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Bacteriophage-mediated reduction of *Salmonella* Enteritidis in swine slurry

Bartłomiej Grygorcewicz[a,*,1], Michał Grudziński[b,1], Agata Wasak[a], Adrian Augustyniak[a], Arkadiusz Pietruszka[b], Paweł Nawrotek[a]

[a] Department of Immunology, Microbiology and Physiological Chemistry, Faculty of Biotechnology and Animal Husbandry, West Pomeranian University of Technology, Szczecin, al. Piastów 45, 70-311 Szczecin, Poland

[b] Department of Pig Breeding, Animal Nutrition and Food, Faculty of Biotechnology and Animal Husbandry, West Pomeranian University of Technology, Szczecin, Doktora Judyma Street 10, 71-460 Szczecin, Poland

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ABSTRACT

Slurry is considered one of the best fertilizers. Nevertheless, it may contain harmful bacteria. Spreading it on the soil may contribute to transfer of these microorganisms in the environment. This could be avoided with effective biocontrol methods Bacteriophage sall_v01 specific to several *Salmonella enterica* serovars was isolated from wastewater and characterized. The lytic activity of bacteriophage sall_v01 showed its effectiveness for the growth reduction of *Salmonella* Enteritidis at the multiplicity of infection = 1, with latent periods = 25 min, and the burst size approx. 107 ± 8 new virions per cell after 45 min at 37 °C. Bacteriophage-mediated treatment of experimentally contaminated swine slurry resulted in 3.8 log CFU/mL reduction in quantity of *Salmonella* Enteritidis. Based on our results, phage sall_v01 could be considered a potential biocontrol agent against *Salmonella* Enteritidis contamination in agriculture and animal production.

1. Introduction

Salmonella enterica ssp. *enterica* ser. Enteritidis (*Salmonella* Enteritidis, *SE*) is the most common serovar of *Salmonella* (Galarce et al., 2014). *SE* may cause mild diarrhoeas as well as severe diseases including typhoid fever, gastroenteritis, and sepsis (Kim et al., 2014). *SE* enters human body, e.g. via food chain, as a result of prior application of organic fertilizers (slurry) on agricultural field (Nicholson et al., 2005; Hölzel and Bauer, 2008).

Swine slurry consists of urine and faeces – waste materials during intensive livestock production, but a valuable fertilizer for crops. Using swine slurry as a fertilizer is considered the most appropriate way of managing it in agriculture. In addition, it is the most economic and practical alternative for the improvement of soil quality (Nicholson et al., 2005; Sánchez and González, 2005). Nevertheless, pig slurry contains high concentrations of bacteria, including *Salmonella* spp., that can cause infections in humans and animals (Guan and Holley, 2003; Levallois et al., 2014; Grudziński et al., 2015). Water-borne outbreaks of *Salmonella* associated with the entering of animal and human waste into groundwater, further dedicated for drinking, were also reported (Kozlica et al., 2010). Many documented outbreaks of *Salmonella* were caused by a consumption of fresh product that had been contaminated with human/animal wastes. Therefore, slurry may pose a health risk when used as a fertilizer for plants (Doyle and Erickson, 2008; Nygård et al., 2008; Jokinen et al., 2015).

Among alternative methods for eradication of bacteria, the use of bacteriophages (viruses infecting and lysing bacterial cells) has appeared as a promising way to control pathogens. As opposed to antibiotics, phages are highly specific to their host. In recent years, the interest in applications of bacteriophages for the control of bacterial pathogens is increasing (Jeon et al., 2016; Kalatzis et al., 2016). In spite of the lytic activity against bacteria, phages show other unique features, such as replication in host cell and relatively easy handling, which support the advantage of their application in miscellaneous fields. Moreover, bacteriophages have been already accepted as natural antimicrobial agents for biocontrol of pathogens and fighting with bacterial infections in medical practice (Mahony et al., 2011; Cheng et al., 2014).

The aim of this study was to evaluate the ability of the newly isolated lytic bacteriophage to reduce *SE* level in pig slurry.

2. Material and methods

The bacteriophage sall_v01 was isolated from wastewater (sample was collected in Stargard, West Pomeranian Voivodeship, Poland),

* Corresponding author at: West Pomeranian University of Technology, Faculty of Biotechnology and Animal Husbandry, Department of Immunology, Microbiology and Physiological Chemistry, al. Piastów 45, 70-311 Szczecin, Poland.
E-mail address: bartlomiej.grygorcewicz@zut.edu.pl (B. Grygorcewicz).
[1] The authors equally contributed to the manuscript.

