Gut solutions to a gut problem: bacteriocins, probiotics and bacteriophage for control of Clostridium difficile infection

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Clostridium difficile infection (CDI) is a major cause of morbidity and mortality among hospitalized patients and imposes a considerable financial burden on health service providers in both Europe and the USA. The incidence of CDI has dramatically increased in recent years, partly due to the emergence of a number of hypervirulent strains. The most commonly documented risk factors associated with CDIs are antibiotic usage leading to alterations of the gut microbiota, age >65 years and long-term hospital stay. Since standard therapies for antibiotic-associated diarrhoea and CDI have limited efficacy, there is now an urgent need for alternative therapeutics. In this review, we outline the current state of play with regard to the potential of gut-derived bacteriocins, probiotics and phage to act as antimicrobial agents against CDI in the human gut.

INTRODUCTION

Since the introduction of antibiotic-based antimicrobial therapies in the middle of the 20th century, antibiotic-associated diarrhoea (AAD) has emerged as an increasing problem. Related to this, one of the more common causes of nosocomially acquired intestinal infections is Clostridium difficile, the aetiological agent responsible for virtually all cases of pseudomembranous colitis and up to 20–30% of cases of AAD or hospital-acquired diarrhoea (Pochapin, 2000; Bartlett, 2002). Mortality due to C. difficile (2–42%) doubled from 1999 to 2004 and continued to rise until 2007, after which a decrease was seen in the UK (Wiegand et al., 2012). However, it is still a significant cause of AAD, posing a considerable financial burden on health service providers in both Europe and the USA (Ghantoji et al., 2010; Wiegand et al., 2012). It is now well recognized that C. difficile infection (CDI) is intrinsically linked to the use of broad-spectrum antibiotics, especially clindamycin, cephalosporins, penicillins and, more recently, fluoroquinolones (Warren & Guerrant, 2011). The argument that it is the disruption of the colonic flora that predisposes an individual to CDI has been reinforced by the ability of normal faecal flora to reverse the symptoms of CDI in very refractory cases when applied via nasogastric feeding/colonoscope (Aas et al., 2003; Rohlke et al., 2010; Grehan et al., 2010). Treatment of CDI almost exclusively involves the use of the broad-spectrum antibiotics vancomycin and metronidazole and, while the resistance to these antibiotics is low, there is a trend towards decreased susceptibility and an increase in recurrences of infection. More recently, a novel oral macrolide antibiotic, fidaxomicin, with a narrow inhibition spectrum was approved in Europe and the USA for the treatment of CDI (Johnson & Wilcox, 2012).

Owing to the frequent use of broad-spectrum antibiotics, the spread of antibiotic resistance, particularly in the hospital environment, is now posing significant problems for healthcare professionals. The emergence of pathogens with resistance to multiple antibiotics is likely to be a major challenge of the 21st century with antibiotics formally used routinely to treat pathogens, such as Staphylococcus aureus, Enterococcus faecalis, Mycobacterium tuberculosis and C. difficile, no longer being effective (Arias & Murray, 2009; Carlet, 2012; Riley et al., 2012). Few new antibiotics have emerged in the recent past due, in part, to the high cost of production and the associated financial risks of the development of such antibiotics (Cotter et al., 2013). Currently, vancomycin, once termed the antibiotic of last resort, and metronidazole are the main antimicrobials used to treat CDI. However, while resistance to these antimicrobials is low, there is a trend towards decreased susceptibility, possibly resulting in an increase in recurrences of infection (Baines et al., 2008; Brazier et al., 2008; Huang et al., 2009; Freeman et al., 2010). It is universally accepted that broad-spectrum antibiotic therapy results in perturbation of the gut flora, which predisposes individuals to CDI. The elderly are most at risk from CDI with carriage rates of C. difficile in hospitals and care homes for the elderly ranging from 2% to 20% (Rupnik et al., 2009; Ryan
et al., 2010; Rea et al., 2012). However, while CDI is a disease induced by broad-spectrum antibiotic usage, broad-spectrum antimicrobials are also used as therapeutics to treat CDI, which in turn may cause further dysbiosis in the distal colon. Indeed, in a murine study by Wlodarska et al. (2011), metronidazole was shown to reduce the depth of the mucus layer, resulting in a reduction of its barrier function, thereby predisposing the host to *Citrobacter rodentium*-induced colitis. The changes in the intestinal microbiota as a result of antibiotic treatment disrupted intestinal homeostasis and the integrity of intestinal defences, which normally protect the host against invading pathogens and intestinal inflammation (Wlodarska et al., 2011). It has also been demonstrated in mice that combinations of neomycin, vancomycin and metronidazole can induce infection by vancomycin-resistant enterococci (Brandl et al., 2008). In this article, we explore novel therapies that may have potential as alternative/adjunct therapies for the treatment of CDI, namely bacteriocins, probiotics and bacteriophage therapy.

