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(54) Titre : TRAITEMENT CONTRE LES BACTERIES RESISTANTES AUX ANTIBIOTIQUES

(54) Title: ANTIBACTERIAL THERAPY FOR MULTI-DRUG RESISTANT BACTERIA

(57) Abrégé/Abstract:

The invention relates to selected bacteriophages, formulations containing same, and their use in killing or inhibiting the growth of bacteria, particularly methicillin-resistant Staphylococcus aureus (MRSA).



ABSTRACT OF THE DISCLOSURE

The invention relates to selected bacteriophages, formulations containing same, and their use
5 in killing or inhibiting the growth of bacteria, particularly methicillin-resistant *Staphylococcus aureus* (MRSA).

MSH File: PHAGE

TITLE: Antibacterial Therapy for Mult-Drug Resistant Bacteria

FIELD OF THE INVENTION

The invention relates to selected bacteriophages, formulations containing same, and their
5 use in killing or inhibiting the growth of bacteria.

BACKGROUND OF THE INVENTION

The rise in the incidence of multi-drug resistant bacterial infections has made the need
for alternative means of treatment more pressing. In particular, the number of nosocomial
infections due to antibiotic resistant bacteria has increased sharply in recent years. Methicillin-
10 resistant *Staphylococcus aureus* (MRSA) has emerged as one of the main causes of such
infections [Voss, A, and B.N. Doebbeling, International Journal of Antimicrobial Agents 5,
1995, 101-106; McGeer, A., et al., LPTP Newsletter, 1996 (190): p. 1-4]. MRSA infections are
normally combated with the administration of the glycopeptide antibiotic, vancomycin. There
have been reports of the development of vancomycin intermediate *Staphylococcus aureus*
15 (VISA) infections in patients being treated with vancomycin for MRSA infections. This strongly
suggests that the continued use of vancomycin to treat MRSA infections could give rise to a
fully glycopeptide resistant population of *Staphylococcus auerus* [Smith, T.L., New. Eng. J.
Med. 1999, 340 7): p. 493-501]. Furthermore, while other antibiotics were used to eradicate the
infections, the resulting complications proved fatal in all the reported cases. Therefore, there is
20 a need for an alternative approach to the treatment of antibiotic resistant infections.

Therapy using bacteriophages is based on the principle of administering phages capable
of killing the bacteria which are the cause of the infection [Parker, M.T. Methods in
Microbiology Vol. 7B, 1972, New York and London: Academic Press]. The phages infect,
replicate and then lyse the target bacteria without affecting the patient's tissues or the normally
25 occurring micro-flora. Phage therapy offers the prospect of an adaptive model of treatment well
suited to fighting antibiotic resistant infections [Smith, H.W., a.H., M.B.. J. of Gen. Microbiol.,
1982, 128 ((Pt 2)): p. 307-318; Merril, C.R., et al, Proc. Natl. Acad. Sci. USA, 1996, 93(8): p.
3188-3192).

The effectiveness of phage therapy was demonstrated against *E. coli* and *S. typhimurium*
30 in animal models of infection (mice, calves and piglets) [Smith, supra; Meril, supra]. A number
of studies of phage therapy in animal models of infection have been carried out targeting *E. coli*,

Salmonella typhimurium, *Pseudomonas*, and *Staphylococcus* with a variety of results, mostly positive. However, these studies did not target clinically relevant strains, particularly those strains implicated in human diseases.

SUMMARY OF THE INVENTION

5 The present inventors have studied the potential of phage therapy for treating antibiotic resistant bacteria. In particular, the present inventors have identified specific bacteriophages which virulently lyse MRSA. The inventors have significantly demonstrated that phages can be used to lyse a broad range of clinically relevant strains of multi-drug or antibiotic resistant bacteria.

10 Therefore, the present invention relates to bacteriophages selected from the species *Myoviridae* for use as active therapeutic substances, particularly in the treatment of infectious diseases caused by bacteria, preferably antibiotic resistant bacteria.

 The invention also relates to formulations comprising isolated and purified bacteriophages from the species *Myoviridae*.

15 Further, the invention provides a method for treating an infectious disease caused by bacteria, preferably antibiotic resistant bacteria, in an animal comprising administering to an animal in need of such treatment a bacteriophage selected from the species *Myoviridae*. The invention also provides a method of reducing virulence of bacteria, preferably antibiotic resistant bacteria in a subject comprising administering to the subject an effective amount of a
20 bacteriophage selected from the species *Myoviridae*.

