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SHORT COMMUNICATION

Viunalikeviruses are environmentally common agents of horizontal gene transfer in pathogens and biocontrol bacteria

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Bacteriophages have been used as natural biocontrol and therapeutic agents, but also as biotechnological tools for bacterial engineering. We showed recently that the transducing bacteriophage φMAM1 is a ViI-like phage and a member of the new genus, 'Viunalikevirus'. Here, we show that four additional ViI-like phages and three new environmentally isolated viunalikeviruses, all infecting plant and human pathogens, are very efficient generalised transducers capable of transducing chromosomal markers at frequencies of up to 10^{-4} transductants per plaque-forming unit. We also demonstrate the interstrain transduction of plasmids and chromosomal markers, including genes involved in anabolism, genes for virulence and genes encoding secondary metabolites involved in biocontrol. We propose that all viunalikeviruses are likely to perform efficient horizontal gene transfer. Viunalikeviruses therefore represent useful agents for functional genomics and bacterial engineering, and for chemical and synthetic biology studies, but could be viewed as inappropriate choices for phage therapy.
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Combined morphological, genomic and phylogenetic analyses have recently led to the proposed creation of a new phage genus, 'Viunalikevirus', within the *Myoviridae* family (Adriaenssens *et al.*, 2012a). The first member of this proposed genus, *Salmonella* phage ViI, was isolated in the 1930s (Craigie and Yen, 1938) and multiple viunalikeviruses have been sequenced and characterised since 2010 (Pickard *et al.*, 2010; Anany *et al.*, 2011; Hooton *et al.*, 2011; Kutter *et al.*, 2011; Matilla and Salmond, 2012; Park *et al.*, 2012; Adriaenssens *et al.*, 2012a, b; Hsu *et al.*, 2013; Luna *et al.*, 2013; Shahrbabak *et al.*, 2013). Viunalikeviruses are characterised as virulent (lytic) phages showing similar genome size, extensive DNA homology, strong gene synteny and a complex adsorption apparatus, which uses tail spike proteins as host-recognition determinants (Adriaenssens *et al.*, 2012a).

We recently isolated the ViI-like phage, φMAM1, that infects several environmental and clinical isolates belonging to *Serratia* and *Kluyvera* genera (Matilla and Salmond, 2012). During the characterisation of φMAM1, we showed that it mediates highly efficient generalised transduction (Matilla and Salmond, submitted for publication). These observations were consistent with a previous report, that the *Salmonella* phage ViI was also capable of transduction (Cerquetti and Hooke, 1993) and we have confirmed that phage ViI can transduce chromosomal markers and plasmids at frequencies of up to 4.6×10^{-5} transductants per plaque-forming unit (p.f.u.; Figure 1a; Supplementary Table 1).

Most generalised transducers utilise a headful packing strategy where phage terminases recognise specific sequences (*pac* sites) in the DNA and perform cycles of packing that result in mature phage particles (Fineran *et al.*, 2009a). Indeed, phage terminases with reduced specificity for *pac* sequences may lead to the evolution of efficient transducing phages (Schmeiger, 1972). Based on the high similarity between the terminases of φMAM1, ViI and those of other previously sequenced viunalikeviruses, we hypothesised that all of these ViI-like phages should be capable of transduction in their respective bacterial hosts. To test this hypothesis, we investigated three additional viunalikeviruses, *Escherichia coli* phage CBA120 (Kutter *et al.*, 2011), and *Dickeya* phages LIMEstone1 and LIMEstone2 (Adriaenssens *et al.*, 2012b). All the bacteriophages, bacterial strains, plasmids and primers used in this study are listed in the Supplementary Tables 2 and 3. Experimental procedures are presented as Supplementary Material.

