Disrupting the mixed-species biofilm of *Klebsiella pneumoniae* B5055 and *Pseudomonas aeruginosa* PAO using bacteriophages alone or in combination with xylitol

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We investigated the potential of bacteriophages alone as well as in combination with xylitol for tackling mixed-species biofilm of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. When mixed-species biofilm was established on polycarbonate discs, *P. aeruginosa* formed the base layer which was physically shielded on the top by *K. pneumoniae*. Thereafter, mixed-species biofilm was treated with bacteriophages. *K. pneumoniae*-specific depolymerase-producing phage KPO1K2 caused significant reduction in the count of *Klebsiella*. In contrast, *P. aeruginosa*-specific non-depolymerase-producing phage Pa29 failed to cause any reduction in the count of *Pseudomonas*. However, application of both phages together resulted in significant reduction in the count of both organisms. This suggests that depolymerase produced by phage KPO1K2 hydrolysed the top layer of *K. pneumoniae* and guided the entry of Pa29 to reach *P. aeruginosa* lying underneath. This phenomenon was confirmed when *K. pneumoniae*-specific non-depolymerase-producing phage NDP was used along with Pa29. Pa29 could not penetrate and reach its host bacterium. Xylitol worked synergistically along with the phage, resulting in a significant decrease in counts of both organisms. Disruption of mixed species biofilm by phage and xylitol was confirmed on the basis of the amount of protein and DNA released. This phage-based approach to altering the structural pattern and disrupting the mixed species biofilm is the first of its kind. It can be used as a topical application, a coating for foreign bodies or for aerosol delivery to tackle infections where both pathogens coexist in a biofilm mode.

**INTRODUCTION**

A biofilm is a dense, highly hydrated cell cluster embedded in a self-produced gelatinous matrix composed of polysaccharides, proteins, DNA, surfactants, lipids, glycolipids, membrane vesicles and ions such as Ca$^{2+}$ (Donlan & Costerton, 2002). It provides protection against phagocytosis, UV radiation, shear stress, dehydration, biocides, antibiotics and the host immune response (Hall-Stoodley & Stoodley, 2005). Bacteria in a biofilm are altered in their genotypic/phenotypic characteristics (Chandrasekar & Manavathu, 2008) and therefore can resist 100–1000 times the antibiotic concentrations resisted by their planktonic counterparts (Ceri et al., 1999). Most natural biofilms consist of multiple microbial species (Luca et al., 2014). Interactions such as competition, commensalism, mutualism and parasitism are common between community members within a mixed-species consortium, which leads to improved survival of microorganisms (Christensen et al., 2002). Palmer et al. (2001) and Filoche et al. (2004) have also reported that bacteria that are unable to form biofilm on their own can synergistically form biofilm with other bacterial species. Polymicrobial communities commonly exist during infections of the oral cavity, middle ear, diabetic foot wounds and cystic fibrosis lungs (Peters et al., 2012).

*Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are two biofilm-forming organisms that can coexist during infections of the urinary tract, respiratory tract and burn wounds and associated with foreign bodies (Childers et al., 2013). Stewart et al. (1997) reported a spatial distribution pattern in dual-species biofilm of *P. aeruginosa* and *K. pneumoniae*. *P. aeruginosa*, owing to its excellent colonizing ability, constructs the base structure while *K. pneumoniae* forms a tower-like structure on the top and persists by virtue of its higher growth rate. A similar spatial distribution has been observed in mixed biofilm of *P. aeruginosa* and *Agrobacterium tumefaciens*, where the former species blankets the latter (An et al., 2006). The recent emergence of antimicrobial resistance
especially among nosocomial pathogens has renewed interest in the use of phages as antibacterial agents. Phages have been used for tackling mixed-species biofilm of Escherichia coli and P. aeruginosa (Kay et al., 2011) and Pseudomonas fluorescens and Staphylococcus lentus (Sillankorva et al., 2010). However, to our knowledge, there has been no report on tackling of P. aeruginosa and K. pneumoniae in a dual-species biofilm. A depolymerase enzyme associated with the tail of certain phages degrades the biofilm exopolysaccharide and enables the phage to reach and lyse its host bacterium (Hughes et al., 1998). Given the distinct pattern adopted by K. pneumoniae and P. aeruginosa in a dual-species biofilm, a depolymerase-producing K. pneumoniae-specific phage KPO1K2 was used in the present study. Its role in disrupting the top K. pneumoniae layer and mediating entry of a non-depolymerase-producing P. aeruginosa-specific phage Pa29 to reach its host bacterium, i.e. P. aeruginosa, lying beneath was evaluated. In addition, sugar alcohols such as xylitol, sorbitol and erythritol exhibit antibiofilm activity. They can either diffuse into the biofilm and accumulate as a toxic, non-metabolizable sugar alcohol phosphate, thus inhibiting bacterial growth, or they can inhibit stress proteins which arise in the biofilm (Ichikawa et al., 2008). Xylitol is a five-carbon sugar alcohol that can be found in birch, plums, strawberries, raspberries and rowanberries (Mäkinen & Söderling, 1980). It is known to decrease the pathogenicity of Streptococcus mutans, Streptococcus pneumoniae and Haemophilus influenzae (Uhari et al., 2000). Therefore, xylitol was used along with K. pneumoniae- and/or P. aeruginosa-specific phages and its synergistic effect in disrupting the dual-species biofilm was evaluated.

