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ning of each regular issue of the PCT Gazette.

(54) Title: TREATMENT OF BACTERIAL INFECTIONS BY MEANS OF LYTIC ENZYMES OF BACTERIOPHAGES

(57) Abstract: The present invention relates to a method for the prophylactic or curative treatment of a bacterial infection of an aquaculture organism, such as crustaceae, mollusca and fish by a new composition which contains a lytic phage enzyme, which substantially does not contain active bacteriophages and which can be produced on a large scale. More specifically, the invention relates to the prophylactic or curative treatment of vibriosis in aquaculture practices, in particular in shrimp (or prawn) cultures. The invention also relates to a composition for use in such a method and to a method for producing such a composition wherein bacteria infected with a lytic bacteriophage are cultured, the infected bacteria are lysed and the bacteriophages present in the phage lysate are inactivated by means of a chemical nucleic acid inactivating agent.



WO 2007/128765 A2

**"Treatment of bacterial infections by means of lytic enzymes of
bacteriophages"**

5 The present invention relates to a method for the prophylactic or curative treatment of a bacterial infection of an organism, in particular of an aquaculture organism such as crustaceae, mollusca and fish. The invention also relates to a composition for use in such a method and to a method for producing such a composition. More specifically, the invention relates to the prophylactic or curative treatment of vibriosis in aquaculture practices, in particular in shrimp (or prawn) cultures.

10 Bacterial diseases are a common problem in aquatic animal farming. *Vibrio spp* is the main genus involved and in particular *V. harveyi* is the species most consistently isolated related to disease outbreaks in the literature. *Vibrio* has a worldwide distribution and it is considered a primary pathogen for shrimp and fish larval stages and a secondary
15 pathogen for juveniles and adults.

The most efficient treatment available is the use of antibiotics. However, their use presents a number of problems. One of them is the increasing development of resistance to antibiotics. Second is the lack of specificity of their action, killing at the same time a wide range
20 of bacteria. Finally, and the most pressing problem is the tighter control and limitation on the residues of antibiotics in products for human consumption.

An alternative treatment is the use of probiotics, which are expected to compete and displace shrimp/fish pathogens. Often, results
25 from probiotics are, however, not consistent.

- 2 -

Therefore, the application of bacteriophages was screened as a possible therapy and prophylaxis against bacterial pathogens in aquaculture. Bacteriophages are widely distributed in the aquatic environment and they are considered to play an important role in maintaining the microbial balance in natural ecosystems.

Bacteriophages have been found to be specific for certain microbial species or even strains. Contrary to antibiotics, phage therapy achieves a very specific effect, working at species level without disturbing the normal flora of the shrimp or the environment. There are no residues after the treatment and phage levels decrease once the target population demises. Karunasagar *et al.* (2005) described the successful use of live lytic phages against *V. harveyi* in aquaculture. However, for phage therapy it would be a disadvantage that a single bacteriophage may not be effective against all or at least against most of the strains of the pathogen.

A first drawback of the use of active phages is therefore that a typical phage is specific for very few bacterial strains even among the same bacterial species. Thus, a typical phage has a small host range and is only capable of killing a small subset of bacteria within a single genus of bacteria. A further drawback is that the introduction of live phages in aquaculture applications, poses risks of transfer of genetic material between bacterial cells, such as pathogenic islands via bacteriophages. Also from a legal point of view, the introduction of live phages as a microbial additive in aquaculture practices requires additional impact assessment on the environment (EC1831/2003).

A further disadvantage is that the preservation and shelf life of the product containing live phages is limited to a few days or weeks as long as it is not frozen at -80°C or freeze-dried, which has a high storage or production cost.