**BACTERIOCINS**

That antibiotics used to treat CDI can cause collateral damage to the gut microbiota prompts the question of what avenues are now open for the development of therapies for the treatment of CDIs. One option currently being explored is the use of bacteriocins, small ribosomally synthesized antimicrobial peptides that have antimicrobial activity against closely related species (narrow spectrum) or across genera (broad spectrum). For a bacteriocin to play a role as an alternative therapeutic for CDI, it would need to be at least as effective as currently used antimicrobials, but ideally would combine high specificity for *C. difficile* with a narrow spectrum of inhibition, resulting in minimal collateral damage to the gut flora. In addition, resistance development by *C. difficile* and related species should be minimal, while the bacteriocin should also be non-toxic for the host. To date, a number of studies have shown *in vitro* activity of a number of bacteriocins active against *C. difficile* (Table 1). The bacteriocins lacticin 3147 and nisin (and bioengineered nisin variants), which are lantibiotics produced by strains of *Lactococcus lactis*, have been shown to be effective *in vitro* against clinically relevant *C. difficile* strains. However, they both have the disadvantage of having a broad spectrum of activity, especially towards Gram-positive bacteria (Bartoloni et al., 2004; Rea et al., 2007; Field et al., 2010). Additionally, we have shown in ex vivo studies using a model of the distal colon that the broad-spectrum lacticin 3147 causes a profound proportional shift in the microbiota from Firmicutes to Proteobacteria (Fig. 1) (Rea et al., 2011b). As nisin is also active against a broad range of Gram-positive bacteria, it is likely that it too would have a similar effect on the gut microbiota. The semi-synthetic broad-spectrum thiopeptide LFE571 inhibits *C. difficile* and demonstrates lower inhibitory activity against *Lactobacillus* and *Bifidobacterium* species than against other Gram-positive bacteria (Citron et al., 2012). A promising semi-synthetic derivative of the lantibiotic actagardine A with a narrow spectrum of inhibition has also been synthesized and inhibits a range of Gram-positive pathogens, including *C. difficile* (Boakes et al., 2012).

However, in an attempt to overcome the problems associated with broad-spectrum antimicrobials for the treatment of CDI, our research group embarked on a search within the gut microbiota for bacteria that produce antimicrobials that specifically target *C. difficile*. Faecal samples were screened for the presence of spore-forming strains that could inhibit *C. difficile* in an overlay assay. This led to the discovery of the sacitibiotic thuricin CD, produced by a strain of *Bacillus thuringiensis*. Thuricin CD exhibits MICs comparable to vancomycin and metronidazole when tested *in vitro* against clinically significant strains, including ribotypes 160, 001 and 027 (Rea et al., 2010, 2011a, b). More significantly, in a model of the distal colon that had been spiked with *C. difficile*, we showed that thuricin CD did not significantly impact on the wider gut microbiota, unlike vancomycin and metronidazole for which there was a major increase in abundance of Enterobacteriaceae at the expense of other families. We also demonstrated that thuricin is very effective in an environment containing a high microbial load as it was able to reduce the *C. difficile* population in the faecal environment ~100 fold (Fig. 2) (Rea et al., 2011b).

