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Characterization of two novel *Rhizobium leguminosarum* bacteriophages from a field release site of genetically-modified rhizobia

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Abstract

Two *Rhizobium leguminosarum* biovar *viceae* bacteriophages with contrasting properties were isolated from a field site in which the survival of genetically modified *R. leguminosarum* inoculants had been monitored for several years. Inoculant strain RSM2004 was used as the indicator for phage isolation and propagation. One phage, RL1RES, was temperate and could not replicate in any of the 42 indigenous *R. leguminosarum* field isolates tested although nested PCR indicated that phage sequences were present in six of the isolates. The second phage, RL2RES, was virulent, capable of generalised transduction, contained DNA with modified cytosine residues, and was capable of infecting all field isolates tested although the GM inoculant strain CT0370 was resistant. Sequence with homology to RL2RES was detected by nested PCR in six of the 42 field-isolates. These were not the same isolates that showed homology to RL1RES. The implication of these findings for the survival of rhizobial inoculants, and the ecology of phages and their host bacteria, are discussed.

Introduction

Bacteriophages are ubiquitous in the environments of their host bacteria. They can be isolated directly, by enrichment, or from bacterial lysogens. Despite this, little is known about their distribution, survival, diversity, properties and ecology. For example, it is not clear whether phages can significantly effect host numbers in soil. Virulent phages have been postulated to cause population crashes in marine cyanobacterial populations (Bratbak et al. 1990). However, the extra mobility associated with aquatic systems allows a much higher probability of new phage-host interactions than the structured and relatively static situation in soil. The possibility that a build-up of phage capable of infecting symbiotic rhizobia was responsible for the decreased growth ('sickness') of host legume plants, was discounted by Kleczkowska (1957) who did, however, show a correlation between the presence of phages and susceptible rhizobia. More recently, it has been suggested that phage numbers may act as an indirect indicator of host population size (Marsh & Wellington 1994; Ashelford et al. 1999), with plaque counts a potential alternative to colony counts for monitoring bacterial populations in soil. However, such approaches rely on what is likely to be an over-simplistic interpretation of the phage-host relationship. For instance, a study of *R. leguminosarum* biovar *trifolii* and its phages in a clover pasture found that, whilst numbers of both were related to vegetation height, phages also fluctuated with soil moisture whereas the rhizobia were affected by solar radiation (Lawson et al. 1987).

Bacteria-phage interactions have other important consequences for microbial ecology. The presence in lysogenic bacteria of the genomes of temperate phages, or of virulent phages in pseudolysogeny, can have a significant phenotypic effect on the host bacteria (Barnet & Vincent 1970). Importantly, phages are a potential mechanism for the dissemination of genes within groups of related bacteria, especially if they are able to survive for long periods in the environment in the absence of hosts, or have a wide host range.

Both temperate and virulent phages have been de-

scribed for *R. leguminosarum,* and a significant number of nodule isolates have been shown to be lysogens. Seven lysogens were identified following induction with UV light, in a collection of 40 *R. leguminosarum* biovar *viciae*, and *trifolii* strains (Scwhinghamer & Reinhardt 1963). One lysogen was detected without induction, in a collection of 98 *R. leguminosarum* biovar *viciae* isolates (Hirsch 1979). Phages were isolated from 27 of 65 soil samples tested (Buchanan-Wollaston 1979a), two of which were capable of generalised transduction (Buchanan-Wollaston 1979b).

Here, we report two novel bacteriophages with contrasting properties, isolated from a field release site of genetically modified rhizobial inoculants (Hirsch 1996). The properties of these phages are described, and also their relationships with native rhizobia isolated from the field and with the introduced inoculants.

Materials and methods

Bacterial strains, media and growth conditions

Strains of *Rhizobium leguminosarum* biovar *viciae* are described in Table 1. Field isolates were obtained from pea (*Pisum sativum*) root nodules and plasmid profiles were assessed as described previously (Hirsch & Skinner 1992). For comparison we included RL38JI, a virulent phage capable of generalised transduction in *R. leguminosarum,* isolated from a Norfolk field soil where peas had been growing (Buchanan-Wollaston 1979a, b).

