutes after their infection. Ever-increasing numbers of infective progeny viruses make their intracellular appearance after more than ten minutes have elapsed. These new infective progeny viruses increased in number until a crop of progeny viruses had been attained, which would have been released by

spontaneous lysis-from-within at the end of the normal latent period. The stage of intracellular bacterial virus growth during which the host cell contains no infective viral particles is called the eclipse. Subsequent studies showed that the actual multiplication of the infecting virus takes place during the eclipse. The phage multiplies in a non-infectious form, the vegetative phage.

As previously mentioned, Schlesinger was the first person to purify a bacterial virus and to determine that it consists of approximately equal proportions of protein and DNA. Subsequent studies by Anderson (4) and by Herriott (37) showed that the viral DNA resides within a proteinaceous head membrane, from which it can be released by osmotic shock.

Hershey and Chase (41) found that practically all of the viral protein remained at the surface of the bacterial cell, and at the outset of intracellular phage growth it was mainly the viral DNA which entered the bacterium. This discovery implicated the viral DNA as being the carrier of the hereditary information.

In order to find out what happens to the viral DNA after its injection into the host cell, Putnam and Kozloff (68) devised an experiment to find out whether any of the atoms of the parental DNA ultimately reappear among the progeny viruses. Using isotopically labeled phage DNA in their "transfer experiment," they found that about half of the atoms of the parental DNA were transferred to the progeny.

For many years the exact nature of phage multiplication was not fully resolved. One theory envisaged that bacteriophage precursors are present within the normal host bacterium and the metamorphosis of these bacteriophage precursors is triggered by the infecting phage particle (52).

In order to settle some of the controversy that surrounded this problem, Cohen (21) designed an experiment to determine the origin of the substance of the phage progeny. He exposed bacterial cultures to P^{32} either prior to or subsequent to their infection with phage. The virus progeny were then analyzed for their relative content of radioisotope. In this manner, Cohen was able to show that most of the phage DNA was synthesized from materials still in the growth medium at the moment of infection; therefore, the phage particles were not derived, to a great degree, from preexisting bacterial precursors.

Further insight into the process of phage multiplication was provided after the discovery of various incomplete phage structures which possessed many of the properties of the virus while being incapable of completing a reproductive cycle. Maaløe and Watson (61) found that premature lysis of infected bacteria at late stages of the eclipse period liberated newly synthesized proteinaceous material which possessed some of the antigenic properties of the intact bacteriophage. Observations of such lysates with the electron microscope revealed the presence of structures which were morphologically similar to mature phage particles (36). Some structures were observed which looked similar to "doughnuts" (54). These doughnut-like structures appeared during the eclipse period and they increased in number at a rate which was similar to that for the formation of complete phage particles. Kellenberger (48) showed that these structures were actually empty phage heads, and the mature infective progeny represent the union of phage precursor DNA with phage precursor protein.

In addition to controlling the synthesis of phage precursor protein and phage precursor DNA, the DNA of the infecting parental virus also presides over the synthesis of other materials. The formation of some

nonprecursor proteins at the outset of intracellular phage growth is essential before replication of the viral DNA can begin. Deoxycytidylate hydroxmethylase is one early protein which was identified by Flaks and Cohen (32). This enzyme is essential for the synthesis of specific viral DNA components; namely, deoxy-5-hydroxymethyl-cytidine-5'-phosphate. According to Stent (78), Kornberg and his collaborators discovered a number of other enzymes which are phage-induced and all of which are involved in the synthesis and replication of phage DNA.

Phage Genetics

Phage genetics is an area of research which has attracted the attention of many prominent phage researchers. Perhaps the greatest emphasis in this area has been placed on the study of mutations in bacteriophage and genetic recombination.

Mutant phages may differ from their parents in a variety of observable characteristics. They may differ with respect to bacterial host specificity, plaque morphology on agar seeded with sensitive indicator bacteria, or in certain chemical or physical properties (39,56).

Luria (58) was among the first of the investigators who studied the nature of self-duplication of the hereditary material of the infecting phage particle. He observed the mutation frequency of the vegetative phage during intracellular growth. From such studies Luria concluded that the replication of phage hereditary material proceeds by a number of successive cycles of self-duplication.

Genetic recombination in phage has been studied by many investigators (26,40). Often the genetic character of phage has been studied by observing the phage progeny from bacterial cells infected with two different but related bacterial viruses. By carefully observing the

recombination frequency for various mutated characters, Hershey and Rotman (40) were able to construct a genetic map of T2 phage by arranging the mutated loci in a linear order. Benzer (7) also constructed a fine structure map based on his observations of phage crosses between parents having closely linked mutant loci.

An exceedingly rapid chain of events is initiated when a bacterial virus collides with a host cell. These events are triggered when the phage becomes fixed or adsorbed to the cell surface. It is thought that the bacterial host cell does not play an active role in the adsorption of phage to the cell surface. This opinion is strongly supported by the fact that virus particles may be irreversibly fixed to cyanide-poisoned or heat-killed bacteria, as well as disrupted cell fragments (78).

The bacterial cell wall is thought to have some phage-specific receptor sites which can undergo irreversible chemical reaction with the phage attachment organs. Weidel, Frank and Martin (83) did chemical fractionation studies on the cell wall of *E. coli* in an attempt to identify the phage receptor site. On the basis of these studies it was proposed that the cell wall of *E. coli* is constructed of at least three distinct layers. The outer layers are composed of a lipoprotein and lipopolysaccharide. The inner layer comprises about ten percent of the cell wall weight and it is a mucopolymer which gives the cell wall its rigidity. Weidel *et al.* found that only the two outer layers carry phage receptors, and the inner layer is devoid of any capacity to adsorb phage. It has been suggested by Stent (78) that the bacterial surface might be imagined as a mosaic of different phage receptor sites, with each receptor having the capability of forming specific bonds with the phage to which it is receptive. The attachment of a phage particle

to a bacterial cell involves the interplay between the detailed configuration of some specific chemical surfaces on the phage proteins and the bacterial cell wall (59).

Some phages attach themselves specifically onto appendages of the bacterial cell. Phage χ adsorbs only to flagella and apparently makes its way to the bacterial surface by sliding down the flagellum (64). According to Zinder (84) the male-specific RNA phages of *E. coli* combine with some fimbriae or pili that are present only in male strains.

Both cells and viruses have surfaces composed of macromolecular structures which have charged groups attached. Chemical studies have been made on the attachment of phages T1 and T2 to *E. coli* and it has been shown that the amino, or substituted amino groups, and the carboxyl group are the primary participants in initial binding. It has been found in the case of the T2 phage that the positively charged nitrogens on the virus form bonds with the negatively charged carboxyl radicals on the cell. Evidence from studies on the binding of T1 phage to *E. coli* indicates that both types of groupings on both surfaces are involved (67).

Adsorption

From all indications, the irreversible adsorption of phage to bacterial surface requires a high degree of stereospecificity. This is probably the leading factor which determines the range of bacterial strains to which a particular virus can adsorb.

In addition to a high degree of stereospecificity which may be required for irreversible adsorption, the composition of the medium in which the two bodies are suspended also determines whether, and how well, adsorption occurs (24,38).

Influence of the Medium

The pH and ionic composition of the medium play an important role in the attachment process (2,15,67,78).