For peptide antimicrobials such as thuricin CD to be effective as a therapeutic many challenges lie ahead, not least in terms of understanding their mode of action, scaling up production and developing stable delivery mechanisms to ensure delivery of active peptides to the distal colon. However, given the pressures currently on the available therapeutics the potential for the commercialization of bacteriocins or their bioengineered derivatives as targeted therapeutics for treatment of gastrointestinal infections such as *C. difficile* is very promising.

**PROBIOTICS**

The evidence for the beneficial role of the gut microbiota in health and disease is mounting. In this respect, it is recognized that perturbations of the intestinal microflora may, in turn, lead to a range of diseases, including inflammatory immune diseases such as Crohn’s disease, insulin resistance, obesity, cancer and infectious diseases such as CDI (Scanlan et al., 2006; Davis & Milner, 2009; Shanahan, 2011; Rea et al., 2012). The fetal gut is sterile and is initially colonized during birth, the microbiota depending largely on the mode of delivery and the hygiene of the surrounding environment, which subsequently develops into the complex ecosystem of the adult gastrointestinal tract (GIT) (Fouhy et al., 2012). In adults, the human microbiota contains ~10^{14} bacteria comprising between 1000 and 1150 different species (Qin et al., 2010)
that exist in a mutualistic relationship with the host and aid in digestion, modulation of the immune system and manufacture of essential nutrients. Disturbances of the gut flora through antibiotic usage can have a profound effect on the health of the host, as is illustrated with CDI. Post-antibiotic treatment, the gut microbiota are altered and can no longer outcompete intestinal pathogens, allowing *C. difficile* to proliferate and produce toxins. In a study of *C. difficile* carriage in an elderly population (Fig. 3) the gut flora of two patients with CDI, from whom ribotype 027 was isolated, showed a much reduced microbial diversity when compared with those of an asymptomatic carrier of the same ribotype (Rea et al., 2012). Both CDI patients had a previous history of antibiotic use, in contrast to the asymptomatic carrier, which suggests that the gut microbiota exerted a protective effect on the subject who did not develop CDI (Rea et al., 2012).

Researchers have investigated whether the dysbiosis that occurs as a result of antibiotic usage can be prevented or reduced by the consumption of probiotics and have provided Table 1.

### Table 1. Referenced antimicrobial peptides with activity against *C. difficile*

<table>
<thead>
<tr>
<th>Bacteriocin</th>
<th>Description</th>
<th>Spectrum of inhibition</th>
<th>Producing strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nisin A</td>
<td>Lantibiotic</td>
<td>Broad</td>
<td><em>Lactococcus lactis</em></td>
<td>Bartoloni et al. (2004)</td>
</tr>
<tr>
<td>Nisin V</td>
<td>Lantibiotic, bioengineered derivative of nisin A</td>
<td>Broad, Gram-positive</td>
<td><em>Lactococcus lactis</em></td>
<td>Field et al. (2010)</td>
</tr>
<tr>
<td>Thuricin CD</td>
<td>Sactibiotic</td>
<td>Narrow</td>
<td><em>Bacillus thuringiensis</em> DPC 6431</td>
<td>Rea et al. (2010, 2011b)</td>
</tr>
<tr>
<td>Lacticin</td>
<td>Lantibiotic</td>
<td>Broad</td>
<td><em>Lactococcus lactis</em></td>
<td>Rea et al. (2007)</td>
</tr>
<tr>
<td>LFF571</td>
<td>Thiopeptide</td>
<td>Broad, Gram-positive</td>
<td><em>Planobispora rosea</em> ATCC 53773</td>
<td>Citron et al. (2012)</td>
</tr>
<tr>
<td>Actagardine A (DAB)</td>
<td>Semi-synthetic derivative of lantibiotic of actagardine</td>
<td>Narrow</td>
<td><em>Actinoplanes liguriensis</em> ATCC 31048</td>
<td>Boakes et al. (2012)</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Reduction in phylum level diversity in a model of the distal colon when the model is treated with lacticin 3147 compared with the control (pie charts). The results are expressed as a percentage of the total population of assignable tags. The corresponding bar charts show the decrease in *C. difficile* numbers in the presence of lacticin 3147. *T*<sub>0</sub>=time zero; *T*<sub>12</sub>=after 12 h incubation in the absence (control) or presence of lacticin 3147. [Redrawn from Rea et al. (2010).]