Rhizobia were cultured routinely at 28 °C on TY media (Beringer 1974); for work with bacteriophages, yeast-sucrose phage broth (YSPB) (Vincent 1970) was used. Solid media contained 1.5% agar (BiTek, Difco Laboratories, USA) and soft agar overlays contained 0.6% agarose (SeaKem, FMC Co., USA). Rhizobial lawns were made in 90 mm Petri dishes overlaid with 3 ml soft agar at 42 °C to which had been added 200 μl rhizobial culture in late logarithmic phase (ca. 10^8 cfu ml^{-1}) together with phage preparations. For large scale phage preparation, 3 ml YSPB aliquots were added to plates with confluently-lysed lawns and shaken for 2 h. The liquid was decanted and centrifuged at 8000 *g* for 10 min and then passed through a 0.22 μm Nalgene filter (Techmate Ltd., UK). These cell-free phage preparations were stored at 4 °C.

Isolation of bacteriophages from soil

Phage, were isolated from soil using a modification of the enrichment method described previously (Patel & Craig, 1984). Soil samples from the field release site, where the genetically modified rhizobial inoculants CT0370 and RSM2004 had been introduced, were suspended in 500 ml YSPB at 1 g ml^{-1}, shaken for 1 h and passed through muslin. The suspension was added to 500 ml YSPB broth, inoculated with 10 ml of a late log-phase RSM2004 culture and incubated for 48 h. From the enrichment culture, 100 ml was removed and centrifuged at 5000 *g* for 20 min, the supernatant was decanted and spun at 9,000 *g* for 30 min, 50 ml of this supernatant was passed through a 0.22 μm filter. Aliquots of 1 ml were added to soft agar overlays containing RSM2004, and incubated until plaques became visible. Plaques were picked, placed in 200 μl YSPB, resuspended by vortex-mixing, spun in a microcentrifuge for 5 min at 12,000 rpm and the supernatant passed through a 0.22 μm filter and then added to an overlay containing RSM2004.

Host range assesment

R. leguminosarum biovar *viciae* field isolates with unique plasmid profiles were incorporated into overlays onto which 10 μl of phage serial dilutions in YSPB were spotted. Plates were assessed for lysis or plaques in the area of the spot when the lawns had grown.

Electron microscopy

Phages were transferred to EM grids by placing a grid on a drop of high-titer phage preparation. They were negatively-stained by transferring the grid to a drop of 2% uranyl acetate for 15 min and then rinsed by two sequential transfers to H_2O. Grids were examined in a Joel 1200 Electron Microscope.

Generalized transduction

An equal volume of late log-phase JI248 TY culture and phage prepared from RSM2004 overlays (c. 10^9 pfu ml^{-1}) were mixed and incubated for 2 h. This mix was then plated onto selective TY plates containing neomycin (100 μg ml^{-1}). A control culture of JI248 was plated on the selective agar, and the phage preparation was plated onto non-selective TY media to confirm that it did not contain viable bacterial cells. Colonies growing on neomycin were replica-plated