According to Puck (67) there is a strict requirement for each virus that the medium contain cations of a certain valence and concentration range to facilitate contact between the active chemical groups of both surfaces. It should be remembered, however, that fulfillment of this condition alone will not insure that adsorption occurs. The T-series phages which are normally receptive to *E. coli* are not fixed in distilled water or in buffered saline at a pH below five, or above twelve. In distilled water or in very dilute buffer at physiological pH's, most phages do not attach. It is thought that under these conditions both virus and cell are strongly negatively charged, thus creating a repulsive force that prevents the close approach of the two particles which is necessary for bond formation. Inorganic cations neutralize this repulsive potential so that the critical chemical groups can react with each other. Evidence from Puck's studies indicates that the effect exerted by the inorganic cations is more important on the virus than the cell. There are optimum ionic concentrations that permit maximal adsorption rates and these are characteristic for each phage and for each ion. At concentrations above optimum there is reduction in the adsorption rate. At low cation concentrations adsorption is sometimes reversible either by dilution or by transfer to distilled water; however, at higher ionic concentrations the trend is in favor of fast irreversible fixation.

Influence of Cell Concentration and Temperature

Studies on the kinetics of adsorption of phage T4 indicated that the rate of phage adsorption increases proportionally to the bacterial

concentration at low cell densities (78). These studies also revealed that a maximum adsorption velocity was reached when more than about 5×10^8 bacteria/ml were present in the adsorption mixture.

When the temperature of the adsorption mixture was lowered from 25 C to 5 C, a ten-fold reduction in the adsorption rate constant was observed.

Adsorption Cofactors

Some phages such as T4 cannot attach in a synthetic medium unless certain cofactors have been added. The most effective cofactor appears to be L-tryptophan (3). Some strains require tryptophan for attachment at 15 but not at 37 C; others require calcium ions in addition to the amino acid (27). Activation by tryptophan requires that five molecules of tryptophan be bound to the phage particle. It is thought that tryptophan exerts its activating effect by opening or weakening some of the bonds in the phage tail proteins. The tail fibers of phage T4 must be extended for attachment to occur, and it has been found that magnesium ions and tryptophan are necessary for extension of the tail fibers (47). Electron micrographs show that the tail fibers of cofactor-requiring strains of T-even phages are tightly wound around the tail sheath in the absence of cofactor. In this restricted fashion the virus particle cannot attach itself to the host cell; however, reaction of the tail with tryptophan cofactor molecules frees the tail fibers from embracing the sheath, and the phage becomes adsorbable.

Phage Receptors

By using a critical point technique for avoiding surface tension artifacts in specimens prepared for viewing under the electron

microscope, Anderson (5) was able to successfully demonstrate just how the virus attaches itself to a bacterial receptor site. For these studies Anderson used the T5 phage which adsorbs to *E. coli*. He was able to clearly show that bacteriophages are adsorbed by their tail and that the phage tail carries the organelles which participate in the specific fixation of phage to host cell receptors. For phages with no tail or no fibers, the attachment structure has not yet been clearly elucidated (5).

Largely through the work of Brenner and his collaborators (11), the tail structures of the T-even phages have been described in detail. The tail of the T-even phage is made of at least four protein components: a core, a sheath, a base plate, and six tail fibers. These proteins differ from the head proteins; they also differ chemically from each other.

Phage Tail Enzymes

Enzymatic activity has been associated with the tail of the T-even bacteriophages. These phages are able to hydrolyze the mucopeptide of the *E. coli* cell wall. This enzymatic activity is due to a phage lysozyme which is synthesized during phage multiplication. Some of the enzyme molecules remain attached to the phage tail after its release from the cell and aid in the injection of viral DNA by boring a hole in the mucopeptide layer of the cell wall. There are, however, some lysozyme-less phages that can still inject their DNA (22).

If the multiplicity of infection is great, i.e., the ratio of phage to bacteria is high, lysis of the bacteria will occur prior to the production of new phage. In this case the host cells are killed by "lysis from without", which is different from the "lysis from within" caused by multiplication of the virus within the cell.

Injection of Nucleic Acid

Most of the studies to explore the mechanism for injection of viral nucleic acid into the host cell have been done using the T-series bacteriophages. Injection is initiated when the tail sheath of the T-even coliphages contracts, followed by the penetration of the tail tube into the outer layers of the bacterial surface, thus facilitating the entry of DNA into the cell (48). The exact mechanism for the sheath contraction is not known; however, adenosine triphosphate is present in the phage tail and it becomes hydrolyzed to adenosine diphosphate upon injection of DNA (51).

It has been found that about ten percent of the T5 phage DNA is rapidly transferred to the host cell and the remainder enters more slowly. This first portion of DNA passes into the cell in the absence of protein synthesis while the remainder passes into the cell only after the synthesis of new protein within the bacterium (53). It has been suggested that the first portion of DNA injected into the cell contains the genetic information for this protein synthesis.

Maturation and Lysis

The latent period of productive phage development terminates with the release of mature virions from the cell. Usually the cell lyses and the mature phage particles, along with any unassembled phage components produced during the latent period, are released in a burst. Cell lysis which is observed following maturation of the phage particles appears to be caused by a lysozyme-like enzyme or lysin which is specified by the phage genes. This phage-induced lysin can attack the rigid mucopeptide layer of the cell wall, and it makes its intracellular appearance about halfway through the latent period.

There is, however, one known class of filamentous, male-specific phages which contain single-stranded circular DNA molecules and whose newly produced virions are released from the cell as they are completed; without lysis of the host cell. After the new phage particles have been produced, the host cell can produce colonies as before infection (42).

Lysogeny

Those bacteriophages which have been discussed thus far usually kill the bacteria that they infect. They are called virulent phages. There is, however, another kind of phage-cell interaction in which the viral genetic material becomes incorporated into the bacterial host genome. This intimate phage-cell relationship can persist for prolonged periods even while the cell goes through many division cycles. Phages which can undergo this type of interaction are characterized as temperate, and a bacterial cell that participates in this type of relationship is said to be lysogenic.

Historical

Within a few years of the discovery of bacteriophage by Twort and d'Herelle, some workers observed that bacterial strains could be found which appeared to "carry" bacteriophages, in the sense that phage particles are always present in the culture fluid of such strains (9).

The first to attack the problem of how this capacity to produce phage is actually propagated within lysogenic bacteria were Burnet and McKie (14). These investigators induced lysis of a lysogenic culture of *Salmonella enteritidis* with an extrinsic phage unrelated to the "carried" phage. They found that only 0.1 percent of the lysogenic bacteria liberated any intracellular phage. Burnet and McKie later proposed that lysogenic bacteria perpetuate the power to produce infectious

phage as a noninfectious anlage of the phage that multiplies in step with the cell. They further stated that it was necessary to activate the anlage in order to produce infectious phage.

The nature of phage production in lysogenic cultures was still an enigma by the late 1930's. It was generally observed that in growing cultures of lysogenic bacteria, the bacteriophage titer runs parallel with the number of bacteria in the culture. It was not known whether phage production in such cultures resulted from a continuous secretion in the course of bacterial growth or from the discontinuous lysis of a small but constant fraction of the bacterial population.

The Prophage

These problems were reinvestigated by Lwoff and Gutmann (60). They observed the multiplication of lysogenic strains of *Bacillus megatherium* and found that lysogenic bacteria can multiply without phage production; however, in some clones the sudden disappearance of cells due to lysis was observed. It was in these instances that the presence of phage was detected. They concluded that the production of phage by lysogenic bacteria is a discontinuous process which is the consequence of the lysis of a small fraction of the lysogenic population. Lysogenic bacteria survive and grow only when they do not produce phage. Their capacity for producing phage is only a potential character. Lwoff and Gutmann coined the name prophage for the noninfectious form of bacteriophage which is the bearer of this potentiality. In a small fraction of a population of growing lysogenic bacteria the prophage becomes induced to produce a crop of infective phage.