 Methods for killing or inhibiting the growth of bacteria are also provided comprising contacting the bacteria with an effective amount of a formulation of the invention. A medium that can be treated by this method may be a food product, substances used in making food products, medical instruments, skin, surgical implants, or metallic, plastic, tile, porcelain, or
25 glass surfaces. The medium may be an inert carrier and such a formulation may be used in a conventional bactericide manner.

 The invention also provides a novel bactericide prepared with one or more isolated and purified bacteriophages from the species *Myoviridae* for disinfecting or sterilizing anything to be protected against infection with pathogenic bacteria, including but not limited to food
30 products, substances used in making food products, areas where there is preparation of foodstuffs, surgical implants, metallic, plastic, tile, porcelain, or glass surfaces, medical devices

and instruments, and skin.

Strains of the sub species *Twort* that are capable of lysing about 99% of MRSA strains, (for example, C-MSRA1 to C-MSRA4 inclusive, Belgian, Swiss, and EMRSA1 to EMRSA inclusive), are particularly useful in the formulations and treatments of the invention. In preferred embodiments of the invention the formulations and methods include one or more of the following phages: $\phi 812$, $\phi 131$, *SK311*, Mu50, and *U16*. These bacteriophage have extremely high specificity for MRSA.

The formulations, compositions, bactericides, and methods of the invention are useful against strains of pathogenic bacteria, particularly multi-drug resistant bacteria. For example, they are suitable against strains of staphylococci such as *Staphylococcus aureus* and *Staphylococcus epidermidis*. The formulations and methods are particularly useful against strains of staphylococci that are of reduced sensitivity to glycopeptides such as vancomycin or teicoplanin. In an embodiment of the invention, the staphylococci strains are also methicillin resistant.

These and other aspects, features, and advantages of the present invention should be apparent to those skilled in the art from the following drawings and detailed description.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention there may be employed conventional molecular biology and microbiology techniques within the skill of the art. Such techniques are explained fully in the literature. See for example, Sambrook, Fritsch, & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y; and Parker, M.T. *Methods in Microbiology* Vol. 7B, 1972, New York and London: Academic Press.

Bacteriophages that can be used in the methods of the invention are strains that are capable of doing direct or indirect harm to the bacteria. Suitable bacteriophages may include lytic bacteriophages, bacteriophages that are lysogenic and later become lytic (e.g. phages genetically modified to become lytic), and nonlytic bacteriophages that produce products that are harmful to the bacteria. Preferably lytic bacteriophages are used in the present invention. Bacteriophages that can be used in the formulations and methods of the invention include those belonging to the family *Myoviridae* [Pantucek, R. et al, *Virology* 1998, 245(2): p. 241-252]. In particular, strains of the species *Twort* that are capable of lysing about 99% of MRSA strains

may be used in the present invention. In a preferred embodiment of the invention the formulations and methods include one or more of the following bacteriophages: $\phi 812$, $\phi 131$, *SK311*, and *U16*.

A bacteriophage may be modified to enable the bacteriophage to delay inactivation by
5 any and all parts of the host defense system that may reduce the numbers of bacteriophage
and/or the efficiency of the bacteriophage at killing the host bacteria in an infection. Modified
bacteriophages that are able to delay inactivation by the host defense system can be obtained by
selection of modified strains by serial passage of the phage, or by genetic engineering of a
phage, so that the modified phage will remain active in the body for longer periods of time than
10 the wild-type phage. (See U.S. Patent No. 5,811,093 U.S. 5,766,892, U.S. 5,688,501.)

The bacteriophages can be used in combination with other anti-bacterial agents. Suitable
antibacterial agents that can be used in combination with the bacteriophages include but are not
limited to antibiotics and chemotherapeutic agents. Examples of such agents include the
penicillins, cephalosporins, glycopeptides (e.g. vancomycin), aminoglycosides (e.g. amikacin,
15 tobramycin), imipenem, erythromycin, carbapenems (WO9920638), penicillinase-resistant
penicillin, anthraquinone derivatives, clavulanic acid, or combinations thereof (WO9622105).