**Fig. 2.** The effects of vancomycin (Van), metronidazole (Met) andthuricin CD (all at 90 µM) on the viability of *C. difficile* in a distal colon model (bar chart). The corresponding pie charts show the effect of vancomycin, metronidazole and thuricin CD on the phylum level diversity in the distal colon. The antibiotic-treated model shows a reduction in microbial diversity compared to the control or thuricin CD. [Redrawn from Rea et al. (2011b).]
Fig. 3. Genus level diversity of gut communities of three subjects carrying C. difficile ribotype 027. (a) Asymptomatic carrier. (b) and (c) Patients with active CDI at the time of sampling. The results are expressed as a percentage of the total population of assigned tags. [Redrawn from Rea et al. (2012).]

Evidence that probiotics have potential as prophylactics in the prevention of CDI. Probiotics are defined as ‘live microorganisms which when consumed in adequate amounts confer a health benefit on the host’ (Pineiro & Stanton, 2007). Probiotics may play a role in improving epithelial barrier function, secretion of inhibitory substances (such as bacteriocins and hydrogen peroxide), immunomodulation, inhibition of expression of virulence factors and competitive exclusion – possibly through colonization resistance (McFarland, 2000; Corr et al., 2009). Most probiotics are lactic acid bacteria, belonging mainly to the genera Lactobacillus and Bifidobacterium. However, in the case of CDI, Saccharomyces boulardii has also been suggested as a potential probiotic. The mechanism of action of most probiotics in the context of prevention of gastrointestinal infection is poorly understood, albeit that some evidence exists for a limited number of probiotic strains. For example, Corr et al. (2007) showed that mice were protected against infection by the intestinal pathogen Listeria monocytogenes when orally fed a strain of Lb. salivarius producing the bacteriocin APB118. That the inhibition of infection was due to the bacteriocin-producing capability of the strain in the GIT was shown through the failure of an isogenic non-bacteriocin-producing variant of the same strain to prevent infection by L. monocytogenes. In addition, when the infecting L. monocytogenes strain was made immune to APB118 no protection against the infection was observed. Interestingly, this strain also was effective in preventing Salmonella infection, albeit through a different mechanism, as the non-producing variant was also effective in preventing infection (Corry et al., 2007). Three possible mechanisms for the probiotic effect of bacteriocin production in vivo have been proposed. These include the bacteriocin (1) acting as colonizing peptides by successfully allowing the probiotic strain to compete with the resident flora, (2) killing peptides, resulting in the eliminating of the pathogen, or (3) acting as signalling peptides through recruitment of other bacteria in the gut or the immune system to fight and eliminate the infectious organism (Dobson et al., 2012). Bacteriocin-producing probiotic cultures may therefore function by facilitating the introduction of a producer into an established niche, directly inhibiting the invasion of competing strains or pathogens into an established community, or modulating the composition of the microbiota and the host immune system. Therefore, the identification of probiotic strains capable of producing bacteriocins within the GIT targeting specific pathogens, such as C. difficile, may be one avenue in the search for probiotics for CDI.