The LIMEstone phages specifically infect some strains of the emerging plant pathogen, *Dickeya solani* (Adriaenssens *et al.*, 2012b), and here we showed that they also infect the recently sequenced

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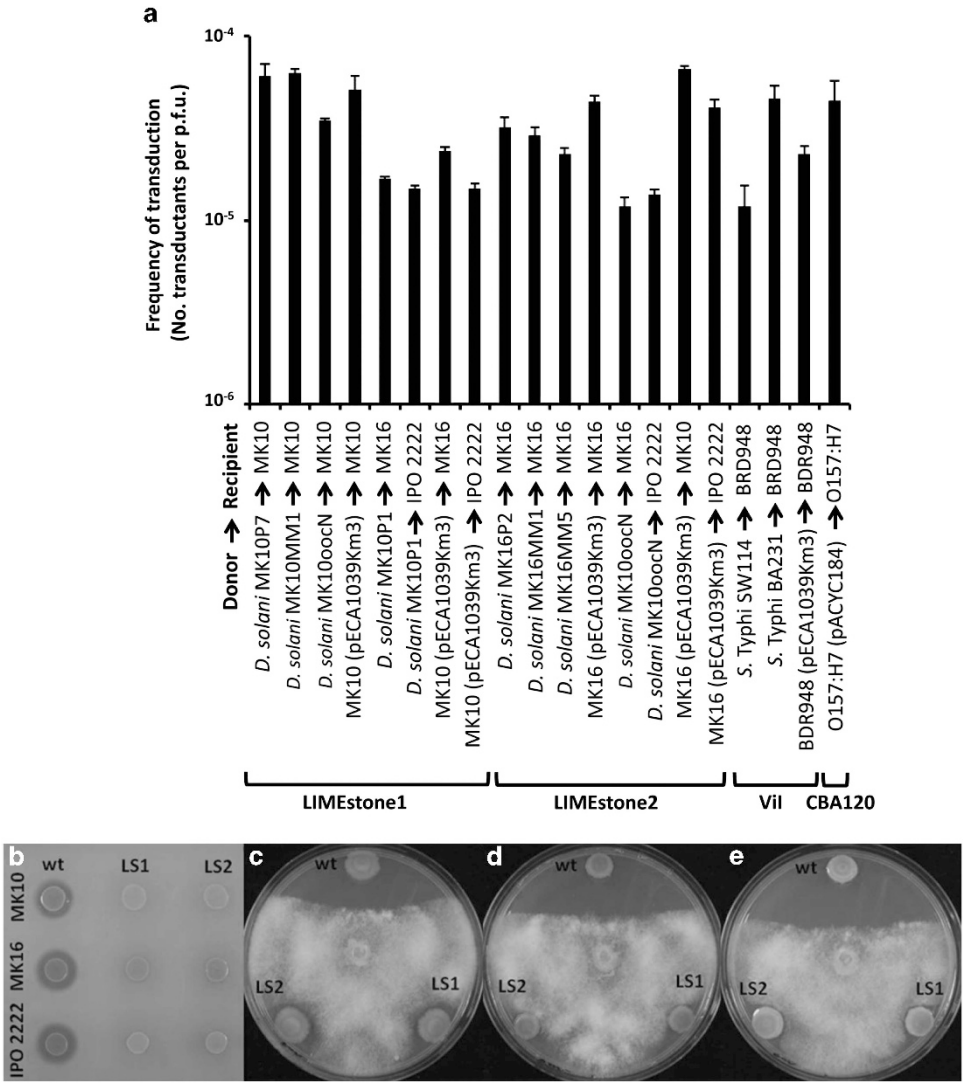

