Elevated Abundance of Bacteriophage Infecting Bacteria in Soil

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Received 5 June 2002/Accepted 1 October 2002

Here we report the first direct counts of soil bacteriophage and show that substantial populations of these viruses exist in soil (grand mean = 1.5 × 10^7 g^-1), at least 350-fold more than the highest numbers estimated from traditional viable plaque counts. Adding pure cultures of a Serratia phage to soil showed that the direct counting methods with electron microscopy developed here underestimated the added phage populations by at least eightfold. So, assuming natural phages were similarly underestimated, virus numbers in soil averaged 1.5 × 10^7 g^-1, which is equivalent to 4% of the total population of bacteria. This high abundance was to some extent confirmed by hybridizing colonies grown on Serratia and Pseudomonas selective media with cocktails of phage infecting these bacteria. This showed that 8.9 and 3.9%, respectively, hybridized with colonies from the two media and confirmed the presence of phage DNA sequences in the cultivable fraction of the natural population. Thus, soil phage, like their aquatic counterparts, are likely to be important in controlling bacterial populations and mediating gene transfer in soil.

Direct counts made with electron and epifluorescence microscopy have shown bacteriophage to be abundant in water from marine (5, 6, 16) and freshwater (18) habitats. They occur at densities of up to 2.5 × 10^8 ml^-1 and are an average of 10-fold more abundant than their bacterial hosts. Fewer direct counts have been done on sediments, and results have been more variable. Numbers of bacterial viruses in sediment are higher than in water, and counts of up to 2 × 10^9 ml^-1 have been recorded (11). However, bacterial counts are also higher, and in some cases, bacteriophage in sediments are more abundant than bacteria (11, 13) and sometimes less abundant (10).

Although it is difficult to grow bacteriophage from soil without enrichment (31), some viable counts have been reported (1, 7, 8, 21, 22, 26, 27). These range from 0 to 4 × 10^7 g^-1 and have been determined from a wide variety of host bacteria. To date, no direct counts of bacterial viruses in this environment have been reported, so in this study we developed methods for counting the total numbers of bacteriophage in soil. We chose to count soil viruses by using transmission electron microscopy (TEM), as a direct observation of phage morphology would allow us to be confident of counting bacterial viruses. We believed that epifluorescence counting would be problematic because there are so many fine particles in soil that interact nonspecifically with most of the DNA stains used for direct virus counts in water and sediment.

**MATERIALS AND METHODS**

**Sampling and sample preparation.** Three samples of rhizosphere soil were taken in November 1999 from a mature sugar beet (Beta vulgaris var. Amythest) growing in the open at Cardiff University (Cardiff, United Kingdom). A further three soil samples were taken from bulk soil >2 m away from the growing beet. Additional samples were taken in quadruplicate in March 2000 from the University of Oxford (Oxford, United Kingdom) Wytham farm site (1, 3, 4) when sugar beet was not growing there. These samples were rhizosphere soil from Poa pratensis L. (meadow grass). Epilobium tetragonum (L.) Griseb. (square stalked willow herb), Senecio jacobaea L. (ragwort), and Cardamine flexuosa With. (greater bitter cress) and bulk soil from molehills. Suspensions were made from soil and rhizosphere samples (1 g [wet weight]) by homogenization in 10 ml of water with 5-mm-diameter glass beads first by vortex mixing for 1 min and then by shaking for 10 min on an orbital shaker. All values are expressed as wet weights, and the average water content of the soil used was 56.2% (coefficient of variation, 29%).

**Total direct bacterial counts were done by acridine orange direct counting with (greater bit...**

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PU21 phage DNA. The resulting mixtures contained phage with varied genomic homology and transducing ability (3, 24).

DNA from the soil and rhizosphere samples was dot blotted onto positively charged nylon membranes (Roche) along with control DNA from pure cultures of CP6 and PU21. DNA was extracted from the soil and rhizosphere by lysis in a high-salt buffer with extended heating in the presence of sodium dodecyl sulfate, hexadecyltrimethylammonium bromide, and proteinase K (35). An equivalent method was used to prepare DNA from colonies grown on SSM and PSIA plates and from the pure cultures of CP6 and PU21 used as controls (25). The labeled phage DNA cocktails were separately incubated with the membranes in 0.22-μm-pore-size membrane-filtered digoxigenin-Easy-Hyb buffer at 42°C for medium stringency and at 50°C for high stringency according to the manufacturer's instructions (Boehringer). Positive and negative controls gave the expected results.

Statistical analysis. Counts of bacteria and phage were compared with standard analysis of variance methods after logarithmic transformation to ensure that variances were homogenous and errors were normally distributed (14). Statistical calculations were done using the Minitab, version 12, computer package (Minitab Inc., University Park, Pa.). Significant differences are quoted at a P value of 0.05 unless stated otherwise.

RESULTS AND DISCUSSION

Methodological development was required to obtain reliable counts of bacteriophage in soil by using TEM. Untreated samples were impossible to count reliably due to large amounts of debris obscuring phage.

