

## Population dynamics of bacteriophages infecting *Serratia* and *Pseudomonas* spp. associated with sugar beet

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### ABSTRACT

Bacteriophages present in the phytosphere of field-grown sugar beet (*Beta vulgaris* var. Amethyst) were investigated over two successive growing seasons, under agriculturally relevant conditions. Numerous genetically distinct phages, capable of infecting the indigenous strains *Serratia liquefaciens* CP6, *Pseudomonas fluorescens* SBW25 or *P. fluorescens* MCP1, were identified, and their population fluctuations followed over the two nine month periods. Unique temporal changes were exhibited by phages for all three bacterial strains. Of particular interest was a distinct temporal succession between two phages antagonistic towards *S. liquefaciens* CP6. These two phages have morphological, physiological, as well as genetic differences, providing an important insight into how phage physiology may reflect their natural ecology.

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### Introduction

Bacteriophages (phages) are thought to play a significant role within natural environments, through their control of bacterial populations, their impact on nutrient and energy cycles and possibly even as agents of gene transfer [11, 14]. Thus their temporal population fluctuations need to be investigated if their environmental significance is to be understood fully.

The population dynamics of specific phages, in concert with their host bacteria, have already been successfully followed within chemostats (for a review see [10]). The resulting predator-prey interactions have enabled the development, and testing, of mathematical models for predator-prey interactions generally. However, the relevance of these experiments to phage populations within natural environments is yet to be fully assessed since there has been relatively few sufficiently long-term *in situ* investigations. What studies that there have been have typically relied on counting virus-like particles within aquatic environments using transmission electron microscopy (TEM) [6-9, 16].

This approach has been shown to be highly useful in determining gross phage population changes, even of differences in abundance of specific phage-morphology-types [7]. However, it is yet to find an equivalent use in the terrestrial environment, due to problems in reliably distinguishing phages from background detritus. There are other limitations to using TEM. It is not possible to distinguish between viable and non-viable phages and then only those phage morphologies that are relatively easily distinguishable under TEM can be

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reliably monitored. In addition, morphology alone is not a reliable guide to specific species or indeed whether the virion observed is even a phage.

An alternative is to assess phage abundance by plating environmental samples onto overlay-agar plates inoculated with a specific host-bacterium [2, 12, 13]. In concert with molecular approaches such as restriction fragment length polymorphism (RFLP) analysis and DNA-DNA cross-hybridization, this simple approach can be highly effective in following the changing populations of specific bacteriophages within a natural environment over prolonged, ecologically relevant time-scales. Here we describe the results of just such an approach when applied to the phytosphere (i.e. plant surfaces) of field-grown sugar beet.

Phytosphere-dwelling bacteria can impact on plant health both positively and negatively, hence anything which potentially impacts on plant associated bacteria, both in terms of their ecology and evolution, is of interest. To this end, we have been investigating phage population dynamics on the surfaces of field-grown sugar beet over the last few years. The phytosphere is a particularly good place to start looking for phages in the terrestrial environment, since the nutrient-rich exudates which plants release enable the establishment of a diverse, dense population of metabolically active microflora and hence potential host bacteria.

## Methodology

What follows is a summary of work carried out at our field-site at Oxford University farm in Wytham, Oxford (UK), from May 1996 to February 1998, during which time we followed the development of two consecutive crops of sugar beet, grown under agriculturally relevant conditions [3, 4 and unpublished]. Our methodology was simple, and based on the overlay agar method for assaying and enumerating phages for a specific host-bacterium [2]. Representative sugar beet were collected from the field-site at regular time intervals throughout the two nine-month growing seasons. Rhizosphere samples were taken from these plants and homogenates were prepared [3]. These were centrifuged to separate the bulk of the plant tissue and associated bacteria from any phages present in the resulting supernatant. Overlay agar aliquots (2.5 ml of 0.65% w/v bacteriological agar, Oxoid L11; 1.3% w/v nutrient broth, Oxoid CM1) were inoculated with 100  $\mu$ l of these supernatants along with 100  $\mu$ l of an overnight culture of one of three potential-host bacteria, and then poured onto nutrient agar (Oxoid CM3) plates and allowed to set. The resulting overlay plates were incubated overnight at 15°C and plaques counted.

