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(54) Title: BACTERIOPHAGES SPECIFIC TO PAK AND CHA STRAINS OF PSEUDOMONAS AERUGINOSA AND THEIR APPLICATIONS

(57) Abstract: The present invention relates to isolated bacteriophage specific to PAK and CHA strains of Pseudomonas aeruginosa and therapeutic compositions comprising said phage. The invention is also directed to their use for the treatment or prevention of bacterial diseases caused by *P. aeruginosa*, in particular for phage therapy of lung infections. The present invention also relates to human or pet food products prepared thereof.



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BACTERIOPHAGES SPECIFIC TO PAK AND CHA STRAINS OF PSEUDOMONAS AERUGINOSA AND THEIR APPLICATIONS

The present invention relates to isolated phage specific to PAK and CHA strains
5 of *Pseudomonas aeruginosa* and therapeutic compositions comprising said phages. The invention is also directed to their use for the treatment or prevention of bacterial diseases caused by *P. aeruginosa*, in particular for phage therapy of lung infections. The present invention also relates to human or pet food products prepared thereof.

Pseudomonas aeruginosa causes acute pneumonia with a high mortality rate in
10 immuno-compromized patients, while in cystic fibrosis patients, it triggers chronic inflammation leading to destruction of the lungs (1). This bacterium as well as an increasing number of opportunistic pathogens is now resistant to multiple antibiotics, urging us to look for other therapeutic approaches, among which phage therapy has been proposed (2). Phage therapy has been considered since the late 80s (3-5) mainly
15 because: 1) It has been used for decades in Eastern Europe and is still used to treat humans infections (6); 2) Phage biology is much better understood today than it was in the mid-1900s when it was overlooked in favour of antibiotics (7-9); 3) Phages are known to be the most specific bacterial killer. Curiously, a large number of *P. aeruginosa* bacteriophages have been characterised (10), but few papers report on using
20 these phages in therapy experiments, and none test them on a lung infection model (11, 12).

There is a need to develop compositions including phage therapeutics in acceptable vehicles that can be administered to subjects infected with bacterial microorganisms.

25 It is therefore an object of the present invention to develop new and useful phage therapeutics which can be used to prevent or to treat infectious disorders caused by pathogenic bacteria, preferably by *Pseudomonas aeruginosa* infections, in particular lung infection diseases in humans or animals.

With the use of a bioluminescent *P. aeruginosa* strain the inventors have
30 monitored and quantified the efficacy of a phage treatment in live animals using phages specific to the PAK strain of *P. aeruginosa*. For example, they have demonstrated that an intranasal phage treatment is effective in saving animals. It has also been shown that

phages given 24 hours (24H) before the initiation of the infection are able to prevent the development of this infection demonstrating the use of phages for a preventive treatment. Both curative and preventive treatments were also demonstrated as active against the CHA strain.

5 Thus the present invention is directed to isolated and/or purified bacteriophages that are specific to PAK and CHA strains of *Pseudomonas aeruginosa*.

 In a preferred embodiment, said isolated and/or purified bacteriophages according to the invention are bacteriophages of the Myoviridae family, more preferably capable to reach 100 % survival of infected mice with *Pseudomonas aeruginosa*
10 bacteria when administering said bacteriophages 2 hours (2H) post-infection, as shown for example in figure 3 herein.

 Preferably, said bacteriophages are isolated from sewage water.

 In a more preferred embodiment, said isolated and/or purified bacteriophages according to the invention are selected from the group of bacteriophages consisting of:

- 15 - PAK-P1 which has been deposited at the Institut Pasteur, 28 rue du Docteur Roux, F-75024 Paris Cedex 15, FRANCE, on July 11, 2008, under the deposit number CNCM I-4040;
- PAK-P2 which has been deposited at the Institut Pasteur, 28 rue du Docteur Roux, F-75024 Paris Cedex 15, FRANCE, on July 11, 2008, under the deposit number CNCM I-
20 4041;
- PAK-P3 which has been deposited at the Institut Pasteur, 28 rue du Docteur Roux, F-75024 Paris Cedex 15, FRANCE, on July 11, 2008, under the deposit number CNCM I-4042;
- PAK-P4 which has been deposited at the Institut Pasteur, 28 rue du Docteur Roux, F-
25 75024 Paris Cedex 15, FRANCE, on July 11, 2008, under the deposit number CNCM I-4043;
- PAK-P5 which has been deposited at the Institut Pasteur, 28 rue du Docteur Roux, F-75024 Paris Cedex 15, FRANCE, on July 11, 2008, under the deposit number CNCM I-4044; and
- 30 - PAK-P3 CHA which has been deposited at the Institut Pasteur, 28 rue du Docteur Roux, F-75024 Paris Cedex 15, FRANCE, on March 30, 2009 under the deposit number CNCM I-4139.

Are also more preferred isolated and/or purified bacteriophages according to the present invention that are selected from the group of bacteriophages consisting of the bacteriophages having the sequences SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5, or a specific fragment thereof capable to reach 100% survival of infected mice with *Pseudomonas aeruginosa* bacteria when administering said specific sequence fragment 2 hours (2H) post-infection as shown in figure 3.

SEQ ID NO: 1 corresponds to the complete nucleic sequence of the PAK-P1 phage which has been deposited at the Institut Pasteur, 28 rue du Docteur Roux, F-75024 Paris Cedex 15, FRANCE, on July 11, 2008, under the deposit number CNCM I-4040;

SEQ ID NO: 2 corresponds to the complete nucleic sequence of the PAK-P2 phage which has been deposited at the Institut Pasteur, 28 rue du Docteur Roux, F-75024 Paris Cedex 15, FRANCE, on July 11, 2008, under the deposit number CNCM I-4041;

SEQ ID NO: 4 corresponds to the corrected complete nucleic sequence (2 contigs assembled) of the PAK-P2 phage, compared to the SEQ ID NO:2;

SEQ ID NO: 3 corresponds to the complete nucleic sequence of the PAK-P3 phage which has been deposited at the Institut Pasteur, 28 rue du Docteur Roux, F-75024 Paris Cedex 15, FRANCE, on July 11, 2008, under the deposit number CNCM I-4042; and

SEQ ID NO: 5 corresponds to the complete nucleic sequence (5 contigs assembled) of the PAK-P3 CHA (also named "PAK CHA") which has been deposited at the Institut Pasteur, 28 rue du Docteur Roux, F-75024 Paris Cedex 15, FRANCE, on March 30, 2009 under the deposit number CNCM I-4139.

In a second aspect, the invention is directed to a pharmaceutical composition comprising isolated and/or purified bacteriophage according to the present invention.

In a preferred embodiment, the present invention comprises the use of isolated and/or purified bacteriophages according to the invention as a medicament.

In a more preferred embodiment, the present invention relates to a composition comprising isolated and/or purified bacteriophages according to the present invention as a medicament or pharmaceutical composition for the prevention or the treatment of an infectious disease caused by bacteria in a mammal, preferably in human.

In a more preferred embodiment, said composition of the present invention is intended for the prevention or the treatment of lung infection, preferably caused by pathogenic *Pseudomonas aeruginosa*.

In an also preferred embodiment, said composition according to the invention
5 further comprises depolymerising enzymes to facilitate the bacteriophage infection of bacteria growing in biofilms.

In a more preferred embodiment, said depolymerising enzyme is an alginate lyase.

In a particular aspect, said composition according to the invention is an aerosol
10 formulation for administration to the lungs of a mammal.

For the composition of the invention, said bacteriophages can be initially prepared in lyophilized form.

In one embodiment, said pharmaceutical composition contains at least one of the bacteriophages of the present invention in an amount sufficient to achieve the desired
15 effect in an animal or human individual. This composition may be a tablet, a liquid, a dried oral supplement, a wet oral supplement, dry tube-feeding, wet tube-feeding etc. The pharmaceutical composition will further contain carriers and excipients that are suitable for delivering the respective active molecule of different nature to the target tissue. The kind of the carrier/excipient and the amount thereof will depend on the
20 nature of the substance and the mode of drug delivery and/or administration contemplated. It will be appreciated that the skilled person will, based on his own knowledge, select the appropriate components and galenic form.

The routes of administration include but are not limited to: oral, aerosol or other device for delivery to the lungs, nasal spray, intravenous, intramuscular, intraperitoneal,
25 intrathecal, vaginal, rectal, topical, lumbar puncture or intrathecal. Excipients which can be used as a vehicle for the delivery of the phage will be apparent to those skilled in the art. For example, the free phage could be in lyophilized form and be dissolved just prior to administration. The dosage of administration is contemplated to be in the range of 1 to 10 ml of 10^3 to about 10^{13} pfu/per ml, twice a day. The phages are administered until
30 successful elimination of the pathogenic bacteria is achieved.

With respect to the aerosol administration to the lungs, the isolated phages of the present invention can be incorporated into an aerosol formulation specifically designed

for administration to the lungs by inhalation. Many such aerosols are known in the art, and the present invention is not limited to any particular formulation. An example of such an aerosol is an aerosol wherein the propellant of which contains trichloromonofluoromethane, dichlorodifluoromethane and oleic acid. The concentrations of the propellant ingredients and emulsifiers are adjusted if necessary based on the phage being used in the treatment. The number of phages to be administered per aerosol treatment will be in the range of 10^3 to 10^{13} pfu.

In another aspect, the present invention is directed to a food or pet food product, a nutritional composition or a supplement for nutritional composition which comprises at least one isolated and/or purified bacteriophage of the invention.

Finally, the present invention is directed to the use of a least one isolated and/or purified bacteriophage of the invention for the preparation of a food or pet food product, a nutritional composition or a supplement for nutritional composition for the prevention or the treatment of infections caused by pathogenic bacteria, preferably by pathogenic *Pseudomonas aeruginosa* in humans or animals, or for decreasing the degree of contamination of food product contamination.

For example, a usual food product may be enriched with at least one isolated and/or purified bacteriophage according to the present invention, particularly food products or nutritional supplements for clinical nutrition of animals or human.

In another embodiment, said at least one isolated and/or purified bacteriophage of the present invention may be prepared so as to improve pet food quality. As dietary adjuncts, they may be encapsulated or may be provided in powder form and packaged in conjunction with or separately from a main meal, be it wet or dry. By way of example, a powder containing bacteriophages according to the invention may be packed in sachets in a powder form or in a gel or lipid or other suitable carrier.

The nutritional formula is preferably enterally administrable. For example in the form of a powder, a liquid concentrate, or a ready-to-drink beverage. If it is desired to produce a powdered nutritional formula, the homogenized mixture is transferred to a suitable drying apparatus such as a spray drier or freeze drier and converted to powder.

The following examples preceded by the legends of the figures are given by way of illustration only and in no way should be construed as limiting the subject matter of the present application.

Figures legends

Figures 1A-1C: A phage treatment cure deadly infected animal.

Figure 1A: Survival curves of infected animals treated by PBS or phages at indicated phage/bacterium ratios.

Figure 1B: Example of time-course pictures of mice infected by bioluminescent *P. aeruginosa* and treated by PBS (left) or treated by the PAK-P1 phage at a ratio phage/bacterium of 10/1 (right).

Figure 1C: Quantification of emitted light given as a ratio between two time points (4H vs 2H and 6H vs 4H). The chest area from infected mice (n=12 from two independent experiments) treated by PBS (white) or treated by the PAK-P1 phage at a ratio 10/1 (black) was quantified.

Figure 2: A phage treatment reduces inflammation.

Cytokine levels were measured in BAL 24H after instillation of PBS (black), or PBS+ PAK-P1 phage (white), or bacteria + PBS 2H later (▼), or bacteria + PAK-P1 phage 2H later (grey)

Figure 3: Time-course of a phage treatment.