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propagated, and stored with chloroform (5% v/v) at 4 °C until used. The host strain used for the propagation of bacteriophage was *Salmonella* Enteritidis ATCC®13076™. Five reference serovars of *Salmonella enterica* and three wild isolates were used in this study for the host range determination. Stock cultures of all bacteria were stored frozen at −20 °C in Tripticase Soy Broth (TSB) containing 20% glycerol (v/v). All strains were aerobically grown at 37 °C in Lysogeny Broth (LB) or agar (LBA, LB containing 1.5% agar). Pig slurry was collected from storage tank at a swine farm located in West Pomeranian Voivodeship (Poland) and stored at 2 °C for no longer than 24 h. Pig slurry was tested for presence of rods from *Salmonella* genus and lytic *S*E-specific phages.

The phage activity in terms of bacteriophage titre was determined by diluting the phage solution in SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-Cl [pH 7.5], 0.01% of gelatin) and subjecting them to plaque assay using the double agar layer method (Flynn et al., 2004; Kropinski et al., 2009). 30–300 Numbers of plaques formed by phage activity, were considered as valid and calculated as plaque forming units (PFU/mL).

In order to determine specificity of the isolated bacteriophage, the host range was determined by spotting 5 µL of phage lysate (~10^9 PFU/mL) on lawn cultures of various *Salmonella* sp. serovars. Plaquing was examined following an overnight incubation at 37 °C.

The one-step growth kinetics curve of isolated bacteriophage was determined according to Pajunen et al. (2000) with minor modifications. Culture of *Salmonella* Enteritidis ATCC®13076™ (optical density (OD_{600nm}) at the wavelength of 600 nm of 0.5 in a mid-exponential phase) was infected with bacteriophage sall_v01 at the multiplicity of infection (MOI) = 0.01, and left for adsorption for 10 min at 37 °C. Afterwards, the mixture was centrifuged (8000 × *g* for 5 min), suspended in 5 mL of BHI medium, and cultured at 37 °C. Sampling was performed at 5-min intervals, with subsequent dilution and plating for phage titration.

Evaluation of the sensitivity of bacteriophage to temperature and pH was examined according to Rattanachaikunsopon and Phumkhachorn (2007) with minor modifications. Temperature stability of bacteriophages (in final concentration approx. 10^8 PFU/mL) was determined by addition to preheated PBS (135 mM NaCl, 1.3 mM KCl, 0.5 mM KH_2PO_4, 3.2 mM Na_2HPO_4, pH 7.4) at temperatures ranging from 40 °C–90 °C, incubation for 30 min, and cooling on ice. In order to examine the pH stability, bacteriophage solution was incubated for 18 h at 37 °C in PBS buffer with pH adjusted (with HCl or NaOH) to value between 2 and 12. Afterwards, pH was re-adjusted to 7.4 and phages titre was measured by the double agar layer assay.

Fifty millilitres of LB (containing 10 mM $CaCl_2$ and 10 mM $MgSO_4$) were inoculated (2%) with the overnight culture and grown to reach OD_{600nm} = 0.2. Measurements were performed in 96 well plates with 200 µL of each sample using Infinite 200 PRO NanoQuant microplate reader (Tecan, Männedorf, Switzerland). The culture was divided into parts and the phage was added to reach multiplicity of infection of 0.01, 0.1 and 1, and then incubated at 37 °C. The OD_{600nm} was measured every 30 min for further 5 h. Samples without phages (containing only bacteria) and bacteria-free samples (containing only bacteriophage) were considered as controls.

Swine slurry was mixed with deionized water at ratio 1:2, accordingly to a common practice in agriculture (Himathongkham et al., 1999). The overnight cultures of *S*E ATCC®13076™ were cooled to 25 °C, centrifuged (8000 × *g* for 5 min) and pellet was resuspended in sterile PBS before mixing into slurry. Experimental contamination was prepared to result in 5 Log_{10} CFU/mL of *S*E, the phage was added to cultures in concentration consistent to MOI = 10, and incubated at 25 °C. Samples were being taken every 2 h and subsequently serially diluted in PBS. *Salmonella* Enteritidis counts in swine manure were determined (in triplicate) on selective X.L.D. agar (Oxoid), and expressed as Log_{10} CFU/mL. The stability of phage in the slurry was examined by incubation of samples at 4 °C and 25 °C for 7 days. Phage titre was measured every 24 h.