Figure 1 Transduction capabilities of viunalikeviruses. (**a**) Transduction frequencies of LIMEstone1, LIMEstone2, VilI and CBA120 phages. The graph also shows transduction efficiencies of LIMEstone phages within and between *Dickeya solani* strains. Transduction efficiency was defined as the number of transductants obtained per p.f.u. In all cases, error bars represent the standard deviations ($n = 3$). (**b**) Skimmed milk agar plates showing protease production in the wild-type (wt) *Dickeya solani* strains MK10, MK16 and IPO 2222. LIMEstone1- (LS1) and LIMEstone2- (LS2) mediated transduction of the *spp*::Km marker from the protease negative mutant strain MK10P1 to the wild-type strains MK10, MK16 and IPO 2222 result in a protease-negative phenotype. (**c**–**e**) LIMEstone-mediated transduction of the *oocN*::Km marker from the oocydin A-negative mutant strain MK10oocN to the wild-type strains MK10 (**c**), MK16 (**d**) and IPO 2222 (**e**) results in an oocydin A-negative phenotype and, consequently, in the generation of strains defective in their antimicrobial activity against the plant pathogenic oomycete, *Pythium ultimum*. The anti-oomycete assays were performed as described previously (Matilla *et al*., 2012).

D. solani strains MK10, MK16 and IPO 2222. As predicted, we confirmed that the LIMEstone phages effected efficient transduction of various auxotrophic markers between *Dickeya solani* strains (Figure 1a; Supplementary Table 4). To our knowledge, only one *Dickeya* transducing phage, φEC2, has been isolated previously (Resibois *et al*., 1984). Additional mutant strains were constructed and the generalised nature of the transduction was confirmed by transfer of multiple chromosomal markers, including mutations in the gene cluster encoding biosynthesis of the anti-oomycete haterumalide, oocydin A (Matilla *et al*., 2012) and in the locus for synthesis and secretion of protease virulence factors. Transduction frequency was higher at an multiplicity of infection (m.o.i.) of 0.1 and 0.01 with efficiencies of up to 10^{-4} transductants per p.f.u. (Figure 1a; Supplementary Tables 4 and 5).

We also demonstrated transduction of a kanamycin resistance-marked plasmid pECA1039-Km3 between strains MK10, MK16 and IPO 2222 at frequencies of up to 8.6×10^{-5} (Supplementary Table 4). Plasmid pECA1039 (originally isolated from the phytopathogen, *Pectobacterium atrosepticum*) encodes a bifunctional type III Toxin-Antitoxin (TA) system, ToxIN, with abortive infection

capacity. Although ToxIN aborts infection of various enterobacteria by diverse phages (Fineran *et al.*, 2009b) it did not protect against infection by the tested viunalikeviruses, φMAM1, ViI, CBA120, LIMEstone1 or LIMEstone2 (not shown). Furthermore, another type III TA system, TenpIN, from the insect pathogen, *Photorhabdus luminescens* (Blower *et al.*, 2012), failed to protect against any of the five ViI-like phages (not shown).

In addition, we also tested the transduction capacity of the *E. coli* phage, CBA120, and confirmed transduction of plasmid-borne antibiotic resistances at a frequency of up to 10^{-4} transductants per p.f.u. (Figure 1a; Supplementary Table 6).