To establish counting efficiency, we used S. quinivorans phage CP6-1 (3) as a test phage. When grown in liquid culture, this phage appeared under TEM as either complete virions with transparent or opaque heads (ca. 40%) (Fig. 1a to c) or as transparent or opaque head capsids without tails (ca. 55%) (Fig. 1d to e). A small number of tails were also visible (5%), but these were not counted. Most of the heads were opaque (66% of complete virions, 88% of heads without tails), and there were no significant differences between TEM counts of diluted culture filtrates ($1.5 \times 10^8$ virions ml$^{-1}$) and the plaque counts ($1.3 \times 10^8$ PFU ml$^{-1}$; $P = 0.395$). This indicates that almost all of the virions counted were viable phage particles with DNA-filled head capsids and that few nonviable ghost particles were present. Homogenization of CP6-1 suspensions did not reduce TEM counts significantly (mean before treatment = $5.51 \times 10^7$ ml$^{-1}$, mean after treatment = $7.48 \times 10^7$ ml$^{-1}$). Low-speed centrifugation removed most cellular debris and was too slow to sediment phage. Filtration through 0.22-μm-pore-size membrane filters further reduced debris without
reducing CP6-1 phage counts (mean before filtration = \(2.6 \times 10^9\) ml\(^{-1}\), mean after filtration = \(2.3 \times 10^9\) ml\(^{-1}\)).

Spiking rhizosphere soil samples with viable CP6-1 phage gave TEM counts of tailed CP6-1-like particles that were about 40-fold less than the viable plaque count (2.5%) (Table 1). However, in this experiment, counts of VLPs, which would probably have been CP6-1, were about 13% of the plaque count. This approximately eight-fold loss of CP6-1 phage could not be reduced, and so it was accepted as an inevitable consequence of the method used. Several other approaches to counting phage particles were tried, but these did not increase the counts. Centrifugation directly onto electron microscopy grids, which works well with seawater (5, 6), deformed the phage particles too much for accurate counting and hindered enumeration enormously (data not shown). This was not unexpected as similar problems have been observed previously with water samples containing large amounts of particulate matter (33). So homogenization, centrifugation, and filtration were used as the standard procedure for TEM counts of phage throughout this study.

The counts of bacteria and their phage from the rhizosphere and bulk soils at Cardiff and Oxford are given in Table 2. The viable bacterial counts were not significantly different between soil types, or between sites, and were consistent with those obtained previously (1, 3, 4). Viable counts of phage done by plaque counting on S. quinivorans CP6 and P. aeruginosa PU21, both before and after enrichment, did not give any counts of CP6 phage CP6-1 (plaque count) ND, none detected (limits of detection were \(1.00 \times 10^5\) for viable counts and \(<1.70 \times 10^5\) for TEM phage particle counts).

**Table 1. Testing recovery of the S. quinivorans CP6 phage CP6-1 from homogenized rhizosphere soil samples from Cardiff**

<table>
<thead>
<tr>
<th>Bacteria or phage</th>
<th>Count (g of rhizosphere soil(^{-1}))(^a) of bacteria or phage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
</tr>
<tr>
<td>Viable bacteria on TSBA</td>
<td>(7.30 \times 10^6)</td>
</tr>
<tr>
<td>CP6-like bacteria</td>
<td>(1.67 \times 10^7)</td>
</tr>
<tr>
<td>Phage CP6-1 (plaque count)</td>
<td>ND</td>
</tr>
<tr>
<td>Phage CP6-1-like particles</td>
<td>ND</td>
</tr>
<tr>
<td>with tails (TEM count)</td>
<td>(4.43 \times 10^6)</td>
</tr>
<tr>
<td>VLPs (TEM count)</td>
<td>(6.43 \times 10^6)</td>
</tr>
</tbody>
</table>

\(^a\) Counts are means from triplicate experiments; average coefficients of variation were 47% for colony and plaque counts and 16% for TEM counts.

\(^b\) ND, none detected (limits of detection were \(<1.00 \times 10^5\) for viable counts and \(<1.70 \times 10^5\) for TEM phage particle counts).