The host-bacteria used were *Serratia liquefaciens* CP6, *Pseudomonas fluorescens* SBW25 and *P. fluorescens* MCP1. *P. fluorescens* SBW25 had previously been isolated from the surface of a sugar beet taken from the same site in 1992 [5]. *P. fluorescens* MCP1 and *S. liquefaciens* CP6 were similarly isolated in 1996 [3]. Plaques produced on these strains were used to produce phage lysates from which DNA was extracted and subsequently cut with restriction enzymes, typically *Eco*RI [4]. When run on agarose gels the resulting restriction digest fragments were analysed and if necessary transferred to positively charged membranes by Southern blotting for probing with suitable phage-specific DIG-labelled probes [4]. In this way genetically distinct bacteriophages were identified and thus, by extrapolating back to overlay plate results, their relative abundance followed over time.

## Results

### *Temporal changes to indigenous phage populations*

Figure 1 shows that three populations of phages, each antagonistic towards a different host-bacterium, varied in relative abundance, in terms of their distribution within the field site, over the two growing seasons. Titers were typically in the region of  $10^3$  pfu per g of rhizosphere, during 1996/7, but an order of magnitude lower the following season.

Note that the occurrence of phage-population blooms varied according to host-strain and that this pattern is repeated over two successive growing seasons. Thus, *S. liquefaciens* CP6-antagonistic phages appeared relatively early during July in both seasons whilst *P. fluorescens*

phages occurred much later, being most abundant in November to February. This, presumably, reflects a variation in the relative abundance of the three host strains *in situ*.

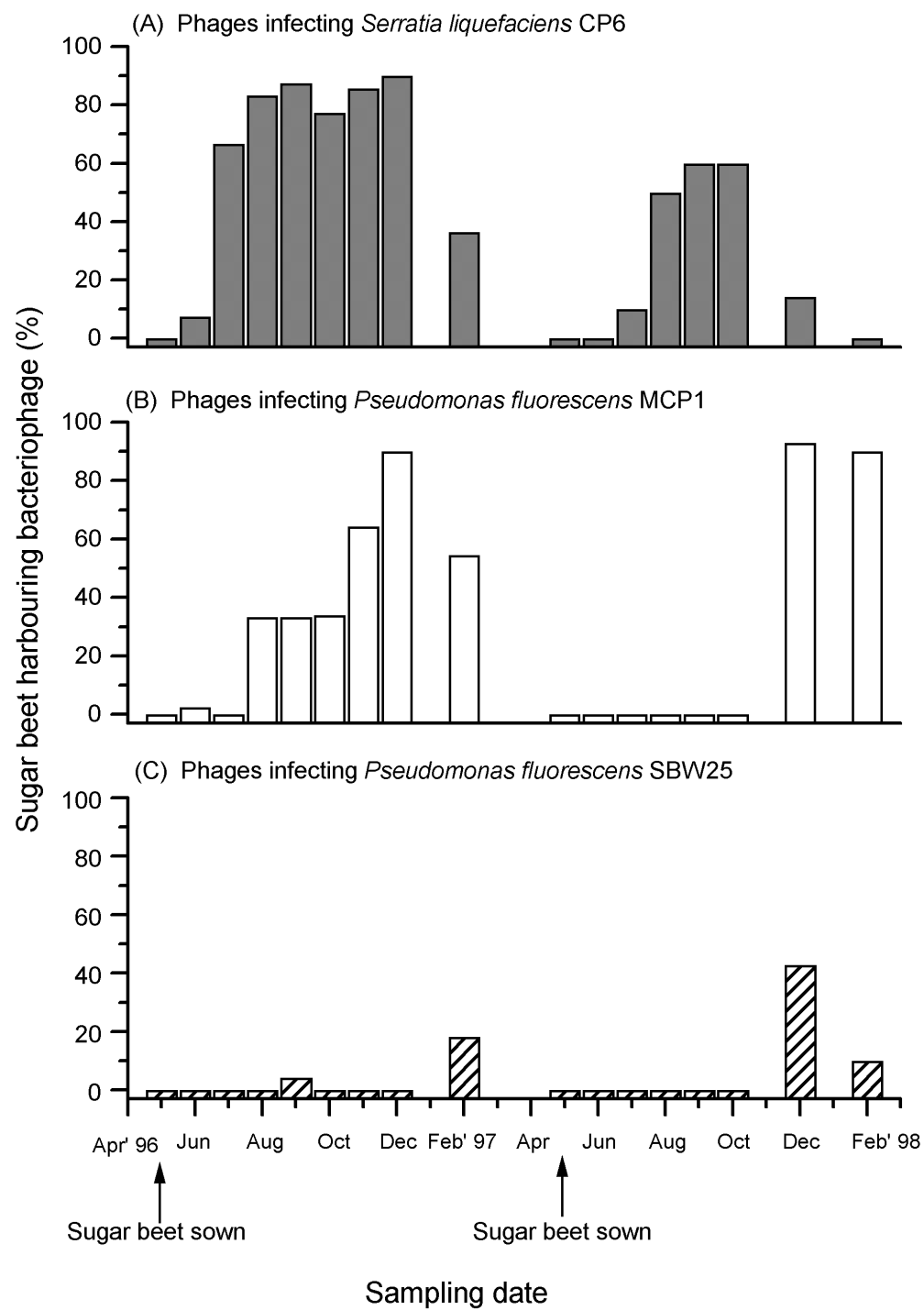
During the 1996/7 season, in an attempt to follow *P. fluorescens* SBW25 population-changes concurrent with that of its antagonistic phages, half of the sugar beet crop was inoculated with a marked strain of SBW25 [3]. As with previous analogous release experiments, the bacterium survived well on these inoculated beet [3]. Unfortunately, as Figure 1 illustrates, very few SBW25-phages were isolated that year, preventing any meaningful analysis of phage-host population-dynamics. Paradoxically, the following year, when no such inoculation was made, considerably more SBW25-phages were isolated.