Survival curves of infected mice treated by PBS (♦) or by the PAK-P1 phage at 10/1 ratio at, 2H (■), 4H (▲) or 6H (○) after the infection was initiated.

Figure 4: Efficiency of a 24H phage pre-treatment.

Time-course of light emitted (photons/s) from the chest area of mice pre-treated by PBS (white) or PAK-P1 phage (black) 24H before the infection by *P. aeruginosa* (n=4 for each group).

Figures 5A-5C: Phages characterisation

Figure 5A: Electron microscopy of phage PAK-P1 (scale bar = 100 nm).

Figure 5B: Restriction profile of phages PAK-P1, PAK-P2 and PAK-P3.

Figure 5C: Lysis kinetics of phages PAK-P1, PAK-P2, PAK-P3 (average of 7 measures with s.d.).

Figure 6: An active phage is required for treating infected mice.

Survival curves of mice infected by *P. aeruginosa* and treated by PBS (), by inactivated PAK-P1 phage (◆), or by active PAK-P1 phage (Δ), the two latest at the 10/1 ratio.

Figure 7: Determination of the optimal delay before phage treatment

- 5 Pictures of animals treated by the 10/1 ratio of the PAK-P1 phage at 2, 4 or 6 H post-infection by bioluminescent *P. aeruginosa*.

Supplementary Figure 4: Comparison of three phage efficiencies

- A) Survival chart of infected mice treated with phages PAK-P1, PAK-P2 and PAK-P3 at 3 phage/bacterium ratios (1/10; 1/1; and 10/1).
- 10 B) Comparison of the efficiencies of phages PAK-P1, PAK-P2 and PAK-P3 at the ratio 1/10 determined by the ratio of light emitted between 2H and 6H post-infection (ie 4H after phages were added).

Figures 8A-8B: Comparison of three phage efficiencies

- Figure 8A: Survival chart of infected mice treated with phages PAK-P1, PAK-P2 and PAK-P3 at 3 phage/bacterium ratios (1/10; 1/1; and 10/1).
- 15

Figure 8B: Comparison of the efficiencies of phages PAK-P1, PAK-P2 and PAK-P3 at the ratio 1/10 determined by the ratio of light emitted between 2H and 6H post-infection (ie 4H after phages were added).

- Figures 9A-9B:** A treatment with the PAK-P3-CHA phage cure deadly infected animals by the CHA strain.
- 20

Figure 9A: Survival curves of infected animals treated 2H later by PBS or by the PAK-P3-CHA phage at indicated amounts.

Figure 9B: Survival curves of animals pretreated by the PAK-P3-CHA phage 4 days before to be infected by the CHA strain.

- 25 **Figures 10a-10f:** Bacteriophages cure and prevent lung infections caused by a CF *P. aeruginosa* strain.

- (a, b) Survival curves of mice infected with the CHA strain and treated or pre-treated with P3-CHA bacteriophage. (a) PBS (◆), 3×10^6 (●) and 3×10^7 (□) pfu of bacteriophage were given intranasally 2h after bacteria (3×10^6 cfu) were administered. This curative treatment appears to be dose dependent. (b) Four days before infection with 3×10^6 bacteria, mice were given either 3×10^7 (●), or 3×10^8 (□) pfu of P3-CHA or 3×10^8 pfu of heat-inactivated P3-CHA solution (◆). These survival curves indicate that the
- 30

preventative treatment is dose dependent. (c, d, e, f) 20h after infection with strain CHA, mice were euthanized and BAL fluids were assayed for bacteria (c), bacteriophages (d), and cytokines (e, f). In the curative treatment protocols, mice were treated with PBS or bacteriophage (phage 2h pi) 2h post infection. In the preventative treatment protocols, mice were intranasally administered bacteriophage solution (4d phage) or heat-inactivated bacteriophage solution (4d phage 80°C) four days before infection. (c) Bacterial counts were significantly lower in the BAL fluids from mice that had received either curative or preventative bacteriophage treatment than the respective control treatment (* $P < 0.05$, and ** $P < 0.01$). (d) Bacteriophage counts were significantly lower in the BAL fluids from mice that had bacteriophage treatment than that from non-infected animals (** $P < 0.01$) or non-infected animals pre-treated four days earlier with the bacteriophage P3-CHA (* $P < 0.05$). (e, f) IL-6 (e) and KC (f) concentrations in BAL fluids from mice that had received either curative or preventative bacteriophage treatments were significantly lower than the values for the corresponding untreated controls ((e) * $P < 0.05$, (f) * $P < 0.05$) and for animals pre-treated with heat-inactivated bacteriophage solution ((e) ** $P < 0.01$, (f) ** $P < 0.01$). $n = 5$ to 7 for each condition (c, d, e, f).

Figure 11: LDH activity in bacteriophage-treated and untreated mice.

BAL fluids were collected after 20h from infected mice (3×10^6 cfu) treated with PBS (second column), or treated with bacteriophage P3-CHA (3×10^7 pfu) given 2h after infection (phage 2h pi), or pre-treated with heat-inactivated bacteriophage P3-CHA (3×10^8 pfu) solution 4 days before (4d phage 80°C), or pre-treated with bacteriophage P3-CHA (3×10^8 pfu) 4 days before (4d phage) infection. LDH activity reflects the evaluation of tissue damage and results are expressed as multiples of the value for LDH in BAL fluids from non-infected mice (first column; arbitrarily defined as 1). LDH values for BAL fluids from bacteriophage-treated mice were significantly lower than those for untreated mice (* $P < 0.05$). Although LDH values for BAL fluids from mice pre-treated with bacteriophage were lower than those for mice pretreated with heat-inactivated bacteriophage solution, the difference was not significant ($P < 0.5$). $n = 4$ for each data set.

Figures 12a-12m: Histological and immunohistochemical analyses of lung sections from mice treated or pre-treated with bacteriophage P3-CHA.

(a, b, c, d; scale bar 200µm) Thin sections of lungs obtained after 20h from, (a) mice infected with the CHA strain, (b) infected mice treated with bacteriophage P3-CHA, (c) mice pre-treated with bacteriophage P3-CHA four days before infection, and (d) uninfected mice were stained with hematoxylin-eosin and Gram. Histological analyses included the determination of a lesion severity score (see materials and methods) to allow comparison (m). This score was significantly lower for mice given curative or preventive bacteriophage treatments than for untreated mice (* P<0.05). (e to l). Immunohistochemistry was performed with anti-*Pseudomonas* antibodies on sections obtained from the same samples as above (e, f, g, h; scale bar 100µm and i, j, k, l; scale bar 50µm). In lung sections from infected mice, bacteria are both intracellular and extracellular (e, i) whereas no signal can be seen in lung sections from uninfected mice (h, l). The signal was lower following either curative (f, j) or preventive (g, k) bacteriophage treatments and bacteria were only observed in macrophage cells.

Figures 13a-13d: Characterization of PAK-P3 and P3-CHA bacteriophages.

(a, c) Electron micrographs of PAK-P3 (a) and P3-CHA (c). Scale bar: 100nm. (b) SDS-PAGE of PAK-P3 and P3-CHA proteins; only the most abundant proteins give visible signals (MW: molecular weight markers, the arrow points to the major capsid proteins). (d) Clustal alignment of the three major capsid proteins of PAK-P1, PAK-P3 and P3-CHA bacteriophages with their closest homologs in the database with known function (the major capsid protein of Felix 01 bacteriophage; NP_944891).

Figure 14 Survival curve of infected mice treated with bacteriophage PAK-P3 compared to P3-CHA.

Mice were infected intranasally with 3×10^6 cfu (CHA strain) and 2h later were treated with either PBS (♦) or 3×10^7 pfu of bacteriophage PAK-P3 (●), or 3×10^7 pfu of

bacteriophage P3-CHA (□) also administered intranasally.

Figure 15: Persistence of P3-CHA bacteriophage in lungs of uninfected mice.

Three groups of five mice were given 3×10^8 pfu of bacteriophage P3-CHA, administered intranasally. The number of bacteriophages in BAL fluids was determined 1, 4 and 5 days post-administration.

Figures 16a-16b: Bacteriophage treatment of infected CFTR -/- mice.

(a, b) CFTR-/- mice were infected with 1×10^6 cfu of strain CHA and were treated 2h later with either PBS (♦) or 1×10^7 pfu of bacteriophage P3-CHA (●), both administered intranasally. Weight (a) and survival (b) were recorded twice a day. The dotted line

represents the weight limit (80% of the initial weight) below which animals were euthanized.

5

EXAMPLES

EXAMPLE 1: Materials and Methods

P. aeruginosa strains

10 A) The bioluminescent PAK strain (PAK lumi) was obtained as described in the publication Ramphal. et al, page 587, First column, paragraph “ Bacterial Strains” of Materials and Methods (21: J Immunol 181, 586-592, 2008 “Control of *Pseudomonas aeruginosa* in the Lung Requires the Recognition of Either Lipopolysaccharide or Flagellin”) (21). 4 serotyped clinical *P. aeruginosa* strains were used to determine
15 phage host range. 10 primo-colonization and 10 chronic clinical strains of *P. aeruginosa* were obtained from the French center of cystic fibrosis strain collection (P. Plésiat, Grenoble, France).

B) The CHA strain was cultivated at 37°C in LB medium with shaking. When bacteria reached the log phase of growth, they were centrifuged, washed twice with
20 PBS, and resuspended in the appropriate volume to obtain the desired concentration of bacteria.

Phages isolation, preparation and characterisation

A) Phages were isolated from sewage water (provided by the Laboratoire
25 d’Hygiène de la Ville de Paris) as follows. First, sewage water was filtered at 0.2 µm and mixed with an equal volume of 2X Luria-Bertani (LB) medium. Second, this mixture was inoculated with a fresh culture of PAK lumi and incubated on a shaker at 30°C overnight. The next day chloroform (1/10 volume) was added to the flask and placed on a shaker for one more hour. The medium was then centrifuged at 10,000 g for
30 10 min. 1ml of the supernatant was collected and 1/10 vol. of chloroform was added. After a brief mix by vortex, the eppendorf tube was centrifuged at 7,500 g for 5 min. To determine if phages were present in this extract, a drop (10 µl) of the supernatant was

applied on a LB agar plate and let dry. Using a palatine wire, the plate was streaked from the drop through the rest of the plate to isolate individual phages. Then, 1 ml of a growing culture of PAK lumi was applied to cover the entire plate; the excess was removed and the plate was incubated at 37°C overnight. Most of the time a drop from a
5 100 fold dilution of the original supernatant was enough to obtain isolated plaques. The following day, one or two plaques were picked up and resuspended in 200 µl of SM buffer (10mM Tris HCl pH7, NaCl 200 mM, gelatine 0.03%). 20 µl of chloroform was added in each tube and tubes were briefly mixed by vortex and centrifuged at 7,500 g for 5 min. Afterwards, 10 µl of the supernatant was applied on a LB plate and let dry
10 and the previous procedure was repeated at least three times. Once the majority of plaques were homogenous, 10 µl of the last resuspended plaque were added to 1 ml of growing culture of PAK lumi at OD 0.1 at 600 nm. This culture tube was incubated at 37°C for 3 to 4 hours until lysis occurred. After addition of 1/10 vol. of chloroform, the culture was transferred to an eppendorf tube, centrifuged at 7,500 g for 5 min and put at
15 4°C. This was considered as the primary stock. Several dilutions of this stock were kept at 4°C and used to infect larger volume of culture in order to prepare larger amounts of phages.

Large preparations of phages were performed using cesium chloride ultracentrifugation technique as described in Boulanger (22).

20 Plaque assay (from not diluted to 10^{-6} dilution) were performed to determine host range and activity levels in comparison with the PAK lumi strain. A high activity was defined as an activity equivalent to the PAK lumi strain; a weak activity as 10^2 to 10^4 less active than the PAK lumi strain and a null activity when no lysis was observed with the not diluted phage solution.