These hosts (<10 PFU g\(^{-1}\)). This was surprising because large numbers of phage growing on CP6 have been recorded previously, when sugar beet was growing at the Oxford site (1, 4). TEM phage counts revealed tailed phage of varied morphology (Fig. 1f to i) in most soil samples, but VLPs (Fig. 1j to m) were more abundant. The average numbers (grand means) of VLPs and tailed phage were \(1.46 \times 10^7\) and \(4.89 \times 10^7\) g\(^{-1}\), respectively. This amount of tailed phage was 3.3% of the VLP count, and VLPs were 0.4% of the total direct bacterial count (mean = \(3.64 \times 10^7\) g\(^{-1}\)). There were significant differences between the tailed phage and VLP counts (\(P < 0.001\)). Although there was no difference in phage counts between rhizosphere and bulk soil samples (\(P = 0.386\), the Cardiff counts (tailed phage count = \(9.8 \times 10^7\) g\(^{-1}\); VLP count = \(2.1 \times 10^7\) g\(^{-1}\)) were significantly greater than the Oxford results (tailed phage count = \(1.2 \times 10^7\) g\(^{-1}\); VLP count = \(9.0 \times 10^6\) g\(^{-1}\); \(P = 0.022\)). These results contrasted with the total direct counts of bacteria, where means for soil type were not different, but for these counts, Cardiff results (\(2.6 \times 10^7\) g\(^{-1}\)) were significantly less than those from Oxford (\(4.3 \times 10^7\) g\(^{-1}\)). This indicates that different soil types might harbor different populations of bacteriophage irrespective of the size of the population of bacteria.

The overall average (grand mean) of all the total counts we have made of viruses in soil was \(1.5 \times 10^8\) g\(^{-1}\). However, spiking the soil with phage CP6-1 showed that this overall count probably underestimated the real count by 40-fold for tailed phage and 8-fold for VLPs. Although storage in fixative can reduce total counts of viruses to about half after 7 days (9), this does not account for the reduction in counts we have observed. So, in view of the abrasive nature of soil, this underestimation was probably due to viral damage during sample processing. Assuming natural phage was similarly underestimated, virus numbers in soil averaged \(1.5 \times 10^8\) g\(^{-1}\), which is equivalent to 4% of the total population of bacteria (\(3.6 \times 10^9\) g\(^{-1}\)). The overall best estimate we can make of the virus-to-bacterium ratio in our soil is 0.04. This ratio is lower than almost all the values from either sediment (0.1 to 55; \(n = 4\)) (10, 11, 13, 28) or water (0.03 to 76; \(n = 26\)) (32). However, the high total count of bacteriophage confirms that they have an important role in soil.

All samples of DNA extracted from the soil and rhizosphere hybridized very strongly at medium stringency with the cocktails of both S. quinivorans CP6 and P. aeruginosa PU21
phages, indicating the presence of *Serratia* and *Pseudomonas* phage DNA. The numbers of colonies from SSM and PSIA plates hybridizing with the two phage cocktails was highly variable (0 to 23%), but overall there were more bacteria containing CP6 phage sequences on the SSM plates than there were containing PU21 phage sequences on the PSIA plates (Table 3). DNA was then extracted from 49 strongly hybridizing colonies from each medium and hybridized with the phage cocktails at high stringency. The results confirmed that both sets of colonies contained both types of phage but that more SSM than PSIA colonies probed positive. Large numbers of the SSM colonies contained sequences hybridizing with CP6 (29%) and PU21 (37%) phages, whereas the PSIA colonies predominantly contained *Pseudomonas*, rather than *Serratia*, phage DNA (8 and 2%, respectively, hybridizing). In only two cases, both from SSM, did single colonies probe positive for both phages, but these were not CP6-like colonies. However, many of the SSM colonies probed positive with the *Pseudomonas* phage cocktail. These results support the idea that phage genomes are constructed in a modular way, as mosaics of related gene cassettes, with access, by horizontal transfer, to a large environmental common genetic pool (17, 19, 29). Genetic remnants of past phage infections can be present as, for example, bacteriocin genes (23) and pathogenicity islands within host chromosomes. So, the presence of phage sequences in soil DNA and bacteria isolated from soil does not prove the presence of lytic or temperate phage.

The only previous phage hybridization study of this type showed, like our results, that 2 to 37% of soil bacteria harbored DNA homologous to *P. aeruginosa* phage genomes (24). So, if the proportion of hosts containing phage DNA is similar for other bacteria, a high proportion of soil bacteria could be infected by phage. More research needs to be done to determine the proportion of cultivable natural bacteria hybridizing positively with phage DNA that actually contain viable bacteriophage. Infected hosts will release many phage progeny, where nutrient and other environmental conditions favor host growth. So, our hybridization results go some way towards confirming the abundance and importance of bacteriophage in terrestrial ecosystems. In view of the large biomass of bacteria in the biosphere (30), our results indicate that predation of bacteria by viruses will be an important factor in controlling and stimulating the growth of bacterial populations in soil.

Similar discoveries in marine waters over 10 years ago (5) have now been incorporated into many oceanic carbon cycle models (20). So, such interactions between phage and bacteria should be incorporated into the detailed models of the carbon cycle in water and soil used to predict the extent of global warming (34). Furthermore, some of our test viruses in the hybridization experiments were transducing phage (3, 24), so our results support the importance of bacteriophage for mediating gene transfer in soil.

**ACKNOWLEDGMENTS**

The research described here was done with the support of a research grant from the United Kingdom Natural Environment Research Council. Technical assistance is gratefully acknowledged to Aaron R. Jeffries for help with some of the phage-counting development and from William Parkes for the acridine orange direct counts of bacteria in soil.

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