Table 1. R. leguminosarum biovar *viciae* strains

Strain	Origin	Source* (Reference)
JI248	*Vicia faba* root nodule, Norfolk, UK	(Hirsch & Spokes 1994)
RSM2001	JI248 derivative, str^r, rif^r	
RSM2004	RSM2001 derivative, Tn*5* insertion in pSym (neo^r) i.e pRL1JI::Tn*5*	
VF39	*Vicia faba* root nodule, Bielefeld, Germany	W. Selbitschka Bielefeld
LRS39401	VF39 derivative, cured of pSym, str^r	(Selbitschka et al. 1995)
CT0370	LRS39401 derivative, str^r sp^r, *gusA* insertion	
CT0370 (pRL1JI::Tn*5*)	CT0370 transconjugant, pSym from RSM2004	
P262Sp^r	*P. satvium* nodule isolate, sp^r, Dijon, France, cured of pSym	N. Amarger, Dijon
T2	P262Sp^r transconjugant, pSym::Tn*5* from RisoA10	(Amarger & Delgutte 1990)
JI307	*P. satvium* nodule isolate, lysogenic, Peterborough, UK	(Buchanan-Wollaston 1979a)
B10Str^r	*P. satvium* nodule isolate, str^r, lysogenic, Erlangen, Germany	W. Lotz, Erlangen (Tichy & Lotz 1985)

*Where no source is designated, strain is from our collection.
str^r – streptomycin resistant, rif^r – rifampicin resistant, neo^r – neomycin resistant, sp^r – spectinomycin resistant.

onto TY plates containing rifampicin (50 μg ml^{-1}) to confirm that they were not RSM2004 contaminants.

Lysogen generation

A small number of colonies were able to grow on confluently-lysed plates. These were picked and used as indicator strains to test for susceptibility as described for the field isolates. Cultures of these potential lysogens grown in TY for 48 h were centrifuged at 8,000 *g* for 15 min, then the supernatant was passed through a 0.22 μm filter and added to RSM2004 overlays. Other putative lysogen cultures were exposed to 300 μJ UV irradiation (Stratalinker, Stratagene, UK) after 24 h incubation in TY and then incubated for a further 24 h. A cell free extract was prepared and assayed as above.

Isolation and cloning of bacteriophage DNA

Phage DNA was prepared using Qiagen (Qiagen Ltd, UK) lambda purification kits with phage suspensions of more than 10^9 pfu ml^{-1}. To generate fragments for cloning, phage DNA was digested with *Sau*3A or *Mbo*1 and heated at 65 °C for 20 min to denature the enzymes before purifying with phenol/chloroform followed by ethanol precipitation. The digests were ligated using T4 ligase to *Bam*H1-digested pBluescript$^{(TM)}$ (Stratagene Europe, UK), that had been alkaline phosphatased and purified as for genome digests. The ligation mix was used to transform library-efficiency competent *E. coli* DH5α (Gibco BRL Life Technologies Ltd, UK). Potential inserts were PCR-amplified with M13 forward and reverse 18-mers (Promega UK) and visualised after agarose gel electrophoresis. The PCR fragments were transferred to positively-charged nylon membrane (Boehringer Mannheim GmbH, Germany) and hybridized with DIG-labelled phage DNA (Boehringer Mannheim) according to the manufacturer's protocol (this system was also used for plaque lifts). Cloned phage DNA (Qiagen) was used to generate DIG labelled probes, and 500 ng used in PCR sequencing reactions (PE Applied Biosystems, USA) as recommended by the manufacturer, with M13 forward and reverse 18mers.

PCR detection of phage specific sequences in field isolates

Phage-specific PCR primers were designed to amplify regions from two of the phage genomic clones, 1-9fl derived from RL1RES and 2-8 derived from RL2RES. Primers showed no significant homology to sequences in the GenEmbl database. Nested primers were designed to reduce the possibility of PCR products arising from spurious primer homologies.

The following primers were used to detect RL1RES homologous DNA in *R. leguminosarum* biovar *viciaes* trains and field isolates, generating products of 902 bp and 607 bp, respectively: 5'-GATCGGTAGCGGCGCTATTGCTG-3' and 5'-GATCTTGGCGCCGAGCCACGTG-3'; nested primers 5'-CAATGTCGCGCGCCATTATCC-3' and 5'-GATCTTGGCGCCGAGCCACGTG-3'. PCR was performed in a 20 μl reaction volume containing 2.5 ng DNA for the first reaction (or 0.1 μl of the first reaction for the second, nested reaction), 4 pmol of

each primer, 200 μM dNTPs, 1.5 mm mg$Cl_2$and 0.4 U Taq DNA polymerase (Boehringer Mannheim). The reaction mixture was incubated for 2 min at 94 °C and then subjected to 25 cycles consisting of 30 s at 94 °C, 30 s at 57°C and 1.5 min at 72°C, with a final step of 5 min at 72°C.