Currently, the efficacy of probiotics for the treatment or prevention of CDI is controversial to say the least. In 2008, a Cochrane review was published on the use of probiotics for the treatment of C. difficile-associated colitis (Pillai & Nelson, 2008). The inclusion criteria for this analysis were randomized prospective studies using probiotics alone or in conjunction with conventional antibiotics for the treatment of documented C. difficile colitis. Only four studies from articles published from 1966 to 2007 were deemed eligible for this study (Table 2). Criticisms of the studies included small sample size, considerable heterogeneity relating to antibiotic usage and initial disease state. The limitations were such that the authors concluded there was insufficient evidence for the use of probiotics as adjuncts to conventional antibiotic therapy and no evidence to support the use of probiotics alone as therapeutics for CDI. In an interesting study (n=57 test group, n=56 placebo group) Hickson et al. (2007) showed that the consumption of a probiotic drink containing Lb. casei, Lb. bulgaricus and Streptococcus thermophilus twice a day during the course of antibiotic treatment and for 1 week following treatment reduced the incidence of AAD and CDI in hospital patients. In the largest study (255 subjects) to date, a combination of Lb. acidophilus and Lb. casei reduced the risk of hospitalized patients on antibiotics contracting CDI (Gao et al., 2010). Other reviewers have concluded that there may be some merit in the use of probiotics for the treatment of CDI, but again point to
Technological hurdles that will have to be overcome to redress the imbalance of gut microflora, and a more effective approach may be to use a cocktail of strains from a number of genera. This will pose a challenge in terms of how alterations in the host–microbiota relationship as a result of antibiotic therapy impact on the health of the host. It is unlikely that a single probiotic strain of Lactobacillus or Bifidobacterium will be sufficient to redress the imbalance of gut microflora, and a more effective approach may be to use a cocktail of strains from a number of genera. This will pose a challenge in terms of identification of the most suitable mixes and the technological hurdles that will have to be overcome to produce stable probiotic mixes containing anaerobic organisms.

### PHAGE THERAPY

Given the rise in antibiotic resistance, non-antibiotic therapies such as phage therapy are now being revisited as a possible therapeutic. Advantages of using phage for the control of infectious agents include (1) their ability to attack specifically target cells and replicate inside the cell by hijacking the DNA replication machinery, and ultimately killing the host, (2) the multiplying effect (given that they are capable of replicating in the presence of the target organism), (3) the specificity of phage against the target pathogen and (4) their inability to attack mammalian host cells or tissue. Phage therapy has been practised in Eastern European countries for decades, although largely neglected by the more-developed world after the advent of antibiotics in the middle of the 20th century. Although work on phage therapy continues in Eastern Europe, this was not conducted to a standard allowing it to support clinical uses in areas regulated by the European Medicines Agency or the US Food and Drug Administration (Harper et al., 2011).

Bacteriophages have been used successfully to control infections in animals and animal models of infection. Efficacy of bacteriophage to control *Pseudomonas* infections in dogs, humans and mice has been reported (Wright et al., 2009; Debarbieux et al., 2010; Burrowes et al., 2011; Harper et al., 2011; Saussereau & Debarbieux, 2012). Very recently, we demonstrated the effective use of bacteriophage to control *Pseudomonas* lung infections (a major cause of lung infections in patients with cystic fibrosis) in a validated mouse model and on biofilms formed on human airway cells by using a cocktail of two phages, φMR299-2 and φNH-4 (Alemayehu et al., 2012). Efficacies of bacteriophages to control *E. coli* O157 in sheep, steers and on cowhides have also been reported (Raya et al., 2009; Rozema et al., 2009; Coffey et al., 2011). In 2011 in Germany, a large outbreak of haemolytic uraemic syndrome and bloody diarrhoea caused by the Shiga toxin-producing *E. coli* O104:H4 was reported. The strain

<table>
<thead>
<tr>
<th>Study</th>
<th>Probiotic</th>
<th>c.f.u. g⁻¹</th>
<th>No. subjects</th>
<th>Additional treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lawrence et al. (2005)</td>
<td>Lactobacillus rhamnosus GG</td>
<td>2.8 x 10¹⁰ twice daily during antibiotic therapy and 21 days post-cessation</td>
<td>15</td>
<td>Vancomycin or metronidazole (concentration at discretion of clinician)</td>
</tr>
<tr>
<td>McFarland et al. (1994)</td>
<td>Saccharomyces boulardii</td>
<td>3 x 10¹⁰ daily for 4 weeks</td>
<td>124</td>
<td>Vancomycin or metronidazole</td>
</tr>
<tr>
<td>Surawicz et al. (2000)</td>
<td>Saccharomyces boulardii</td>
<td>1 g day⁻¹ for 30 days</td>
<td>168</td>
<td>Vancomycin (high dose: 2 g day⁻¹ or low dose: 500 mg day⁻¹) or metronidazole</td>
</tr>
<tr>
<td>Wullt et al. (2003)</td>
<td>Lactobacillus plantarum 229v</td>
<td>5 x 10¹⁰ for 38 days</td>
<td>21</td>
<td>Metronidazole 400 mg three times a day</td>
</tr>
</tbody>
</table>

