Development of smart probiotics against *Clostridium perfringens*

T. Gervasi,[1,2] M.J. Mayer,[1] R.B. Lo Curto,[2] G. Dugo,[2] A. Narbad[1]

[1]Institute of Food Research, Norwich Research Park, Norwich, UK; [2]Department of Food and Environmental Sciences, University of Messina, Italy

Probiotics are living microorganisms which confer health benefits to the host. Our work was initially focused on investigating the possibility of producing *smart* probiotics, which are probiotics with modified extra functions, such as the heterologous expression of an antimicrobial. Our first aim was to identify antimicrobial activities or agents which could act against *Clostridium perfringens. C. perfringens*, one of the most pathogenic species in the *Clostridium* genus, is causing increasing concern because it is responsible for severe infections both in human and animals, especially poultry. It is considered the third leading cause of food poisoning death in the UK and USA and causes necrotic enteritis (NE) in poultry.[1] Bacteriophages and their endolysins have been used to treat human infections and to control antibiotic-resistant pathogenic bacteria in animal models.[2-4] Bacteriophages infecting *C. perfringens* are both lysogenic and virulent and show either long tails if members of the Siphoviridae family or short tails if members of the Podoviridae, both in the order of Caudovirales. Several putative bacteriophage endolysins have been identified, both from *C. perfringens* bacteriophages and by genome mining, producing a rich resource of enzymes.[5] The use of endolysins as antimicrobials has been explored. In fact recent studies showed the efficiency of these proteins in killing or controlling pathogenic bacteria when used alone, by a synergistic action with antibiotics or also in combination with other proteins such as the holin. The first test conducted to observe the presence of bacteriophages which contain an endolysin, was a plaque assay test. The appearance of a plaque is the oldest, but at the same time the most useful and direct confirmation way of a phage presence.[6] The nutrient agar layer method was first described by Gratia to enumerate phage particles.[7] A thin layer of soft agar, containing host bacteria and bacteriophages, is poured on a thick layer of higher concentrated agar, used as nutrient medium by bacteria. The phages infect the bacteria and after the production of new phage particles, which are released after bacterial lysis, start a new infectious cycle. To investigate the presence of prophages in *C. perfringens* strains, bacteriophage release was induced by mitomycin C. The supernatants were then concentrated by PEG precipitation then both observed by TEM and used for plaque assays. In the anaerobic cabinet 25 μl aliquots of filtered mitomycin C-induced supernatant were spotted on plates of BHI agar which had been overlaid with 4 ml BHI top agar (0.7% agar) seeded with 100 μL of *C. perfringens* overnight culture. Plates were incubated for up to 48 h and checked regularly for plaque formation. TEM observations on PEG-precipitated supernatants obtained after bacteriophage induction showed the presence of bacteriophages both in *C. perfringens* strains 54116-97 and 6081-97 (Figure 1). The bacteriophages in these supernatants did not produce plaques on any of the 25 strains tested. However, the mitomycin C-induced supernatant from strain 6081-97 did show antimicrobial activity on several *C. perfringens* strains, evident because of the zones of clearance around the supernatant dropped on plates (Figure 2). This activity appeared to be variable so to elucidate this behavior, strain 6081-9736 was streaked close to other *C. perfringens* strains such as 562118-98, 4519-98, 2151-88, and DP3. Bacteria which were potential producers of antimicrobials were streaked across BHI agar plates and potential sensitive strains were cross-streaked at a 90° angle with regard to the first streak of the indicator organism or parallel to this one and incubated overnight. The aim was to check if this strain was able to inhibit the growth of other *C. perfringens* strains. Among the strains tested, strain 2151-88 was shown to be able

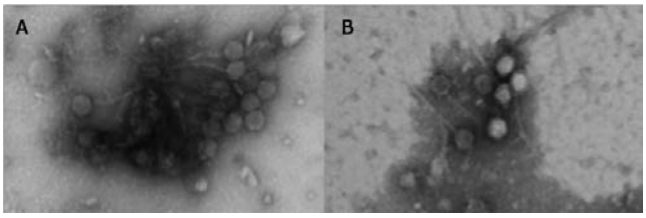

Figure 1. Tailed bacteriophages found in mitomycin-C induced supernatants of strain 6081-97 (A) and 5416-97 (B).[10] Scale bar represents 100 nm.

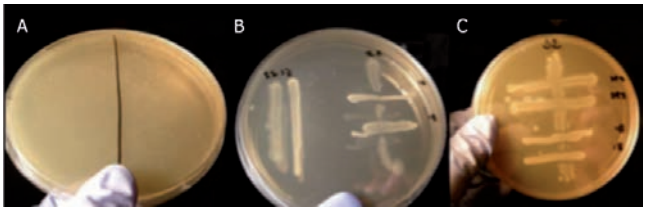

Figure 2. A) Antimicrobial effect of *C. perfringens* 6081-97 mitomycin-C induced supernatant on *C. perfringens* NCTC3110 and (B-C) on *C. perfringens* 2151-88 and 6081-97.

Correspondence: Teresa Gervasi, Department of Food and Environmental Sciences, University of Messina, viale Ferdinando Stagno D'Alcontres 31, 98166 Messina, Italy.
E-mail: tgervasi@unime.it

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to inhibit the growth of strain 6081-97 (Figure 2B,C), but its antimicrobial activity was not constant in repeat tests. In the same way strain 5416-97 showed an antimicrobial activity against other *C. perfringens* strains, but again with non-constant responses. It has been previously reported that mitomycin C can induce the production of bacteriocins from *C. perfringens*.[8] The genomes of both *C. perfringens* strains have been sequenced using Illumina technology and are currently being mined for genes associated with bacteriocin production. Further studies are in progress to investigate bacteriocin production and to assess the meaning of the observed variability in the antimicrobial activity of *C. perfringens* strains tested. The genome sequencing also allowed the identification of an active endolysin against *C. perfringens* from strain 5146-97[9] and we will investigate the possibility of a further lysin from strain 6081-97.

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