The bacteriophage can be grown using appropriate bacteria (e.g. SA812) in suitable
media. The resulting lysates are treated to provide a preparation that has no live organisms and
toxins such as bacterial cell wall. For example, the resulting lysates can be sterilized using
20 conventional methods such as filtration, and purified, using for example ultrafiltration, to
remove bacterial cell wall. The bacteriophages, or formulations thereof as described herein, can
be packaged and sealed into ampoules or otherwise prepared and packaged for administration.
Approximate titers can be determined by checking the dilution that would produce lysis after
coinoculation with specific numbers of bacteria of standard test strains, and each batch can be
25 tested for any surviving bacterial contaminants. In an embodiment, preparations with a
minimum concentration of between 10^8 and 10^9 pfu are prepared. Where the bacteriophage is
to be injected, it can be concentrated (e.g. lyophilized) and resuspended in buffers such as
physiological saline. The bacteriophage preparations can be tested for toxicity, (e.g. in laboratory
animals such as guinea pigs) to ensure that no residual bacterial surface fragments are present.

30 Accordingly, the bacteriophages may be formulated into compositions or formulations
for administration to subjects in a biologically compatible form suitable for administration *in*

vivo. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the active substance to be administered in which any toxic effects are outweighed by the therapeutic effects. The active substances may be administered to animals including humans, domestic pets, livestock, pisciculture, zoo animals, and animals in aquatic parks. Administration of a therapeutically active amount of a formulation of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual. Dosage regima may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The dosage may be in the range of about 10^6 to about 10^{13} pfu/kg/day, preferably about 10^8 to 10^{11} pfu/kg/day. The bacteriophage can be administered until successful elimination of the pathogenic bacteria is achieved.

The active substance may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, pulmonary (e.g. aerosol or by other devices for delivery to the lungs), nasal spray, intramuscular, intraperitoneal intrathecal, intravitreal, vaginal, rectal, topical, lumbar puncture, and direct application. Depending on the route of administration, the active substance may be coated in a material to protect the substance from the action of enzymes, acids and other natural conditions that may inactivate the substance (e.g. enteric coated tablets or pills). The bacteriophage may be incorporated into an aerosol formulation specifically adapted for aerosol administration to the lungs by inhalation. Suitable means for aerosol administration are well known in the art and include the Proventil™ inhaler (Schering-Plough). The types and concentrations of the propellants in the device are adjusted based on the type of phage.

The pharmaceutical formulations described herein can be prepared by per se known methods for the preparation of pharmaceutically acceptable formulations which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985). On this basis, the formulations include, albeit

not exclusively, solutions of the active substances in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

Excipients which can be used as delivery vehicles are apparent to those skilled in the art.

5 The bacteriophage can be lyophilized and dissolved prior to administration by intravenous injection.

The bacteriophage can be dissolved in a suitable carrier for example an aqueous solvent or buffer or suspended in any suitable liquid, colloidal, or polymeric matrix to create bactericides. The bactericides can be incorporated into ointments, or coatings for medicinal uses
10 such as the treatment of infections as described herein, wound dressings, or surgical implants, and as a broad spectrum disinfectant for skin or oral rinses, disinfectant scrubs, wipes, or lotions. The bactericides can be used for cleaning medical instruments, in pre-operative surgical scrubs, and the like.

The formulations and methods of the invention are suitable against strains of
15 staphylococci for example, *Staphylococcus aureus* and *Staphylococcus epidermidis*. The formulations and methods are particularly useful in the prevention and treatment of infections caused by strains of staphylococci which are of reduced sensitivity to glycopeptides such as vancomycin or teicoplanin. In an embodiment of the invention, the staphylococci strains are also methicillin resistant. In a preferred embodiment of the invention the compositions and methods
20 are used to prevent and treat MSRA infections, in particular those caused by C-MSRA1 to C-MSRA4 strains inclusive, Belgian strain, Swiss strain, and EMRSA1 to EMRSA strains inclusive.

The foregoing embodiments of the invention are further described in the following
25 example. However, the present invention is not limited by the Example, and variations will be apparent to those skilled in the art without departing from the scope of the present invention. The following non-limiting example is illustrative of the present invention:

Example

Phage therapy for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA)
30 was developed by first identifying bacteriophages capable of lysing a selection of MRSA isolates representative of the variations found *in vivo*. 8 candidate phages were identified which

had been shown to be capable of killing a high percentage of *Staphylococcus aureus* strains: phages 44AHJD, 2638A, Twort, P68, ϕ 812, ϕ 131, SK311, and U16. The selected isolates were representative of the variations in phage typing patterns and each of the four clones responsible for the majority of MRSA infections in Ontario.