Lysogenization

Sensitive bacteria can respond to infection with temperate phages in different ways. In a certain fraction of the population the infecting phage may enter the vegetative state where the viral genes direct the synthesis of new viral components which are assembled into virions, the cell lyses and virions are released into the medium. This is called the productive response which is typical of virulent phages (2,34,45). Another fraction of the infecting phages may enter into the prophage state. In this case the bacteria survive and each clone contains lysogenic cells (lysogenic or reductive response) (2,34). In some rare cells the infecting material disappears after injection. These cells may survive without becoming lysogenic (refractory response), or they may die without releasing phage (55).

The type of response that a bacterial cell gives following infection with a temperate phage is determined within a critical period (59). Jacob and Monod proposed a model to explain the intracellular events which influence the type of response given by the infected cell. After infection, the temperate phage genes begin to function. The "early genes" direct the synthesis of enzymes which start to transcribe messenger RNA in order to direct the synthesis of a repressor substance. Next, there is a race between the production of repressor substance and the early proteins. The lytic cycle results if the early enzymes become sufficiently concentrated and viral DNA is synthesized. On the other hand, the lysogenic response ensues if the repressor substance reaches a certain concentration in the cytoplasm (44).

The lysogenic response is favored by such environmental factors as low temperature, inhibition of protein synthesis, and high multiplicity of infection (MOI) (59).

Prophage Attachment

Two general models have been suggested to account for the connection between prophage and bacterial chromosome. The attachment hypothesis proposes that the prophage pairs with its homologous region on the bacterial chromosome. On the other hand, the insertion hypothesis asserts that the prophage becomes integrated into the bacterial chromosome by some type of recombination event, and the two form a continuous structure (35). So far, only DNA phages have been found to participate in the lysogenic response. A widely accepted version of the establishment of lysogeny is as follows: The infecting phage DNA enters the cell and provided enough repressor is made so that the lytic cycle does not ensue, the phage DNA becomes inserted into the bacterial chromosome at a specific region and becomes prophage. Lambda (λ) phage has been used in most of the studies on the mechanism of prophage integration into the bacterial chromosome. One model proposed by Campbell states that after infection the λ phage DNA molecule forms a circular structure which is covalently bonded. This structure then becomes associated with the host chromosome at a region of specific genetic homology. A single crossing over then results in linear insertion of the phage genome. Prophage detachment after induction could occur through a reversal of the above process (16,35).

Immunity and the Repressor

Immunity may be thought of as a state in which the vegetative multiplication of prophage and of superinfecting related phages is prevented (59). A common mechanism is thought to restrain both the prophage and the superinfecting phage. A lysogenic cell is immune to superinfection by a closely related phage.

The prophage multiplies as part of the chromosome in immune bacteria, whereas the genome of a superinfecting phage fails to multiply because it is deprived of such a chromosomal connection. It has been found that the superinfecting phage genome does undergo a stage of intracellular circularization. The repressor substance immediately combines with the DNA of a superinfecting phage, thereby rendering it unable to replicate.

On the other hand, the virulent T-even phages prevent superinfection by causing the production of deoxyribonuclease which breaks down the DNA of a superinfecting phage.

The repressor substance which is responsible for immunity in lysogenic bacteria is specified by the immunity gene of temperate phages (46). In phage λ the repressor gene is called cI and it is identified by the fact that its mutations lead to either a loss of immunity or to production of a noninducible mutant.

The repressor substance has been characterized as a protein molecule with a molecular weight of about 30,000 daltons. It is a gene product that can diffuse away from its site of production to function at other locations in the bacterium (66).

Phage Production by Lysogenic Bacteria

Spontaneous

Cultures of lysogenic bacteria may contain free phage particles. The rate of spontaneous production of free phage particles by a lysogenic strain of bacteria is a constant for a given set of conditions. Depending upon the strain and the prophage propagated, the rate of spontaneous production of phage may vary between 10^{-2} and 10^{-5} (45).

Some observations have been made on polylysogenic strains in which every bacterium is capable of producing two or more phage types, and each type of phage is produced at a characteristic rate. It was found that the rate of spontaneous production of one type is apparently independent of the production of the other type when the newly produced phages are unrelated (8).

Induced

Lwoff and his associates found that when certain strains of *B. megatherium* were irradiated with suitable doses of ultraviolet light (UV), all the cells in the culture lysed and produced phage. The cells did not lyse immediately after UV treatment, but continued growing without division for a time period corresponding to one or two generations. After this latent period the cells lysed, releasing virus particles in the process (60).

After this observation was reported, it was soon discovered that other agents could also cause induction. These agents include nitrogen mustard, x-rays, λ-rays, organic peroxides, epoxides, ethyleneimines, mitomycin C, 5-fluorouracil, and thymine starvation (45,62,63).

Many investigators believe that the inducing agents act by inhibiting the production of repressor substance. Once the concentration of

repressor molecules falls below a certain critical value, the prophage enters the vegetative cycle (34). Some investigators believe, however, that prophage induction is caused by a change in activity of the repressor substance possibly due to accumulation of some DNA precursor (59).

Zygotic induction is another example of prophage induction. Jacob and Wollman (45) noted that conjugation between lysogenic bacteria often resulted in induction. Using *E. coli*, they found that the conjugation culture was induced after the genome from a λ phage lysogenic male, along with the prophage, was introduced into a nonlysogenic female cell. There was apparently no exchange of cytoplasmic substances; therefore, the prophage from the male cell was introduced into an environment with no repressor substance. The female cell then became induced, and the cell lysed releasing a crop of phage after a latent period.

Factors Controlling Induction

According to Adams (2), there are at least three factors that control the induction process:

A. There seems to be a genetic factor involved since only certain lysogenic systems are inducible. Inducibility is apparently a property of the prophage rather than the bacterial strain.

B. Exposure of lysogenic strains to inducing agents is required for induction. Most of the inducing agents also exhibit mutagenic and carcinogenic activity.

C. Finally, the physiological condition of the culture plays an important role in determining the bacterial response to the inducing agent.

Detection of Lysogeny

In order to recognize the lysogenic character of a bacterial strain it is necessary to have available a sensitive indicator strain. When tests are run to determine whether a particular bacterial strain is, or is not, lysogenic, the usual procedure is to assay cultures of the strain by commonly practiced phage techniques on a variety of other strains of the same or closely related species. If plaques are observed on any of these strains and are consistently formed by cultures grown from serial isolations of single colonies, then the strain being tested may be considered lysogenic (45).

Defective Lysogenic Bacteria

In some lysogenic strains certain mutations of the prophage prevent synthesis of infectious phage. The prophage is still perpetuated; however, this prophage is defective and the bacterial strains that carry such mutant prophages are called defective lysogenic strains (2,22).

Curing

Lysogeny is usually a very stable hereditary property of a bacterial strain. Therefore, in a given population of lysogenic cells only a small percentage of individuals appear to lose their prophage. When a lysogenic cell does lose its prophage, the cell is then said to be cured (18).

Lysogeny Associated with *Pasteurella multocida*

For many years it has been known that some cultures of *Pasteurella multocida* are lysogenic (20). There have been a number of studies to determine the incidence of lysogeny among isolates of *P. multocida* from animal infections. Rifkind and Picket (69) tested 118 strains of

P. multocida for lysogeny and were able to associate phage activity with 16 of the strains. They reported that 84 of the 118 strains were sensitive to one or more of the ly bacteriophages. In these same experiments they found that some of the phages required calcium or magesium ions to cause lysis in broth. In another study Kirchner and Eisenstark (50) screened 25 *P. multocida* isolates from diseased fowl to determine the extent of lysogeny. They detected 11 lysogenic strains among the 25 isolates. Dhanda (28) examined 38 strains and found that 4 were lysogenic. Saxena and Hoerlein (72) have also examined lysogeny among strains of *P. multocida*.