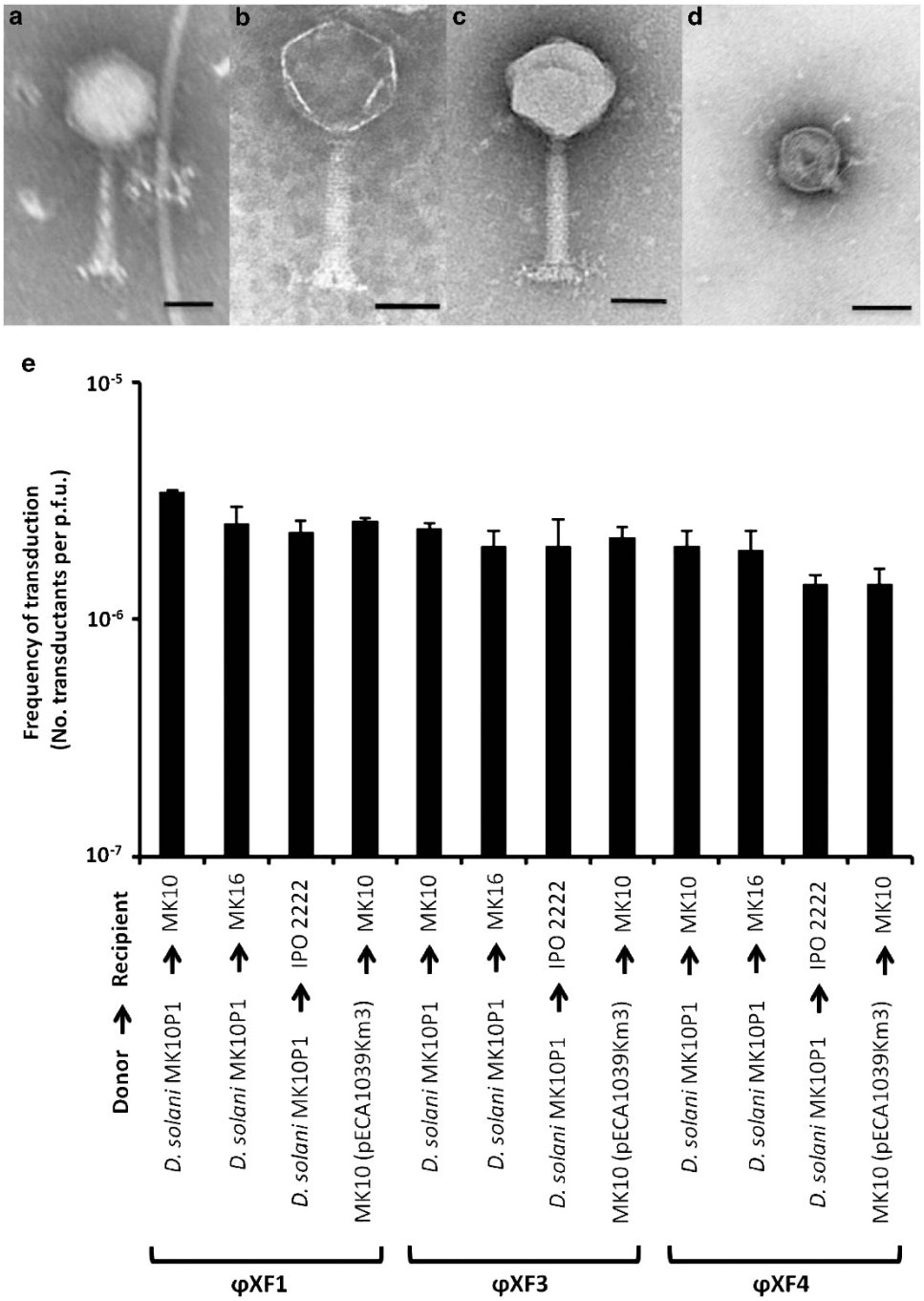

Figure 2 Environmental isolation and characterisation of new viunalikeviruses with generalised transduction functionality. Transmission electron micrographs of phages φXF1 (**a**), φXF3 (**b**), φXF4 (**c**) and φXF28 (**d**) are shown. As an internal control, φXF28 was an example of a new lytic phage isolated from the same environment but showing no transduction capabilities. Bars, 50 nm. (**e**) Transduction frequencies of the new viunalikeviruses φXF1, φXF3 and φXF4. Transduction experiments were performed using 10^9 cells with φXF1, φXF3, φXF4 at an m.o.i. of 0.01. Transduction efficiency was defined as the number of transductants obtained per p.f.u. Error bars represent the standard deviations ($n = 3$).