- 3 -

Instead of using the active phages it is also possible to use only the lytic phage enzymes. These lytic enzymes could be produced by sequencing the genome of the phage, determining the gene coding for the lytic enzyme, inserting it in a vector and over-expressing the gene in a host. This is however a time-consuming procedure which is moreover expensive and not always successful. Another procedure for producing the lytic enzyme is disclosed in US 2005/0136088 and in WO 2004/064732. In this known procedure bacterial cells infected with the bacteriophage are lysed and the cell debris and phages are removed from the lysate by ultracentrifugation, more particularly by centrifugation at 100 000 g for 5 hours. A drawback of this method is thus that an ultracentrifuge is needed and since production with this centrifuge is about 1000 times less than by standard centrifuge, the method is not suited for bulk production and is more particularly not applicable for the production of products for use in the aquaculture field.

An object of the present invention is therefore to provide a new composition for the therapeutic or prophylactic treatment of a bacterial infection which contains a lytic phage enzyme but substantially no active bacteriophages and which is easier to produce on a larger scale.

To this end, the composition according to the invention, which comprises at least a fraction of a phage lysate produced from bacteria which are infected with a lytic bacteriophage and containing at least one lytic enzyme having the ability to lyse the bacteria causing the bacterial infection, is characterised in that said bacterial lysate fraction further contains said bacteriophage which is inactivated by means of a chemical nucleic acid inactivating agent. The method for producing the composition according to the invention comprises the additional step of inactivating the bacteriophages present in the phage lysate by means of a chemical nucleic acid inactivating agent.

- 4 -

The lytic enzymes (most probably lysin and/or holin enzymes) present in the composition according to the invention allow a significant reduction of *Vibrio* bacterial numbers, especially of *Vibrio harveyi*, *Vibrio campbelli* and other relevant shrimp bacterial pathogens, without requiring the presence of the active bacteriophages. It has been found quite by surprise that the antibacterial effect could be maintained during the chemical inactivation process of the phage, notwithstanding the fact that during this inactivation process they are also subjected to inactivation compounds that react not only with nucleic acids but also with proteins. Furthermore, tests performed with the chemically inactivated phage lysate showed that, it even had a lytic effect against variants of bacterial strains of *Vibrio harveyi* which showed a resistance against the active phages themselves. Moreover, even a diluted suspension of inactivated phage, still has a growth inhibition effect against the wild or resistant *Vibrio* strains. These findings are quite surprising especially in view of the teachings of Wang Jing et al. (2006) disclosing that heat-inactivated bacteriophages achieve only 20% of the survival rate of *Pseudomonas aeruginosa* infected mice, compared to virulent phages. It has thus been found according to the present invention that bacteriophages which are inactivated by means of a chemical nucleic acid inactivating agent instead of by means of heat remain active against bacterial infections and are even more active than the non-inactivated bacteriophages.

In a preferred embodiment of the composition according to the invention, the nucleic acid inactivating agent, i.e. the RNA or DNA inactivating agent by means of which the bacteriophage is inactivated, comprises preferably an alkylating agent, in particular at least one alkylating agent selected from the group consisting of alkyl halides, dialkyl sulfates, alkyl sulfonates, alkyl leads, β -propiolactone (BPL), butyrolactone, alkyl iodides, alkyl bromides, alkylidenamines, including

substituted alkylenimines, the alkylating agent being preferably selected from the group consisting of β -propiolactone (BPL), butyrolactone, methyl iodide, ethyl iodide, propyl iodide, methyl bromide, ethyl bromide, propyl bromide, dimethyl sulphate, diethyl sulphate, binary ethylenimine and acetyl ethylenimine, and comprises more preferably β -propiolactone.

Alkylating agents appeared to be effective for inactivating vibrio phages. Such alkylating agents are mutagenic chemicals that react with nucleic acid and modify it by adding alkyl groups. They enable to inactivate viruses, i.e. to modify viruses in such a manner that they are no longer able to reproduce themselves. Although the inactivation of viruses by alkylation is primarily the result of reaction with the nucleic acid (RNA or DNA) than with the proteins of the virus (Herriot 1948), the alkylating agents also react with proteins / enzymes thus affecting their enzymatic activity (Herriot 1946). With respect to BPL, Broo et al. (2001) have found that BPL would even react with viral capsid proteins. Quite surprisingly, it has thus been found that the lytic enzymes present in the phage lysate were not affected by the alkylating agent used to inactivate the phage, in particular not by BPL.