MATERIALS AND METHODS

Cultures

Thirty-five strains of *Pasteurella multocida* were used in this study. Eighteen were from the Michigan State University Clinical Microbiology Laboratory. The other seventeen were obtained from Dr. G. R. Carter, Michigan State University.

All stock cultures were maintained on Bacto stock culture agar and kept at room temperature. The cultures were transferred to fresh stock culture agar at intervals of 4 months.

Determination of Culture Purity

Initially all strains were examined for possible contamination. Each strain was streaked on blood agar (BBL-Trypticase Soy Agar with 7% bovine blood) to obtain discrete colonies. Plates were incubated at 37 C for 24 hours, at which time the colonial morphology was studied. Subcultures were made from characteristic colonies which were composed of small Gram negative coccobacilli. The numbering scheme that was employed is shown in Table 1.

Screening for Phage Carriers

The following media were used for the screening procedures. Tryptose agar (TA) was prepared by adding 41 g of the dehydrated medium (Difco) to one liter of distilled water. The tryptose soft agar contained one-half the concentration of agar contained in the TA just referred to.

The concentrations of the hard and soft agars were 1.5% and 0.75%, respectively. TA medium was poured into each Petri dish and, after the agar hardened, all plates were inverted and dried at 37 C overnight. When not being used the plates were placed in a plastic bag and stored in the cold room to prevent loss of moisture. Tryptose Phosphate Broth (TPB) was prepared according to the directions on the bottle (Difco) and dispensed in test tubes prior to autoclaving.

The following modification of the Fisk method (31) was used in the screening procedures. Overnight (O/N) broth cultures were initiated for each strain by touching a sterile streaking loop to the surface of a colony growing on a TA plate and then inoculating a tube containing 5 ml of TPB with the organisms on the loop. The tubes were incubated at 37 C for 12 hours (approximately 1.4×10^8 cells/ml). A sufficient amount of the broth cultures of *P. multocida* strains to be tested was spread over the entire surface of TA plates. After allowing these to dry the underside of each plate was marked into sectors and numbered, and a drop of supernatant from each culture was spotted on the sector of the inoculated agar. It was predetermined that each plate would hold a maximum of twelve drops without the drops merging. These were allowed to dry at room temperature, then incubated at 37 C. The plates were checked for lytic activity after 12 to 14 hours incubation. Some plates required longer periods of incubation and were examined later. This method allowed for combinations of each potential lysogenic culture and indicator strain. The presence of phage could be detected either by the formation of plaques or by the presence of a narrow zone of clearing around the periphery of the spots.

UV Treatment

The following soft-agar-overlay technique was used with the modified Fisk method to screen for lysogeny after UV induction. Tubes containing 3.0 ml of melted soft agar were kept in a 45 C water bath until used. Two-tenths milliliter of each overnight (O/N) broth culture was added to a separate tube of soft agar. The contents of the tubes were mixed by rotating in the hands, then poured over the TA to make lawns. These were then set aside to dry.

Two milliliters from each broth culture were transferred to 50 x 12 mm disposable Petri plates. The plates were placed on a cardboard box located in a Hamilton control cabinet. The cultures were irradiated for 3 minutes with a germicidal ultraviolet light (Westinghouse mercury vapor sterilamp No. 782L-20) located 14 cm from the organisms. The treated cultures were transferred to sterile test tubes and incubated in a 37 C water bath. An effort was made to minimize exposure to light. After 2 hours incubation the tubes were centrifuged (ca. 2000 rpm) for 10 minutes and the lawns were spotted with drops of supernatant. Plates were allowed to dry at room temperature, then incubated at 37 C for 10 to 14 hours.

Mitomycin C Treatment

A stock solution of mitomycin C* (100 µg/ml) was employed. A working solution was prepared by diluting the stock solution to a final concentration of 1 µg/ml. A few drops of chloroform were added to the solution, which was stored in the refrigerator. An O/N broth culture of each strain was initiated according to the method described earlier.

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*Nutritional Biochemical Corporation.

Two-tenths milliliter of each O/N broth culture was added to a separate tube of soft agar. These were mixed and lawns were then made by pouring the soft agar mixture over the entire surface of the TA plates.

One milliliter of the working solution was added to each tube of broth culture which contained about 2.3 ml of cell suspension. The contents were mixed, then left to stand for 3 minutes. All tubes were then centrifuged for 10 minutes (2000 rpm). The supernatant was decanted from each tube and the pellet resuspended in 2.5 ml of fresh TPB. All cell suspensions were incubated at 37 C for 2 hours, followed by centrifugation for 10 minutes at the same speed as before.

A drop of supernatant from each treated suspension was spotted on a sector of the seeded soft agar lawn. The plates were left to dry at room temperature, then inverted and incubated at 37 C for 10 to 14 hours.

Screening for Plaque Formation after UV Treatment

Broth cultures incubated overnight were initiated from each strain that showed signs of lytic activity after the Fisk screening procedure.

Cultures were grown in tubes containing 2.5 ml of TPB, and 2 ml of cell suspension from each potential producer strain were treated with UV in the same manner as described previously. After final treatment, 3 drops of supernatant from the treated cell suspensions were added with Pasteur pipettes to 0.2 ml of the appropriate indicator strains. The tubes were shaken and then placed in a 37 C water bath for 20 minutes. Each suspension was then added to a tube of melted soft agar, mixed by rotating, and poured over a TA plate. Plates were incubated at 37 C for 10 to 14 hours.

Screening for Plaque Formation after Mitomycin C Treatment

The strains used in this test showed signs of lytic activity after the previous mitomycin C treatment. Overnight broth cultures of potential producer strains were treated with mitomycin C in the same manner described earlier. Three drops of supernatant from each potential producer strain were mixed with 0.2 ml of the appropriate indicator strain and placed in a 37 C water bath for 20 minutes. The mixture from each tube was then added to a tube of melted soft agar and mixed before being poured over the surface of a TA plate. The plates were incubated at 37 C for 10 to 14 hours.

Isolation of the Lytic Agent

The procedure that was used to isolate the lytic agent is outlined below.

I. Grow culture O/N in 70 ml TPB.
↓
II. Add 15 ml mitomycin C solution (1 μg/ml), shake, allow to stand for about 3 minutes.
↓
III. Centrifuge for 10 minutes at 7000 x g.
↓
IV. Decant supernatant and resuspend pellet in 50 ml fresh TPB.
↓
V. Incubate for 1 to 2 hours at 37 C on gyrotory shaker.
↓
VI. Add 6 to 8 drops of chloroform and continue shaking for 30 to 40 minutes.
↓
VII. Transfer to centrifuge tube and centrifuge for 10 minutes at 7000 x g (avoid chloroform in bottom layer).
↓
VIII. Decant the supernatant and repeat step VII. Discard both pellets.
↓
IX. Transfer the crude isolate to a sterile bottle and store in the refrigerator.

Viable Cell Count

Results from the screening exercises were analyzed and it was decided that the producer-indicator system which demonstrated the most lytic activity would be used in subsequent qualitative experiments. Strain B568-69 was chosen as the best indicator strain.