**METHODS**

**Bacterial strains.** Standard strains of K. pneumoniae B5055 (O1:K2, MTCC 5832) obtained from Dr Mathia Trautmann, University of Ulm, Germany, and P. aeruginosa PAO (MTCC 3541) obtained from Dr Barbara H. Iglewski, University of Rochester, New York, USA, stored in our laboratory in 60 % glycerol at 80 °C were used in the present study. Trypticase soy broth (TSB) was routinely used for the establishment of biofilms, overnight cultures of K. pneumoniae and P. aeruginosa, centrifuged and washed twice with PBS (0.1 M, pH 7.2). Stock solution of xylitol (Sigma) was stored in our laboratory in 60 % glycerol at 4 °C. It has a genome of 42 kb, a 10 nm short non-contractile tail and a T7-like structural protein pattern, suggesting its inclusion in the family Podoviridae with a designation of T7-like lytic bacteriophage. Depolymerase produced by the phage gave a turbid halo around a clear plaque on the bacterial lawn as a result of the degradation of capsular polysaccharide of K. Pneumoniae (Verma et al., 2009a).

(i) KPO1K2: K. pneumoniae B5055-specific depolymerase-producing phage. It has an icosahedral head with a head diameter of about 39 nm. It has a genome of 42 kb, a 10 nm short non-contractile tail and a T7-like structural protein pattern, suggesting its inclusion in the family Podoviridae with a designation of T7-like lytic bacteriophage. Depolymerase produced by the phage gave a turbid halo around a clear plaque on the bacterial lawn as a result of the degradation of capsular polysaccharide of K. Pneumoniae (Verma et al., 2009a).

(ii) NDP: K. pneumoniae B5055-specific non-depolymerase-producing phage. It produced no halo around plaques. It has an icosahedral head with a head diameter of about 44 nm. It has a genome of 49 kb and a 15.7 nm tail, suggesting its inclusion in the family Podoviridae, order Caudovirales (Kassa & Chhibber, 2012).

(iii) Pa29: P. aeruginosa PAO-specific non-depolymerase-producing phage. It produced no halo around plaques. It has an icosahedral head with a head diameter of about 42.13 nm. It has a genome of 23 kb, a very short 4.76 nm non-contractile tail and a T7-like structural protein pattern, suggesting its inclusion in the family Podoviridae, order Caudovirales (Kumari et al., 2009).

The titre of bacteriophage preparations was estimated by the soft agar overlay method (Adams, 1959, pp. 450–456) and expressed as p.f.u. ml⁻¹. Dilutions of each bacteriophage were made in sterile PBS (0.1 M, pH 7.2).

A schematic representation of the strategy adopted for the present study is depicted in Fig. 1.

**Establishment of mixed-species biofilm on polycarbonate discs.** Colony biofilm of K. pneumoniae and P. aeruginosa was grown on black polycarbonate membranes (Millipore; diameter: 47 mm, pore size: 0.4 μm) following the method described by Anderl et al. (2000) with some modifications. Briefly, the membranes were sterilized by exposure to UV light (15 min per side) prior to inoculation and placed gently on MacConkey agar plates using sterile forceps. They were then inoculated with a 5 μl drop each of K. pneumoniae (10⁶ c.f.u. ml⁻¹, OD₆0₀ of 0.3) and P. aeruginosa (10⁵ c.f.u. ml⁻¹, OD₆0₀ of 0.1) in the centre. The drop was allowed to dry and plates were incubated at 37 °C. Membrane-containing biofilm was transferred to fresh MacConkey agar plates daily for 7 days and photographed (refer to data in the online Supplementary Material for details on optimization of K. pneumoniae and P. aeruginosa inoculum for establishing mixed biofilm).