In a further preferred embodiment of the composition according to the invention, the bacteriophage is a lytic *Vibrio* phage, in particular a lytic *Vibrio harveyi* or a *Vibrio campbelli* phage.

Three of the *Vibrio* phages tested by the present inventors could be classified under *Siphoviridae* whilst the fourth one could be classified under *Myoviridae*. All of the phages had no envelope. According to Scheidler et al (1998), non-enveloped viruses would be highly resistant against inactivation processes. According to the present invention, it has however been found that such non-enveloped viruses, more particularly the lytic *Vibrio* phages, could be inactivated whilst maintaining the activity of the lytic enzymes which are also present in the phage lysate treated with the inactivation agent.

- 6 -

In an advantageous embodiment of the composition according to the invention, the phage lysate is substantially free of bacteria and bacterial debris.

5 Such a lysate can easily be obtained by filtering and/or especially by centrifuging the phage lysate, more particularly at relatively low centrifugal accelerations lower than 25 000 g, and preferably lower than 15 000 g. Due to the absence of bacteria, there is no risk on infecting the organism with possibly pathogenic bacteria.

10 In a further advantageous embodiment of the composition according to the invention, said fraction of the phage lysate is freeze-dried. In this way, it can be stored easily.

The composition according to the invention is further preferably in the form of a feed for the organism.

15 To produce the feed, said fraction of the phage lysate can be mixed, either in liquid or in solid form (f.e. freeze-dried form) with other feed components which are normally used for feeding the organism. The invention can be brought in inclusion in liposomes in an oil emulsion, so that they for instance could be delivered to filter feeding organisms like life food organisms such as rotifers or *Artemia* or to bivalve organisms.

20 The invention can also be encapsulated in a fatty solution to protect the active enzymes from interference with other enzymes or from physical or chemical aggression during the feed process. Said incapsulation could be useful for incorporation in feeds such as pellets. The freeze-dried form is useful to incorporate the invention in solid feeds, preferably as a coating,

25 mixed or not with other temperature unstable additives like for instance vitamins, micro-organisms.

Although it is also possible, at least for aquatic organisms, to add the phage lysate to the aqueous medium, an advantage of this embodiment wherein the composition is in the form of a feed is that more

- 7 -

of the composition will actually be taken in by the organism so that a smaller amount is required to treat the bacterial infection.

The present invention also relates to the first medical use wherein the composition described hereabove is used for the manufacture of a medicament for the therapeutic or prophylactic treatment of a bacterial infection of an organism, in particular of an aquaculture organism such as crustaceae, mollusca and fish, and more particularly for the manufacture of a medicament for the therapeutic or prophylactic treatment of a *Vibrio* infection, in particular of a *Vibrio harveyi* or a *Vibrio campbelli* infection of an aquaculture organism such as shrimp.

The invention further also relates to a method for producing the composition described hereabove, which method comprises the steps of culturing bacteria infected with said lytic bacteriophage, lysing said bacteria to produce a phage lysate, and inactivating the bacteriophages present in said phage lysate by means of a chemical nucleic acid inactivating agent, in particular by means of the alkylating agents as described hereabove. The bacteria used to produce the phage lysate are preferably *Vibrio* bacteria, and are more particularly *Vibrio harveyi* or *Vibrio campbelli* bacteria.

Lysis of the bacteria can be achieved by means of the lytic enzymes produced by the bacteriophages themselves and/or can be achieved by the addition of lytic additives.

After the lysing step, any remaining bacteria and bacterial debris are preferably removed from the phage lysate, in particular by centrifuging the lysate, preferably at a centrifugal acceleration smaller than 25 000 g and preferably smaller than 15 000 g. This separation step can be performed after the inactivation step, in which case a first preliminary separation step is preferably carried out before the

- 8 -

inactivation step to remove some of the bacteria and bacterial debris. In this way, the inactivating agent can be used more efficiently.