The following primers were used to detect RL2RES homologous DNA in *R. leguminosarum* biovar *viciae* strains and field isolates, generating products of 173 bp and 134 bp, respectively: 5'-ATCAATCCTGGTGTGATCG-3' and 5'-TAAGGTCGTCGAACTGTCTC-3'; nested primers 5'-TGGTAGCACGAACCCAGC-3' and 5'-TTATCGCACTGGTGGACG-3'. The reaction and cycling conditions were as above, except for an annealing temperature of 55°C and an extension time of 30 s.

Soil from the field release site was seeded with different amounts of RL2RES before extracting DNA from the soil microbial community and subjecting it to PCR (Cullen & Hirsch 1998).

The sequences of 1-9fl and 2-8 have been submitted to the GenEmbl database under the accession numbers AJ249281 and AJ249282, respectively.

Results and discussion

Isolation and characterisation of bacteriophage

Following the enrichment and isolation proceedure with RSM2004, 18 plaques were identified on RSM2004 lawns. Two distinct phage types were seen: 17 clear plaques and one smaller, turbid plaque. The process of enrichment made it impossible to compare the relative numbers originally present in the soil sample or to exclude the posssibility that the 17 plaques arose from one original phage particle. Phages from the turbid plaque, and from one of the clear plaques, were picked for further investigation, and designated RL1RES and RL2RES, respectively. RL1RES formed cloudy, plaques with a diameter of less than 1 mm (Figure 1A); RL2RES was lytic, forming 2–3 mm, clear plaques surrounded by cloudy haloes (Figure 1B), similar to those described by Barnet (1972).

Host ranges differed markedly: RL1RES formed plaques only on RSM2004 and related strains, although it was able to inhibit growth of some strains when high titers were spotted on lawns (Table 2). This phenomenon is presumed to be abortive infection, in which cells are lysed but the phage is

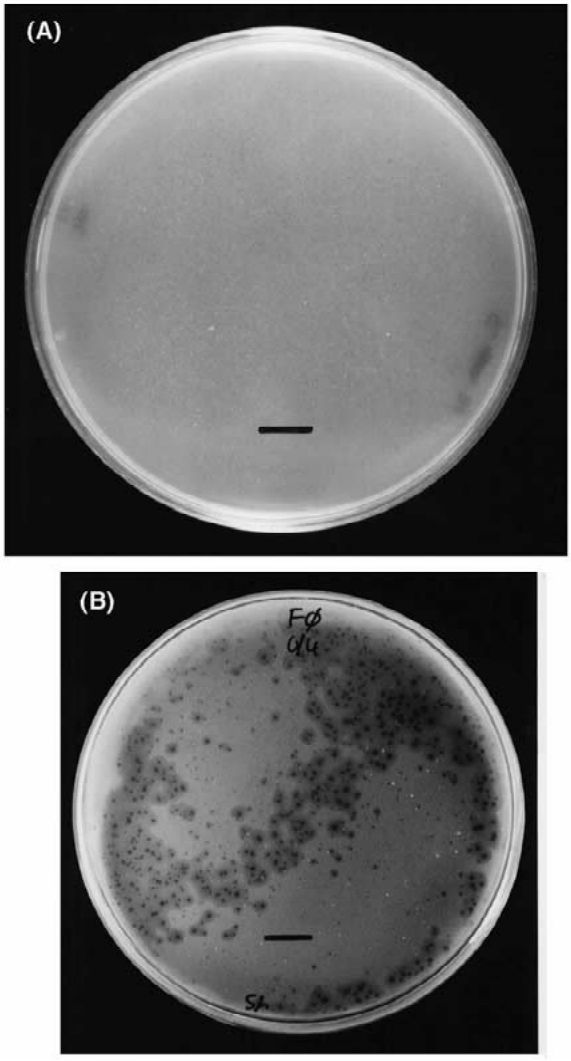

Figure 1. Plaque morphology on RSM2004 A – RL1RES, B – RL2RES. Bar represents 5 mm.