25 Lysis kinetics were performed at 37°C in 96 wells microplates in a final volume of 100 µL with a ratio of 1 phage for 1000 bacteria. OD was automatically recorded every 5 minutes with a microplate reader (Biotek, USA).

Phage DNA was prepared using the Qiagen DNA lambda mini-prep kit from 5ml fresh liquid lysate. Restriction profiles by AccI enzyme were performed using
30 manufacturer's recommendations (NEBiolabs, USA).

Electron microscopy was performed from cesium chloride phage preparations (23). Observations were performed on a Jeol 1200 EXII.

For animal experiments, phages prepared by cesium chloride ultracentrifugation were diluted in PBS.

B) Bacteriophage PAK-P3 was isolated from sewage water as described in Debarbieux *et al.*¹³. To adapt the PAK-P3 bacteriophage to the CHA strain, a liquid culture of the CHA strain was infected with PAK-P3 (MOI of 1/1000) and incubated at 37°C with shaking. After 4 hours, the culture, showing signs of lysis, was stopped by adding few drops of chloroform. The culture was then centrifuged at 8000g for 10 min, and the supernatant was stored at 4°C before use in the next round of amplification. This was repeated five times and the final supernatant was diluted and plated onto a Petri dish overlaid with the CHA stain. A few individual plaques were picked and resuspended in SM buffer (10mM Tris HCl pH7, 200mM NaCl, 0.03% gelatine) and subsequently used for four cycles of plaque purification. A final set of 10 isolated plaques were chosen and tested for their host range against a set of 20 CF strains of *P. aeruginosa*¹³. All plaques showed the same host range, and one was chosen and named bacteriophage P3-CHA. Liquid cultures of 1 litre were used for large-scale preparation of PAK-P3 and P3-CHA as described by Boulanger¹⁷. Bacteriophage solutions were either diluted in PBS for the curative treatment or passed five times through an endotoxin-removal column (EndoTrap blue, Hyglos, Germany) before use for the preventive treatment. Endotoxin concentrations were determined using the QCL-1000® Chromogenic LAL endpoint assay (Cambrex, Walkersville, MD, USA). The endotoxin-free bacteriophage solution contained 1×10^{-10} endotoxin units per pfu.

Heat-inactivated bacteriophage solution was obtained by 10 min incubation at 80°C.

Electron microscopy observations were performed on a Jeol 1200 EXII machine after uranyl acetate staining of caesium chloride bacteriophage preparations¹⁸.

Genomic characterisation of bacteriophages

Genome sequencing (20 to 25x coverage) was performed using 454 technology with DNA prepared by standard procedures. The complete genome sequences of the PAK-P3 and P3-CHA bacteriophages are respectively SEQ ID NO:3 and SEQ ID NO:5. Sequence analysis to search for temperate bacteriophage markers (similar genes) was

performed as described previously¹³. PAK-P3 and P3-CHA genomes were compared to search for mutations.

Total bacteriophage proteins were run on an SDS gel and subjected to in-gel trypsin digestion; microsequencing of the major capsid proteins was then performed by the Institut Pasteur microsequencing facility. None of the peptide sequences corresponded to any protein sequences in the databank before inclusion of our bacteriophage sequence data. Alignments shown in supplementary figure 2 were performed with Clustal X.

10 **Animal infections and Ethics Statement**

Mice (8 weeks old Balb/c males) were supplied by the Centre d'Elevage R. Janvier and housed in animal facility following Institut Pasteur guidelines in agreement with European recommendations. Food and drink were provided ad libitum. Mice were anesthetized by intra peritoneal injection of a mixture of ketamine-xylazine before infection. Then, 1×10^7 luminescent bacteria resuspended in 50 μ l of PBS were instilled intranasally. Two hours after bacterial instillation, bioluminescence was recorded and, while mice were still asleep (isoflurane inhalation), phages were applied intranasally in a final volume of 30 μ l.

Mice were then observed twice a day.

20

Luminescence measurements

Photon emission of the luminescent bacteria in the lungs of infected mice was quantified using the IVIS 100 system (Xenogen Biosciences). After infection, luminescence record was performed in mice under isoflurane inhalation in an IVIS charge-coupled device camera coupled to the LivingImage software package (Xenogen). A digital false-colour photon emission image of the mice was generated, and photons were counted within a constant defined area corresponding either to the surface of the chest encompassing the whole lung region or to the entire animal. Photon emission was expressed as photons per second emitted.

30

Bronchoalveolar lavage and inflammation analyses

Bronchoalveolar lavage (BAL; 4x0.5ml) were performed at the indicated time periods (i.e. 20h after infection) after infection following pentobarbital euthanasia (300mg/kg). The BAL fluids were diluted and plated on LB agar plates to obtain viable bacterial counts in the BAL fluid. BAL were centrifuged 10 min at 1,400 rpm and then
5 murine cytokine concentrations were determined using DuoSet ELISA kits obtained from R&D Systems.

LDH activity was estimated using the Cytotox96 kit from Promega. Another aliquot of the BAL fluids was centrifuged at 6000 rpm for 10 minutes. To determine the amount of free bacteriophages, supernatants were diluted and spotted onto plates
10 overlaid with the CHA strain. To determine viable bacterial counts, pellets from BAL fluids centrifugations were resuspended in PBS, serially diluted and plated onto LB agar plates.

Histological and immuno-histochemistry analyses

15 Lungs were removed from euthanized animals, fixed in 4% buffered formaline for at least 24h at 4°C, and then embedded in paraffin. Serial 4mm-thick sections were stained with haematoxylin-eosin (HE) and Gram. Histological scoring of elementary lesions of alveolitis, bronchitis, necrosis, and consolidation were evaluated by an investigator blind to the treatment group from 0 to 5 (0: none, 1: mild, 2: moderate
20 focal, 3: moderate focal and diffuse, 4: severe focal, 5: severe focal and diffuse extending to the whole lobule). Recruitment of lymphocytes, alveolar macrophages - monocytes and polymorphonuclear cells was similarly evaluated from 0 to 3 (0: no, 1: weak, 2: moderate and 3: severe). Immunohistochemistry of infected lungs was performed with a primary polyclonal anti-*Pseudomonas* antibody raised in rabbit
25 against the PcrV protein²⁴. Affinity-purified antibodies were diluted 1/4000 before use. The method used was a classical Envision Dako protocol performed with amino-ethyl – carbazol as a chromogen.

Statistical analysis

30 P values were calculated with unpaired t-test using XLStat software (France). A one-tailed Mann-Whitney paired test in Prism 4.0 software (France) was used to compare groups. Error bars are s.e.m.

EXAMPLE 2: Isolation and characterisation of new phages specific to the PAK strain of *P. aeruginosa* and its susceptibility to *in vivo* phage infection: PAK ability to cure and prevent bacteria infection

5 New phages specific to the PAK strain of *P. aeruginosa* were isolated from sewage water. 3 phages named PAK-P1, PAK-P2 and PAK-P3 were chosen for this study. Electron microscopy observations revealed that these three phages are part of the *Myoviridae* family (10). By studying their lysis kinetics in liquid culture, their host range against a panel of 4 clinical *P. aeruginosa* strains and their DNA restriction
10 profiles, It has been established that these three phages are genetically distinct (see Figures 5A-5C and Table 1 below).

Table 1: Phages host range was determined against a panel of 4 clinical strains serotypes O:4; O:9; O:10; O:11) and compared to the PAK strain (serotype O:6). N = no
15 activity; W = weak activity; H = high activity.

D)

Serotype	PAK-P1	PAK-P2	PAK-P3			
O: 4		W		W		W
O: 6		H		H		H
O: 9		W		N		N
O: 10		N		N		N
O: 11		W		W		W

To ascertain the affect of phage on infection in a live animal lung infection model, mice were both infected by the bioluminescent PAK strain and treated with
20 phage by intranasal instillation. After a preliminary experiment which showed that the PAK-P1 phage was active *in vivo* by delaying the death of highly infected animals (data not shown), we designed an experiment to determine the amount of this phage required to fully cure infected mice (see Figures 1A-1C). While non phage-treated mice (NPT-mice) died within 48H after the PAK inoculation (the majority were still alive after
25 24H), phage-treated mice (PT-mice) using a ratio of 1 phage/10 bacteria, died within 5 days. PT-mice using higher phage to bacterium ratios (1/1 and 10/1), survived until the end of the experiment (12 days). In two independent experiments, 100% of animals

treated by the dose 10/1 survived, while only 80% of animals survived among those treated by the 1/1 ratio, leading us to choose the ratio 10/1 as a standard condition for future experiments.

An active phage was required to cure animals since giving infected mice a heat-killed phage PAK-P1 solution (shown to be inactive on a bacterial lawn) did not cure them when given 2H after infection, animals dying at the same rate as untreated animals (see Figure 6). Moreover, to determine if PAK-P1 phage treatment was harmless to animals, we applied intranasally a dose 10 times higher than that previously defined as a standard dose, and followed the behaviour of a group of mice (n=8) for 10 days. These mice did not show erratic behaviour, their fur stayed regular and they gained weight, suggesting the phage did not have an adverse affect on these animals.

The rate at which the PAK-P1 phage was able to eliminate bacteria *in vivo*, was estimated by quantifying the light emitted by the bioluminescent bacteria in live animals during the first hours of infection (see Figures 1A-1C). Animals were first inoculated with PAK and then with PAK-P1 phage 2H later. Between 2H and 4H post-bacterial infection, the variation of light emitted from PT-mice by 10/1 dose and NPT-mice showed no significant difference, showing that the initial evolution of the infection is similar in both groups during the first 4 hours. However, at the 6H time point (ie 4H after phage was added), the variation of light emitted from phage treated mice was highly significantly reduced compared to NPT-mice, suggesting a rapid killing of bacteria by phages. 24H after the infection started, PT-mice presented none or weak spots of light, while NPT-mice were dead or highly luminescent (data not shown), suggesting that phage treated animals can survive the lethal bacterial challenge.

It is hypothesised that if phages are able to kill bacteria *in vivo* leading to a reduction in the amount of these bacteria in the lungs, then the inflammatory response (the first line of host defence against invading pathogens) should be diminished. By performing broncho-alveolar lavages (BAL) on NPT-mice and PT-mice, we evaluated the levels of two inflammatory markers, TNF- α and IL-6 (known to be induced by a bacterial challenge (13-15)) 24H after phage addition (see Figure 2). Both IL-6 and TNF- α increased in PAK infected animals (either with or without phage) compared to non PAK infected controls confirming that the infection had started in both groups. These levels were significantly reduced however, in the phage-treated group in

comparison to the untreated group confirming that bacteria/ml (n=4). In contrast only 1
10 reducing the number of bacteria by phage resulted in an attenuation of the host
inflammation response. 48H after the infection IL-6 and TNF- α levels returned to basal
values in the PT group (data not shown). We also measured the bacterial load and phage
5 amounts in BAL after 24H of infection. As expected in the BAL of NPT-mice no phage
was detected and the average amount of bacteria was $1 \cdot 10^8$ bacteria/ml (n=4). In
contrast only $1 \cdot 10^2$ bacteria/ml were recovered from BAL of PT-mice together with 1
 10^7 phages/ml (n=4). In comparison, in BAL from uninfected PT-mice, PAK-P1 phage
was still present at a concentration of $5 \cdot 10^5$ phages/ml (n=2), which confirmed that
10 phages did multiply inside the lungs of infected and phage treated animals.

