Coverage of diarrhoea-associated *Escherichia coli* isolates from different origins with two types of phage cocktails

Gilles Bourdin,1 Armando Navarro,2 Shafiquil A. Sarker,3 Anne-C. Pittet,1 Firdausi Qadri,3 Shamima Sultana,2 Alejandro Cravioto,2,3 Kaisar A. Talukder,3 Gloria Reuteler1 and Harald Brüssow1*

1Nutrition and Health Department, Nestlé Research Center, Lausanne, Switzerland
2Department of Public Health, Faculty of Medicine, Universidad Nacional Autónoma de México, Mexico City, Mexico, Mexico
3International Center for Diarrheal Disease Research, Bangladesh, Dhaka, Bangladesh

Summary

Eighty-nine T4-like phages from our phage collection were tested against four collections of childhood diarrhoea-associated *Escherichia coli* isolates representing different geographical origins (Mexico versus Bangladesh), serotypes (69 O, 27 H serotypes), pathotypes (ETEC, EPEC, EIIE, EAEC, VTEC, *Shigella*), epidemiological settings (community and hospitalised diarrhoea) and years of isolation. With a cocktail consisting of 3 to 14 T4-like phages, we achieved 54% to 69% coverage against predominantly EPEC isolates from Mexico, 30% to 53% against mostly ETEC isolates from a prospective survey in Bangladesh, 24% to 61% against a mixture of pathotypes isolated from hospitalized children in Bangladesh, and 60% coverage against *Shigella* isolates. In comparison a commercial Russian phage cocktail containing a complex mixture of many different genera of coliphages showed 19%, 33%, 50% and 90% coverage, respectively, against the four above-mentioned collections. Few O serotype-specific phages and no broad-host-range phages were detected in our T4-like phage collection. Interference phenomena between the phage isolates were observed when constituting larger phage cocktails. Since the coverage of a given T4-like phage cocktail differed with geographical area and epidemiological setting, a phage composition adapted to a local situation is needed for phage therapy approaches against *E. coli* pathogens.

Introduction

The antibiotic crisis has rekindled the interest in phage therapy approaches that were developed in Eastern Europe (Brüssow, 2012). Phage therapy has theoretical advantages over antibiotic therapy. Phage is a self-amplifiable antibacterial agent that increases in titer only when it meets in vivo its target (Payne et al., 2000). Phage is quickly eliminated from the body in the absence of the pathogen as shown in pioneer work in mice (Merril et al., 1996) and later in humans (Bruttin and Brüssow, 2005). In addition, phages are mostly species-specific bacterial viruses. Exceptions are phages infecting *Listeria innocua* and *Listeria monocytogenes* or *Salmonella* and *Escherichia coli* (Santos et al., 2010). Consequently, and in notable contrast to antibiotics, no collateral damage on the commensal microbiota is to be expected. However, the full scale of ecological relationships between coexisting bacterial species in a given microbial community is quite complex and is not fully described or understood yet.

Data from human healthy subjects do not suggest phage-induced gut microbiota changes induced by oral phage (Sarker et al., 2012; McCallin et al., 2013). The specificity of phages has also a downside: many phages are not only species-specific, but are also strain-specific. This property becomes a coverage problem when phage therapy targets a disease caused by a complex pathogen like diarrhoea-associated *E. coli*, which is represented by many different pathotypes and serotypes (Chibani-Chennoufi et al., 2004a,b). The constitution of complex phage cocktails then becomes also a commercial problem of producing a standardized preparation. For some bacterial pathogens, broad host-range phages have...
been described (Salmonella: Santos et al., 2010; Staphylococcus aureus: Kelly et al., 2011). However, no such broad host-range phages have so far been isolated for E. coli. Recent data suggest that coliphage phi92, isolated from an encapsulated K1 E. coli strain and belonging to the novel genus of rV5-like phages, might have a broad host range (Schwarzer et al., 2012) like the multivalent Salmonella phage PVP-SE1 infecting both Salmonella and E. coli (Santos et al., 2010). These phages have not yet been tested against large collections of pathogenic E. coli strains. Phage therapy against E. coli must therefore rely on phage cocktails. The structure of host–phage interaction matrices has recently become an active research area (Flores et al., 2011; Weitz et al., 2013). Different types of interaction were identified: specialist phages that may infect a unique host leading to a nearly diagonal matrix when columns indicate phage and rows host isolates; phage–host interactions that may describe block-like matrices, which exhibit high degrees of modularity; generalist phages and phage–host interaction that are random.

For regulatory reasons, we worked with a cocktail containing only a single phage genus, reasoning that it might be easier to demonstrate safety for a single phage group than for a cocktail containing numerous different phage genera. We settled for the T4-like genus of Myoviridae (Bruttin and Brüssow, 2005; Sarker et al., 2012) because professional virulent phages that destroy the host genome during the infection process are considered by many researchers as best candidates for phage therapy (Brüssow, 2012). Early on, it was reported that T4-like phages recognize lipopolysaccharides (LPS) in the outer membrane of E. coli (O antigens) as primary phage receptors (Tamaki et al., 1971). However, LPS synthesizing genes represent a hotspot of genetic diversity in E. coli (Heinrichs et al., 1998). In fact, the specificity of LPS became the basis for the definition of more than 150 different O serotypes, although not all O serotypes are encountered in pathogenic E. coli (Robins-Browne, 1987). If LPS is the receptor for T4-like phages on E. coli, one would predict problems for achieving high coverage of a phage cocktail against diarrhoea-associated E. coli. Other researchers identified the outer membrane protein OmpC as receptor (Hashemolhosseini et al., 1994; Yu et al., 2000) for T4 phages. Since this gene is well conserved across different genera of Enterobacteriaceae when using the E. coli K-12 gene in BLAST searches, multivalent T4-like phages should be expected. In the present report, we explored the coverage of phage cocktails composed of T4-like phages against E. coli isolates from different epidemiological settings compared with a commercial Russian phage cocktail containing many different genera of coliphages (McCallin et al., 2013). We conclude that pathogen coverage is dependent on the epidemiological context requiring flexible and locally adapted phage cocktails.

**Materials and methods**

**Phages**

Our T4-like phage collection was described in Sarker and colleagues (2012), and the commercial Russian ColiProteus phage cocktail from Microgen (product number 460502100204; batch numbers 16/ 0905/09. 06 and 3/0307/03. 09) was described in McCullin and colleagues (2013). ColiProteus contains phages directed against Proteus and E. coli. Sextaphage was another commercial phage cocktail from Microgen (product number 4602784002612 batch numbers 531/ 0211 /II13 and 196–17 /1205/I 08). Sextaphage (also called Pyobacteriophage Polyvalent by Microgen) contains phages directed against six pathogens: Staphylococcus aureus, Streptococcus pyogenes, Proteus mirabilis and Proteus vulgaris, Pseudomonas aeruginosa, Klebsiella pneumoniae, and E. coli.

