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Effects of storage conditions on the stability of spray dried, inhalable bacteriophage powders

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ABSTRACT

This study aimed to develop inhalable powders containing phages active against antibiotic-resistant *Pseudomonas aeruginosa* for pulmonary delivery. A *Pseudomonas* phage, PEV2, was spray dried into powder matrices comprising of trehalose (0–80%), mannitol (0–80%) and L-leucine (20%). The resulting powders were stored at various relative humidity (RH) conditions (0, 22 and 60% RH) at 4 °C. The phage stability and *in vitro* aerosol performance of the phage powders were examined at the time of production and after 1, 3 and 12 months storage. After spray drying, a total of 1.3 log titer reduction in phage was observed in the formulations containing 40%, 60% and 80% trehalose, whereas 2.4 and 5.1 log reductions were noted in the formulations containing 20% and no trehalose, respectively. No further reduction in titer occurred for powders stored at 0 and 22% RH even after 12 months, except the formulation containing no trehalose. The 60% RH storage condition had a destructive effect such that no viable phages were detected after 3 and 12 months. When aerosolised, the total lung doses for formulations containing 40%, 60% and 80% trehalose were similar (in the order of 10^5 pfu). The results demonstrated that spray drying is a suitable method to produce stable phage powders for pulmonary delivery. A powder matrix containing ≥40% trehalose provided good phage preservation and aerosol performances after storage at 0 and 22% RH at 4 °C for 12 months.

1. Introduction

The use of bacteriophages to treat bacterial infections has recently regained significant attention owing to the rapid emergence of multidrug-resistant (MDR) bacterial strains and the slow development of new antibacterial compounds. With these increased research efforts, a large number of novel phages have been isolated and characterised to target broad-spectrum antimicrobial resistance of *Pseudomonas aeruginosa*,*Staphylocccus aureus* and *Burkholderia cepacia* complex (*BCC*) (Ackermann, 2007; Larche et al., 2012; Essoh et al., 2013), which are major pathogens in chronically infected cystic fibrosis (CF) patients. In addition, the potential of phages to treat respiratory infections caused by MDR bacteria has recently been demonstrated in animal models (Chhibber et al., 2008; Carmody et al., 2010; Debarbieux et al., 2010; Morello et al., 2011; Alemayehu et al., 2012; Semler et al., 2014; Singla et al., 2015; Cao et al., 2015; Pabary et al., 2016). These promising results have made phage therapy one of the most promising alternatives to conventional antibiotics for respiratory infectious diseases.

Carmody et al. (2010) compared the efficacy of delivery routes in a mouse model of acute *B. cenocepacia* pulmonary infections. They reported that the bacterial density was significantly reduced in lungs of mice treated with intraperitoneal phages, while no significant difference was observed between untreated mice and mice treated with phages delivered using intranasal instillation. Chhibber et al. (2008) showed a significant reduction of *Klebsiella*

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pneumoniae in the lung to below the initial infectious dose, when the phages were administered intraperitoneally immediately or 3 h prior to the bacterial challenge. However, opposite results were reported by Semler et al. (2014) who showed significant bacterial reduction in the lung in *BCC* infected mice treated with aerosolized phages using a jet nebulizer attached to a Nose-Only Inhalation Device, but not in the intraperitoneal treatment group. The different treatment outcomes in intraperitoneal phage delivery could be attributed to different systemic clearance rates of phages used in these studies. On the other hand, the disparity of the pulmonary phage treatments may be accounted by the efficiency of delivery methods, with Semler et al.'s use of aerosol delivery achieving better pulmonary targeting efficiency. Traditionally, it has been considered that the more effective route for phage delivery should be the one that results in direct delivery to the site of infection, enhancing the chance of phages attaining and sustaining an efficacious concentration in the vicinity of target bacteria. Thus, for pulmonary infections, direct lung delivery would be a more rational and clinically relevant approach. The effectiveness of intranasal administration of phages to treat mice infected with *P. aeruginosa* has also been demonstrated (Debarbieux et al., 2010; Morello et al., 2011; Alemayehu et al., 2012; Cao et al., 2015). Debarbieux and coworkers (Debarbieux et al., 2010; Morello et al., 2011) showed that the survival rate of the infected mice was increased with higher phage-to-bacteria ratios, with 100% survival rate observed at a 10:1 ratio (with dose of the order of 10^8 pfu). Similar results were also reported by Cao et al. (2015).