A broth culture of the indicator strain was incubated at 37 C until turbidity was first evident. Ten-fold dilutions of the culture were made in 0.85% saline. One-milliliter amounts were removed from the prepared dilutions and spread over the surface of TA plates by rapidly rotating the plates over the table surface in a "figure 8" pattern. Two samples were tested for each dilution. The plates were dried at room temperature, then incubated for 18 to 24 hours at 37 C. Colonies were counted with a "Quebec" colony counter.

Plaque Formation by the Bacteriolytic Agent

Ten-fold dilutions of the isolated bacteriolytic agent were made. After diluting to $1:10^{10}$, 0.2 ml was removed from each dilution tube and mixed with 0.4 ml of the indicator broth culture. The mixtures were placed in a 37 C water bath for 20 minutes, after which duplicate 0.3 ml aliquots from each tube were added to tubes of melted soft agar. These were mixed and poured over TA plates. The plates were incubated at 37 C for 10 to 14 hours.

Serial Transfer of Lytic Agent

The crude isolate was tested for its ability to produce lysis after a series of transfers. The method used to test the transferability of the lytic agent is outlined below:

I. Add 0.2 ml sensitive cells to 2 tubes containing 10 ml of TB. Incubate at 37 C for 2 hours on a gyrotory shaker.

↓

II. Add 0.1 ml of lytic agent to one tube, using the other as a control. Incubate at 37 C for 6 to 10 hours, making periodic observations to note any clearing of broth.

↓

III. When clearing is observed, add 3 or 4 drops of chloroform to the tube and continue shaking for about 45 minutes.

↓

IV. Centrifuge the mixture for 10 minutes at 7000 x g and save supernatant.

↓

V. By adding 0.2 ml of indicator cells, inoculate 5 tubes containing the following amounts of broth: one-10 ml and four-9 ml. Incubate at 37 C for 2 hours on the gyrotory shaker.

↓

VI. Add 0.1 ml of supernatant to the tube containing 10 ml of broth. Set aside one tube with 9 ml of broth for a control and make ten-fold dilutions from the 10 ml tube into the remaining 9 ml broth cultures.

↓

VII. Incubate at 37 C for 6 to 10 hours, making frequent observations to note clearing.

↓

VIII. Select the highest dilution of lytic agent which shows visible signs of clearing when compared to the control.

↓

IX. Continue at Step III and repeat the procedure for the desired number of transfers.

Concentration of Bacteriophage

The crude phage suspension was concentrated in the following manner: The suspension was transferred to two 1 x 3-1/2 inch cellulose nitrate tubes which were placed in a precooled No. 30 rotor head. The loaded rotor was seated on the shaft of a Beckman Model L ultracentrifuge and the sample was centrifuged for 3 hours with an average force of approximately 105,000 x g (ca. 30,000 rpm). After the run, the supernatant was decanted and 0.5 ml of 0.01 M acetate buffer pH 7.5 was slowly added to the tubes. A drop of chloroform was added to each tube and both were placed in a cold room where the pellets were allowed to disperse without agitation.

Electron Microscopy

Samples of the concentrated suspension were examined in the electron microscope.* The samples were held on grids coated with a solution of 0.4% parlodion** (a nitrocellulose plastic) in amyl acetate.

Influence of Divalent Cations

In order to determine if divalent cations were required for lysis to take place 0.02 M solutions of $CaCl_2$, $MgSO_4$, $MnCl_2$, and $Zn(C_2H_3O_2)\cdot 2H_2O$ were prepared.

Each cation solution was added to two tubes of TB so that each tube of broth was supplemented 5%. Two unsupplemented tubes of broth were also used (see Table 8). The final volume in each tube was 10 ml. Two-tenths milliliter of an overnight broth culture of indicator strain B568-69 was added to each tube and these were incubated for 2 hours at 37 C on a gyrotory shaker. After this 0.1 ml of phage 6604 suspension was added to one of the tubes supplemented with each cation solution. In all, 10 tubes were used, i.e., 5 tubes with bacteriophage and 5 tubes as controls without bacteriophage. All tubes were incubated on a shaker and examined for clearing after 6 to 10 hours.

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*Phillips 300 electron microscope.

**Mallinckrodt Chemical Works, St. Louis, Mo.

RESULTS

Table 1 lists the species origin and numbering arrangement for the strains used in this study.

Screening for Phage Carriers

Thirty-five strains of *P. multocida* were examined initially by the Fisk method for phage activity. The results of this procedure are listed in Table 2. No zones of inhibition were observed around any of the combinations. This indicated one of the following possibilities: (1) no free phage particles were present in the cultures, (2) if free phage particles were present, their concentration was too low to be detected, (3) free phage particles were present but undetected because no sensitive indicator strain was available.

Plaque Formation after UV Treatment

Table 3 shows the results of the spot tests after UV treatment. The results from these tests provided presumptive evidence of possible plaque formers. Fourteen combinations showed zones of inhibition around the periphery of the drops of supernatant from treated suspensions. A plus sign (+) was recorded for each combination that produced a zone of inhibition. These 14 combinations were then tested by the soft-agar-overlay procedure to see whether or not they would produce plaques. All attempts to demonstrate plaque formation with these combinations were unsuccessful.

Table 1. Species origin, specimen source, and numbering arrangement for the various *P. multocida* strains

No.	Strain No.	Species Origin	Specimen Source and/or Infected Organ
1	6604	Bovine	Cutter
2	702	Bovine	Unknown
3	656	Bison	Unknown
4	P1284	Cattle	Namioka
5	P1235	Bovine	Africa
6	B568-69	Bovine	Lung, kidney
7	19427	Unknown	ATCC, Indol neg. (India)
8	B175-69	Bovine	Lung
9	154	Bovine	Lung
10	B220-68	Bovine	Lung
11	B378-67	Bovine	Spleen
12	151	Unknown	Unknown
13	Insein	Bovine	Burma
14	PM	Bovine	Sepsis (Philippines)
15	989	Bovine	Wound (Australia)
16	R473	Bovine	Egypt
17	704	Bovine	Pitman-Moore
18	P1234	Bovine	Africa
19	P15-71	Porcine	Lung
20	P9-71	Porcine	Lung
21	P19-71	Porcine	Lung
22	932A	Bison	H.S. 1305-1

Table 1 (cont'd.)

No.	Strain No.	Species Origin	Specimen Source and/or Infected Organ
23	P30-71	Porcine	Lung
24	B365-61 (F45)	Bovine	Nasal swab
25	B218	Bovine	Lung
26	B365-71 (E46)	Bovine	Nasal swab
27	B365-71 (E49)	Bovine	Nasal swab
28	Type III	Deer	Roberts (Gr. Brit.)
29	Type IV (731)	Deer	Roberts (Gr. Brit.)
30	Type IV (739)	Deer	Roberts (Gr. Brit.)
31	Type V	Goat	Roberts (Gr. Brit.)
32	P36-71	Porcine	Lung
33	P48-71	Porcine	Lung, spleen
34	P49-71	Porcine	Nasal sinus
35	P64-71	Porcine	Lung

Table 2. Results of tests for the presence of phage carriers by culturing strains of *P. multocida* in combination