It is worth noting that, although *P. fluorescens* MCP1 and SBW25 are both closely related strains of the same species (as determined from their fatty acid methyl-ester profiles; [3]) their associated phage-populations do not cross-infect. This inevitably raises the question as to exactly how many strains of *P. fluorescens* are present within the sugar beet phytosphere, each with their own phage-population. Indeed, there is evidence to suggest a similar situation with *S. liquefaciens* CP6 (unpublished data), indicating this phage-host polymorphism to be widespread, at least on the surface of sugar beet.

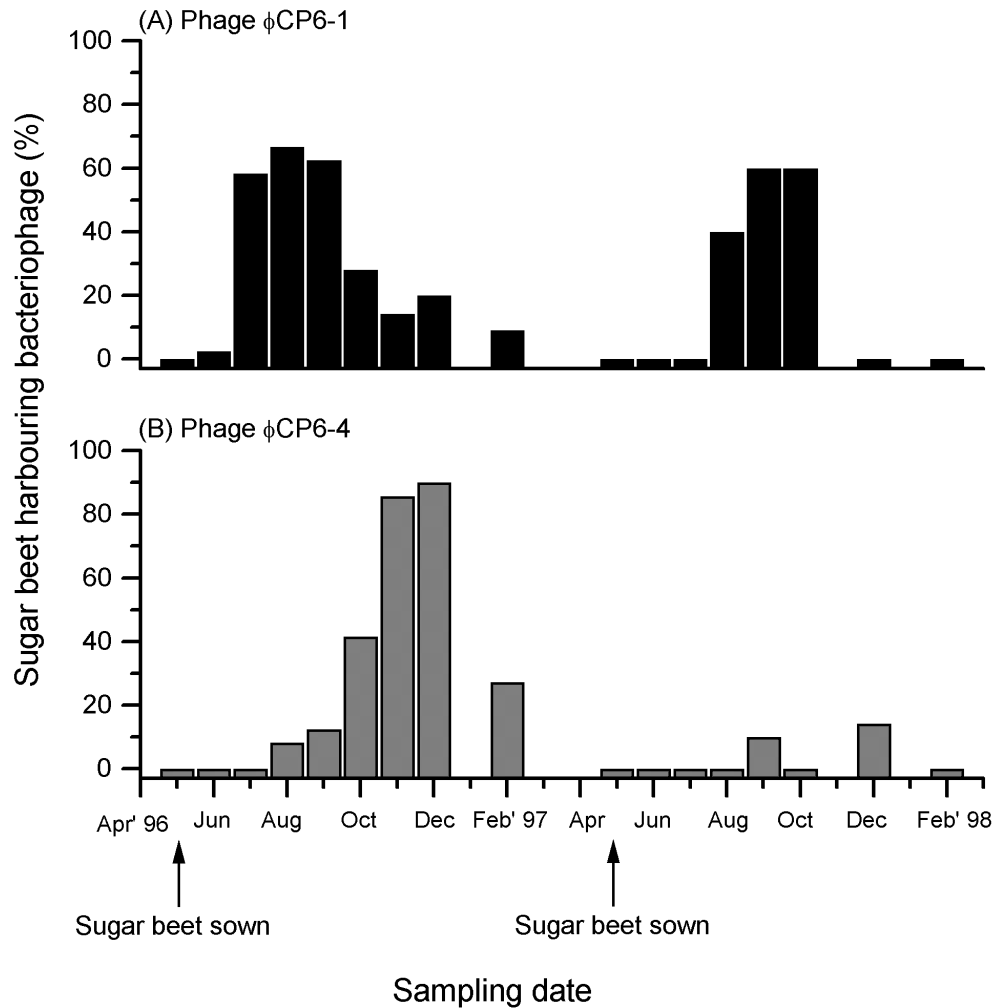
### *Temporal changes of individual types within a phage population*