5 **Methods and Materials:**

Selection and Propagation of Bacteriophages:

Samples of the phages Twort, 2638A, P68, and 44AHJD and their bacterial propagating strains were obtained from Dr. H.W. Ackermann, director of the Felix d'Herelle Reference Centre for Bacterial Viruses at the University of Laval. Samples of the phages ϕ 812, ϕ 131, 10 SK311, and U16 were obtained from Dr. L. Valicek of the Czech Collection of Animal Pathogens at the Veterinary Research Institute, Brno, Czech Republic. Upon receipt, each propagating strain was sub-cultured onto a Columbia base blood agar (5% sheep blood) plate (BA plate)(Oxoid) for purity and incubated overnight at 37°C. A single colony was used to inoculate 5ml of trypticase soy broth (TSB)(Difco), which was incubated overnight at 37°C with 15 shaking. 1.5ml of the overnight culture was added to 150ml of TSB and grown at 37°C with shaking for 3hrs. At the end of the 3hrs, by which point the cultures had become somewhat turbid with bacterial growth, 500 μ l of the phage solutions (10^{5-8} pfu/ml) were added to the cultures. The cultures were left without shaking for 10-15min to allow phage adsorption, and then grown under the same conditions until lysis was observed or 6hrs passed. The cultures were 20 then divided into 50ml Falcon tubes and centrifuged at 2000g for 20min to pellet the bacterial cells and debris. The supernatant was filtered through a 0.22 μ m pore size vacuum driven filter (Millipore Stericup) to sterilise the solution and remove as much bacterial debris as possible. The final phage solution was titrated on lawns of the appropriate propagating strain, and the concentration calculated in plaque forming units per ml (pfu/ml) [Ackerman, H.W.a.D., M.S., 25 Viruses of Prokaryotes Vol 1. 1987, Boca Raton, Florida: CRC Press].

Selection of MRSA Isolates:

The study targeted the four MRSA strains which have been responsible for most of the MRSA cases in Ontario for the last seven years (Ontario Epidemic, North American, British Empire and Historic). These strains have been delineated on the basis of macro-genetic analysis 30 (*Sma*I digestion and pulse field gel electrophoresis). A selection of 92 isolates, representative of each of the strains, were plated from freezer stocks maintained in the Microbiology

Department of Mount Sinai Hospital, Toronto, Ontario. Their classification as methicillin-resistant was based on the determination of the minimum inhibitory concentration of a variety of antibiotics, in accordance with the guidelines set out by the National Centre for Clinical Laboratory Standards.

5 In addition, most of the isolates had been phage typed by the Laboratory Centres for Disease Control (Winnipeg, Manitoba, Canada) according to the standard protocols Parker, M.T., *Methods in Microbiology*, Vol 7B, 1972, New York and London: Academic Press). The isolates chosen represented a wide variety of phage types, including 20 classified as non-typable. Finally, to test the specificity of phages, 5 coagulase-negative *Staphylococcus aureus* (CNST) 10 clinical isolates, and ATCC strains of *S. saprophyticus* (ATCC #15305) and *S. epidermidis* (ATCC #12228) were also tested for their susceptibility.

Screening of MRSA Isolate Susceptibility to Phages:

The MRSA isolates were plated onto BA plates and incubated overnight at 37°C. Lawns of bacterial growth were created by making up 0.5 MacFarland standard solutions in sterile 15 0.9% NaCl solution (0.5 MacFarland is equivalent to 1.5×10^8 cfu/ml). Sterile swabs soaked in the 0.5 MacFarland solutions were used to spread bacteria evenly across the plates. Dilutions of each phage were made up, ranging in concentration from 10^9 - 10^3 , inclusively. 10µl of each dilution was spotted onto the bacterial lawns and the solution allowed time to be absorbed. The plates were then incubated overnight at 37°C. The formation of plaques exhibiting confluent 20 lysis was taken as evidence of virulent infection and successful lysis of the target MRSA strain (Parker, M.T., *supra*). The degree of lysis was classified as either not susceptible (no visible plaques), weakly susceptible (very few isolated plaques), or strongly susceptible (total lysis at higher concentrations, and clearly defined plaques at lower concentrations). The isolates were screened in batches of variable size (10-30) and control plates of the propagating strains were 25 run in parallel with each batch to ensure the activity of the phage dilutions. One isolate which possessed a very strong capsule and which was initially resistant to the phages was re-tested on Trypticase Soy Agar (TSA).