Super-natants	←Indicator Strains→ 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
1		H[1]	M[2]	H	M	M	M	M	M	M	M	M	H	M	H	M	M	M	M	H	M	H	H	H	H	H	H	M	M	H	M	H	M	H	H
2	H		M	H	M	M	H	H	M	H	H	H	H	H	M	M	M	M	H	H	H	H	H	H	M	H	H	H	M	M	H	H	H	M	H
3	H	H		M	M	H	M	M	M	M	M	M	H	M	M	M	M	M	H	H	M	H	H	H	M	M	H	H	H	H	M	M	M	H	M
4	M	H	M		M	M	H	H	M	H	H	H	H	H	H	M	M	M	H	H	H	H	H	H	M	H	M	H	M	H	H	M	M	H	H
5	H	H	M	H		M	H	H	M	H	M	H	H	H	M	M	M	M	H	H	H	H	H	H	M	H	M	M	H	H	H	H	M	H	H
6	M	H	M	H	M		M	H	M	M	M	M	H	M	H	M	M	M	H	H	H	H	H	H	M	M	H	M	H	M	M	M	H	H	M
7	M	H	M	M	M	M		M	M	M	M	M	H	M	H	M	M	M	H	H	H	H	H	H	M	H	M	H	M	M	M	H	M	H	H
8	M	H	M	H	M	M	M		M	M	M	M	H	M	H	M	M	M	M	H	H	H	H	H	M	H	H	H	M	M	M	H	H	H	M
9	M	M	M	H	M	M	M	M		M	M	M	H	M	H	M	M	M	H	H	H	H	H	H	M	H	H	H	M	M	H	H	H	M	M
10	M	H	M	H	M	M	M	M	M		M	M	H	M	H	M	M	M	M	H	H	H	H	H	M	M	H	H	M	M	M	H	M	M	M
11	M	H	M	H	M	M	M	H	M	M		H	H	M	M	M	M	M	H	H	H	H	H	H	M	H	H	H	H	M	M	H	M	M	M
12	M	H	M	H	M	M	M	M	M	M	M		H	M	H	M	M	M	M	H	H	H	M	H	M	H	M	H	M	M	M	M	M	H	H
13	M	H	M	M	M	M	M	M	M	M	M	M		M	M	M	M	M	H	H	H	H	H	H	M	H	H	H	M	M	M	H	M	H	M
14	M	M	M	H	M	M	H	M	M	H	H	M	M		M	M	M	M	H	H	H	H	H	H	M	H	H	H	M	M	M	M	M	H	M
15	H	M	M	M	M	M	H	H	M	H	H	H	M	H		M	M	M	H	H	H	H	H	H	M	H	H	H	M	M	H	M	M	H	H
16	H	H	M	M	M	M	H	H	M	M	M	H	H	M	M		M	M	H	H	M	H	H	H	M	H	H	H	M	M	H	H	M	H	M
17	H	H	M	H	M	M	H	H	H	M	H	H	H	M	H	M		M	H	H	H	H	H	H	M	H	H	H	M	H	M	H	M	H	H
18	M	H	M	H	M	M	H	M	M	M	H	M	H	M	H	M	M		H	H	H	H	H	H	M	H	H	H	M	H	M	H	M	H	H

Table 2--cont'd.

Super-natants	←Indicator Strains→ 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
19	M	H	M	M	M	M	H	H	H	M	H	M	M	M	M	M	M	M		H	H	H	H	H	M	H	H	M	H	M	M	H	M	H	H
20	H	H	M	H	M	H	H	H	H	M	H	M	H	H	M	M	M	M	H		H	H	H	H	M	H	M	M	M	H	H	H	H	H	H
21	M	H	M	H	M	M	M	M	M	M	H	M	M	M	M	M	M	M	H	H		H	H	H	M	H	H	M	M	M	H	M	M	H	M
22	H	H	M	H	M	H	H	H	M	M	H	H	M	H	H	M	M	M	H	H	H		H	H	H	H	H	H	H	H	H	H	H	H	H
23	H	H	M	H	M	H	H	H	H	M	H	H	H	H	M	M	M	M	H	H	H	H		H	M	H	H	H	H	H	H	H	H	H	H
24	H	H	M	H	M	H	M	H	H	M	H	H	H	M	H	M	M	M	H	H	H	H	H		M	H	M	H	H	H	H	H	H	H	H
25	M	H	M	H	M	M	H	H	M	M	H	M	M	M	M	M	M	M	H	H	H	H	H	H		M	H	H	M	M	M	M	M	M	M
26	H	H	M	H	M	H	H	H	H	M	H	H	M	M	M	M	M	M	H	H	M	H	H	H	H		H	M	H	H	H	M	H	H	H
27	H	H	M	H	M	H	M	H	H	M	M	M	H	H	H	M	M	M	M	H	H	H	H	H	H	H		H	M	H	M	M	H	H	H
28	H	M	H	M	M	M	H	H	H	H	H	H	H	H	M	M	M	M	H	H	M	H	H	H	M	M	M		M	H	H	H	M	M	H
29	H	H	M	H	H	H	H	H	H	H	H	H	H	H	H	M	M	M	H	H	H	H	H	H	H	M	M	H		M	H	M	M	M	M
30	M	H	M	H	H	H	H	H	H	H	H	H	H	H	H	M	M	M	H	H	H	H	H	H	M	M	H	H	H		H	M	H	H	M
31	M	H	M	H	M	H	H	H	H	H	H	H	H	H	H	M	M	M	H	H	H	H	H	H	H	M	M	M	H	M		M	H	M	M
32	H	H	M	H	M	H	H	H	H	M	M	M	H	H	H	M	M	M	H	H	H	H	H	H	H	H	H	H	M	H	M		M	M	H
33	M	H	M	H	M	M	H	H	H	H	H	H	M	H	H	M	M	M	H	H	H	H	H	H	H	H	H	H	H	M	H	H		H	M
34	M	H	M	H	M	H	H	H	H	H	H	H	H	H	H	M	M	M	H	H	H	H	H	H	M	M	H	H	H	H	M	M	H		M
35	M	H	M	H	M	H	H	H	H	H	H	H	H	H	H	M	M	M	H	H	H	H	H	H	H	H	M	H	H	H	M	H	H	M	

These notations were used to record growth around the periphery of the drops.

[1]H = heavy, [2]M = moderate.

Table 3. Surveillance of 35 strains of *P. multocida* for lytic activity after UV treatment

Indicators	Supernatants (producer strains) 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3--cont'd.

Indicators	Supernatants (producer strains) 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
33	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
34	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-
35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ = zone of inhibition surrounding drop of treated suspension.

- = no zone of inhibition.

Plaque Formation after Mitomycin C Treatment

In the initial screening procedure the cells were treated with mitomycin C and a drop of supernatant was spotted on a Petri plate previously inoculated confluently with the indicator strain. Inhibition of growth was evidenced by a zone of clearing around the drop after incubation. Table 4 shows the results of this procedure. A plus sign (+) was recorded for each combination that produced a zone of inhibition. Fourteen potential producer strains were found that showed signs of lytic activity when cultured in combinations with 20 indicator strains.

In an effort to demonstrate plaque formation, the lytic agents were mixed directly with sensitive cells in soft agar and poured over TA plates. Thirty-seven potential producer vs. indicator combinations were tested. Only those potential producer and indicator strains which showed a positive result in the spotting procedure were used. The results are summarized in Table 5. The following combinations of producer vs. indicator strain produced plaques: 1 vs. 6, 1 vs. 26, 1 vs. 35, and 8 vs. 6.

The plaques were very difficult to observe; however, they were best observed in a room lighted only by a desk lamp. Very tiny, turbid plaques (ca. 0.5 mm) were observed when the plates were held between the viewer and the light source. An attempt was made to obtain clear plaques by varying the ratio of lytic agent vs. indicator strain added to the soft agar, and by varying the conditions of incubation. Both of these variations failed to result in the formation of clear plaques.