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Small sample sizes and inadequate study design, indicating the need for large-scale well-designed clinical trials across a number of sites (McFarland, 2009; Johnson et al., 2012). One very critical review by Miller (2009) contends that probiotics play no role at all in the prevention or therapy of CDI and advise against the use of *Sc. boulardii* as a probiotic due to the danger of fungaemia not only in the patient being treated but also in patients in close proximity in intensive care facilities (McFarland, 2009; Miller, 2009; Johnson et al., 2012).

However, the question needs to be posed as to why particular probiotic strains were chosen for these trials. Most likely, they were chosen without any sound rationale for their selection other than that these strains were easy to produce or were commercially available. There is no evidence that in vivo trials were carried with the probiotics selected in animal models of CDI. As stated previously, current probiotics for human use belong almost exclusively to the genera *Lactobacillus* and *Bifidobacterium* as they are considered to contribute significantly to human health and have the added advantage of a long history of safe use, thereby overcoming many of the hurdles that may exist for other genera when included in foods for human nutrition. However, it is now recognized that probiotic properties being assigned to these genera are not only species specific but are also much more likely to be strain specific. The importance of strain selection was highlighted in a recent murine study investigating the effect of two strains of *B. breve*, NCIMB 702258 and DPC 6630, on gut microbiota composition which showed that only *B. breve* DPC 6630 modulated the gut microflora (Wall et al., 2012). The future of probiotic design for CDI will involve a deeper understanding of the role of the commensal flora of the gut and how alterations in the host–microbiota relationship as a result of antibiotic therapy impact on the health of the host. Bacteriophages have been used successfully to control infections in animals and animal models of infection. Efficacy of bacteriophage to control *Pseudomonas* infections in dogs, humans and mice has been reported (Wright et al., 2009; Debarbieux et al., 2010; Burrowes et al., 2011; Harper et al., 2011; Saussereau & Debarbieux, 2012). Very recently, we demonstrated the effective use of bacteriophage to control *Pseudomonas* lung infections (a major cause of lung infections in patients with cystic fibrosis) in a validated mouse model and on biofilms formed on human airway cells by using a cocktail of two phages, φMR299-2 and φNH-4 (Alemayehu et al., 2012). Efficacies of bacteriophages to control *E. coli* O157 in sheep, steers and on cowhides have also been reported (Raya et al., 2006; Rozema et al., 2009; Coffey et al., 2011). In 2011 in Germany, a large outbreak of haemolytic uraemic syndrome and bloody diarrhoea caused by the Shiga toxin-producing *E. coli* O104:H4 was reported. The strain
responsible for the epidemic was resistant to all penicillins and cephalosporins (Merabishvili et al., 2012). Recently, bacteriophages that efficiently lyse the E. coli O104:H4 outbreak strains have been suggested as potential therapeutics against such antibiotic-resistant pathogens (Merabishvili et al., 2012; Muniesa et al., 2012). Experimental data have also shown that both bacteriophages and their lysins could be effective in the treatment of methicillin-resistant St. aureus, one of today’s most prevalent pathogens associated with nosocomial infections (Fischetti, 2008; Fenton et al., 2010; Borysowski et al., 2011). Recently, the potential of bacteriophage therapy against experimental bubonic plague was highlighted (Filippov et al., 2012). The report shows that administration of phage φA122 provided 40% protection to BALB/c mice against 1000 LD₅₀ of Yersinia pestis CO92 and extended the mean time to death by 84%, showing that φA122 bacteriophage is a promising alternative therapy against multidrug-resistant plague strains (Filippov et al., 2012).