We decided to test our hypothesis that the viunalikeviruses may all be generalised transducers by first isolating new viunalikeviruses from the environment. From treated sewage effluent, we isolated three new bacteriophages infecting *Dickeya solani*, φXF1, φXF3 and φXF4, as defined initially by their very characteristic ViI-like morphology in electron microscopy (Figures 2a–c). As predicted, all of these new phages were able to transduce chromosomal markers and plasmids at frequencies of up to 3×10^{-6} transductants per p.f.u. (Figure 2e; Supplementary Table 7). Sequencing of structural and non-structural protein-encoding genes of φXF1, φXF3 and φXF4 showed high nucleotide homology (between 80% and 100%) with the corresponding orthologs in LIMEstone1 (Supplementary Figure 1), indicating that these virgin environmental isolates also clade within the Viunalikevirus genus.

Although we did not have access to other ViI-like *Salmonella* phages SFP10 (Park *et al.*, 2012), φSH19 (Hooton *et al.*, 2011) and Marshall (Luna *et al.*, 2013), *Escherichia* phage PhaxI (Shahrbabak *et al.*, 2013), *Shigella* phage φSboM-AG3 (Anany *et al.*, 2011) and *Klebsiella* phage 0507-KN2-1 (Hsu *et al.*, 2013), our results allow us to predict that all of these phages will mediate generalised transduction. Importantly, these phages would be expected to contribute to the horizontal gene transfer of virulence factors and antimicrobial-resistance determinants in diverse environments.

Viunalikeviruses do not seem to be limited to the enterobacteria as bacteriophages showing ViI-like morphology have been isolated in *Acinetobacter* (Ackermann *et al.*, 1994), *Bordetella* (Adriaenssens *et al.*, 2012b) and *Sinorhizobium* (Werquin *et al.*, 1988). Furthermore, another ViI-like morphotype phage (φM12 of *Sinorhizobium meliloti*) has also been shown to be an efficient transducer (Finan *et al.*, 1984). Taken together, these results suggest that, even in the absence of strongly predictive comparative genomic detail, a characteristically discrete ViI-like morphology in electron microscopy may be sufficient to identify new phages as strong candidates for possession of generalised transduction capacity.

The emergence and dissemination of antibiotic-resistant pathogens coupled with low discovery rates for new antimicrobials, plus increasing legal constraints on the use of chemical pesticides, have (re)focussed attention on the potential use of bacteriophages for 'natural biocontrol' of human, animal and plant pathogens. Several viunalikeviruses have been proposed as candidate therapeutic agents for the control of bacterial infections (Anany *et al.*, 2011; Hooton *et al.*, 2011; Park *et al.*, 2012; Hsu *et al.*, 2013; Shahrbabak *et al.*, 2013) and the LIMEstone phages have been used in successful field trials for biocontrol of *D. solani* infections (Adriaenssens *et al.*, 2012b). However, their efficient transduction capacities could provide a route for dissemination of virulence factors, such as proteases (Marits *et al.*, 1999). In fact, we have demonstrated the interstrain transduction of plasmids and oocydin A, auxotrophy and protease markers between three different *D. solani* strains, at high frequencies (Figures 1 and 2; Supplementary Tables 4 and 7). Also, the irregular distribution of the oocydin A gene cluster within the *Dickeya* genus and the fact that its genomic context varies between strains raises the possibility of phage-mediated horizontal gene transfer between bacterial strains. These results emphasize strongly that when considering the genomics of phages for 'phage therapy' the absence of genes readily defined as playing roles in lysogeny or bacterial virulence may be insufficient to inspire confidence that use of a particular therapeutic phage presents no risk–particularly among the high efficiency-transducing viunalikeviruses.

Concluding remarks

Our results predict that all viunalikeviruses are likely to be capable of highly efficient horizontal gene transfer between their cognate bacterial hosts. This capacity could be exploited for use in fundamental research in bacterial functional genomics, and biotechnologically, for genetic engineering in chemical biology and synthetic biology applications. However, phages that show efficient horizontal gene transfer capacity could present biosafety implications for manipulation of bacterial pathogens. Obviously, the transduction capabilities of viunalikeviruses should encourage a cautious reconsideration of their appropriateness for phage therapy in human, animal or plant pathology.

Conflict of Interest

The authors declare no conflict of interest.

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