**Establishment of mixed-species biofilm on microtitre plates.** Mixed-species biofilm was established in 96-well microtitre plates following the method of Bedi et al. (2009) with some modifications. Briefly, 100 μl TSB and 50 μl each of K. pneumoniae (10⁶ c.f.u. ml⁻¹) and P. aeruginosa (10⁵ c.f.u. ml⁻¹) were added to wells of microtitre plates and the plates incubated overnight at 37 °C. After every 24 h, spent medium containing unadhered bacteria was removed and a set of five wells was washed three times with 0.85 % NaCl. Adherent biofilm was scraped from these wells, suspended in 200 μl of 0.85 % NaCl and vortexed for 3 min using a Remi Cyclomixer (Remi Instruments & Appliances). Bacterial counts of K. pneumoniae and P. aeruginosa were determined by viable cell counting on MacConkey agar. In the remaining wells, spent medium was replaced with fresh, sterile 200 μl TSB and each microtitre plate was re-incubated at 37 °C overnight. The above process was repeated each day for 7 days. Control wells containing sterile TSB were also included as a plate sterility control (refer to the supplementary data for details on use of MacConkey agar for estimating the count of K. pneumoniae and P. aeruginosa).

**Effect of phage (KPO1K2/Pa29/NDP) on mixed-species biofilm.** Mixed-species biofilm of K. pneumoniae and P. aeruginosa was established on microtitre plates as described above. On each day, after removal of unadhered bacteria, phage (KPO1K2/Pa29/NDP; n = 5 each) was added either alone or in different combinations (KPO1K2 + Pa29 or NDP + Pa29; n = 5 each) to mixed-species biofilm at an m.o.i. of 1 (m.o.i.: ratio of infectious agent, i.e. phage, to infection target, i.e. bacteria). The microtitre plate was incubated for 3 h at 37 °C. Following treatment, the wells were washed thoroughly with 0.85 % NaCl, scraped and viable cell counts were determined as described above. In the
remaining wells, spent medium was replaced with fresh medium as described above. On each day, a set of five untreated control wells was also processed in a similar way. This procedure was repeated for 7 days (refer to supplementary data for details on optimization of treatment duration and m.o.i. of the bacteriophage).

**Estimation of cell viability.** The uptake of fluorescent dyes, Syto 9 (stains live cells green) and propidium iodide (PI; stains dead cells red) by biofilm cells was determined using a BacLight viability kit (Invitrogen) following the method of Jung et al. (2008). Briefly, mixed-species biofilm was established in 96-well microtitre plates as described earlier. A set of 15 wells with biofilm was treated with three phage combinations (Pa29, KPO1K2 + Pa29 or NDP + Pa29; n = 5 each) for 3 h. After thorough washing with 0.85% NaCl, biofilm was scraped from wells and placed in sterile Eppendorf tubes. Then, 1.5 μl ml⁻¹ each of Syto 9 (3.34 mM) and PI (20 mM) (BacLight reagents) were added to each sample and incubated in the dark for 15 min. The percentage of live and dead cells was determined by flow cytometry using a FACSCanto II system (BD Biosciences) and analysed with FACSDiva software.

**Interaction of labelled phage and biofilm.** Bacteriophage KPO1K2 was labelled with Syto 9 (Sigma), NDP with Syto 62 (Sigma) and Pa29 with DAPI (Sigma) using a modification of the method of Furukawa et al. (1983). Briefly, to exponential phase cultures of Klebsiella or Pseudomonas, their respective phage KPO1K2/NDP or Pa29 and fluorescent dye (final concentration: 5 μg ml⁻¹) was added simultaneously. The mixture was incubated at 37 °C for 2 h. Thereafter, bacterial cell debris was removed by centrifugation at 8000 r.p.m. for 10 min. The supernatant was ultracentrifuged at 20 000 g for 60 min and labelled phage was collected from the precipitate. The supernatant was suspended in a minimal volume of sterile PBS (0.1 M; pH 7.2). The viability of labelled bacteriophages was checked by the soft agar overlay method (Adams, 1959, pp. 450–456) before their further use.