Viable Cell Count

In the previous procedure it was found that four pairs of interacting strains showed signs of bacteriolytic activity. After repeating the

Table 4. Surveillance of 35 strains of *P. multocida* for lytic activity after mitomycin C treatment

Indicator	Supernatants (producer strains) 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	+	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
6	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
10	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
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17	-	-	+	-	-	+	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4--cont'd.

Indicator	Supernatants (producer strains) 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
19	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	-	-	+	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
22	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26	+	-	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
27	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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34	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35	+	-	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

+ = zone of inhibition surrounding drop of supernatant from treated suspension.

- = no zone of inhibition.

Table 5. Plaque formation after mitomycin C treatment

Producer Strains	Indicator Strains: 2	6	7	14	16	17	21	22	26	35
1	-	+	-	-	-			-	+	+
3	-					-	-		-	-
6	-		-	-	-	-	-	-	-	-
8	-	+	-	-	-	-	-	-	-	-
23				-						
30	-				-			-	-	

Legend: + = plaque formation

- = no plaque formation

Shaded areas represent combinations that were not tested.

same experimental procedure with only the four interacting pairs it was found that strain No. 1 (6604) and strain No. 6 (B528-69) showed the most lytic activity when used in combination as producer and indicator, respectively.

For possible future reference, colony counts were made to determine the average number of viable cells present in a broth culture of indicator strain No. 6. An overnight culture was grown in TPB under the conditions described in the Methods section.

Table 6 shows the results of the colony counting procedure. Duplicate samples were tested for each dilution and average values were recorded. Three values were obtained in this manner and these were again averaged to give an average number of cells per milliliter. The average number of viable cells per milliliter was calculated to be 1.4×10^8.

Table 6. The average number of host (strain B568-69) cells per milliliter in the screening procedure

Dilution	Avg. Colony Count	No. cells/ml	Avg. No. cells/ml[a] All Dilutions
10^{-6}	65	0.65×10^8	1.4×10^8
10^{-7}	16	1.60×10^8	
10^{-8}	2	2.00×10^8	

[a]Represents an average of the three values in the preceding column.

Lytic Activity of the Bacteriolytic Agent

All attempts to determine the number of plaque forming units (PFU's) per milliliter were unsuccessful. No quantitative data were obtained because of the difficulty involved in locating and counting the tiny turbid (almost pin point) plaques.

The steps that were undertaken to isolate the lytic agent were given in the Methods section. The crude preparation was tested for lytic activity after isolation by adding three drops to 0.2 ml of a broth culture of sensitive indicator cells followed by incubation at 37 C for 20 minutes, then adding the mixture to soft agar and pouring over TA plates.

The plates were examined periodically after 10 to 12 hours incubation at 37 C and very small plaques were observed. Some plates showed plaques when examined after 10 hours but failed to show any signs of plaque formation when examined after 20 hours of incubation. The disappearance of plaques was attributed to the proliferation of resistant cells. The crude isolate was bacteriolytic since plaque formation was observed.

Serial Transfer of Lytic Activity

This experiment was included to offer further evidence in support of the premise that the bacteriolytic activity which had been observed was caused by bacteriophage and not by bacteriocin.

Like virulent phages, bacteriocins can kill sensitive strains of bacteria; however, they differ from bacteriophages by the fact that they are not reproduced after infection, and their lytic activity is not transmissible in series (10). The method for preparation of broth cultures before adding lytic agent was adapted from a similar procedure

used by Rifkind and Pickett (69). An initial 0.1 ml of lytic suspension was added to tubes of broth culture and serially diluted to a final dilution of 10^{-11}.

Table 7 lists the highest dilution that showed signs of lytic activity after each transfer, and the resulting dilution of the initial input after each transfer. It also shows that after five transfers lytic activity or clearing of the broth culture was still evident in the tube containing a 10^{-2} dilution of the fluid from the previous transfer. This indicated that lytic activity was transferable in series. It further demonstrated that the bacteriolytic agent was a bacteriophage rather than a bacteriocin.

Table 7. Determination of the transferability of lytic activity in series

No. of transfers	Highest dilution showing activity	Dilution of initial 0.1 ml of lytic agent
1	10^{-2}	10^{-2}
2	10^{-4}	10^{-5}
3	10^{-2}	10^{-7}
4	10^{-2}	10^{-9}
5	10^{-2}	10^{-11}

Electron Microscopy

Figure 1 shows micrographs of the bacteriophage produced after induction of strain 6604 with mitomycin C. The micrographs show the presence of phage-like particles with heads connected to tails which

Figure 1. Electron micrographs of bacteriophage produced after mitomycin C induction of *P. multocida* strain 6604. PTA, x 540,000, scale 1000 Å.