Next, the maximum possible delay of phage treatment has been determined to
maintain a 100% survival rate of animals (2H, 4H and 6H after the infection). While in
the PT-mice 2H post-infection group 100% of mice survived, at 24H only 75% of mice
were still alive in the 4H and 6H groups. At 72H, survival was close for the 2H (100%)
15 and 4H (75%) groups but dropped to 25% for the 6H group. These results could actually
be anticipated when looking at the bioluminescence pictures at early time points (see
Figure 7). In such experiment giving phage 2H post-infection was then required to reach
100% survival of infected animals (see Figure 3).

This set of experiments demonstrates that non-invasive bioluminescence
20 technology is remarkably useful to assess the efficiency of a phage treatment and
especially to study the kinetics of infection at early time points without sacrificing
animals. For example, in the time-course experiment to determine the optimal time to
give phage after the infection was started, bioluminescence images helped us to
understand why early phage inoculation (2H) was so important in resolving a PAK
25 infection (see Figure 7). It is during this early time after infection that the multiplication
of bacteria is most rapid. In such conditions bacteria susceptibility to phage infection is
the highest. Then, rapidly the infection will be reduced and consequently, the host will
diminish its inflammatory response which is a benefit since an excess of inflammatory
response can be deleterious (13-15).

30 Results obtained with the PAK-P1 phage encouraged us to examine the ability of
other PAK-killing phages to cure infected animals or to prevent their infection. Next, 2

new phages PAK-P2 and PAK-P3 have been compared with the PAK-P1 by varying phage/bacterium ratios 10/1, 1/1 and 1/10 (see Figure 7). In terms of survival, animals of the three groups treated by phages at the ratio of 10/1 or 1/1 survived to the end of the experiment (12 days). However, at the dose of 1/10 of PAK-P1 phage 66% of mice
 5 died on the 3rd day and 100% on the 9th day, while a ratio of 1/10 for PAK-P2 and PAK-P3 allowed the survival of 100% of infected animals. Examination of the bioluminescent data of the 1/10 dose between 2H and 6H time points, revealed that the ratio of the light emitted from animals is more reduced by phages PAK-P3 and PAK-P2, than by phage PAK-P1 (see Figures 8A-8B). A similar efficiency is observed both *in*
 10 *vivo* and *in vitro* (PAK-P3 and PAK-P2 faster than PAK-P1) suggesting that inside the lungs there is no specific cellular factor (proteases for example) which could have enough activity to prevent phages from infecting bacteria.

The efficiencies of our phages were also studied against a panel of twenty *P. aeruginosa* clinical strains from cystic fibrosis patients. Ten strains from primo-colonisation and ten strains from chronically colonized patients were also studied. Our
 15 three phages were able to efficiently lyse up to 40% of primo-colonisation strains but only moderately lyse 30% of chronic ones (see Table 2).

Table 2: Phages efficacies on *P. aeruginosa* clinical strains

20 Numbers represent the percentage of strains sensitive to phages based on plaque assays from non diluted to 10⁻⁶ dilution.

Strains / Phages	PAK-P1			PAK-P2			PAK-P3		
	<i>H</i>	<i>W</i>	<i>N</i>	<i>H</i>	<i>W</i>	<i>N</i>	<i>H</i>	<i>W</i>	<i>N</i>
Primo-infection	40%	20%	40%	10%	50%	40%	0%	40%	60%
Chronic-infection	0%	10%	90%	0%	20%	80%	0%	30%	70%

This is in accordance with the fact that the phages were isolated from planktonic
 25 cultures, a bacterial life-style closer to the primo-colonisation state than the chronic state. In the case of chronic infection, phages described here are most likely not the most appropriate. For these situations more adequate phages should be specifically

isolated (16, 17) or existing phages could be “selected”, by cultivating them on bacteria growing in biofilms, to become more efficient. Actually, Hanlon *et al.* (18) demonstrated at least for one case that a *P. aeruginosa* phage was active on biofilms.

As phages given in uninfected animals persisted in reasonable amounts inside
5 the lungs at least for 24H, we tested whether a phage pre-treatment would prevent a subsequent infection. Two groups of mice which were administered either 10^8 PAK-P1 phage or buffer intranasally, were infected 24H later by 10^7 bacteria. Quantifications of light emitted from these 2 groups of mice, showed that 2H after bacteria inoculation the light emitted in the phage pre-treated group was approximately
10 5 times lower than the buffer pre-treated group (see Figure 4). Following time points confirmed that the bacterial load decreased in phage pre-treated animals and increased in the buffer pre-treated animals (see Figure 4). Finally, 100% of phage pre-treated animals survived while 100% of untreated animals died. This full protective effect was also observed when a 1000 fold dilution of phages (i.e 10^4 of PAK-P1 phage alone or a
15 cocktail of the three phages) was given 24H before infection (data not shown).

This last experiment demonstrates that low doses of phages can actively prevent an infection from occurring and provide 100% protection against a deadly bacterial challenge. Such an effect was not anticipated since it is generally reported that phages are rapidly eliminated from the body (19). This does not seem to be the case for the
20 lungs since we could still detect traces of phage on lungs of uninfected mice 5 days after instillation (data not shown). Recently Golshahi et al. (20) provided evidences that phages given by nebulization should be efficiently distributed in lungs. Taken together, these results demonstrate the possibility of using phages in prevention of bacterial lungs infection. For example one could propose to pre-treating populations at risk (immuno-
25 compromised or cystic fibrosis patients) with moderate dosage of phage in order to decrease the probability of infection when going to places where patients are more likely to be infected by bacteria (care centres, hospitals etc). This is particularly relevant in situation where an epidemic has been identified where a preventive treatment with specific phages could be used to limit the spread of the epidemic strain.

EXAMPLE 3: Successful curative and preventive phage therapies against a clinical CF *P. aeruginosa* strain using a lung infection model both in wild-type and CFTR -/- mice

An acute lung infection in mice was initiated by intranasal administration of a lethal dose of *P. aeruginosa* strain CHA (a clinical isolate from a CF patient in Grenoble hospital France²⁵). Development of the infection was assessed by quantification of bacteria, inflammatory markers and lactate dehydrogenase (LDH) levels at 20h post-infection time point (figures 10a-10f). The number of bacteria in the lungs had increased by two orders of magnitude compared with the initial infectious dose (fig. 10c). The levels of two cytokines (IL-6 and KC) and the level of LDH were highly elevated (fig. 10e, 10f and figure 11). Histological analysis, also at 20h post-infection, revealed severe lesions, consistent with acute pneumopathy, combined with focal and diffuse alveolitis and bronchitis, consolidation and necrosis (fig. 12a). Disease severity was scored at 16 out of 25 (fig. 12m). These findings confirmed that the *P. aeruginosa* strain CHA is able rapidly to infect lungs and induce severe damages leading to death in these mice, thereby making it a suitable model to study the curative potential of bacteriophages.

We then screened our collection of natural *P. aeruginosa* bacteriophages (initially isolated using the *P. aeruginosa* strain PAK¹³) and found that the PAK-P3 bacteriophage was able to establish a moderate infection in strain CHA (plating efficiency of 10 % compared with strain PAK). After five consecutive passages in liquid culture with the CHA strain we obtained a bacteriophage stock that showed the same plating efficiency on both CHA and PAK strains. Subsequently, four steps of plaque purification were performed to obtain a preparation of a single phage isolated that we named P3-CHA. Electron microscopy showed that both bacteriophages (P3-CHA and its parental isolate) belong to the Myoviridae family of bacterial viruses (figures 13a-13d). Their genomes were sequenced and analysed revealing that they are closely related to the PAK-P1 bacteriophage. Major capsid proteins were identified by mass spectrometry (figures 13a-13d). Putative proteins encoded by their genomes were analysed and no significant similarities to proteins considered to be markers of temperate bacteriophages or toxins were identified (see materials and methods). This

confirmed that both PAK-P3 (vB_PaeM_PAK_P3) and P3-CHA (vB_PaeM_P3_CHA) are virulents bacteriophages.

We administered two different doses of P3-CHA bacteriophage prepared by classical techniques to two groups of mice that had received a lethal dose of the CHA strain 2h earlier and followed survival (fig. 10a). Both doses of bacteriophage improved survival and the high dose was associated with a greater rate of survival than the low dose throughout the experiment (16 days). Twenty hours after inoculation (i.e. 18h after bacteriophage treatment was given) we quantified bacteria, bacteriophages, cytokines and LDH and also performed histological analyses. In the group treated by the high dose the number of bacteria was over two orders of magnitude lower than in the untreated group (fig. 10c), and the number of bacteriophages was ten times higher (fig. 10d). Cytokines (fig. 10e, 10f) and LDH (fig. 11) concentrations were markedly lower in the bacteriophage-treated group than the untreated group. Histological analysis confirmed that lung damages in the treated group were less severe than in the untreated animals. In the bacteriophage-treated mice, moderate or mild lesions of alveolitis and bronchitis were observed without necrosis or consolidation; the severity of these histological lesions was scored, in a blind protocol, 5 out of 25 (fig. 12b, 12m). Immunohistochemistry, using a polyclonal anti-*Pseudomonas* antibody, detected few bacteria (entire cells or debris) in bacteriophage-treated animals mainly in the cytoplasm of macrophages in the lungs (fig. 12f). By contrast, bacteria were detected in macrophages, alveolae, and extracellular spaces of the lungs from untreated animals (fig. 12j). These observations are consistent with the fact that bacteriophages target only extracellular bacteria and also demonstrate the role of phagocytosis in bacterial removal. These experiments indicate that a curative bacteriophage treatment acts cooperatively with the immune response to eliminate acute lung infection caused by a CF strain of *P. aeruginosa*. We also used survival curve analysis to assess the curative efficacy of the parental bacteriophage PAK-P3 (used in the same conditions as P3-CHA; figure 14). After 8 days, only 20 % of mice had survived. Thus, protection in this model *in vivo* correlated with *in vitro* plating efficiency, as previously hypothesised¹³.

To determine whether the P3-CHA bacteriophage could be used to prevent an infection by the CHA strain, we used an endotoxin-free bacteriophage solution (see materials and methods). This reduced the possibility of stimulating an immune response

in the host, which could mask the effect of bacteriophage treatment. We first determined the rate of elimination of the P3-CHA bacteriophage from the lungs of uninfected mice and found that its concentration decreased by about half-log/day over five days (figure 15). 20 % of animals (n=5) given 3×10^7 bacteriophages four days prior to infection with 3×10^6 bacteria were protected while all animals (n=10) given 3×10^8 bacteriophages were protected (fig. 10b). Thus, administration of a suitable dose of bacteriophage four days before initiation of infection fully protected mice. Mice were administered a fully protective dose of bacteriophage four days before to receive a lethal dose of the CHA strain, and then twenty hours later, animals were euthanized to perform histological analyses and quantify bacteria, bacteriophages, cytokines, and LDH (figures 10a-10f, figure 12). Bacteria counts were lower and bacteriophage counts were higher in P3-CHA pre-treated mice than in heat-killed P3-CHA pre-treated mice (fig. 10c, 10d). Cytokines concentrations were also significantly lower for P3-CHA pre-treated mice, whereas LDH concentrations were similar in both groups (fig. 10e, 10f and fig. 2). Histological analyses showed that lung damages were markedly less severe in P3-CHA pre-treated than the control animals, with histological lesions being scored at 7 out of 25 (fig. 12c, 12m). Immuno-histochemistry with lungs from mice that were pre-treated with P3-CHA bacteriophages showed a pattern that was similar to curative treatment. Bacterial antigens were detected only in the cytoplasm of alveolar macrophages (fig. 12g, 12k). These data demonstrate particularly that bacteriophage pre-treatment, given four days prior to infection, effectively reduces the infectivity of the CF strain of *P. aeruginosa*. The concentrations of IL-6 and KC in the lungs of mice four days after intranasal administration of bacteriophage solution alone were low (fig. 10e, 10f). Therefore, the main cause of bacterial destruction can be attributed to the bacteriophage itself. However, the cytokines concentrations were still significantly higher than those measured twenty four hours after administration of a PBS control solution. Therefore, partial involvement of a weak immune response stimulated by the bacteriophage solution cannot be ruled out. Indeed, the quality of the bacteriophage preparation, and in particular elimination of endotoxins (data not shown), influenced the extent of the immune response following pre-infection treatments. This is in contrast to post-infection treatments where the immune system has already been primed by bacteria when bacteriophages are administered.