5 The invention thus provides a simple and cheap method to treat the phage lysate, containing the active phage and the products encoded by its genome, without any risk of transfer of (virulent) genetic material, without the need of additional assessment impact on the environment and which can be used to treat the animals infected with the target bacteria or to prevent infection of said bacteria.

10 The present invention finally also relates to a method for the prophylactic or curative treatment of a bacterial infection of an organism, in particular of an aquaculture organism such as crustaceae, mollusca and fish, which method comprises the step of administering an effective amount of the above described composition to the organism, in particular to shrimp.

15 In a preferred embodiment, the composition contains a lytic enzyme of a lytic *Vibrio* phage and is used for the prophylactic or curative treatment of vibriosis, in particular of luminous vibriosis caused by *Vibrio harveyi* or vibriosis caused by *Vibrio campbelli*.

20 Further advantages and particularities of the present invention will become apparent from the following description of some specific examples of the method, composition (feed) according to the invention.

Isolation of the *Vibrio* bacteriophages:

25 Samples of water from shrimp farm, hatcheries and seawater were taken for phage isolation. 25 ml of water sample was centrifuged at 10 000 g for 15 min. 1 ml of the supernatant was added to an 8 h old *V. harveyi* culture grown in 5 ml Tryptic soya broth with 1% NaCl (TSBS) and further incubated at room temperature for 8-12 h. The mixture was
30 centrifuged at 10 000g for 15 min at 4°C. The supernatant was filtered

- 9 -

through 0.45 µm filters and collected in a sterile container. The presence of phage was tested by inoculating 25 µl of the filtrate on lawns of *V. harveyi* in tryptic soya agar. Areas of clearing were cut out to inoculate fresh *V. harveyi* culture in TSBS for enrichment of phages. Bacteria and debris were separated by centrifugation at 10 000 g for 15 min at 4°C. The supernatant was filtered through 0.45 µm filters and inoculated on lawns of *V. harveyi* to confirm the presence of bacteriophages.

Propagation of phages

Propagation of phages was done according to the method described by Su *et al.* (1998). Phage lysates were first prepared by flooding a confluent lysed plate with 5 ml of SM buffer and placed on a shaker incubator maintained at 28°C for 2h. The suspension was transferred to a tube and the bacterial debris was cleared by centrifugation at 4000 g for 10 min, and the entire supernatant was used to infect a 50 ml host bacterial culture (*V. harveyi*) in mid-exponential growth phase ($A_{600} = 0.5$). The host-phage mixture was incubated in a shaker incubator at 28°C for 3 h and the degree of lysis was observed by reduction in A_{600} to about 0.2. The bacterial debris was pelleted by high speed centrifugation at 10 000 g for 30 min at 4°C, and the supernatant containing phage was filtered through 0.22 µm membrane filter (Millipore Corporation, USA) and the filtrate stored at -80°C for further studies.

Inactivation of the phage with an alkylating agent

A high titre suspension of phages (10^9 pfu /ml) obtained as describe hereabove was treated with β-propiolactone to give a final concentration of 0.25 %. The suspension was then centrifugated for 8 hours at 30°C to remove any remaining bacteria and/or bacterial debris. The reaction was stopped by adding adding sodium thiosulphate to a final

- 10 -

concentration of 35 mM. The suspension was then stored at 4°C until further analyses.

Experiment 1: Effect of the inactivated phage suspension on the growth of *Vibrio harveyi*

The suspension with the inactivated phage was added to a bacterial culture (*V. harveyi*) in a liquid growth medium. A 1:10 dilution of this preparation was also tested in the bacterial culture.

Figure 1 shows the *Vibrio* counts after 24 hours and 48 hours when no inactivated phage preparation is added (control), compared to the bacterial growth when the inactivated phage suspension was added directly (1ml) and in a dilution of 1:10 (0.1ml).