Studies on C. difficile bacteriophages, however, are limited and early reports deal mainly with their use in strain typing and morphological characterization (Fortier & Moineau, 2007). Recent work has focused more on the isolation, morphotyping, molecular characterization and genome sequencing of C. difficile bacteriophages (see Table 3 for a list of C. difficile bacteriophages whose genome sequences are available). Almost all C. difficile phages reported so far were isolated exclusively from the host cell following mitomycin induction (Table 3). Shan et al. (2012) performed an analysis of prophage carriage and diversity on 16 clinically isolated C. difficile ribotypes and found that 15 of the ribotypes were myovirus positive whereas two carry additional siphovirus prophages, while only one ribotype was negative for prophages. Recently, Nale et al. (2012) reported that they induced prophages from 91 clinical C. difficile 027 isolates and identified myophages and siphophages in 63 and three of the isolates, respectively, whereas dual phage type carriage was observed only in four isolates. Both reports showed convincing data regarding the very lysogenic nature of C. difficile phages and their coexistence through integration into the host genome.

Table 3. Referenced C. difficile phages with complete genome sequence data

<table>
<thead>
<tr>
<th>Phage ID</th>
<th>Family</th>
<th>Genome size (kb)</th>
<th>G+C (%)</th>
<th>Source of viral genome, strain type, and prophage inducing method</th>
<th>Reference</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>φXM02</td>
<td>Myoviridae</td>
<td>48.4</td>
<td>29.6</td>
<td>C. difficile 027 infected in C. difficile host</td>
<td>Misra et al. (2012)</td>
<td>JX145342</td>
</tr>
<tr>
<td>φXM04</td>
<td>Myoviridae</td>
<td>31.6</td>
<td>30.0</td>
<td>Human stool (viral genome located in C. difficile host)</td>
<td>Misra et al. (2012)</td>
<td>JX145342</td>
</tr>
<tr>
<td>φ38-2</td>
<td>Siphoviridae</td>
<td>41.1</td>
<td>30.8</td>
<td>Mitomycin induced</td>
<td>Sekulovic et al. (2011)</td>
<td>YP_004508400</td>
</tr>
<tr>
<td>φCK-356</td>
<td>Myoviridae</td>
<td>37.6</td>
<td>30.4</td>
<td>Mitomycin induced</td>
<td>Horgan et al. (2010)</td>
<td>YP_002929999</td>
</tr>
<tr>
<td>φCD6356</td>
<td>Myoviridae</td>
<td>37.6</td>
<td>29.4</td>
<td>Mitomycin induced</td>
<td>Moy et al. (2008)</td>
<td>YP_000874521</td>
</tr>
<tr>
<td>φCD630</td>
<td>Myoviridae</td>
<td>56.5</td>
<td>29.1</td>
<td>Mitomycin induced</td>
<td>Goh et al. (2007)</td>
<td>YP_001087452.1</td>
</tr>
<tr>
<td>φC2</td>
<td>Myoviridae</td>
<td>53.3</td>
<td>28.7</td>
<td>Mitomycin induced</td>
<td>Govind et al. (2006)</td>
<td>YP_529653.1</td>
</tr>
</tbody>
</table>

Studies on functional analysis of prophages revealed that they can influence the phenotype of their host, e.g. expression of potent toxins to make the pathogen more aggressively virulent. This is the case, for instance, with the Shiga toxin in E. coli, cholera toxin in Vibrio cholerae and the botulinum neurotoxins in C. botulinum (Brussow et al., 2005) where their expression is positively affected by the presence of prophage. A possible role of prophages in influencing the TcdA and TcdB exotoxin expression in C. difficile has also been reported (Goh et al., 2005; Sekulovic et al., 2011). Introducing the genome of phage CD38-2, a pac-type temperate siphophage, into the C. difficile CD274 host stimulated TcdA and TcdB expression over twofold, demonstrating a possible role for the phage genome in toxin production in C. difficile (Sekulovic et al., 2011).
Contrary to this, however, a very recent study by Meessen-Pinard et al. (2012) reported a natural induction of prophage in CDIs. In the study, four Myoviridae phages, φMMP01, φMMP02, φMMP03 and φMMP04, were recovered as free viral particles in filter-sterilized stool supernatants of patients suffering from CDI, which provides evidence that prophages are naturally induced and can kill the host (*Clostridium difficile*) during episodes of CDI (Meessen-Pinard et al., 2012).