CFTR $-/-$ mice are more sensitive than wt mice to infection with the CHA strain (L. Touqui, personal communication). However, using the standard dose of P3-CHA bacteriophage (10 fold the infectious dose) we were able to show that all of the bacteriophage-treated CFTR $-/-$ mice survived the lethal infection. This indicates that the CFTR $-/-$ genotype does not impair bacteriophage treatment (figures 16a-16b).

Our experiments demonstrate that bacteriophages isolated from the environment can be used to treat lung infections caused by a clinical CF strain, and that the CFTR $-/-$ genotype does not interfere with this treatment. In our model, bacterial debris released during bacteriophage treatments were not pro-inflammatory as production of cytokines was not strongly stimulated in both curative and preventive treatments. We have now shown for lung infections that two different bacteriophages that target two different bacterial strains are effective *in vivo*. These findings support that nothing in the lungs prevents bacteriophage/bacteria interactions. However, this is not sufficient to conclude that in general phage therapy in lungs will always be as positive, since different lung pathogen / bacteriophage pairs might give rise to different conclusions²⁶. This highlights the need for a detailed understanding of the bacterial processes in each given infectious model before concluding whether treatment with bacteriophages is effective. For example taking into account the chronically infected status of CF patients is an issue that has not yet been addressed with bacteriophage treatments. It is well known that *P. aeruginosa* forms biofilms inside lungs and this protects the bacteria from treatment with antibiotics. Nevertheless, several publications have reported that the use of depolymerising enzymes facilitates bacteriophage infection of bacteria growing in biofilms^{27, 28}. The use of alginate lyase to improve the dispersion of bacteriophages may also overcome the protection conferred by biofilms²⁹. Therefore, bacteriophages either alone or associated with other agents such as alginate lyase, DNases, or antibiotics, may be useful for the treatment of chronic bacterial infection in CF patients. Our study also suggests that pre-treatment with bacteriophages may allow long-term prevention of lung infection in CF patients. Due to the specificity of bacteriophages, any such pre-treatment requires preliminary identification of the pathogen and could be useful in particular situations, for example to limit the spread of epidemic strains in CF care centres.

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CLAIMS

1. Isolated and/or purified bacteriophages that are specific to PAK and/or CHA strains of *Pseudomonas aeruginosa*.
- 5 2. Isolated and/or purified bacteriophage according to claim 1 of the Myoviridae family.
3. Isolated and/or purified bacteriophages according to claim 1 or 2, selected from the group of bacteriophages consisting of:
 - PAK-P1 which has been deposited at the Institut Pasteur, 28 rue du Docteur Roux, F-
10 75024 Paris Cedex 15, FRANCE, on July 11, 2008, under the deposit number CNCM I-4040;
 - PAK-P2 which has been deposited at the Institut Pasteur, 28 rue du Docteur Roux, F-75024 Paris Cedex 15, FRANCE, on July 11, 2008, under the deposit number CNCM I-4041;
 - 15 - PAK-P3 which has been deposited at the Institut Pasteur, 28 rue du Docteur Roux, F-75024 Paris Cedex 15, FRANCE, on July 11, 2008, under the deposit number CNCM I-4042;
 - PAK-P4 which has been deposited at the Institut Pasteur, 28 rue du Docteur Roux, F-75024 Paris Cedex 15, FRANCE, on July 11, 2008, under the deposit number CNCM I-
20 4043;
 - PAK-P5 which has been deposited at the Institut Pasteur, 28 rue du Docteur Roux, F-75024 Paris Cedex 15, FRANCE, on July 11, 2008, under the deposit number CNCM I-4044; and
 - PAK-P3 CHA which has been deposited at the Institut Pasteur, 28 rue du Docteur
25 Roux, F-75024 Paris Cedex 15, FRANCE, on March 30, 2009 under the deposit number CNCM I-4139.
4. Isolated and/or purified bacteriophages according to one of claims 1 to 3, selected from the group of bacteriophages consisting of the bacteriophages having the sequences SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID
30 NO: 5 or a specific fragment thereof
5. Pharmaceutical composition comprising at least one isolated and/or purified bacteriophage according to one of claims 1 to 4.

- 6 Composition according to Claim 5 capable to reach 100 % survival of infected mice with *Pseudomonas aeruginosa* bacteria when administering said specific sequence fragment 2 hours (2H) post-infection as shown in figure 3.
7. Composition according to claim 6 for the prevention or the treatment of an
- 5 infectious disease caused by bacteria in a mammal, preferably in human.
8. Composition according to claim 6 or 7 for the prevention or the treatment of lung infection.
9. Composition according to one of claims 6 to 8 for the prevention or the treatment caused by pathogenic *Pseudomonas aeruginosa*.
- 10 10. Composition according to one of claims 6 to 9, wherein said composition is an aerosol formulation for administration to the lungs of a mammal.
11. Composition according to one of claims 6 to 9, wherein said composition further comprises a depolymerising enzyme.
12. Composition according to claim 11, wherein said depolymerising enzyme is an
- 15 alginate lyase.
13. Composition according to claim 10, wherein said composition contains bacteriophages which have been initially prepared in lyophilized form.
14. A food or pet food product, a nutritional composition or a supplement for nutritional composition comprising at least one phage isolate according to one of claims
- 20 1 to 4.
15. The use of a least one phage isolate preparation according to one of claims 1 to 4, for the preparation of a food or pet food product, a nutritional composition or a supplement for nutritional composition for the prevention or the treatment of infections caused by pathogenic bacteria, preferably by pathogenic *Pseudomonas aeruginosa* in
- 25 humans or animals.