**Phage propagation and lysis tests**

The test phages were propagated on the E. coli strain K-12 derivative K803, which lacks prophage lambda, obtained from E. Kutter, Evergreen College, Olympia, Wa, USA. Luria-Bertani (LB) broth was inoculated with 1% of an overnight bacterial culture and incubated at 37°C for 2 h to reach 1 x 10^8 cfu/ml. Phage lysate was added to the culture to get 1 to 5 x 10^8 pfu/ml and the infected culture was incubated at 37°C for 3 to 5 further hours with agitation (100 r.p.m.). After removal of bacterial cells and cellular debris by centrifugation for 15 min at 14 000 x g and passing the lysate through a 0.22 μm Millipore filter, the phage titers in the lysates were determined by the plaque assay as described previously (Bruttin and Brüssow, 2005). In small experimental series 100 μl of an appropriate phage dilution and 100 μl of an exponentially growing test pathogenic E. coli culture to achieve an moi of 3 were incubated at 37°C in 10 ml tubes containing LB broth, and the OD development was read at different time points against the uninfected test cell as control. For larger experimental series (100 phage isolates against about 100 E. coli isolates at three moi in duplicates resulting in 60 000 tests) we used the microtiter lysis test; 96-well plates with 10 μl of centrifugation-cleared and sterile-filtered (0.22 μm pore size) phage lysate and 180 μl of fresh LB broth were inoculated with 20 μl of the test E. coli target strains to obtain an moi of 0.1, 0.01 and 0.001 respectively. The plate was incubated at 37°C for 4 h under agitation (200 r.p.m.). Every hour the OD595 was meas-
ured in a Dynex reader (MRX model, Magellan Biosciences, Chantilly, VA). The E. coli strain without phage and non-inoculated LB medium were used as positive and negative controls respectively.

E. coli strains from Mexico

Several hundred E. coli test strains were collected between 1985 and 2003 from children who experienced an episode of diarrhoea in two regions of Mexico (Mexico City and the State of Morelos). Eighty-nine strains representing the diversity of O and H serotypes encountered were selected for phage susceptibility testing. The E. coli strains isolated from children in the State of Morelos belonged to a field study reported previously (Cravioto et al., 1990). In short, the isolates were characterized as E. coli strains by the Vitek automated bacterial identification system from BioMérieux. The strains were then serotyped by agglutination assays (Ørskov and Ørskov, 1984) using 96-well microtitre plates and rabbit serum (SERUNAM) obtained against 187 somatic antigens and 53 flagellar antigens for E. coli, as well as against 45 somatic antigens for Shigella species. Polymerase chain reaction (PCR) assays were used to determine the presence of virulence genes with primers specified in Supplementary Table 1. The presence of the following genes was investigated: for enteropathogenic E. coli (EPEC), intimin gene (eae) (Karch et al., 1993) and bundle forming pilus (bfp) gene (Gunzburg et al., 1995); for enterotoxigenic E. coli (ETEC), heat-labile enterotoxin LTh (Schults et al., 1994), heat-stable enterotoxins STh and STp genes (Schults et al., 1994); and for enterohemorrhagic E. coli (EHEC), the Shiga-like toxin Stx genes (Pollard et al., 1990). The bacterial strains were stored at –20°C on ‘Protect Bacterial Preservers’ beads (Technical Service Consultants, Lancashire, UK).

E. coli strains from Bangladesh

A prospective community-based study was conducted in an urban slum in Mirpur, Dhaka, Bangladesh, from April 2002 to October 2004. Pregnant mothers were screened and 300 newborn children were followed prospectively for diarrhoeal diseases (Qadri et al., 2007). E. coli colonies were isolated as described previously (Albert et al., 1995; Qadri et al., 2000). In case of diarrhoea, six lactose-fermenting E. coli colonies from MacConkey agar were inoculated on GM1-coated microtiter plates containing LB broth for 18 h. The supernatant was tested for ST using an inhibition ELISA procedure (Svennerholm et al., 1986) and for LT by using an anti-LT monoclonal antibody (Svennerholm and Wiklund, 1983). ETEC strains were cultured on CF antigen agar (McConnell et al., 1989) and tested for the expression of CFA/I, CS1 to CS8, CS12, CS14, CS17, CS19, and CS21 by monoclonal antibody-based dot blot assay (Qadri et al., 2000).

Results

E. coli pathogens from a community survey of childhood diarrhoea in Mexico

Since LPS, the chemical determinant of the somatic O antigen, was identified as receptor for T4-like phages on E. coli cells by some authors (Tamaki et al., 1971; Thomassen et al., 2003), we first tested our T4-like phage collection against a collection of O serotype-defined, diarrhoea-associated E. coli strains. E. coli strains were isolated from children with diarrhoea living in two regions of Mexico (Cravioto et al., 1990). From this collection, we chose 89 E. coli strains for screening against our T4-like phage collection (Table 1). The test E. coli strains represented 69 different O serotypes. Six O serotypes were associated with two or three different H serotypes. Overall, 26 different H serotypes were observed, several H serotypes were associated with up to 4 O serotypes (the strains were selected for maximal serotype diversity). Fifty isolates showed the EPEC-specific eae1 gene. Only three of them also showed the bfp gene characterizing them as classical EPEC strains, making the majority of the Mexican isolates atypical EPEC. Fourteen isolates showed enterotoxin genes—the most prevalent combination were strains with STh+LTh genes (n = 7). Only few strains showed STp+LTh gene combination (n = 2), only LTh (n = 2), only STp (n = 2) and STp+STh+LTh genes (n = 1). Compared to the 50 EPEC isolates, only 14 isolates were thus diagnosed as ETec strains. Shiga-like toxin was found in a single isolate.

Coverage of the Mexican E. coli pathogens with coliphages

The susceptibility of the individual 89 E. coli strains for the 98 T4-like phages from our collection (Sarker et al., 2012) was tested in a lysis test at three different multiplicities of infection (moi = 0.1, 0.01, 0.001). The lytic effect of the phage was rated according to the observed lysis: grades 1, 2 and 3 lysis represented growth inhibition only at the highest moi, at two and all three moi levels, respectively (Fig. 1). Most T4-like phages had only a narrow host range on the 89 serotyped test strains, but no clear correlation with O serotypes was seen (data not shown). A few phage isolates lysed many E. coli strains independent of the O serotype (Supplementary Table 2). The T4-like phage with the broadest host range showed lytic activity on 44 bacterial strains (49% of the total). When combining three T4-like phages with the broadest individual host
Table 1. Host-range determination of different T4-like coliphage cocktails and a commercial Russian phage cocktail on a collection of *E. coli* strains isolated from children with diarrhoea in Mexico.