Once all 92 isolates had been screened separately with each phage, a selection of MRSA isolates, representative of both the strongly susceptible and not susceptible groups, were tested 30 with a combination of all four phages. 10µl of a concentration of each phage were spotted together onto the same lawn, so that the concentration of each phage was the same as when

tested individually.

Results:

92 MRSA isolates, representative of the four strains responsible for the majority of the MRSA cases in Ontario over the last 7 years, were screened with 4 phages from the family of *Myoviridae*. The results are shown in the table below.

Relative Susceptibility of MRSA Isolates to each Phage:

Phage	Resistant (%)	Weakly Susceptible (%)	Strongly Susceptible (%)
44AHJD	29 (36.25)	22 (27.5)	29 (36.25)
P68	24 (30)	24 (30)	32 (40)
2638A	80 (100)	0 (0)	0 (0)
Twort	80 (100)	0 (0)	0 (0)
ϕ 812	6 (6.5)	5 (5.5)	81 (88)
ϕ 131	2 (2.2)	9 (9.7)	81 (88)
SK111	5 (5.4)	8 (8.7)	79 (85.9)
U16	3 (3.2)	11 (12)	78(84.8)

10

The percentage of isolates of each strain that were susceptible was compared to determine whether the macro-genetic characteristics analysed by pulse field gel electrophoresis is a predictor of susceptibility to phages.

The isolate displaying the strong capsule formation that was initially resistant to phages ϕ 812, ϕ 131, SK311, and U16, was re-tested on TSA and found to be strongly susceptible.

Discussion:

Of the 8 phages, ϕ 812, ϕ 131, SK311, and U16 collectively proved capable of lysing ~99% of the isolates screened, most of which were strongly susceptible. These 4 phages have therapeutic value against MRSA. Phage therapy's advantages over conventional antibiotics for the treatment of MRSA include its specificity of action, its relatively non-specific mechanism

20

and its natural adaptability. While conventional antibiotics affect all bacteria, including the normal micro-flora, bacteriophages only destroy the host bacteria. Since there are no commensal strains of *S. aureus*, there would be no ill effects due to the elimination of such organisms as the non-pathogenic *E.coli* found in the gastrointestinal tract.

5

The present invention is not to be limited in scope by the specific embodiments described herein, since such embodiments are intended as but single illustrations of one aspect of the invention and any functionally equivalent embodiments are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and
10 described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

All publications, patents and patent applications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the, methodologies etc. which are reported therein which might be used in connection with the invention. Nothing herein is
15 to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise.

We Claim:

1. Isolated and purified bacteriophages from the species *Myoviridae* that are used in the treatment of infectious diseases caused by bacteria.
- 5 2. A pharmaceutical formulation comprising an isolated and purified bacteriophage from the species *Myoviridae*.
3. A pharmaceutical formulation as claimed in claim 2 wherein the bacteriophage is selected from the group consisting of $\phi 812$, $\phi 131$, *SK311*, and *U16*.
4. A method for treating an infectious disease caused by bacteria in an animal comprising
10 administering to an animal in need of such treatment an isolated and purified bacteriophage as claimed in claim 1.
5. A method as claimed in claim 4 wherein the infectious disease is caused by methicillin-resistant *Staphylococcus aureus* (MRSA).
6. A method of reducing virulence of bacteria in a subject comprising administering to the
15 subject an effective amount of an isolated and purified bacteriophage as claimed in claim 1.
7. A method as claimed in claim 6 wherein the bacteria is a methicillin-resistant *Staphylococcus aureus* (MRSA).
8. Methods for killing or inhibiting the growth of bacteria comprising contacting the bacteria
20 with an effective amount of an isolated and purified bacteriophage as claimed in claim 1.
9. A method as claimed in claim 8 wherein the bacteria is a methicillin-resistant *Staphylococcus aureus* (MRSA).
10. A method as claimed in claim 8 wherein the method is used to treat a food product, a substance used in making a food product, a medical instrument, skin, a surgical implant,
25 or metallic, plastic, tile, porcelain, or glass surface.
11. A bactericide comprising one or more isolated and purified bacteriophages from the species *Myoviridae*.
12. A bactericide as claimed in claim 11 comprising one or more of $\phi 812$, $\phi 131$, *SK311*, and *U16*.