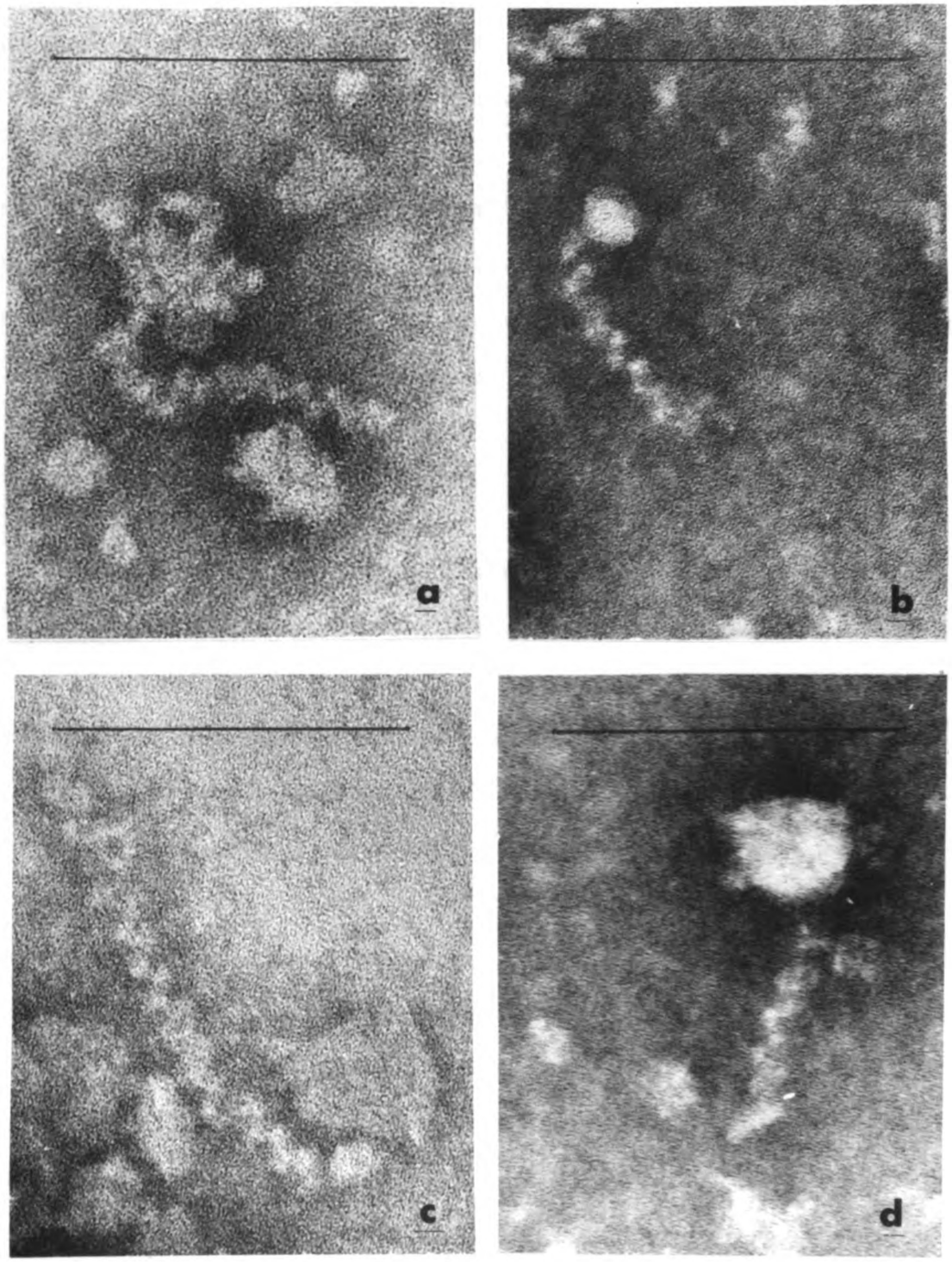

Figure 1

resemble corkscrews. Two-dimensionally, the head-like structure has a pentagonal shape with dimensions which are within the range of 333 x 444 Å. The tail structure seems to be formed of spirally coiled strands which gives it a corkscrew appearance. Intervals between adjoining turns of the tail helix are approximately 111 Å. The length of the tail structure varied with different particles between 740-1665 Å, whereas the diameter of the tail was found to be 111 Å in each case. Because of inadequate resolution it was not possible to determine whether or not the tail structure had an end plate.

Influence of Divalent Cations

The tubes of broth containing the cation supplements (Mg^{++}, Ca^{++}, Mn^{++}, Zn^{++}) and the unsupplemented broth cultures were observed after 8 hours of incubation on the shaker. Visual observation was used to make a qualitative determination of whether or not the broth cultures had cleared as a result of bacteriolytic activity. In Table 8 a minus sign (-) was used to indicate a turbid culture in which cells were growing without appreciable lysis. A positive sign (+) was recorded for those tubes that appeared to be appreciably clearer than the turbid controls. A separate control was prepared for each salt solution. This was done to allow for any differences of growth rate in the various supplemented broth cultures. It was also useful as a check for nonspecific growth inhibition caused by the salt solution. Unsupplemented tubes were included to check for bacteriolytic activity in the absence of any cation supplement. The final concentration of cation supplement in each tube was .001 M. Table 8 shows that appreciable lysis occurred in the broth culture which contained no added divalent cations. This indicated that the bacteriophage isolated from *P. multocida* strain 6604

Table 8. Influence of divalent cations on the lysis of *P. multocida* by bacteriophage 6604

TB with 10^{-3} M:	Cells	Cells and Phage
Mg^{++}	-	+
Ca^{++}	-	+
Mn^{++}	-	-
Zn^{++}	NG	NG
(Unsuppl.)	-	+

+ = lysis

- = no lysis

NG = no growth

did not require divalent cations as lysis cofactors. The table also shows that cell growth was inhibited in the broth supplemented with zinc ions. Manganous ions inhibited bacterial lysis in this experiment.

DISCUSSION

A modification of the Fisk method was used to screen thirty-five strains of *P. multocida* for lysogeny. The cultures were screened before treatment to see whether or not phage was spontaneously produced by any of the strains. No signs of bacteriolytic activity were detected as indicated in Table 2. Broth cultures of the strains were later treated with UV and mitomycin C to induce bacteriophage production. After treatment the cultures were screened to determine the extent of lysogeny (Tables 3 and 4). It was found that UV treatment induced 10 producer strains which reacted with 4 indicator strains. This indicated 28.5% lysogeny among the 35 strains. Mitomycin C treatment induced 14 producer strains which reacted with 20 indicator strains. This indicated that 40% of the 35 strains were lysogenic. Rifkind and Pickett (69) screened 118 strains of *P. multocida* and found that 13.5% were lysogenic. Eighty-four of the 118 strains were sensitive to bacteriophages that they isolated. Kirchner and Eisenstark (50) studied 25 strains of *P. multocida* and reported that 11 were lysogenic. These investigators detected lysogeny without inducing the bacteria. Other studies mentioned in the literature review also reported percentages of lysogeny in the same general range (28,72).

The results of this study indicated that mitomycin C was more effective than UV in inducing bacteriophage production. Six producer strains (1, 6, 8, 17, 22, and 23) were induced by both UV and mitomycin C.

Bacteriolysis is usually detected by the following: plaque formation, a zone of inhibition surrounding a drop of the producer strain (Fisk method) or by the clearing of a broth culture indicating the lysis of sensitive cells by bacteriophage. In any of the above the detection of bacteriolysis may be impeded by the proliferation of phage-resistant cells in the test medium (15). On an agar medium the resistant cells may grow over the areas of the agar where plaques were produced, thus making it very difficult, if not impossible, to locate the plaques. After the lysis of indicator cells in a broth culture, any remaining phage-resistant cell can proliferate and result in turbidity. It was for these reasons that precautions were taken to eliminate the producer strain after production of bacteriophage.

In a review Adams (1) pointed out that a phage which completely lyses broth cultures may produce very tiny plaques and may be unsuitable for quantitative work by plaque-counting techniques. He also stated that a phage which produces "beautiful" plaques may not bring about lysis in broth cultures. The bacteriophages which were examined in this study were in the former category. Because it was very difficult to locate plaques, many of the quantitative tests carried out are not recorded in this study.

One producer vs. indicator combination was found which produced better plaques than the other combinations observed. This combination included producer strain 6604 and indicator strain B568-69. The phage isolated from this combination was selected for further characterization.

Since bacteriocins and temperate phages are detected in the same way, it was necessary to identify the bacteriolytic agent. Bacteriolytic activity caused by bacteriocins is diluted out by serial transfer, whereas

lytic activity caused by bacteriophages continues after high dilution (see Table 7). The bacteriolytic agent isolated from strain 6604 was tested to determine if the lytic activity was affected by serial dilution. After five ten-fold transfers in broth from 10^{-1} to 10^{-11} bacteriolytic activity was still apparent. This strongly indicated that the bacteriolytic agent was a bacteriophage rather than a bacteriocin.

Some bacteriophages require the presence of certain divalent cations to lyse host cells (69). It was found that divalent cations were not required for phage 6604 to lyse indicator cells in broth. Lysis was inhibited in the tube supplemented with manganous ions, and growth of indicator strain B568-69 was inhibited in the tubes supplemented with zinc ions. Rifkind and Pickett (69) tested the influence of 0.001 M divalent cations on the ability of 11 bacteriophages to lyse *P. multocida* in broth. They found that certain of the bacteriophages required either calcium or magnesium ions as a lysis cofactor and, in a few instances, no cofactor was required. They also found that manganous ions inhibited bacterial lysis.

Since this is the first study on *P. multocida* bacteriophages to include information on phage morphology, no direct comparisons could be made concerning morphological characteristics. There are, however, two bacteriophages which showed morphological characteristics similar to the phage isolated from strain 6604. Phage No. 1 of *Bacillus mycoides* has head and tail features similar to the bacteriophage shown in Figure 3. Phage No. 1 (70) has an end plate on the tail structure; however, inadequate resolution made it impossible to distinguish an end plate on the tail structure of the 6604 phage. The bacteriophage shown in Figure 1 also resembles Coliphage E1 described by Bradley (10).

It is considered that the methods developed in this study can be applied by other investigators to isolate and concentrate temperate bacteriophages from lysogenic strains of *P. multocida*.

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