The results showed that by using the β -propiolactone treated phage preparation a reduction of 3-4 logs in the bacterial count could be recorded after 48 hours. After diluting the β -propiolactone treated phage preparation 1:10, a reduction of 2-3 logs in bacterial counts still could be recorded. This proves that the lytic enzymes in the suspension after inactivation by β -propiolactone, still have their lytic effect. When the clearance zone test (on *V. harveyi* growing on a solid growth medium) was repeated with these suspensions, no effect on the bacterial cultures was observed, proving that the phages had been inactivated and that they couldn't reproduce themselves in the bacterial cultures.

Experiment 2: Effect of the inactivated phage suspensions on the resistant strains of *Vibrio harveyi*

In this experiment, 9 strains of *V. harveyi* and 4 lytic *V. harveyi* phages (isolated as described hereabove) were used. The bacterial strains (wild type: strains W1 to strain W9) were exposed to the live phages and resistant colonies (resistant to all four phages) were found to grow in the clearing zones (resistant variants: strain R1 to strain

- 11 -

R9). These resistant variants were picked up with a loop and cultured in a microbiological medium. Then, the isolates were tested against four inactivated phage preparations.

5 The inactivated phage preparations were produced according to the method described above. The inactivated phage preparations (designated Viha1, Viha2, Viha3 and Viha4) produced from live phages with a titre of 10^9 pfu / ml and tenfold dilutions (10^{-1} , 10^{-2} , 10^{-3} and 10^{-4}) of the inactivated phage preparations were applied on a lawn of the isolated culture. Growth inhibition was recorded for the highest dilution of the
10 inactivated phage suspension.

Table 1: Highest dilution of inactivated phage preparation (Viha1, Viha2, Viha3 and Viha4) at which growth inhibition of 9 resistant strains of *V. Harveyi* (Strain R1 to R9) was recorded.

Resistant Vibrio isolates	Viha 1	Viha 2	Viha 3	Viha 4
Strain R1	10^{-2}	10^{-1}	10^{-1}	10^{-2}
Strain R2	10^{-2}	10^{-2}	10^{-2}	10^{-1}
Strain R3	10^{-1}	10^{-2}	10^{-3}	10^{-1}
Strain R4	10^{-1}	10^{-2}	10^{-1}	10^{-2}
Strain R5	10^{-1}	10^{-1}	10^{-2}	10^{-1}
Strain R6	10^{-1}	10^{-2}	10^{-2}	10^{-2}
Strain R7	10^0	10^0	10^0	10^0
Strain R8	10^{-1}	10^{-2}	10^{-2}	10^{-2}
Strain R9	10^0	10^0	10^0	10^0

15 10^{-0} , 10^{-1} , 10^{-2} , 10^{-3} : dilutions of the inactivated phage preparation that could inhibit growth of the resistant variant of the selected strain of *V. Harveyi*.

- 12 -

The growth of all wild types was affected by the live phages and the four inactivated phage preparations in a dilution till 10^{-4} .

However, resistant variants of these strains cannot be controlled by the live phages. Nevertheless, when these resistant variants were brought in
5 contact with the inactivated phage preparations, the growth of these variants was inhibited, sometimes even when a dilution of 10^{-2} or 10^{-3} of the inactivated phage preparation was used.

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CLAIMS

1. A composition for use in the therapeutic or prophylactic treatment of a bacterial infection of an organism, in particular of an aquaculture organism such as crustaceae, mollusca and fish, which composition comprises at least a fraction of a phage lysate produced from bacteria which are infected with a lytic bacteriophage, and said fraction containing at least one lytic enzyme having the ability to lyse the bacteria causing said bacterial infection, characterised in that said phage lysate fraction further contains said bacteriophage which is inactivated by means of a chemical nucleic acid inactivating agent.