| O:H type   | VF     | N 1-p | N 3-p | N 6-p | N 9-p | Micro | N 10-p |
|------------|--------|-------|-------|-------|-------|-------|--------|
| O1:H45     | eae1   | 1     | 1     | 3     |       | 1     |        |
| O1:H45     | eae1   | 2     | 3     | 3     |       | 1     |        |
| O2:H4      | eae1   | 3     |       |       |       | 1     |        |
| O4:H16     | eae1   | 3     |       |       |       | 1     |        |
| O5:H11     | bfp    | 2     |       |       |       | 1     |        |
| O6:H16     | STh, LTh | 3     | 1     | 2     |       | 1     | 2      |
| O6:H      | eae1   | 2     | 3     | 3     |       | 1     |        |
| O8:H9      | STh, LTh | 3     |       |       |       | 1     |        |
| O8:H-      | STh, LTh | 3     |       |       |       | 1     |        |
| O8:H11     | nt     | 2     |       |       |       | 1     |        |
| O9:H25     | nt     | 1     |       |       |       | 1     |        |
| O11:H27    | eae1   | 2     | 3     | 3     |       | 1     |        |
| O11:H-     | eae1   | 3     | 3     | 3     |       | 1     |        |
| O11:H7     | nt     | 2     | 1     | 2     |       | 1     |        |
| O11:H10    | nt     | 3     | 3     | 3     |       | 1     | 2      |
| O15:H18    | eae1   | 1     |       |       |       | 1     |        |
| O15:H1     | nt     | 2     | 1     | 3     |       | 1     |        |
| O15:H32    | nt     | 3     | 1     | 2     |       | 1     |        |
| O16:H-     | eae1   | 3     | 3     | 3     | 3     | 3     |        |
| O18ac:H-   | eae1   | 3     | 3     | 3     | 3     | 1     |        |
| O20:H13    | eae1   | 3     | 3     | 3     | 3     | 1     |        |
| O22:H1     | nt     | 1     |       |       |       | 1     |        |
| O23:H15    | eae1   | 3     | 2     | 3     |       | 1     |        |
| O25:H-     | eae1   | 3     | 3     | 3     |       | 1     |        |
| O25:H4     | nt     | 3     | 3     | 3     | 3     | 3     |        |
| O26:H1     | eae1   | 3     | 3     | 3     | 3     | 3     |        |
| O26:H8     | nt     | 3     | 3     | 3     |       | 1     |        |
| O27:H20    | STp    | 1     | 3     | 3     |       | 1     |        |
| O28ac      | bfp    | 3     | 1     | 2     |       | 1     |        |
| O29:H21    | eae1   | 3     | 1     | 1     |       | 1     |        |
| O38:H9     | eae1   | 3     | 3     | 3     | 3     | 3     |        |
| O39:H12    | eae1   | 3     | 3     | 3     |       | 1     |        |
| O45:H-     | eae1   | 2     | 3     | 3     | 3     | 1     |        |
| O48:H30    | nt     | 1     |       |       |       | 1     |        |
| O55:H6     | eae1   | 3     |       |       |       | 1     |        |
| O63:H-     | eae1, bfp | 1     | 3     | 3     | 3     | 1     |        |
| O75:H5     | eae1   | 1     | 3     | 3     | 3     | 1     |        |
| O78:H10    | STh, LTh | 3     | 3     | 3     | 3     | 1     |        |
| O78:H10    | STh, LTh | 3     | 3     | 3     | 3     | 1     |        |
| O80:H26    | STp, LTh, eae1 | 3     |       |       |       | 1     |        |
| O82:H8     | eae1   | 3     |       |       |       | 1     |        |
| O84:H?     | eae1   | 3     |       |       |       | 1     |        |
| O85:H10    | eae1   | 3     |       |       |       | 1     |        |
| O86:H18    | eae1   | 3     |       |       |       | 1     |        |
| O86:H18    | eae1   | 3     |       |       |       | 1     |        |
| O88:H10    | eae1   | 3     |       |       |       | 1     |        |
| O100:H25   | STh, LTh | 3     |       |       |       | 1     |        |
| O101:H-    | eae1   | 3     |       |       |       | 1     |        |
| O103:H43   | eae1   | 3     |       |       |       | 1     |        |
| O103:H-    | STh, LTh | 3     |       |       |       | 1     |        |
| O104:H-    | eae1   | 3     |       |       |       | 1     |        |
| O105ab:H5  | bfp    | 3     |       |       |       | 1     |        |
| O110:H16   | eae1   | 2     | 3     | 3     |       | 1     |        |
| O111ab:H-  | eae1   | 3     | 3     | 3     |       | 1     |        |
| O112ac:H9  | eae1   | 3     |       |       |       | 1     |        |
| O113:H21   | LTh    | 3     |       |       |       | 1     |        |
| O113:H-    | eae1   | 3     |       |       |       | 1     |        |
| O113:H-    | eae1, bfp | 3     |       |       |       | 1     |        |
| O114:H49   | nt     | 1     |       |       |       | 1     |        |
| O117:H27   | SLT    | 1     | 2     | 3     |       | 1     |        |
| O118:H-    | bfp    | 3     |       |       |       | 1     |        |
| O119:H4    | nt     | 3     |       |       |       | 1     |        |
| O120:H27   | eae1   | 1     |       |       |       | 1     |        |
| O121:H19   | eae1   | 1     |       |       |       | 1     |        |

© 2014 The Authors. *Microbial Biotechnology* published by John Wiley & Sons Ltd and Society for Applied Microbiology, *Microbial Biotechnology*, 7, 165–176
ranges, an arithmetical coverage of 87% should have been achieved. However, the physical combination of these three T4-like phages only resulted in the lysis of 54% of the *E. coli* strains suggesting interference between the phages (Table 1). When six T4-like phages were combined into a cocktail, we could increase the coverage to 69%. Further increasing the number to nine phages did not result in enlarged coverage (Table 1). A commercial Russian phage cocktail (Coli-Proteus phage cocktail from Microgen) only lysed 17 of the 89 test bacteria (19% coverage) (Table 1).

Coverage of *E. coli* pathogens from a community diarrhoea survey in Bangladesh

The commercial Russian phage cocktail was presumably selected for good coverage on *E. coli* strains from Russia. Its low coverage on the Mexican *E. coli* pathogens raises the question whether phage cocktails display geographical specificities. We do not know the selection process of phages for the Microgen cocktail, but at the Eliava Institute in Tbilisi/Georgia where Soviet phage therapy took its origin, new phages against *E. coli* pathotypes not covered by the previous stock are in regular intervals added to the cocktail (Chanishvili, 2012).

Table 1. cont.

| O:H type     | VF           | N 1-p | N 3-p | N 6-p | N 9-p | Micro | N 10-p |
|--------------|--------------|-------|-------|-------|-------|-------|--------|
| O125ab:H33   | eae1         |       |       |       |       |       |        |
| O125ab:H18   | nt           | 1     | 3     | 3     | 2     |       | 2      |
| O125ac:H-    | nt           |       |       |       |       |       |        |
| O126:H21     | nt           | 2     | 2     | 1     |       |       |        |
| O127:H11     | eae1         | 3     | 3     | 3     | 2     | 1     |        |
| O128:H27     | eae1         | 3     | 3     | 3     | 3     |       |        |
| O132:H8      | bfp          | 1     | 3     | 3     | 3     |       | 2      |
| O135:H?      | bfp          |       |       |       |       | 3     |        |
| O141:H32     | eae1         | 1     | 1     |       |       |       | 1      |
| O141:H26     | nt           | 3     | 1     | 2     | 2     |       |        |
| O142:H?      | eae1         |       |       |       |       |       |        |
| O145:H45     | eae1         |       |       |       |       |       |        |
| O146:H8      | eae1         | 2     | 3     | 3     | 3     |       | 3      |
| O147:H32     | nt           | 3     | 3     | 3     | 3     |       | 3      |
| O148:H28     | STp, STh, LTh| 3     | 3     | 3     | 3     |       | 1      |
| O149:H-      | eae1         |       |       |       | 3     | 2     |        |
| O153:H12     | eae1         |       |       |       |       |       | 1      |
| O158:H9      | eae1         |       |       |       |       | 2     | 1      |
| O159:H25     | STp, LTh     | 2     | 3     | 3     | 3     |       | 3      |
| O163:H19     | eae1         | 1     | 3     | 3     |       |       | 3      |
| O165:H18     | eae1         | 1     | 3     | 3     | 3     |       | 3      |
| O166:H5      | eae1, bfp    | 3     |       |       |       |       | 1      |
| O167:H5      | LTh          |       |       |       |       |       |        |
| O169:H-      | STp          |       |       |       |       |       | 2      |

Notes: All isolates were *E. coli* strains identified by the Vitek automated bacterial Identification system (BioMerieux) O:H. The O and H serotypes were identified by reference sera VF (virulence factors): The presence of the following virulence genes from diarrhoea-associated bacteria were assessed by PCR: enterotoxigenic *E. coli* (ETEC) were diagnosed by the presence of the heat-labile enterotoxin LTh (encoded by the eltA/B genes) or heat-stable enterotoxins STh (encoded estA gene) or STp (encoded by the st1 gene); enteropathogenic *E. coli* (EPEC) were diagnosed by the presence of the intimin (encoded by the eae gene) and the bundle forming pilus (bfp gene); the strains were also tested for the presence of the gene encoding the Shiga-like toxin (SLT); nt: not tested N1-p to N10-p: The indicated *E. coli* strains were tested for lysis graded by 1, 2 and 3 (according to the OD decrease observed at different moi, see Fig. 1) by the following phage cocktails: N1-phage (NPC1001), N3-phage (NPC1001, NPC 1005, NPC1006), N6-phage (NPC1001, NPC 1005, NPC 1006, NPC 1002, NPC 1008, NPC 1003), N9-phage (NPC 1000, NPC 1002, NPC 1003, NPC 1006, NPC 1007, NPC 1008, NPC 1009, NPC 1024, NPC 1031), N10-phage cocktail (NPC 1000, NPC 1001, NPC 1002, NPC 1003, NPC 1004, NPC 1005, NPC 1006, NPC 1007, NPC 1008, NPC 1009) (for the phage codes see Sarker et al., 2012) and the commercial Microgen ColiProteus phage cocktail Micro (containing an unknown number of phage isolates representing according to metagenomic analysis at least ten different phage genera, representing all three major families of tailed phages, McCallin et al., 2013).
For a subset of the *E. coli* strains, the phage susceptibility of the strain was confirmed by the plaque assay (Table 2).