To study the interaction of labelled bacteriophage with biofilm, biofilm was established on glass coverslips following the Tipbox batch culture method of Hughes et al. (1998). Coverslips were washed in sterile PBS (1 mM; pH 7.2) and treated with two phage combinations, i.e phage KPO1K2 (Syto 9 labelled) + phage Pa29 (DAPI labelled) or phage NDP (Syto 62 labelled) + phage Pa29 (DAPI labelled) at an m.o.i. of 1.0, for 15 min in the dark. Coverslips were then examined under a fluorescence microscope (Olympus America). All images were obtained at a magnification of 200 ×. Multiple images were collected for each set of experiments.

**Effect of xylitol on mixed-species biofilm.** Mixed biofilm of *K. pneumoniae* and *P. aeruginosa* was established in 96-well microtitre plates as described above. On each day, a set of five wells was selected and xylitol added at a final concentration of 20 mg ml⁻¹. After incubation for 24 h at 37 °C, the wells were washed with 0.85% NaCl, scraped and viable cells enumerated as described above. In the remaining wells, spent medium was replaced with fresh medium as described above. This procedure was repeated until day 7. On each day, a set of five control wells without treatment was also processed in a similar way (refer to supplementary data for details on optimization of xylitol concentration for biofilm treatment).

**Effect of various combinations of phage with xylitol on mixed-species biofilm.** Mixed biofilm of *K. pneumoniae* and *P. aeruginosa* was established in microtitre plates as already described. On each day, a set of five wells each was treated with various combinations (xylitol alone, Pa29 alone, KPO1K2 alone, KPO1K2 + xylitol, Pa29 + xylitol or KPO1K2 + Pa29 + xylitol). After washing the wells with 0.85% NaCl, viable counts were determined on all 7 days as described above. On each day, a set of five control wells without treatment was also processed in a similar way.

**Leakage of DNA and protein.** Mixed-species biofilm was established on microtitre plates as already described. The wells were treated with various combinations of phage and xylitol (KPO1K2 alone/Pa29 alone/NDF alone/xylitol alone/KPO1K2 + Pa29/NDF + Pa29/KPO1K2 + xylitol/Pa29 + xylitol/KPO1K2 + Pa29 + xylitol; n = 5 each) on days 2 and 5. After incubation for 24 h at 37 °C, the well suspension was collected in sterile Eppendorf tubes. Their DNA and protein content was

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**Fig. 1.** Diagrammatic representation of the scheme employed in the present study. Treatment efficacy was determined by estimating the viable count in various groups. *In addition to viable count, percentage protein and DNA content was determined for all subgroups (for details see text). In addition to viable count, FACS was performed for these subgroups (for details see text). In addition to viable count, fluorescence microscopy was performed for these subgroups (for details see text).
determined based on absorbance at 260 and 280 nm using a spectrophotometer according to the method of Cox et al. (2001). For complete release of DNA and protein, biofilm in a set of five wells was treated with 100 μg lysozyme ml⁻¹ at 37 °C for 120 min, followed by sonication of the cell suspension. The extent of DNA and protein leakage obtained in lysozyme-treated wells was taken as 100 % and the percentage release in test wells was calculated relative to lysozyme-treated wells.

**Statistical analysis.** The effect of different treatments on biofilm eradication was evaluated by applying two-way ANOVA and P<0.05 was considered significant. Data were analysed using SPSS 16.0.

**RESULTS**

**Visualization of mixed-species biofilm on polycarbonate discs**

To observe the spatial distribution pattern of *Klebsiella* and *Pseudomonas*, mixed biofilm was established on polycarbonate membrane discs. On day 1, *Pseudomonas* colonized the polycarbonate membrane while *Klebsiella* was clearly visible as lactose-fermenting pink biomass on top of the basal biofilm structure (Fig. 2a). With progression of biofilm age, on day 3, *Pseudomonas* was visible as non-lactose-fermenting biomass formed beneath *Klebsiella* (Fig. 2b). On day 5, striations appeared on both the surface and the base, indicating formation of a mature biofilm structure (Fig. 2c). From these images, it was clear that *Pseudomonas* colonized the substratum while *Klebsiella* was predominantly present in the superficial layer of dual-species biofilm. As visible in Fig. 2, the area of mixed-species biofilm also increased with increase in biofilm age. On day 1, it was 20 mm², increasing to 40 mm² on day 3 and 100 mm² on day 5 (Fig. 2).

**Formation of mixed-species biofilm in microtitre plates**

Mixed-species biofilm of *K. pneumoniae* and *P. aeruginosa* was established in 96-well microtitre plates. The maximum count for *Klebsiella* was observed on day 2 (7.979±0.14 log10 c.f.u. ml⁻¹) and *Pseudomonas* on day 4 (8.159±0.14 log10 c.f.u. ml⁻¹), followed by a consistent decline (Table 1; see also Table S1). Both organisms persisted in dual-species biofilm up to 7 days.