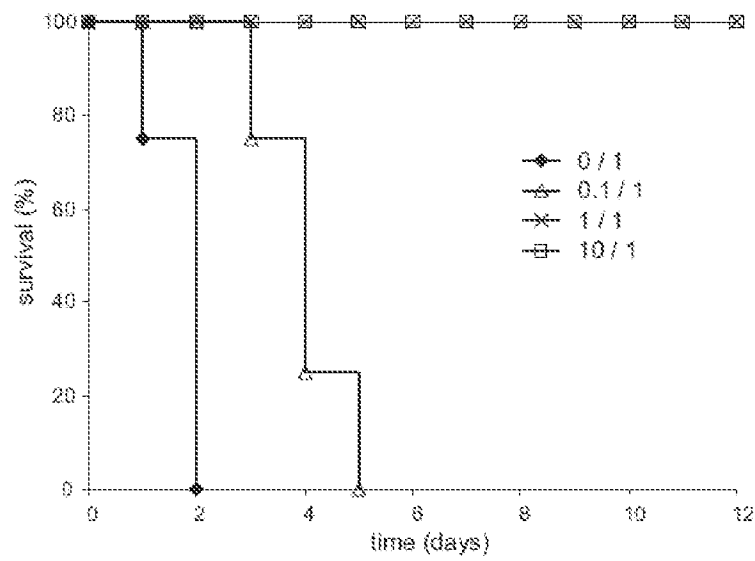


FIGURE 1A

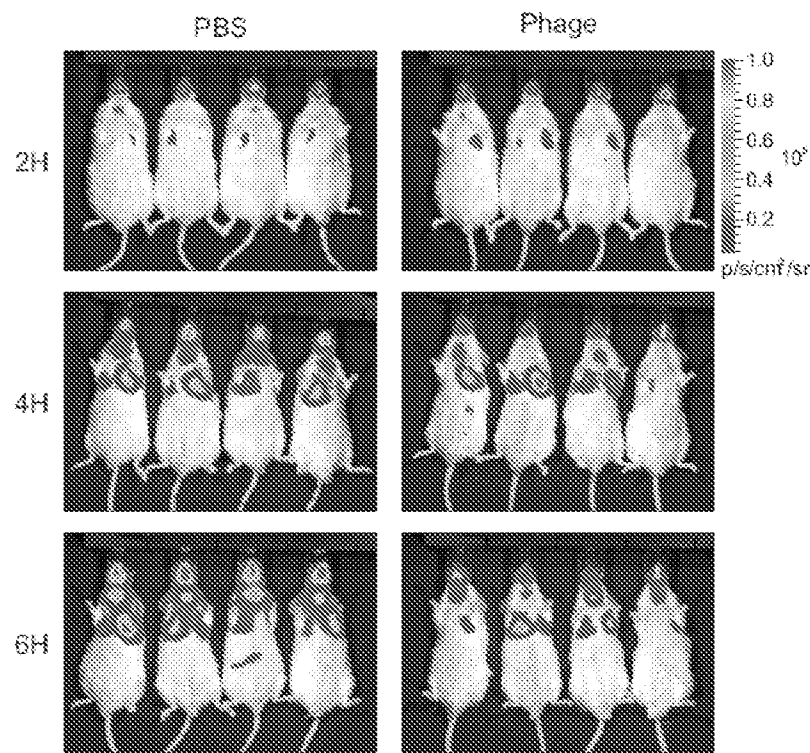


FIGURE 1B

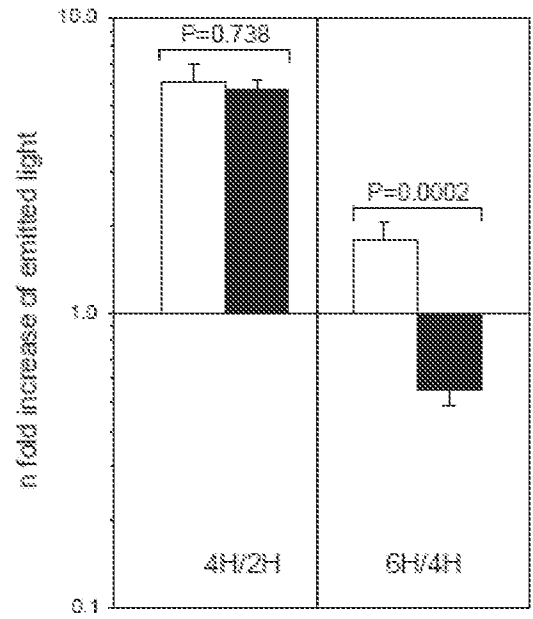


FIGURE 1C

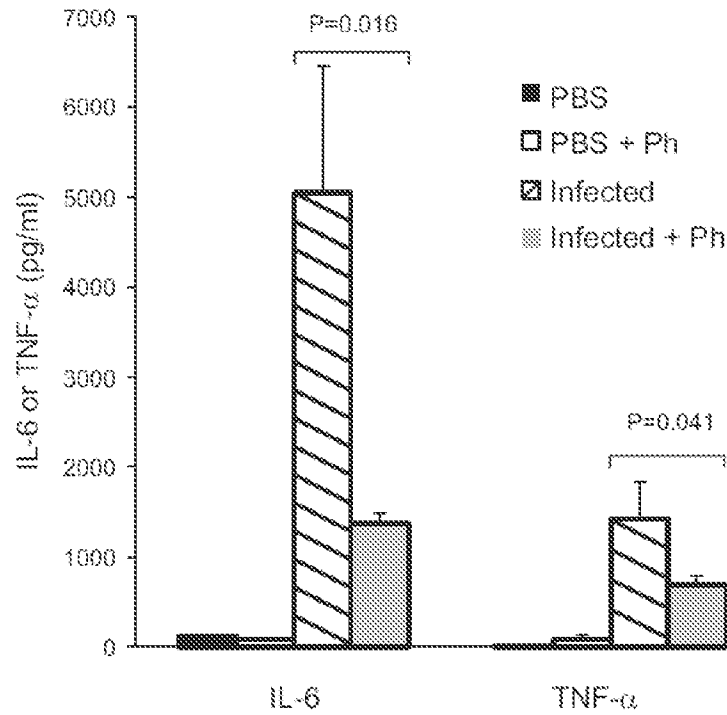


FIGURE 2

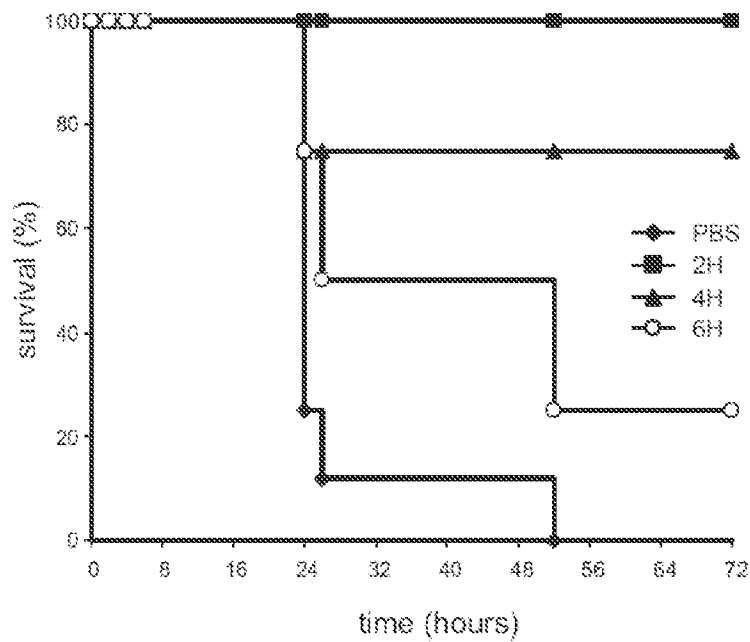


FIGURE 3

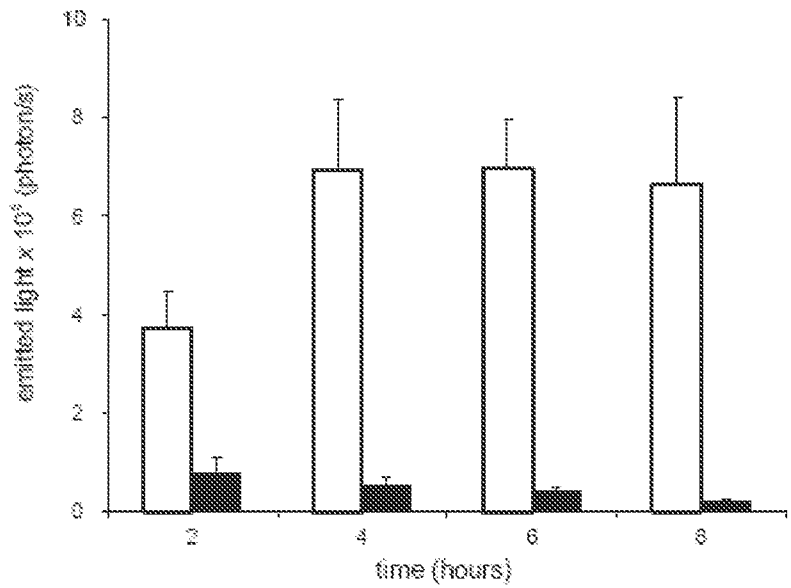


FIGURE 4

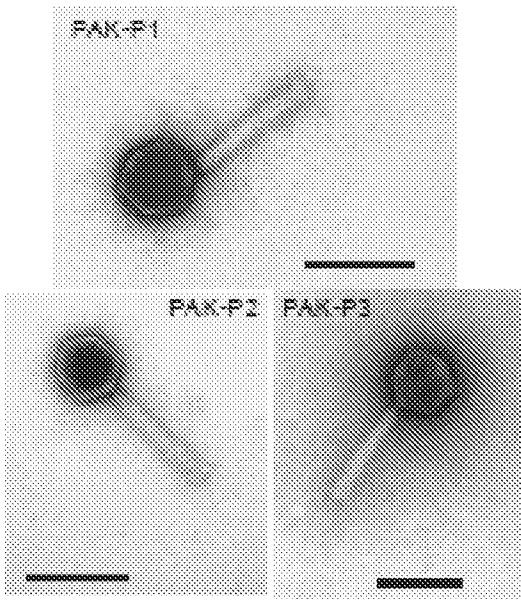


FIGURE 5A

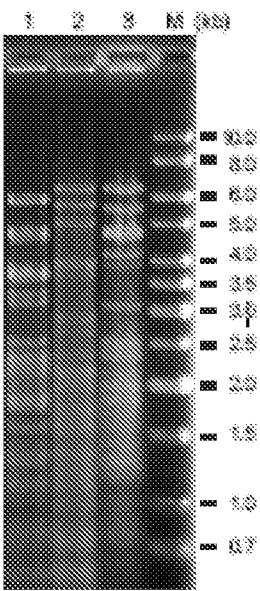