### Coverage of *E. coli* pathogens from diarrhoea patients hospitalized in Bangladesh

The distinct coverage observed with the same phage cocktail on different sets of *E. coli* pathogens could reflect the difference in location (*Mexico versus Bangladesh*) or pathotype (*EPEC versus ETEC*). To separate both effects, we tested the cocktails against different pathotypes of *E. coli* obtained from the same geographical region. From the Enteric Microbiology and Immunology Units of the International Center for Diarrheal Disease Research in Dhaka/Bangladesh (icddr,b) we obtained *E. coli* isolates that represented all major pathotypes, namely 15 EPEC, 15 ETEC, 10 enteroaggregative (EAEC), 3 enteroinvasive (EIEC), and 3 Verotoxin-producing (VTEC) *E. coli* strains, all isolated from diarrhoea patients hospitalized in 2008 at icddr,b. Our T4-like phage cocktail consisting of nine phages lysed 24% of these *E. coli* strains while the Microgen phage cocktail showed 50% coverage (Table 3). Two different batches of Microgen ColiProteus phage cocktails showed a nearly identical lysis pattern on these strains (data not shown) while two different batches from Microgen ‘Sextaphage’ cocktail showed an overlapping, but distinct lysis pattern with an overall lower coverage of 35% (Table 3). Since we were not satisfied with the 24% coverage by our nine-phage cocktail, we modified its composition to obtain a broader coverage. Modification was done on the basis of lytic host range determined for each individual phage strain against the *E. coli* pathogens isolated in 2008 from patients at icddr,b. A new cocktail consisting of 10 T4-like phages led to an increased coverage of 50% on these hospital-derived *E. coli* pathogens. With the 14-phage T4 cocktail, 61% coverage was achieved (Table 3).

Notably, the 10-phage cocktail lysed only 46% of the Mexican strains, less than the 68% lysed by the 9-phage cocktail (Table 1). Changing the phage composition of a cocktail in order to achieve a higher coverage in one geographical region might thus result in lesser coverage in another region. *E. coli* pathogen coverage by a phage cocktail is thus strongly context-dependent, suggesting that phages for therapy should be tested, if not isolated, using the most prevalent pathogenic strains in a given geographical area.

In 2011, we requested again random ETEC and EPEC isolates from children hospitalized with diarrhoea at icddr,b to explore temporal variation of pathogen coverage by given phage cocktails. The 10-phage T4 cocktail showed 29% coverage in the tube lysis test. The T4-like phage cocktail showed phage plaque counts in excess

© 2014 The Authors. *Microbial Biotechnology* published by John Wiley & Sons Ltd and Society for Applied Microbiology, *Microbial Biotechnology*, 7, 165–176
of 10^4 pfu/ml in all cases where lysis was observed (Table 4). The Microgen Coliproteus phage cocktail lysed only 6% of these test strains, but formed plaques on half of them, but plaque counts were low when the cells were not lysed. Apparently, plaque counts higher than 2 × 10^3 pfu/ml are needed to cause lysis of the cells (Table 4). Lysis was occasionally observed when no plaques were seen in the plaque assay. Whether this represents ‘lysis from without’ by phage tail-associated lytic enzymes from phages that cannot complete an infection cycle or ‘abortive infection’ is not clear, but according to the low moi which we used and a theoretical paper (Abedon, 2011), the second diagnosis is more appropriate. Independent of this question of clear terminology,

Table 2. Lysis of pathogenic E. coli strains isolated from children who experienced a bout of diarrhoea in the community, which did not lead to hospitalization (prospective study from Dhaka/Bangladesh).

| No | Toxins | CF | O type | N 9-p | N 14-p | Microgen |
|----|--------|----|--------|-------|-------|----------|
| 1  | LT+ST+ | CS2+CS3 | O6 | L | | |
| 2  | LT+ST+ | CS1+CS3,CS21 | O6 | L | L | |
| 3  | LT+ST+ | CS2+CS3 | O6 | L | | |
| 4  | LT+ST+ | CS5+CS6 | O6 | L | | |
| 5  | LT+ST+ | CS1+CS3,CS21 | O6 | L | L | |
| 6  | LT+ST+ | CS14 | O114 | L | L | |
| 7  | LT+ | CS17 | | L | | |
| 8  | LT+ST+ | CS2+CS3 | O6 | L | | |
| 9  | ST+ | CFA/ \( \alpha \) | O126 | | | |
| 10 | ST+ | CS5+CS6 | O115 | L | | |
| 11 | ST+ | CFA/ \( \alpha \) | O126 | L | L | |
| 12 | LT+ST+ | CS4+CS6 | O25 | | | |
| 13 | LT+ | CS7 | O114 | L | L | |
| 14 | ST+ | CS14 | | L | L | |
| 15 | ST+ | CFA/CS21 | | L | | |
| 16 | LT+ST+ | CS4+CS6 | | L | | |
| 17 | LT+ | CS7 | | | | |
| 18 | LT+ST+ | CS5+CS6 | | L | | |
| 19 | LT+ST+ | CFA/CS21 | O25 | | | |
| 20 | LT+ST+ | CS1+CS3,CS21 | O6 | L | L | |
| 21 | LT+ST+ | CS2+CS3 | O6 | L | | |
| 22 | LT+ST+ | NEG | | L | | |
| 23 | LT+ST+ | NEG | | | | |
| 24 | ST+ | NEG | | L | | |
| 25 | ST+ | NEG | | L | L | |
| 26 | ST+ | NEG | | L | | |
| 27 | LT+ | NEG | | L | | |
| 28 | LT+ | NEG | | L | L | |
| 29 | LT+ST+ | NEG | | | | |
| 30 | LT+ | NEG | | L | | |
| 31 | ST+ | CFA/ \( \alpha \) | O126 | L | L | |
| 32 | LT+ST+ | CFA/ \( \alpha \) | | L | | |
| 33 | LT+ST+ | CS1+CS3,CS21 | | L | | |
| 34 | ST+ | CS6 | | | | |
| 35 | LT+ | NEG | | L | L | |
| 36 | EPEC | | | | | |
| 37 | EPEC | | | | | |
| 38 | EPEC | | | | | |
| 39 | EPEC | | | | | |
| 40 | EPEC | | | | | |

Note: Of the 40 pathogenic E. coli isolates, 35 were identified as ETEC strains by the presence of the heat-stable (ST) or heat-labile (LT) enterotoxin. The strains were further characterized by the presence of colonization factor (CF) antigens and by O serotype. Five strains were EPEC isolates. The lysis (L) as observed in the 10 ml test tube lysis assay on the indicated host strain is indicated for a 9-phage cocktail (Table 1) and 14-phage T4-like phage cocktail (NPC 1000, NPC 1001, NPC 1002, NPC 1003, NPC 1004, NPC 1005, NPC 1009, NPC 1009, NPC 1032, NPC 1040, NPC 1041, NPC 1042, NPC 1043, NPC 1044, NPC 2017) and for the commercial Microgen phage cocktail. Underlined values from the 9-phage cocktail were confirmed by plaque assay, NEG, negative for tested CF.