**Treatment efficacy of various combinations of phages KPO1K2/Pa29/NDP on mixed-species biofilm**

**Viable count method.** Mixed-species biofilm of *K. pneumoniae* and *P. aeruginosa* was treated with phages KPO1K2/Pa29/NDP alone and in combination (KPO1K2+Pa29 or NDP+Pa29). On treatment with KPO1K2 alone, ~3.9 log reduction was observed for *Klebsiella* while Pa29 alone was completely ineffective against *Pseudomonas* (Table 1). By contrast, when both phages (KPO1K2+Pa29) were used, phage Pa29 became effective against *P. aeruginosa*, causing a significant ~2.8 log reduction (P<0.05) on days 1 and 2 in comparison with the Pa29 alone treated group (Table 1). The reduction observed in *Klebsiella* counts in this combination group (~4 log) was similar to that observed in the group treated with phage KPO1K2 alone (~3.9 log). Phages KPO1K2/NDP/Pa29 when used for treating single-species biofilm formed by their respective host bacterium resulted in higher reduction in log bacterial counts compared with when they were used for treating mixed-species biofilm (Figs S1 and S2).

To study the significance of depolymerase-producing phage KPO1K2, mixed-species biofilm was treated with non-depolymerase-producing *K. pneumoniae*-specific phage NDP alone as well as in combination with Pa29. On treatment with phage NDP alone, an average ~3.3 log reduction was observed in the count of *Klebsiella* while no effect was seen in the count of *Pseudomonas* (Table 1). When phages NDP and Pa29 were used in combination, although an ~3 log reduction was seen in the count of *Klebsiella*, no significant reduction (P>0.05) was observed in the count of *Pseudomonas*. The overall reduction in bacterial count in the NDP+Pa29-treated group was less than that observed in the KPO1K2+Pa29-treated group.

**Flow cytometry.** The efficacy of treatment of mixed biofilm with bacteriophages was studied by estimating the percentage of live and dead cells by flow cytometry. In untreated biofilm, 98±0.25 % of cells were viable (Fig. 3a, Q4). When mixed-species biofilm was treated with phage Pa29 alone, 97.4±0.38 % of cells were viable (Fig. 3b, Q4). When phage NDP was used in combination with Pa29, no significant difference (P>0.05) was observed, as 94±0.29 % of cells were viable (Fig. 3c, Q4) and 6±0.14 % were dead (Fig. 3c, Q3). In contrast, treatment with phage KPO1K2 in combination with Pa29 resulted in a significant increase (P<0.05) in the percentage of dead cells (39±0.27 %) (Fig. 3d, Q3) while 60±0.31 % cells were viable (Fig. 3d, Q4).
Tackling mixed biofilms

Table 1. Bacterial counts in mixed-species biofilm of K. pneumoniae (K.pn.) and P. aeruginosa (PAO) treated with phages KPO1K2/Pa29/NDP/xylitol alone as well as in various combinations

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Bacterial count (log_{10} c.f.u. ml^{-1})</th>
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<tbody>
<tr>
<td></td>
<td>1 day old biofilm</td>
</tr>
<tr>
<td></td>
<td>K.pn</td>
</tr>
<tr>
<td>Untreated</td>
<td>7.606 ± 0.24</td>
</tr>
<tr>
<td>Pa29 treated</td>
<td>7.666 ± 0.17</td>
</tr>
<tr>
<td>KPO1K2 treated</td>
<td>5.259 ± 0.18</td>
</tr>
<tr>
<td>NDP treated</td>
<td>5.542 ± 0.32</td>
</tr>
<tr>
<td>Xylitol treated</td>
<td>6.374 ± 0.35</td>
</tr>
<tr>
<td>KPO1K2 + Pa29 treated</td>
<td>5.476 ± 0.11*</td>
</tr>
<tr>
<td>NDP + Pa29 treated</td>
<td>5.624 ± 0.22</td>
</tr>
<tr>
<td>KPO1K2 + xylitol treated</td>
<td>0 ± 0.‡</td>
</tr>
<tr>
<td>Pa29 + xylitol treated</td>
<td>6.079 ± 0.22</td>
</tr>
<tr>
<td>KPO1K2 + Pa29 + xylitol treated</td>
<td>0 ± 0.¶</td>
</tr>
</tbody>
</table>