FIGURE 5B

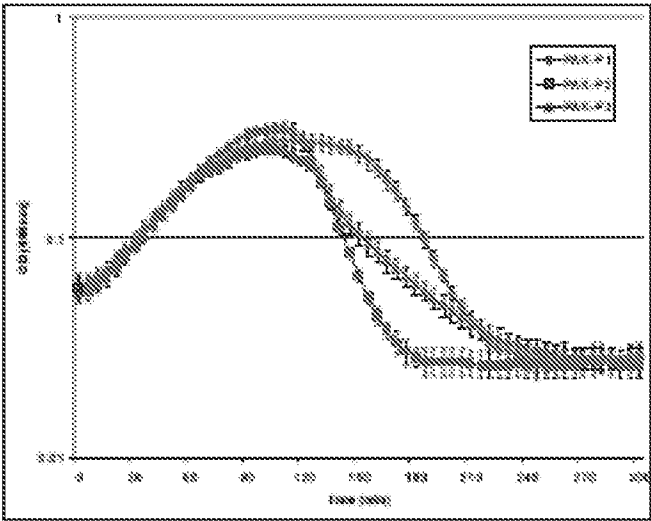


FIGURE 5C

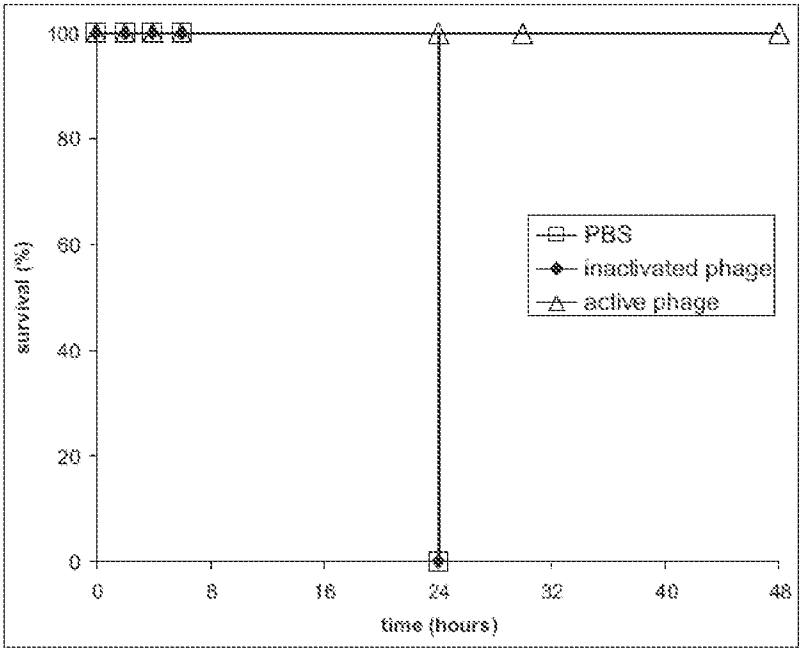


FIGURE 6

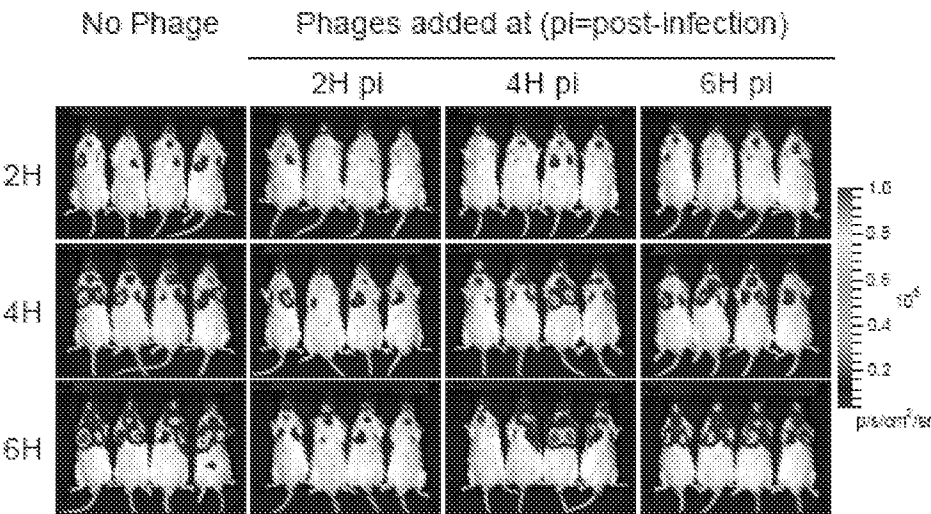


FIGURE 7

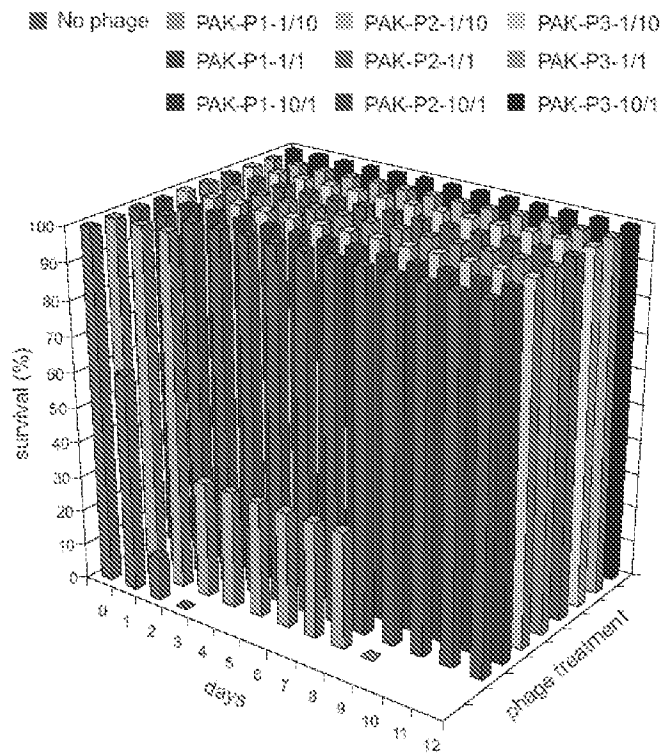


FIGURE 8A

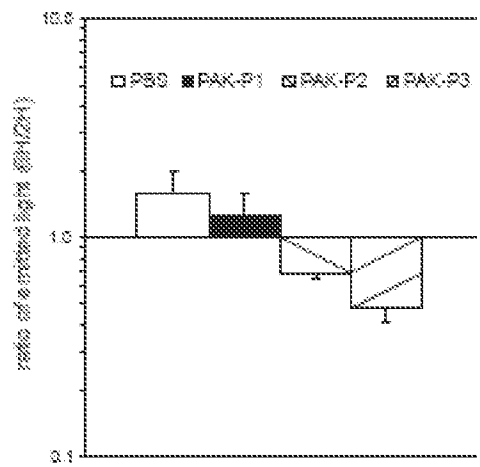


FIGURE 8B

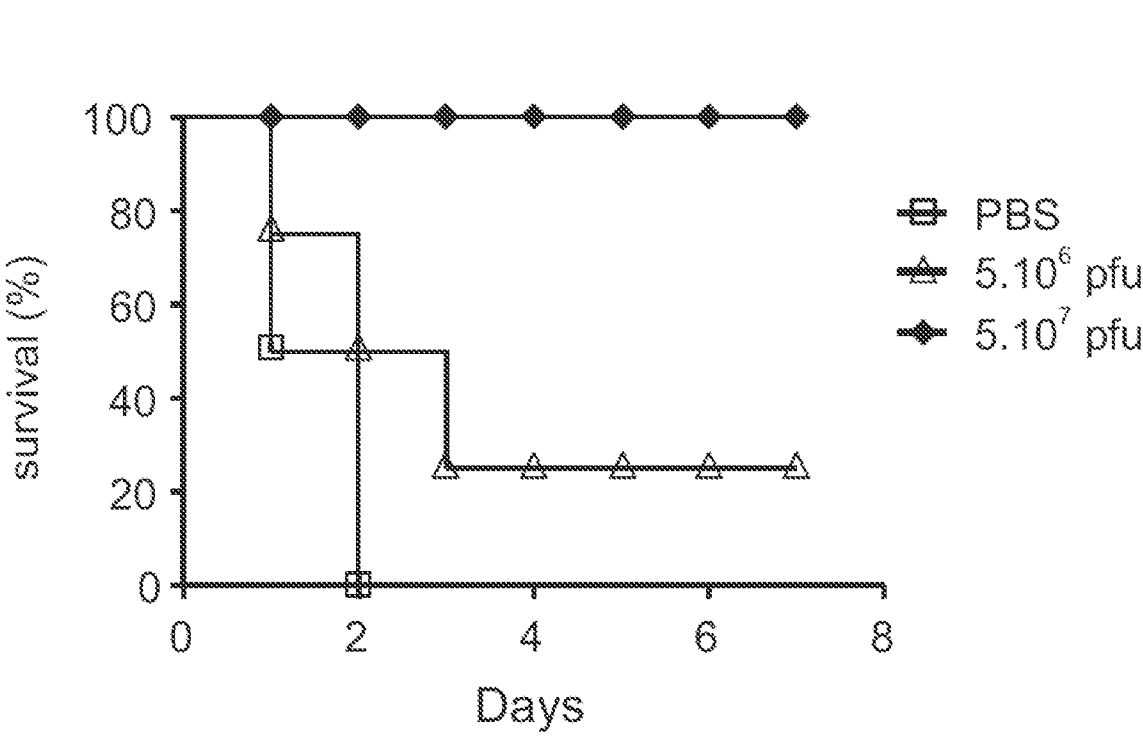


FIGURE 9A

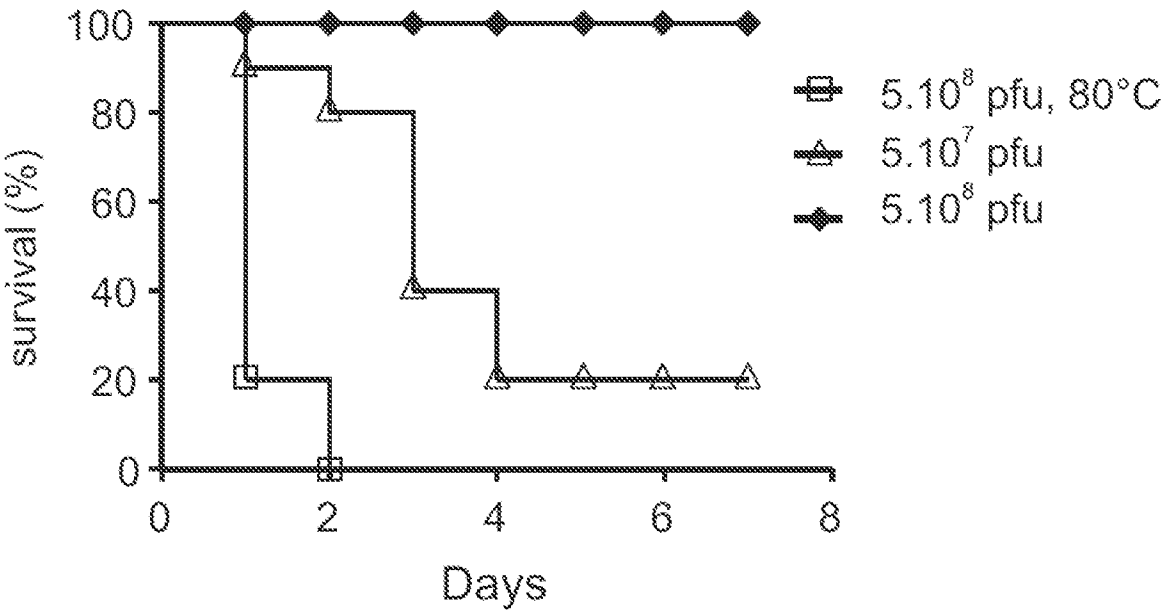
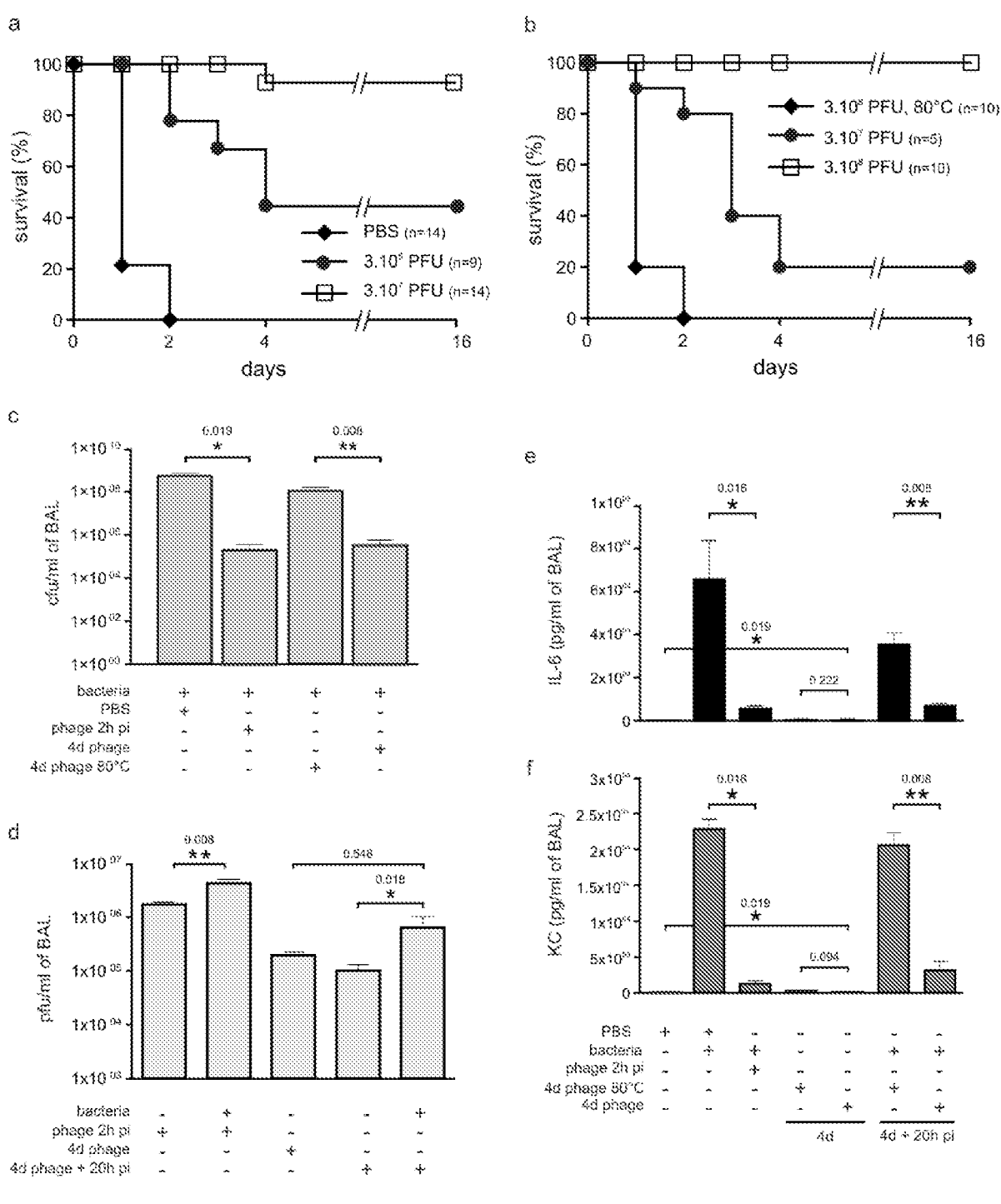