Table 3. Lysis of E. coli strains representing different pathotypes isolated in 2008 from patients hospitalized with diarrhoea at icddr,b hospital in Dhaka, Bangladesh with the indicated phage cocktails.

| No | Pathotype | AGE | N 9-p | N 10-p | N 14-p | Micro | Sexta |
|----|-----------|-----|-------|--------|--------|-------|-------|
| 1  | EAggEC   | P   | | | | | |
| 2  | EAggEC   | P   | | | | | |
| 3  | EAggEC   | P   | | | | | |
| 4  | EAggEC   | P   | L | L | L | L | |
| 5  | EAggEC   | P   | | | | | |
| 6  | EAggEC   | P   | | | | | |
| 7  | EAggEC   | P   | L | L | L | | |
| 8  | EAggEC   | P   | | | | | |
| 9  | EAggEC   | P   | L | L | L | | |
| 10 | EAggEC   | P   | | | | | |
| 11 | EIEC     | L   | L | L | L | | |
| 12 | EIEC     | L   | L | L | L | | |
| 13 | EIEC     | L   | L | L | L | | |
| 14 | EPEC     | L   | | | | | |
| 15 | EPEC     | L   | | | | | |
| 16 | EPEC     | L   | | | | | |
| 17 | EPEC     | L   | | | | | |
| 18 | EPEC     | L   | | | | | |
| 19 | EPEC     | P   | | | | | |
| 20 | EPEC     | P   | L | L | L | | |
| 21 | EPEC     | P   | | | | | |
| 22 | EPEC     | P   | | | | | |
| 23 | EPEC     | P   | | | | | |
| 24 | EPEC     | L   | | | | | |
| 25 | EPEC     | L   | | | | | |
| 26 | EPEC     | L   | | | | | |
| 27 | EPEC     | L   | | | | | |
| 28 | EPEC     | L   | | | | | |
| 29 | VTEC     | L   | L | L | L | | |
| 30 | VTEC     | L   | L | L | L | | |
| 31 | VTEC     | L   | L | L | L | | |
| 32 | VTEC     | L   | L | L | L | | |
| 33 | ETEC O114 | P   | | | | | |
| 34 | ETEC O167 | P   | | | | | |
| 35 | ETEC O6   | P   | L | | | | |
| 36 | ETEC O25  | P   | L | | | | |
| 37 | ETEC O115 | P   | L | | | | |
| 38 | ETEC O25  | P   | L | | | | |
| 39 | ETEC O114 | P   | L | | | | |
| 40 | ETEC O115 | P   | L | | | | |
| 41 | ETEC O6   | P   | L | | | | |
| 42 | ETEC O167 | P   | | | | | |
| 43 | ETEC O6   | P   | | | | | |
| 44 | ETEC O167 | P   | L | | | | |
| 45 | ETEC O114 | P   | | | | | |
| 46 | ETEC O8   | P   | | | | | |

Notes: Pathotypes: Enteropathogenic E. coli (EAEC), Enteroinvasive E. coli (EIIEC), Enteropathogenic E. coli (EPEC), Verotoxin producing E. coli (VT), Enterotoxigenic E. coli (ETEC) – the latter were serotyped for O antigen. Age: P indicates pediatric patient. Phage cocktails: N9-p cocktail consisting of 9 T4-like phages, N10-p cocktail consisting of 10 T4-like phages (for composition see Table 1); N14-p cocktail consisting of 14 T4-like phages (for composition see Table 2); Micro: Collegrots phage cocktail; Sexta: Sextaphage cocktail from Microgen. L: lysis in 10 ml test tube assay.
bacterial cells do not experience the same environment when they are grown in solid medium as compared to liquid that leads to a distinct expression of some surface proteins which may affect the infection process.

Since *Shigella* is taxonomically not even a subspecies of *E. coli*, we extended the testing to *Shigella* isolates from 10 different pediatric patients. The isolates represented two different *Shigella* species; one was represented with four different serotypes. All strains showed the *ipaH* virulence genes, while none contained the Shiga toxin gene. The Microgen ColiProteus phage cocktail formed plaques on 9 *Shigella* isolates while 10 T4-like phage cocktail showed lysis and plaques on 6 *Shigella* isolates (Table 5).

**Discussion**

T4-like phages from our collection were with few exceptions all isolated on a non-pathogenic laboratory *E. coli* strain, more specifically K803, a derivative of the K-12 strain lacking the lambda prophage (Chibani-Chennoufi et al., 2004b). This restriction could be a significant selection bias which might explain the relatively poor coverage observed in the current study. One might object that phages should be isolated using prevalent pathogenic *E. coli* as indicator strains even if this renders the whole experimental work more difficult to handle (need of a safety laboratory, need of many propagating strains). If these phages are subsequently propagated on a non-pathogenic laboratory strain for commercial purposes, then their ability to lyse the most prevalent pathogenic strains should be confirmed. In fact, in the initial phase of our work, we isolated phages from stool samples of diarrhoea patients using in parallel an enteropathogenic *E. coli* strain, which was commonly isolated from childhood diarrhoea cases, and the laboratory K803 *E. coli* strain (Chibani-Chennoufi et al., 2004b). We obtained less phage isolates on the pathogenic strain than on the laboratory *E. coli* strain. Furthermore, the phage isolates made on the pathogenic *E. coli* strain were *Siphoviridae* with 50 kb genomes showing the morphology of *Jersey*-and beta-4 like phages and a narrower lytic pattern on O-serotyped pathogenic *E. coli* strains than phage isolates from the laboratory K803 strain, which yielded *Myoviridae* with a 170 kb genome, the morphology of T4-like phages and a broader host range on O-serotyped pathogenic *E. coli* strains (Chibani-Chennoufi et al., 2004b). We also obtained rather narrow host-range phage isolates when using a few other pathogenic *E. coli* as indicator strains (unpublished observations).

It is not unusual to select and propagate phages for phage therapy purpose against pathogenic bacteria on a non-pathogenic host strain. For example, a multivalent *Salmonella* phage was selected and amplified on a non-pathogenic *E. coli* strain. After serial passage on the two alternative hosts, the researchers observed a smaller burst size and longer rise period for the phage propagated on the heterologous *E. coli* host than on the homologous *Salmonella* host, but the lower cost of producing a safer phage product justified in their opinion these growth disadvantages. Notably, no difference in host range was observed between the phage propagated on the pathogenic *Salmonella* or non-pathogenic *E. coli* host (Santos et al., 2010).