*P<0.05 [KPO1K2 & Pa29 (K.pn.) vs. untreated (K.pn.)/NDP (K.pn./PAO)/KPO1K2 (K.pn.)/xylitol (K.pn.)].
†P<0.05 [KPO1K2 & Pa29 (PAO) vs. untreated (PAO)/Pa29 (PAO)/NDP + Pa29 (PAO)].
§P<0.05 [KPO1K2 & xylitol (K.pn.) vs. untreated (K.pn./PAO)/Pa29 (PAO)/xylitol (K.pn.)].
¶P<0.05 [KPO1K2 & xylitol (PAO) vs. untreated (PAO)/Pa29 (PAO)/xylitol (PAO)].
#P>0.05 [Pa29 & xylitol (K.pn./PAO) vs. KPO1K2 & xylitol (K.pn./PAO)].
&P<0.05 [KPO1K2, Pa29 & xylitol (K.pn.) vs. untreated (K.pn./PAO)/KPO1K2 (K.pn./PAO)].

**Fluorescence microscopy.** To study the interaction of bacteriophages with mixed biofilm, fluorescent microscopy was performed. As shown in Fig. 4(a), when mixed biofilm was treated with KPO1K2 and Pa29, blue regions corresponding to *Pseudomonas*-specific phage Pa29 were visible intermingled with green areas containing depolymerase-producing *Klebsiella*-specific phage, KPO1K2. In contrast, when phage Pa29 (blue) was used along with non-depolymerase-producing *Klebsiella*-specific phage NDP (red), regions of low red as well as blue intensity were observed (Fig. 4b). This was because, in the absence of phage-borne depolymerase, phage NDP (red) could not efficiently attach to the biofilm, which in turn hampered the penetration of phage Pa29 into the biofilm (Fig. 4b).

**Treatment of mixed-species biofilm with xylitol**

Mixed-species biofilm was treated with xylitol at a concentration of 20 mg ml^{-1}. As shown in Table 1, treatment with xylitol resulted in an average ~2 log reduction in the count of *Klebsiella* (*P<0.05*). However, it was ineffective in reducing *Pseudomonas* in mixed biofilm (*P>0.05*). In contrast, when xylitol was used for treating single-species biofilm formed by *Klebsiella* or *Pseudomonas*, it caused a significant reduction (*P<0.05*) in the count of both organisms throughout the 7 day period (Figs S1 and S2). This indicated a reduced efficacy of xylitol in mixed-species biofilm due to spatial heterogeneity.

**Treatment of mixed-species biofilm with various combinations of phage and xylitol**

When phage KPO1K2 was used in combination with xylitol, complete eradication of *Klebsiella* was observed from mixed-species biofilm, indicating a synergistic activity of xylitol and phage (Table 1). After KPO1K2 + xylitol treatment, an average ~4 log reduction was also observed in the count of *Pseudomonas* compared with when xylitol or phage Pa29 was used alone. When Pa29 and xylitol were used in combination, a negligible reduction (*P>0.05*) was observed in counts of the two organisms (Table 1). After treatment with KPO1K2 + Pa29 + xylitol, complete eradication of *Klebsiella* was observed, similar to that observed after treatment with KPO1K2 + xylitol. However, in mixed biofilm treated with KPO1K2 + Pa29 + xylitol there was an ~6 log reduction in *Pseudomonas* compared with that observed in biofilm treated with phage KPO1K2 + xylitol (~4 log reduction).

From day 3 onwards, the average log reduction observed in biofilms belonging to various groups (Table S1) was similar to that observed in 1 and 2 day old biofilm (Table 1).

**Estimation of protein and DNA release**

Young and old mixed biofilms were treated with various combinations of phage and xylitol and release of protein and DNA was estimated. As shown in Table 2, treatment
of 2 day old biofilm with phages (KPO1K2 and Pa29) resulted in a 55.78 ± 0.18 and 43 ± 0.19 % release of DNA and protein, respectively. The role of depolymerase-producing phage was clear as significantly less reduction (P < 0.05) was observed when phage NDP was used along with Pa29 (DNA: 48.12 ± 0.16 %, protein: 37.6 ± 0.12 %). DNA and protein release in mixed biofilm treated with xylitol alone was 10.18 ± 0.30 and 6.4 ± 0.17 %, respectively (Table 2). When 2 day old mixed biofilm was treated with phage KPO1K2 + xylitol, release was 58.9 ± 0.17 % (DNA) and 47.3 ± 0.18 % (protein) whereas when it was treated with Pa29 + xylitol, release was 53.8 ± 0.28 % (DNA) and 44 ± 0.16 % (protein). In contrast, when both phages were used in combination with xylitol (KPO1K2 + Pa29 + xylitol), a significantly higher release (P < 0.05), i.e. 60.7 ± 0.19 % (DNA) and 48 ± 0.17 % (protein), was observed (Table 2). Overall, the pattern observed in old biofilm (day 5) was similar to that observed in young biofilm (day 2).