2. A composition according to claim 1, characterised in that said bacteriophage has been inactivated by means of an alkylating agent, in particular by means of at least one alkylating agent selected from the group consisting of alkyl halides, dialkyl sulfates, alkyl sulfonates, alkyl leads, β -propiolactone (BPL), butyrolactone, alkyl iodides, alkyl bromides, alkylenimines, including substituted alkylenimines, the alkylating agent being preferably selected from the group consisting of β -propiolactone (BPL), butyrolactone, methyl iodide, ethyl iodide, propyl iodide, methyl bromide, ethyl bromide, propyl bromide, dimethyl sulphate, diethyl sulphate, binary ethylenimine and acetyl ethylenimine, and comprises more preferably β -propiolactone.

3. A composition according to claim 1 or 2, characterised in that said bacteriophage is a lytic *Vibrio* phage, in particular a lytic *Vibrio harveyi* or a *Vibrio campbelli* phage.

4. A composition according to any one of the claims 1 to 3, characterised in that said phage lysate fraction is substantially free of bacteria and bacterial debris.

5. A composition according to any one of the claims 1 to 4, characterised in that said fraction of the phage lysate is freeze-dried.

- 15 -

6. A composition according to any one of the claims 1 to 5, characterised in that it is in the form of a feed for said organism, in particular in the form of a solid feed.

5 7. Use of the composition according to any one of the claims 1 to 6 for the manufacture of a medicament for the therapeutic or prophylactic treatment of a bacterial infection of an organism, in particular of an aquaculture organism such as crustaceae, mollusca and fish.

10 8. Use according to claim 7, wherein said medicament is a medicament for the therapeutic or prophylactic treatment of a *Vibrio* infection, in particular of a *Vibrio harveyi* or a *Vibrio campbelli* infection of an aquaculture organism.

15 9. A method for producing a composition according to any one of the claims 1 to 6, which method comprises the steps of culturing bacteria infected with said lytic bacteriophage and lysing said bacteria to produce a phage lysate, characterised in that the method comprises the further step of inactivating the bacteriophages present in said phage lysate by means of a chemical nucleic acid inactivating agent.

20 10. A method according to claim 9, characterised in that said bacteriophages are inactivated by means of an alkylating agent, in particular by means of at least one alkylating agent selected from the group consisting of alkyl halides, dialkyl sulfates, alkyl sulfonates, alkyl leads, β -propiolactone (BPL), butyrolactone, alkyl iodides, alkyl bromides, alkylenimines, including substituted alkylenimines, the alkylating agent being preferably selected from the group consisting of β -propiolactone
25 (BPL), butyrolactone, methyl iodide, ethyl iodide, propyl iodide, methyl bromide, ethyl bromide, propyl bromide, dimethyl sulphate, diethyl sulphate, binary ethylenimine and acetyl ethylenimine, and comprises more preferably β -propiolactone.

30 11. A method according to claim 9 or 10, characterised in that it comprises the further step of removing bacteria and bacterial

- 16 -

debris from said phage lysate, in particular by centrifuging the lysate, preferably at a centrifugal acceleration smaller than 25 000 g and preferably smaller than 15 000 g.

12. A method according to any one of the claims 9 to 11,
5 characterised in that said bacteria are *Vibrio* bacteria, and are more particularly *Vibrio harveyi* or *Vibrio campbelli* bacteria.

13. A method for the prophylactic or curative treatment of a bacterial infection of an organism, in particular of an aquaculture organism such as crustaceae, mollusca and fish, comprising the step of
10 administering an effective amount of the composition according to any one of the claims 1 to 6 to the organism.

14. A method according to claim 13, characterised in that said composition is administered orally to the organism, the composition being preferably in the form of a feed which is fed to the organism.

15. A method according to claim 13 or 14, characterised in that said composition contains a lytic enzyme of a lytic *Vibrio* phage and is used for the prophylactic or curative treatment of vibriosis, in particular of luminous vibriosis caused by *Vibrio harveyi* or vibriosis caused by *Vibrio campbelli*.

20 16. A method according to any one of the claims 13 to 15, characterised in that said organism is a shrimp.