Propagation on a laboratory strain avoids biosafety production conditions and the risk of transfer of bacterial or prophage-encoded toxins (Canchaya et al., 2003) from the pathogenic host into the phage preparation. The fact that K-12 displays only a truncated LPS molecule with a shortened O side chain (Qimron et al., 2006) might not only have changed biological phenotypes like pathogenicity for infection models in worms (Browning et al., 2013). Use of K-12 might have selected against phages recognizing the O side chain-specific sugar residues as recep-

| No | Pathotype | N10-p | N10-p | Micro | Micro |
|----|-----------|-------|-------|-------|-------|
| 1  | ETEC      | lysis | < 10  | lysis | < 10  |
| 2  | ETEC      | < 10  | < 10  | < 10  | < 10  |
| 3  | EPEC      | < 10  | lysis | 4 × 10^5 |
| 4  | ETEC      | < 10  | < 10  | < 10  | < 10  |
| 5  | ETEC      | < 10  | < 10  | < 10  | < 10  |
| 6  | EPEC      | < 10  | < 10  | < 10  | < 10  |
| 7  | ETEC      | < 10  | < 10  | < 10  | < 10  |
| 8  | EPEC      | < 10  | (lysis) | 20   |
| 9  | EPEC      | lysis | 1 × 10^5 | 200  |
| 10 | ETEC      | lysis | 1 × 10^6 | 50   |
| 11 | EPEC      | < 10  | 2000  | 2000  |
| 12 | ETEC      | < 10  | < 10  | < 10  | < 10  |
| 13 | ETEC      | lysis | 5 × 10^4 | 10   |
| 14 | ETEC      | < 10  | < 10  | < 10  | < 10  |
| 15 | ETEC      | lysis | 1 × 10^5 | 10   |
| 16 | EPEC      | < 10  | 300   | 300   |
| 17 | ETEC      | lysis | 1 × 10^5 | 2000  |

**Table 4.** Phage susceptibility of ETEC and EPEC strains isolated from pediatric diarrhoea patients hospitalized in 2011 at icddr,b, Dhaka, Bangladesh as assessed by tube lysis and plaque assay.

**Table 5.** Lysis of Shigella strains from patients at icddr,b, Dhaka, Bangladesh.

| Species            | Virulence gene | Serotype | N10-p | Microgen |
|--------------------|----------------|----------|-------|----------|
| *Shigella flexneri* | *ipaH*         | 1b       | < 10  | 1 × 10^4 |
| *S. flexneri*      | *ipaH*         | 2a       | < 10  | 1 × 10^4 |
| *S. flexneri*      | *ipaH*         | 3a       | < 10  | 2 × 10^4 |
| *S. flexneri*      | *ipaH*         | 6        | < 10  | < 10     |
| *S. flexneri*      | *ipaH*         | 6        | 1 × 10^2 | 2 × 10^2 |
| *Shigella sonnei*  | *ipaH*         | 1 × 10^6 | 3 × 10^6 |
| *S. sonnei*        | *ipaH*         | 9 × 10^7 | 2 × 10^6 |
| *S. sonnei*        | *ipaH*         | 5 × 10^7 | 5 × 10^7 |
| *S. sonnei*        | *ipaH*         | 1 × 10^7 | 1 × 10^7 |
| *S. sonnei*        | *ipaH*         | 9 × 10^7 | 3 × 10^7 |
tor potentially explaining why the phages selected on the laboratory *E. coli* strain showed a broader host range on O-serotyped pathogenic *E. coli* than the phages selected on a pathogenic *E. coli* strain displaying a complete O side chain. While the majority of our phage isolates still showed a rather narrow infection range on our pathogenic *E. coli* strain collection, only few of them were actually O serotype-specific. We do not know what receptor structures our phages recognized. Some researchers had demonstrated that the conserved inner parts of the LPS molecule can serve as receptors for T4 and T7 (Tamaki et al., 1971; Qimron et al., 2006), potentially explaining the infection of many different O serotypes with a single phage. Recognition of alternative T4 phage receptors like the membrane protein OmpC proposed by other researchers (Hashemolhosseini et al., 1994; Yu et al., 2000) is not very likely for the isolated phages since one would expect a much broader cross-reactivity on O-serotypes *E. coli* pathogens than was actually observed.

One might also object that determining the host range of the isolated phages on *E. coli* growing in LB broth as done for enterobacteria propagation in the laboratory has nothing in common with the conditions encountered in vivo. For example, in our experiments, the infected *E. coli* cells were shaken to assure oxygen access while the lower parts of the gut display anaerobic conditions. To address this problem, we tested whether the investigated T4-like phages could infect *E. coli* under anaerobic in vitro growth conditions. This was the case: our T4-like phages yielded in vitro similar titers under aerobic and anaerobic growth (Weiss et al., 2009). Of course, there are many other conditions that differentiate in vitro from in vivo growth (Brüssow, 2013). For example, in their natural environment bacteria grow mostly in biofilms while broth culture represents growth in suspension. Indeed, T4 phage showed lytic infections on biofilms (Doolittle et al., 1995), but nutrient limitation in biofilms delayed the lysis process (Doolittle et al., 1996). One might therefore wonder whether in vitro conditions better adapted to in vivo growth conditions would not be preferable to standard broth culture. Since the in vivo growth conditions of *E. coli* are still poorly characterized, it is unfortunately not yet clear what in vitro conditions best mimic the in vivo condition.

It is widely believed that phage therapy approaches need phage cocktails (Brüssow, 2012). If resistance development is an issue during phage therapy (Cairns et al., 2009), it is less likely to develop against a phage cocktail than against a single phage. However, combining phages into a cocktail can create interference problems making the actual lytic capacity of a phage cocktail less than the sum of its parts. This phenomenon was here observed with cocktails of T4-like phages. Mechanistically, super-

infection of *E. coli* cells with two T4-like phages causes lysis inhibition (Abedon, 1992) preventing an OD decrease, which was the read-out of our test. We tried different combinations of individual T4-like phages hoping to identify specific phage strain combinations displaying antagonistic behavior in order to eliminate them from the cocktail. However, no such specific interference problems between individual phage strains were spotted, suggesting merely random effects when increasing the overall number of T4-like phages in a cocktail. Retrospectively, one might also ask whether limiting the phage cocktail to a single phage genus like T4-like phages for safety reasons was a good strategy. Safety trials showed no difference when using a T4-like phage cocktail (Sarker et al., 2012) or the Russian phage cocktail containing in addition to T4- and T7-like phages a dozen further phage genera (McCallin et al., 2013). As the in vivo phage growth conditions in the human gut are still largely undefined, working with a complex phage cocktail as done by the Eastern European phage tradition might represent a hedge betting strategy for diversifying risk. However, when considering only the coverage of the target pathogen with in vitro lysis tests, no clear superiority of the Microgen phage cocktails over the T4-like phage cocktail was observed.

If we accept the hypothesis that in vitro lysis is a necessary, although not sufficient condition for in vivo lysis, coverage is critical for efficient phage therapy. To illustrate it numerically: with 50% coverage, two *E. coli* patients need to be treated to expect an effect in one of them. If only one out of two to three hospitalized pediatric diarrhoea patients suffers from an *E. coli* diarrhoea as is the case in Bangladesh (Albert et al., 1999), four to six patients need to be treated for one to profit from phage treatment. This calculation neglects the possibility that many patients are infected with similar strains which is actually the case during a given epidemic (Qadri et al., 2005), while this might not be the same strains between epidemics (Chowdhury et al., 2011). This consideration put aside, relatively large numbers of children with diarrhoea need to be enrolled into a controlled clinical trial to observe a treatment effect raising even an ethical conundrum using a therapeutical preparation which only cures a limited proportion of the patients. This problem could be solved when targeting *Shigella* infections. Dysentery symptoms are so characteristic that it can be easily diagnosed clinically and its coverage by phage was much higher than for *E. coli* diarrhoea. The combination of both factors makes it likely that at least every second dysentery patient could profit from phage therapy. In line with other reports (Chang and Kim, 2011), *Shigella* was lysed by coliphages, but this should not be surprising since Shigella qualifies not even as a subspecies of *E. coli* (Sims and Kim, 2011). In support to this conclusion identifying *Shigella* infections as a suitable target for phage therapy,
the only clinical trial published by Soviet phage therapy researcher reported successful prevention of *Shigella* dysentery (Sulakvelidze et al., 2001). Diarrhoea-associated *E. coli* pathogens in contrast are a difficult target for phage therapy since the pathogen coverage by phages varies with geographical region, epidemiological setting and time as demonstrated in the present study. It is not clear whether our observations can be generalized. Due to frequent inundation by flood water, Bangladesh displays an exceptionally high dynamic of diarrhoea pathogens (Harris et al., 2008) making it not a typical, but a worst-case scenario.