**Fig. 3.** Flow cytometry of (a) untreated biofilm cells, and biofilm cells treated with (b) Pa29, (c) NDP + Pa29 and (d) KPO1K2 + Pa29 and stained with Syto 9 and PI. Q4, live cells; Q3, dead cells. PE-A on Y axis refers to bacterial cells stained with propidium iodide (PI).

**Fig. 4.** Syto 9-labelled KPO1K2 (green) and DAPI-labelled Pa29 (blue) (a) Syto 62-labelled NDP (red) and DAPI-labelled Pa29 (blue) (b) were allowed to react with the biofilm for 15 min in the dark and visualized under a fluorescence microscope. Bars, 10 μm.
Table 2. Release of DNA and protein from young (2 days) and old (5 days) biofilm following various treatments

Data are represented as mean ± SD.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>2 day old biofilm</th>
<th>5 day old biofilm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% DNA release</td>
<td>% Protein release</td>
</tr>
<tr>
<td></td>
<td>(260 nm)</td>
<td>(280 nm)</td>
</tr>
<tr>
<td>KPO1K2 treated</td>
<td>52 ± 0.14</td>
<td>45 ± 0.24</td>
</tr>
<tr>
<td>Pa29 treated</td>
<td>51.7 ± 0.26</td>
<td>41.9 ± 0.31</td>
</tr>
<tr>
<td>KPO1K2 and Pa29 treated†</td>
<td>55.78 ± 0.18</td>
<td>43 ± 0.19</td>
</tr>
<tr>
<td>Xylitol (20 mg ml⁻¹) treated</td>
<td>10.18 ± 0.30</td>
<td>6.4 ± 0.17</td>
</tr>
<tr>
<td>KPO1K2 and xylitol (20 mg ml⁻¹) treated</td>
<td>58.9 ± 0.17</td>
<td>47.3 ± 0.18</td>
</tr>
<tr>
<td>Pa29 and xylitol (20 mg ml⁻¹) treated</td>
<td>53.8 ± 0.28</td>
<td>44 ± 0.16</td>
</tr>
<tr>
<td>KPO1K2, Pa29 and xylitol (20 mg ml⁻¹) treated†</td>
<td>60.7 ± 0.19</td>
<td>48 ± 0.17</td>
</tr>
<tr>
<td>NDP treated</td>
<td>49.9 ± 0.17</td>
<td>40 ± 0.19</td>
</tr>
<tr>
<td>NDP and Pa29 treated‡</td>
<td>48.12 ± 0.16</td>
<td>37.6 ± 0.12</td>
</tr>
</tbody>
</table>

*P<0.05 [(KPO1K2 and Pa29 treated) vs. (NDP and Pa29 treated)].
†P<0.05 [(KPO1K2, Pa29 and xylitol treated) vs. (KPO1K2 and xylitol treated/Pa29 and xylitol treated/KPO1K2 treated/Pa29 treated/xylitol treated).
‡P>0.05 [(NDP and Pa29 treated) vs. (NDP treated)].

DISCUSSION

Biofilm-related diseases are responsible for 19 million infections worldwide, resulting in significant fatalities (Wolcott et al., 2010). In nature, various micro-organisms such as E. coli and Candida share a common milieu and coexist harmoniously in microbial niches (Thein et al., 2007). Strains of Burkholderia, Pseudomonas and Bacillus also coexist in polluted environments and cause biodegradation (Yoshida et al. 2009). P. aeruginosa and K. pneumoniae have been known to coexist in cystic fibrosis, burn wound, respiratory tract and urinary tract infections (Childers et al. 2013). As many acute and chronic infections occur due to mixed-species biofilm, there is a need to target multiple organisms in biofilm mode (Peters et al., 2012).