The current knowledge regarding the use of phage therapy for the control of CDI is limited. This is mainly due to the lysogenic nature of *Clostridium difficile* bacteriophages studied and isolated to date. Thus far, no lytic phage specific for *C. difficile* has been isolated, with all reports to date showing that phage were recovered only after induction of the host with mitomycin C (Table 3). The resulting phage can integrate easily during infection of a new host, limiting the lytic cycle and resulting in a very low phage titre. It has been suggested that the temperate lifestyle of *Clostridium difficile* phage is due to (1) the high incidence of prophage genes which may impart resistance to further infections and (2) the spore-forming nature of *C. difficile* which would also favour phage that integrate into the genome of the host (Meader et al., 2010). Revathi et al. (2011) also reported recently the lysogenization of phage CD119 into the genome of *C. difficile* during infection in the hamster model and highlighted the importance of lysogeny of *C. difficile* phages as an evolutionary adaptation for survival. Despite the lysogenic nature of *C. difficile* phages, anti-*C. difficile* bacteriophages and their active endolysins have potential as effective antimicrobial agents. Administration of bacteriophage intragastrically to hamsters challenged with *C. difficile* protected the animals against infection, whereas control animals that did not receive phage died within 96 h (Ramesh et al., 1999). By using an *ex vivo* faecal fermentation model (that simulated the conditions in the human colon), Meader et al. (2010) also demonstrated how effectively phage therapy can reduce/prevent *C. difficile* toxin production and/or colonization.

To overcome the problem of the lysogenic nature of *C. difficile* bacteriophage, cloned biologically active phage endolysins have been suggested as an alternative avenue for the development of phage-based anti-*C. difficile* therapeutics. The cloned endolysin CD27L was active against a diverse range of *C. difficile* strains, including ribotype 027 (Mayer et al., 2008, 2011). We reported previously the isolation and genetic characterization of phage φCD6356, the first report of a *C. difficile* temperate phage belonging to the Siphoviridae family (Horgan et al., 2010). This endolysin has been cloned, and the partially purified protein was shown to have high lytic activity against *C. difficile* ribotype 027 and resulted in a reduced cell count of 2–3 log units (Burke et al., 2012). While lytic bacteriophage have shown potential in their ability to kill a wide range of pathogens involved in infectious diseases, the lysogenic nature of the *C. difficile* phages poses a formidable challenge for their use as therapeutics, and may, in fact, prove counterproductive if they integrate into the genome of the host and impact on toxin production. However, the ability to clone active phage endolysins may be the way forwards to developing a very specific anti-*C. difficile* therapeutic, which may go some way towards overcoming the increasing problem of developing drug resistance within the gut microflora.

**Overall conclusions**

While the discovery of antibiotics in the first half of the 20th century undoubtedly heralded a new age in our ability to fight infections, the emergence of organisms like *Clostridium difficile* highlights many of their limitations and side effects. There is a clear need for a new approach to fight gastrointestinal infections and one that takes into account the need to maintain the integrity of the gut microbiota. What better place to look for such therapeutics than in the complexity of the human gut itself? In this review, we have outlined the current state of play with regard to the potential of gut-derived probiotics, phages and bacteriocins to act as antimicrobial agents against CDI in the human gut.

**ACKNOWLEDGEMENTS**

The authors and their work were supported by the Science Foundation of Ireland (SFI) and funded by the Centre for Science, Engineering and Technology grant 02/CE/B124. The Alimentary Pharmabiotic Centre is a research centre funded by the SFI.

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