Evolutionary biologists have asked whether in vitro phage-host systems are adequate models for the in situ situation in the real ecological niche (Forde et al., 2008). For clinical efficacy, phages must display the appropriate *in vivo* stability to survive the gastro-intestinal passage, a good in situ bioavailability at their site of action and a good replication potential on *E. coli* demonstrating different physiological states in different gut segments. None of these phenotypes can be easily tested in vitro. Some pathogenic *E. coli* strains multiply in the small intestine (e.g. EPEC, Shigella). Data on intestinal phage–host interaction in laboratory mice were equivocal. In our laboratory, a T4-like phage was less efficient than a T7-like phage in replicating on slowly growing commensal *E. coli* in the gut (Weiss et al., 2009). In another laboratory, a T4-like phage was a better replicator than a T7-like phage on a pathogenic *E. coli* strain asymmetrically colonizing the gut of mice (Maura et al., 2012a,b). In both models, oral T7-like phages replicated to high titers in the gut without, however, having a sustained effect on the fecal *E. coli* titer (Weiss et al., 2009; Maura et al., 2012a). When investigating phage–host interaction in different segments of the gut, marked differences were observed (Weiss et al., 2009; Maura et al., 2012b). Our understanding of coliphage *E. coli* interaction in the gut, their natural ecological niche, is apparently so limited (Brüssow, 2013) that testing a phage cocktail for *in vitro* coverage can only be seen as a step towards a successful phage therapy approach. In the absence of an *E. coli* diarrhoea model in small laboratory animals, we study currently the faecal excretion pattern in children hospitalized with *E. coli* diarrhoea who were treated with two different phage cocktails to get data on the *in vivo* replication of oral phage cocktails.

Acknowledgements

We thank our NRC colleagues Enea Rezzonico and Olga Sakwinska in Switzerland for critical reading of the manuscript, Delia Licona, Gabriel Pérez and Luis Antonio León for technical support in the Mexico laboratory by performing *E. coli* serotyping, Azmira Begum, Senior Health assistant for the collection of stool samples and strains from the Bangladesh laboratory and sending it to NRC.

Conflict of interest

None declared.

References

Abedon, S.T. (1992) Lysis of lysis-inhibited bacteriophage T4-infected cells. *J Bacteriol* 174: 8073–8080.

Abedon, S.T. (2011) Lysis from without. *Bacteriophage* 1: 46–49.

Albert, M.J., Faruque, S.M., Faruque, A.S.G., Neogi, P.K.B., Ansaruzzaman, M., Bhuiyan, N.A., *et al.* (1995) Controlled study of *Escherichia coli* diarrheal infections in Bangladeshi children. *J Clin Microbiol* 33: 973–977.

Albert, M.J., Faruque, A.S., Faruque, S.M., Sack, R.B., and Mahalanabis, D. (1999) Case-control study of enteropathogens associated with childhood diarrhea in Dhaka, Bangladesh. *J Clin Microbiol* 37: 3458–3464.

Browning, D.F., Wells, T.J., França, F.L., Morris, F.C., Sevastyanovich, Y.R., Bryant, J.A., *et al.* (2013) Laboratory adapted *Escherichia coli K-12* becomes a pathogen of *Caenorhabditis elegans* upon restoration of O antigen biosynthesis. *Mol Microbiol* 87: 939–950.

Bruttin, A., and Brüssow, H. (2005) Human volunteers receiving *Escherichia coli* phage T4 orally: a safety test of phage therapy. *Antimicrob Agents Chemother* 49: 2874–2878.

Brüssow, H. (2012) What is needed for phage therapy to become a reality in Western medicine? *Virology* 434: 138–142.

Brüssow, H. (2013) Bacteriophage-host interaction: from splendid isolation into a messy reality. *Curr Opin Microbiol* 16: 500–506.

Cairns, B.J., Timms, A.R., Jansen, V.A., Connerton, I.F., and Payne, R.J. (2009) Quantitative models of *in vitro* bacteriophage-host dynamics and their application to phage therapy. *PLoS Pathog* 5: e1000253.

Canchaya, C., Proux, C., Fournous, G., Bruttin, A., and Brüssow, H. (2003) Prophage genomics. *Microbiol Mol Biol Rev* 67: 238–276.

Chang, H.W., and Kim, K.H. (2011) Comparative genomic analysis of bacteriophage EP23 infecting *Shigella sonnei* and *Escherichia coli*. *J Microbiol* 49: 927–934.

Chanishvili, N. (2012) Phage therapy-history from Twort and *d’Herelle* through Soviet experience to current approaches. *Adv Virus Res* 83: 3–40.

Chibani-Chennoufi, S., Sidoti, J., Bruttin, A., Kutter, E., Sarker, S., and Brüssow, H. (2004a) In vitro and *in vivo* bacteriolytic activities of *Escherichia coli* phages: implications for phage therapy. *Antimicrob Agents Chemother* 48: 2558–2569.

Chibani-Chennoufi, S., Sidoti, J., Bruttin, A., Dillmann, M.L., Kutter, E., Qadri, F., *et al.* (2004b) Isolation of *Escherichia coli* bacteriophages from the stool of pediatric diarrhea patients in Bangladesh. *J Bacteriol* 186: 8287–8294.
Chowdhury, F., Rahman, M.A., Begum, Y.A., Khan, A.I., Faruque, A.S., Saha, N.C., et al. (2011) Impact of rapid urbanization on the rates of infection by *Vibrio cholerae* O1 and enterotoxigenic *Escherichia coli* in Dhaka, Bangladesh. *PLoS Negl Trop Dis* 5: e999.

Cravioto, A., Reyes, R., Trujillo, F., Uribe, F., Navarro, A., De la Roca, J., et al. (1990) Risk of diarrhea during the first year of life associated with initial and subsequent colonization by specific enteropathogens. *Am J Epidemiol* 131: 886–904.

Doolittle, M.M., Cooney, J.J., and Caldwell, D.E. (1995) Lytic infection of *Escherichia coli* biofilms by bacteriophage T4. *Can J Microbiol* 41: 12–18.

Doolittle, M.M., Cooney, J.J., and Caldwell, D.E. (1996) Tracing the interaction of bacteriophage with bacterial biofilms using fluorescent and chromogenic probes. *J Ind Microbiol* 16: 331–341.

Flores, C.O., Meyer, J.R., Valverde, S., Farr, L., and Weitz, J.S. (2011) Statistical structure of host-pherage interactions. *Proc Natl Acad Sci U S A* 108: E288–E297.

Forde, S.E., Beardmore, R.E., Gudelj, I., Arkin, S.S., Thompson, J.N., and Hurst, L.D. (2008) Understanding the limits to generalizability of experimental evolutionary models. *Nature* 455: 220–223.

Guznburg, S.T., Tornieporth, N.G., and Riley, L.W. (1995) Identification of enteropathogenic *Escherichia coli* by PCR based detection of the bundle-forming pilus gene. *J Clin Microbiol* 33: 1375.

Harris, A.M., Chowdhury, F., Begum, Y.A., Khan, A.I., Faruque, A.S., Svennerholm, A.M., et al. (2008) Shifting prevalence of major diarrheal pathogens in patients seeking hospital care during floods in 1998, 2004, and 2007 in Dhaka, Bangladesh. *Am J Trop Med Hyg* 79: 708–714.

Hashemolhosseini, S., Montag, D., Krämer, L., and Henning, U. (1994) Determinants of receptor specificity of coliphages of the T4 family. A chaperone alters the host range. *J Mol Biol* 241: 524–533.