FIGURE 9B



FIGURES 10a- 10f

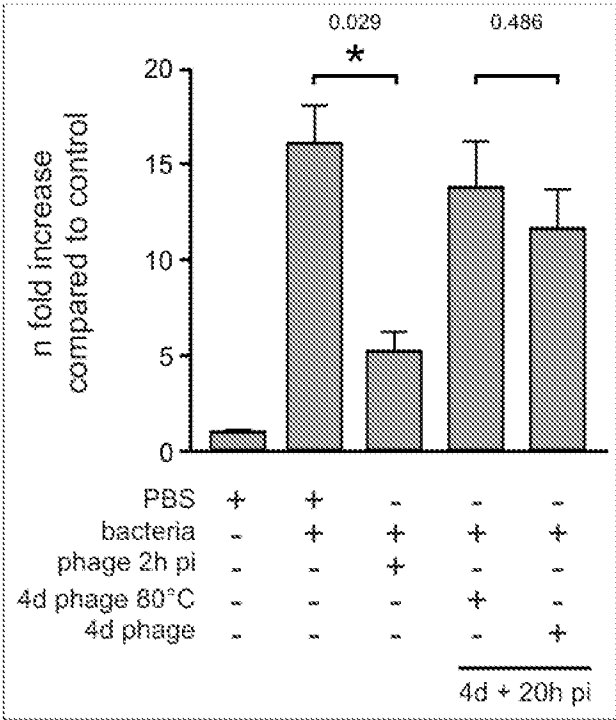
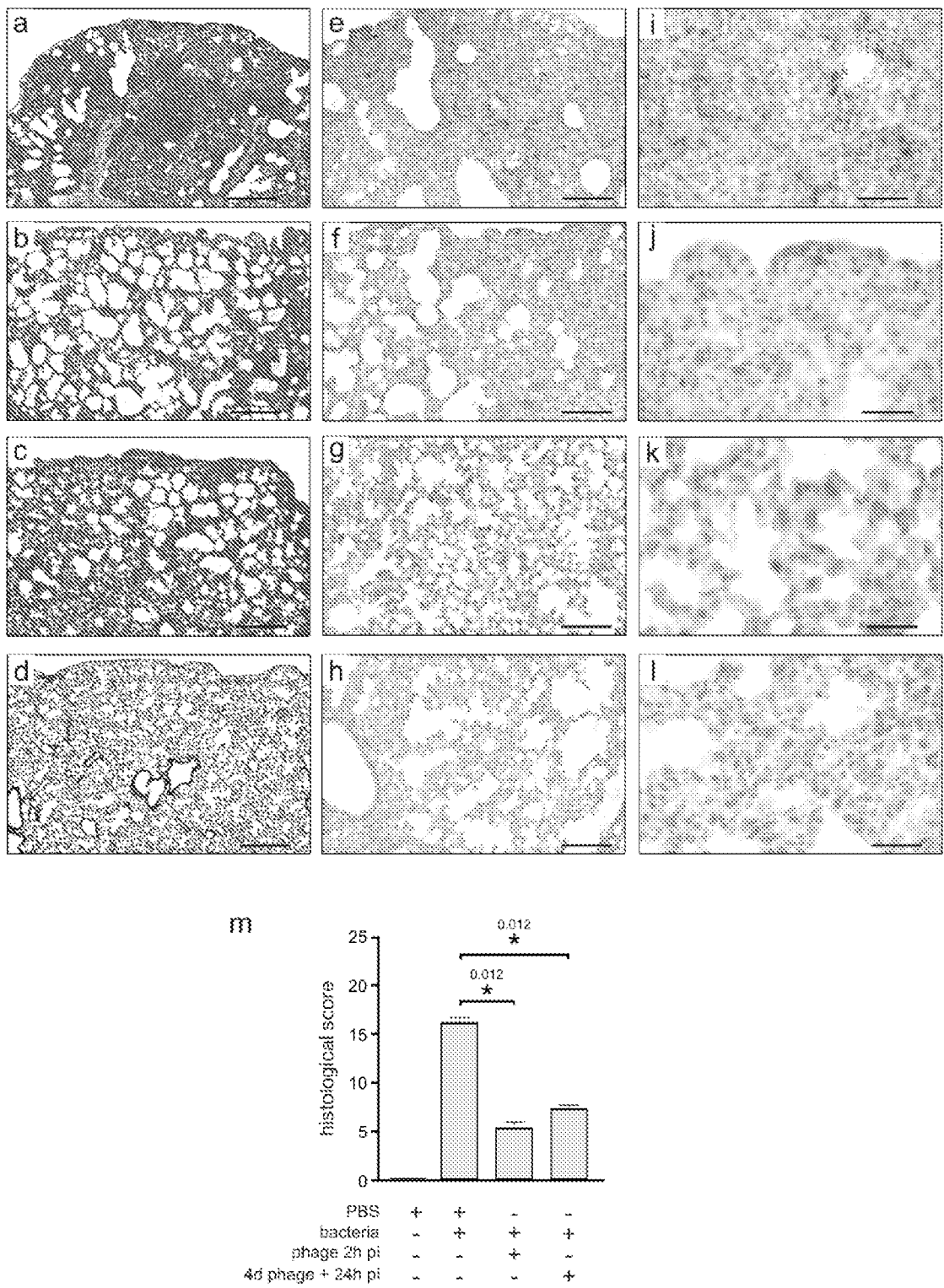
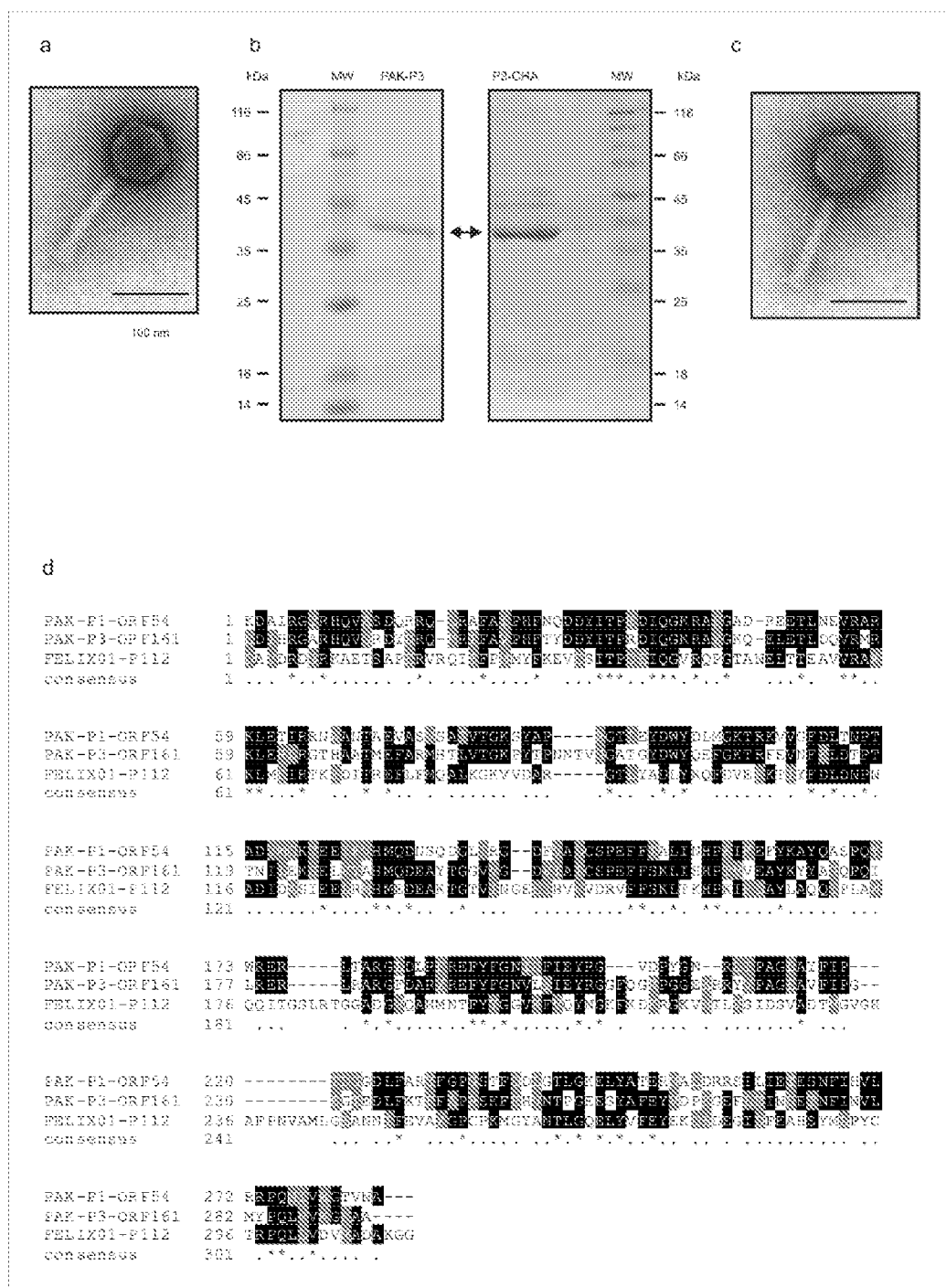


FIGURE 11



FIGURES 12a-12m



FIGURES 13a-13d

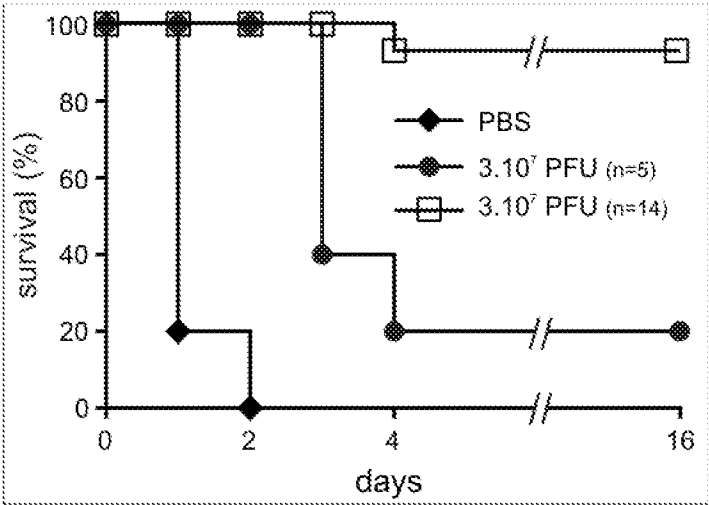


FIGURE 14

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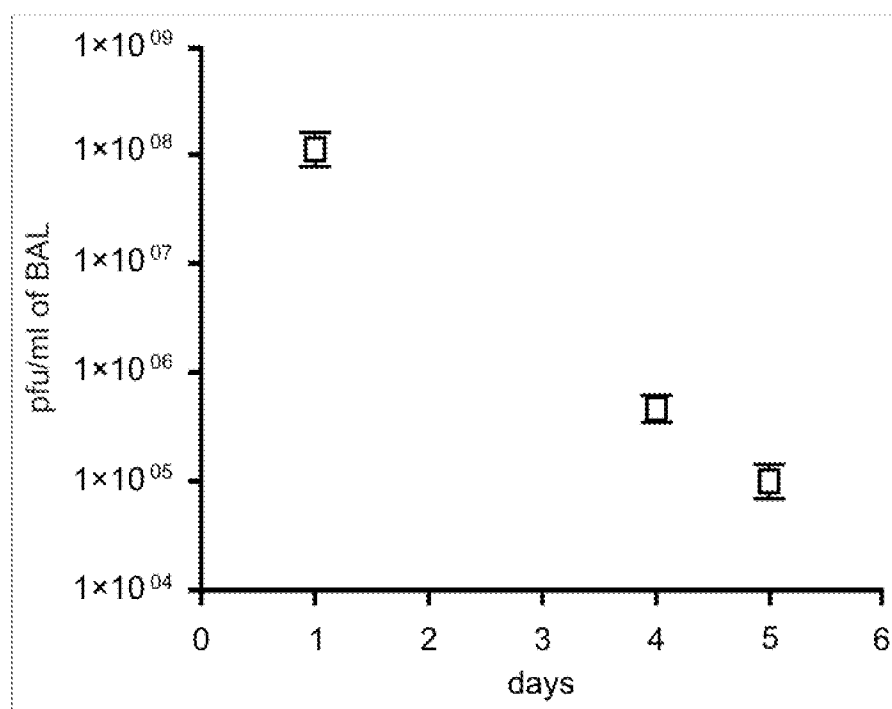
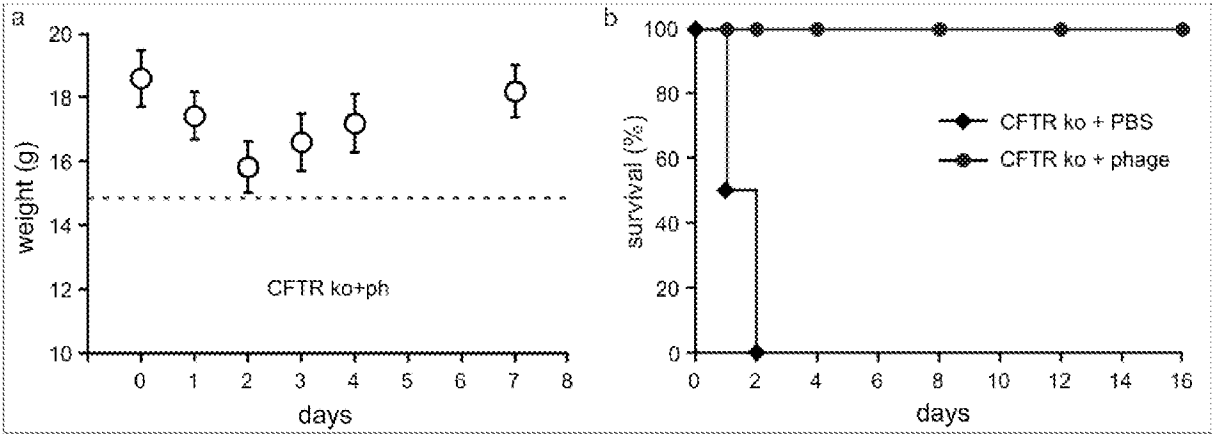


FIGURE 15



FIGURES 16a-16b