The present study was conducted to design a potential strategy for combating mixed biofilm of K. pneumoniae BS055 and P. aeruginosa PAO. Counts corresponding to 10⁸ and 10⁶ c.f.u. ml⁻¹ for K. pneumoniae and P. aeruginosa, respectively, formed stable mixed-species biofilm over a period of 7 days. Siebel & Characklis (1991) have reported appearance of putative ‘towers’ of K. pneumoniae protruding above the base biofilm of P. aeruginosa. A similar structural heterogeneity was observed in our study: when dual-species biofilm was formed on the polycarbonate disc, a top pink film of K. pneumoniae blanketed the colourless basal biofilm of P. aeruginosa.

Phages have been successfully used for treating mixed-species biofilm of E. coli and P. aeruginosa (Kay et al., 2011) and Pseudomonas fluorescens and Staphylococcus lentus (Sillankorva et al., 2010). They caused significant killing of bacteria and prevented the release of biofilm cells to planktonic phase (Sillankorva et al., 2010). In our study, when phage Pa29 was used alone, insufficient reduction was observed in P. aeruginosa counts due to limited penetration of Pa29 to the deeper layers. By contrast, promising results were obtained when depolymerase-producing phage KPO1K2 was used in combination with phage Pa29. Capsular depolymerase produced by phage KPO1K2 hydrolysed the polysaccharide layer formed by K. pneumoniae on the top. This resulted in attachment of KPO1K2 to specific receptors on K. pneumoniae thereby causing its lysis. Disruption of this top layer facilitated penetration of phage Pa29 to reach its host, i.e. P. aeruginosa, lying beneath and cause its lysis.

To confirm the role of depolymerase in facilitating entry of Pa29 into deeper layers, mixed-species biofilm was treated with a Klebsiella-specific non-depolymerase-producing phage, i.e NDP, in combination with phage Pa29. Insignificant reduction in the Klebsiella and Pseudomonas counts was observed in comparison with when KPO1K2 and Pa29 were used for treatment. This observation is in accordance with the previous work done in our laboratory on the role of depolymerase-producing phage in improving antibiotic efficacy and reducing resistant variant formation (Bedi et al., 2009; Verma et al., 2009b). The mechanism followed by bacteriophages for efficiently disrupting the spatial pattern and tackling the two organisms in mixed biofilm was further confirmed by FACS and fluorescence microscopy.

Reports on the emergence of phage-resistant mutants have necessitated a need for combination therapies to tackle pathogens in a mixed community (Knoll & Mylonakis, 2014). Xylitol has been used for inhibiting planktonic cells as well as biofilms formed by various organisms (Ichikawa et al., 2008; Ammons et al., 2009; Abbas et al., 2012; Ghezelbash et al., 2012). In our study, a significant reduction was observed in the counts of Klebsiella and Pseudomonas when xylitol was used for treating single-species biofilm over a period of 7 days (Figs S1 and S2). In contrast, xylitol

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was not very effective when used to treat mixed-species biofilm. Its reduced efficacy might be due to the distinct spatial distribution pattern adopted by the two organisms in mixed-species biofilm.

Xylitol has been used synergistically with other sugar alcohols or antibiotics against the biofilms of Candida albicans, P. aeruginosa, streptococci and Staphylococcus aureus (Ichikawa et al., 2008; Ammons et al., 2009; Abbas et al., 2012; Ghezelbash et al., 2012). In our study, when mixed-species biofilm was treated with phage KPO1-K2 + Pa29 + xylitol or phage KPO1K2 + xylitol, a complete eradication of Klebsiella and significant reduction in the count of Pseudomonas was observed. This can be explained due to the depolymerase-producing ability of KPO1K2, which probably facilitated the penetration of Pa29 and xylitol, leading to disruption of the basal Pseudomonas layer.

Disruption of the 3D architecture of biofilm facilitates the release of cellular constituents of bacterial cells. In this study, treatment with phage KPO1K2 in combination with other agents resulted in a significant release of DNA and protein. This confirmed the role of phage depolymerase in destabilizing the spatial pattern in mixed biofilm.

There are several concerns associated with the use of bacteriophages including their rapid removal from the circulation, neutralization by specific antibodies and their inability to act on intracellular pathogens. However, advantages such as their highly specific, self-replicating and self-limiting nature, their inexpensive and easy production, and their ability to act on antibiotic-resistant bacteria outweigh these concerns (Drulis-Kawa et al., 2012). Xylitol is regularly used in various commercial formulations (Lynch & Milgrom, 2003). Hence, this novel strategy utilizing phage and xylitol can be successfully used for topical application, the coating of indwelling devices or in nebulizers for tackling burn wounds, urinary tract and respiratory tract, respectively.

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