Heinrichs, D.E., Yethon, J.A., and Whittfield, C. (1998) Molecular basis for structural diversity in the core regions of the lipopolysaccharides of *Escherichia coli* and *Salmonella enterica*. *Mol Microbiol* 30: 221–232.

Karch, H., Böhm, H., Schmidt, H., Gunzer, F., Aleksic, S., and Heesemann, J. (1993) Clonal structure and pathogenicity of *Shiga*-like toxin-producing, sorbitol-fermenting *Escherichia coli* O157:H. *J Clin Microbiol* 31: 1200–1205.

Kelly, D., McAuliffe, O., Ross, R.P., O’Mahony, J., and Coffey, A. (2011) Development of a broad-host-range phage cocktail for biocontrol. *Bioeng Bugs* 2: 31–37.

McCallin, S.E., Sarker, S.A., Barretto, C., Sultana, S., Berger, B., Huq, S., et al. (2013) Safety analysis of a Russian phage cocktail: from metagenomic analysis to oral application in healthy human subjects. *Virology* 443: 187–196.

McConnell, M.M., Chart, H., Field, H., Hibberd, M., and Rowe, B. (1989) Characterization of a putative colonization factor (PCFO166) of enterotoxigenic *Escherichia coli* of serogroup O116. *J Gen Microbiol* 135: 1135–1144.

Maura, D., Morello, E., du Merle, L., Bomme, P., Le Bouguënc, C., and Debarbieux, L. (2012a) Intestinal colonization by enterogaugregative *Escherichia coli* supports long-term bacteriophage replication in mice. *Environ Microbiol* 14: 1844–1854.

Maura, D., Galtier, M., Le Bouguënc, C., and Debarbieux, L. (2012b) Virulent bacteriophages can target O104:H4 enterogaugregative *Escherichia coli* in the mouse intestine. *Antimicrob Agents Chemother* 56: 6235–6242.

Merrill, C.R., Biswas, B., Carlton, R., Jensen, N.C., Creed, G.J., Zullo, S., and Adhya, S. (1996) Long-circulating bacteriophage as antibacterial agents. *Proc Natl Acad Sci U S A* 93: 3188–3192.

Orskov, F., and Orskov, I. (1984) Serotyping of *Escherichia coli*. *Meth Microbiol* 14: 43–112.

Payne, R.J., Phil, D., and Jansen, V.A. (2000) Phage therapy: the peculiar kinetics of self-replicating pharmaceuticals. *Clin Pharmacol Ther* 68: 225–230.

Pollard, D.R., Johnson, W.M., Lior, H., Tyler, S.D., and Rozee, K.R. (1990) Rapid and specific detection of verotoxin genes in *Escherichia coli* by the polymerase chain reaction. *J Clin Microbiol* 28: 540–545.

Qadri, F., Das, S.K., Faruque, A.S.G., Fuchs, G.J., Albert, M.J., Sack, R.B., and Svennerholm, A.-M. (2000) Prevalence of toxin types and colonization factors in enterotoxigenic *Escherichia coli* isolated during a 2-year period from diarrheal patients in Bangladesh. *J Clin Microbiol* 38: 27–31.

Qadri, F., Khan, A.I., Faruque, A.S., Begum, Y.A., Chowdhury, F., Nair, G.B., et al. (2005) Enterotoxigenic *Escherichia coli* and *Vibrio cholerae* diarrhea, Bangladesh, 2004. *Emerg Infect Dis* 11: 1104–1107.

Qadri, F., Saha, A., Ahmed, T., Al Tarique, A., Begum, Y.A., and Svennerholm, A.-M. (2007) Disease burden due to enterotoxigenic *Escherichia coli* in the first 2 years of life in an urban community in Bangladesh. *Infect Immun* 75: 3961–3968.

Qimron, U., Marintcheva, B., Tabor, S., and Richardson, C.C. (2006) Genomewide screens for *Escherichia coli* genes affecting growth of T7 bacteriophage. *Proc Natl Acad Sci U S A* 103: 19039–19044.

Robins-Browne, R.M. (1987) Traditional enteropathogenic *Escherichia coli* of infantile diarreal. *Rev Infect Dis* 9: 28–53.

Santos, S.B., Fernandes, E., Carvalho, C.M., Sillankorva, S., Krylov, V.N., Pleteneva, E.A., et al. (2010) Selection and characterization of a multivalent Salmonella phage and its production in a nonpathogenic *Escherichia coli* strain. *Appl Environ Microbiol* 76: 7338–7342.

Sarker, S.A., McCallin, S., Barretto, C., Berger, B., Pittet, A.C., Sultana, S., et al. (2012) Oral T4-like phage cocktail application to healthy adult volunteers from Bangladesh. *Virology* 434: 222–232.

Schultsz, C., Pool, G.J., van Ketel, R., de Wever, B., Speelman, P., and Dankert, J. (1994) Detection of enterotoxigenic *Escherichia coli* in stool samples by using nonradioactively labeled oligonucleotide DNA probes and PCR. *J Clin Microbiol* 32: 2393–2397.

Schwarzer, D., Buettner, F.F., Browning, C., Nazarov, S., Rabsch, W., Bethe, A., et al. (2012) A multivalent adsorption apparatus explains the broad host range of phage phi92: a comprehensive genomic and structural analysis. *J Virol* 86: 10384–10398.

Sims, G.E., and Kim, S.H. (2011) Whole-genome phylogeny of *Escherichia coli/Shigella* group by feature frequency profiles (FFPs). *Proc Natl Acad Sci U S A* 108: 8329–8334.

© 2014 The Authors. *Microbial Biotechnology* published by John Wiley & Sons Ltd and Society for Applied Microbiology, *Microbial Biotechnology, 7*, 165–176
Sulakvelidze, A., Alavidze, Z., and Morris, J.G., Jr (2001) Bacteriophage therapy. *Antimicrob Agents Chemother* **45**: 649–659.

Svennerholm, A.-M., and Wiklund, G. (1983) Rapid GM1-enzyme-linked immunosorbent assay with visual reading for identification of *Escherichia coli* heat-labile enterotoxin. *J Clin Microbiol* **17**: 596–600.

Svennerholm, A.M., Wikström, M., Lindblad, M., and Holmgren, J. (1986) Monoclonal antibodies against *Escherichia coli* heat-stable toxin (STa) and their use in a diagnostic ST ganglioside GM1-enzyme-linked immunosorbent assay. *J Clin Microbiol* **24**: 585–590.

Tamaki, S., Sato, T., and Matsuhashi, M. (1971) Role of lipopolysaccharides in antibiotic resistance and bacteriophage adsorption of *Escherichia coli* K-12. *J Bacteriol* **105**: 968–975.

Thomassen, E., Gielen, G., Schütz, M., Schoehn, G., Abrahams, J.P., Miller, S., and van Raaij, M.J. (2003) The structure of the receptor-binding domain of the bacteriophage T4 short tail fibre reveals a knitted trimeric metal-binding fold. *J Mol Biol* **331**: 361–373.

Weiss, M., Denou, E., Bruttin, A., Serra-Moreno, R., Dillmann, M.L., and Brüssow, H. (2009) In vivo replication of T4 and T7 bacteriophages in germ-free mice colonized with *Escherichia coli*. *Virology* **393**: 16–23.

Weitz, J.S., Poisot, T., Meyer, J.R., Flores, C.O., Valverde, S., Sullivan, M.B., and Hochberg, M.E. (2013) Phage-bacteria infection networks. *Trends Microbiol* **21**: 82–91.

Yu, S.L., Ko, K.L., Chen, C.S., Chang, Y.C., and Syu, W.J. (2000) Characterization of the distal tail fiber locus and determination of the receptor for phage AR1, which specifically infects *Escherichia coli* O157:H7. *J Bacteriol* **182**: 5962–5968.