In addition to the delivery route and phage concentration, the timing of administration has an impact on the efficacy of phage therapy. Debarbieux and coworkers (Debarbieux et al., 2010; Morello et al., 2011) reported that the survival rate of *P. aeruginosa* infected mice was 100% when phages were given 2 h after the bacterial challenge, but it dropped to 75% and 25% in 4 h and 6 h post-treatment groups, respectively. Furthermore, for preventive treatment, 100% protection was achieved when a single dose of phages were given at 24 h (Debarbieux et al., 2010) and 4 day (Morello et al., 2011) before infection with *P. aeruginosa*. Similar results were reported in Pabary et al. (2016). Singla et al. (2015) compared the efficacy of intraperitoneal application of phages alone and when delivered in liposomes in a mouse model of *K. pneumoniae* lobar pneumonia. While the phages alone treatment was only able to prevent the infection up to 6 h prior to bacterial challenge, the liposome-entrapped phages provided complete protection up to 48 h prior treatment. Their results showed that entrapped phages could slow down the systemic clearance rate of phages in the absence of bacteria. It is noteworthy to mention that these animal models used were acute, rapidly progressive infection, and this does not resemble the chronic bacterial infections in CF patients. Therefore, the relevance of these results in translating to a chronic environment would still need to be assessed clinically.

While the quantity of phage research and well-defined preclinical trials has substantially increased, further research on pharmaceutical formulations and their long-term stability is important for both clinical and commercial application (Merabishvili et al., 2013). Currently, phage preparations for therapeutic use are limited to liquid formulations, which are generally recommended to be stored at 4 °C with one year shelf life (Merabishvili et al., 2013). As a result, most phage research for respiratory infections in the past has been confined to liquid aerosols using intranasal instillation (Carmody et al., 2010; Debarbieux et al., 2010; Morello et al., 2011; Alemayehu et al., 2012; Semler et al., 2014) and nebulisation (Golshahi et al., 2008; Sahota et al., 2015; Abedon, 2015). Since many bacteriophage strains may be stabilised in the powder form for years and so facilitate their global distribution (Golshahi et al., 2011), recent efforts have been devoted to develop stabilised dry dosage formulations for phage inhalation (Golshahi et al., 2011; Matinkhoo et al., 2011; Vandenheuvel et al., 2013, 2014; Leung et al., 2016).

Previously, we compared the phage viability and aerosol performance of two dry powder formation techniques, spray freeze drying and spray drying, for the production of inhalable phage powders (Leung et al., 2016). Multi-component excipient systems consisting of various amounts of trehalose, mannitol and leucine were used as the bulking and stabilising agents. We reported that the spray drying method caused less phage titer reduction during the powder formation process and achieved better aerosol performance. In this study, we focused on the effects of storage conditions on the stability of the spray dried powder matrix. After spray drying, the powders were immediately stored at various relative humidity (RH) conditions (0, 22 and 60% RH) at 4 °C. Since storage of biologics (proteins, phages) at ambient conditions is not recommended (Jończyk et al., 2011) and there is no long term storage stability data for phage in powder form, 4 °C was chosen to maximize the chance of phage survival. The phage stability and *in vitro* aerosol performance of the powders were assessed at 0, 1, 3 and 12 months storage.

2. Material and method

2.1. Materials

The phage used in this study was an N4-type, lytic podovirus, PEV2. It was isolated from the sewage treatment plant in Olympia, WA, USA by students in the Evergreen State College Phage Laboratory, who made a detailed analysis of its infection-related properties and genome (Ceyssens et al., 2010). A PEV2 stock with a titer of 2.2×10^9 pfu/ml stored in salt-magnesium buffer (SMB, 5.2 g/l sodium chloride, 2 g/l magnesium sulfate, 6.35 g/l Tris-HCL, 1.18 g/l Tris base and 0.01% gelatin) was supplied via AmpliPhi Biosciences (AmpliPhi Biosciences AU, NSW Australia) and used without further purification.

Various amounts of D-(+)-trehalose dihydrate, mannitol and L-leucine from Sigma–Aldrich (NSW, Australia) were co-spray dried with phage to form a powder matrix to protect the phage particles. Table 1 shows the composition of the five formulations prepared in the present study. The compositions of F2 and F3 were the same as that prepared in our previous study (Leung et al., 2016). Their long term storage stability was assessed in the present study.

2.2. Powder preparation

A volume of 50 ml excipient solution with a total solid concentration of 20 mg/mL was prepared in ultra-pure distilled water with the pH adjusted to 7–7.5 using 1 M hydrochloric acid and 1 M sodium hydroxide. Then 0.5 ml phage suspension was added to the mixture, resulting in 100 times titer dilution (starting titer = 2.2×10^7 pfu/ml). The phage viability in the sugar solution before spray drying was confirmed by using a standard plaque assay (Carlson, 2005). The mixtures were spray dried using a Büchi 290 spray dryer (Buchi Labortechnik AG, Flawil, Switzerland) using

Table 1
Formulation compositions.

Formulation #	Contents% (w/w)		
	Trehalose	Mannitol	Leucine
F1	80	0	20
F2	60	20	20
F3	40	40	20
F4	20	60	20
F5	0	80	20