The data were analysed using the Statistica software package (StatSoft, Tulsa, OK, USA). All experiments were performed in triplicate with triplicate plating. Each phage-treated sample was compared to its control counterpart using one-way ANOVA. The data fulfilled with the suspicion of a normal distribution. *P* values of < 0.05 were considered as significant.

3. Results & discussion

While liquid manure is being commonly used as a fertilizer in agriculture, the necessity to develop alternative methods for eradication and biocontrol of pathogens residing in such material has become a great challenge (Sharma et al., 2013). Additionally, swine slurry being used as a crop fertilizer may transfer antibiotic-resistant bacteria and increase the occurrence of antibiotic resistance in bacteria of agricultural soils. Therefore, novel methods for prevention to the spreading of multiple-drug-resistant (MDR) bacteria are particularly required (Bao et al., 2015; Jokinen et al., 2015). Contamination of the natural environment caused by *Salmonella* Enteritidis poses a potential risk for human health. Hölzel and Bauer (2008) isolated *Salmonella* spp. from Bavarian liquid pig manure. Additionally, Nicholson et al. (2005) have reported that *Salmonella* strains can survive up to three months in slurry and polluted water, at temperatures < 20 °C. Our research has demonstrated that bacteriophages specific to several *Salmonella enterica* serovars can be isolated from the wastewater and can be used as biocontrol agents of these pathogens in pig slurry.

Initially, three phages against *S*E were isolated from collected waste-water samples. Phage sall_v01 produced small (approximately 1 mm in diameter), clear plaques with the absence of halo zone (Fig. 1A). Isolated bacteriophage efficiently infected various *Salmonella* serovars (reference − Enteritidis ATCC®13076™, Typhimurium ATCC®14028™, Pullorum NCTC 5776, Gallinarum NCTC 10532, Paratyphi NCTC 5702, and wild strains – 4750670, liver33, PIW), although no phage activity was observed on other Gram-negative bacilli such as *Escherichia coli* ATCC®11775™, *E. coli* ATCC®8739™, *Shigella flexneri* ATCC®12022™, *Shigella sonnei* ATCC®25931™, *Yersinia enterocolitica* subsp. *enterocolitica* ATCC®27729™, and *Proteus vulgaris* ATCC®6380™ in the experimental conditions. Sall_v01 have showed more extensive host range than bacteriophage PPST1 isolated by Rattanachaikunsopon and Phumkhachorn (2007), which can infect only *Salmonella* Typhi. A one step growth curve of sall_v01 indicated a latent period = 25 min (time between the adsorption and the first phage release), and the burst size of 107 ± 8 PFU per infected cell (Fig. 1B). This parameter was calculated as a ratio of released progeny bacteriophages to infected cell, also was higher than the phage PPST1, which have burst size of approx. 79 PFU per infected cell and longer latent period (30 min) (Rattanachaikunsopon and Phumkhachorn, 2007).

The thermal stability tests showed that sall_v01 can remain active at temperatures up to 60 °C. Higher temperatures (70 °C) inactivated 83% of virions. The heat up to 80 °C resulted in the complete inactivation of bacteriophage after one hour of incubation. Sall_v01 remained active when was incubated in pH ranging from 4 to 11 (Fig. 1C), whereas out of this range infectivity dramatically decreased what resulted in complete inactivation of virions. The highest stability was observed in pH ranging from 7.0 to 9.0. Moreover, we observed that quantity of the phage sall_v01 was constant during storage in pig slurry sample. We did not detect significant changes in the number of viral particles after 7 days in both temperatures (4 °C and 25 °C). Obtained results confirm high stability of bacteriophage and its resistance to many environmental factors including ions, temperature, pH, or organic solvents (Jończyk et al., 2011).

The infection experiment was performed on the *S*E cultures in the exponential growth phase. Sall_v01 was able to completely lyse bacterial culture at MOI = 1. Furthermore, we also observed high lytic activity at MOI = 0.1, although after six hours of incubation