It was noticeable that isolated phages frequently produced very different plaque morphologies on the overlay lawns, suggesting more than one phage was contributing to the observed populations. This was confirmed when restriction digests of DNA from the various isolates revealed a number of distinctly different phages [3, 4]. *S. liquefaciens* CP6 is particularly noticeable in this respect, being parasitized by seven genetically distinct phage-types (six isolated in 1996 a further one isolated in 1997).

When the populations of these phages were examined with respect to time, distinct temporal variations in their distribution within the field site were revealed [3]. Here we shall concentrate on CP6-phages, and, in particular, the two most dominant in both years - phages  $\Phi$ CP6-1 and  $\Phi$ CP6-4 (Fig. 2). For both years,  $\Phi$ CP6-1 reached peak populations between July and October. In contrast,  $\Phi$ CP6-4 predominated between October and February - at least for the 1996/7 growing season. The following year, in concert with the other less dominant CP6-phages,  $\Phi$ CP6-4 was largely absent. Indeed, the difference in overall CP6-phage abundance between the 1996 and 1997 seasons illustrated in Figure 1, is largely due to the virtual absence of all phages except for  $\Phi$ CP6-1.



**Fig. 1.** Temporal variation in phage populations, antagonistic towards three bacterial-strains (panels A, B and C) over two consecutive growing seasons.



**Fig. 2.** Temporal variation in two phage species, both antagonistic towards *Serratia liquefaciens* CP6, over two consecutive growing seasons.

## Discussion

### *Temporal succession?*

The apparent temporal succession between  $\Phi$ CP6-1 and  $\Phi$ CP6-4 during the 1996 season is worth closer examination. The results would seem to suggest that the phages are adapted to two quite separate temporal niches in the rhizosphere. The defining parameters of these niches must remain unknown at present, but clearly host availability, both in terms of absolute numbers and physiological suitability, is likely to be of overriding significance. It is in this context that some of the genetic and physiological differences between  $\Phi$ CP6-1 and  $\Phi$ CP6-4 (Table 1) become pertinent.

**Table 1.** Comparing bacteriophages  $\Phi$ CP6-1 and  $\Phi$ CP6-4.

	$\Phi$ CP6-1	$\Phi$ CP6-4
Family	<b>Siphoviridae</b>	<b>Podoviridae</b>
DNA size (kb)	43.6	44.2
Genetic homology	No detectable cross hybridization	
Temperate?	yes	no
Latent period* (min)	70	26
Burst size*	120	38
Capable of transduction	yes	no
Plaque morphology	Turbid plaques	Clear plaques surrounded by several concentric rings

\*Calculated from single-step growth curve experiments at 30°C. Latent period (to the nearest minute) and burst size (to the nearest virion) were determined from the sigmoidal curve of best-fit for each single-step growth curve graph.

Considerations of phage physiology, in respect to their ecology [15], and in particular, the application of optimal foraging theory to phage biology [1, 17] have highlighted the importance of (i) latent period and burst size, and (ii) the ability to produce lysogens, as phenotypes by which a phage might optimize its capacity for survival. Hence, on the basis of these papers, one would predict that phage  $\Phi$ CP6-4, with its shorter latent period, consequent smaller burst size, and virulent nature, will have a selective advantage over  $\Phi$ CP6-1, when high densities of physiologically ‘suitable’ host are present in the environment. Conversely,  $\Phi$ CP6-1 would be expected to prevail when the reverse is true. Whether this temporal succession between  $\Phi$ CP6-1 and  $\Phi$ CP6-4 does indeed reflect such a change in their host in the phytosphere remains to be determined.

## Summary

Our *in situ* experiments with field-grown sugar beet have enabled us to follow phage population dynamics over two consecutive years. In so doing we have observed some intriguing similarities and differences between years, the discovery of numerous phages predated on the same bacterium, and, most interestingly, a possible temporal succession between two distinctly different phages. We believe this illustrates the importance of *in situ* research carried out over more lengthy time periods than is usually the case in microbial ecology. The results presented here are based on the use of three host strains. The relative ease with which phage population dynamics for these strains were discerned strongly indicates that what we have described here is reflective of phage population dynamics generally within the sugar beet phytosphere.

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