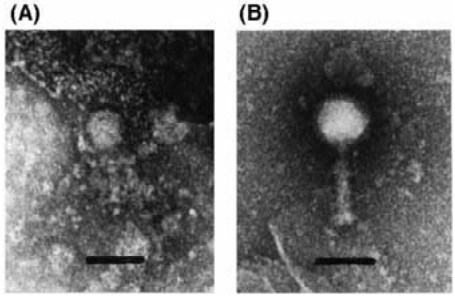

Figure 2. Electron micrographs of phages A – RL1RES, B – RL2RES. Bar represents 100 nm.

unable to replicate, i.e. to form plaques. Similar results have been observed in a previous study on *R. leguminosarum* (Buchanan-Wollaston 1979a). It is interesting that whilst Vf39 is resistant to RL1RES, the phage can lyse, but not replicate in, derivatives of Vf39 that are cured of their symbiotic plasmid, e.g CT0370. Transconjugants of CT0370 carrying the pSym from RSM2004 did not regain resistance to RL1RES, indicating that the phage immunity conferred by the Vf39 pSym is not associated with its symbiotic functions. Such immunity is probably due to one or more functions inhibiting phage attachment, infection or growth. This contrasts with earlier reports that rhizobial mutants resistant to phage had lost symbiotic abilities (discussed by Patel & Craig 1984). Strain P262Sp^r, which lacks a pSym, and the lysogenic strains JI307 and B10Str^r were all resistant to RL1RES. Similar findings were reported for RL38JI, which formed plaques on a derivative of a *R. leguminosarum* biovar *phaseoli* lacking its pSym, although the parental strain carrying pSym was resistant. In this case, the pSym was shown to be necessary but was not the only factor required for phage resistance (Jun et al. 1993).

In contrast to RL1RES, RL2RES was able to form plaques on 47% of *R. leguminosarum* biovar *viciae* field isolates tested, only Vf39 and its derivatives, and the lysogenic strain JI307, were totally resistant (Table 2).

Electron micrographs show RL1RES consisting of an icosahedral head, 60 nm diameter, with a tail of 110 nm (Figure 2A). Attempts to prepare RL1RES over chloroform caused a drop in infectivity from 10^9 pfu ml^{-1} to 10^3 pfu ml^{-1} after approximately 10 sec exposure. When viewed under EM, chloroform-treated preparations contained many fragmented and hollow phage heads (micrograph not shown). The chloroform sensitivity implifies that the head of RL1RES may contain lipid, although chloroform-sensitive phages that do not contain lipids have been reported (Mindich & Bamford 1988). The head size of RL1RES is similar to that of the temperate *E. coli* phage Lamda which has a genome of 48.5 kb. It appears to belong to the morphological type A of Bradley (1967), or family Myoviridae (Murphy et al. 1995).

RL2RES was similar in appearance to RL1RES, but larger, with an icosahedral 90 nm diameter head, a 140 nm tail, and a base plate structure (Figure 2B). The head size is similar to the virulent T4 phage of *E. coli* which contains 160 kb DNA. The size and appearance of RL2RES are similar to that of RL38JI (Buchanan-Wollaston 1979a). Both phages can be also assigned in the morphological type A of Bradley (1967) or the family Myoviridae (Murphy et al. 1995).

Generalised Transduction

No putative transductants were obtained with RL1RES, but when JI248 was infected with RL2RES isolated from RSM2004, neomycin resistant, rifampicin-sensitive colonies arose at a frequency of 1.2×10^{-8} transductants per JI248. No spontaneous neomycin-resistant JI248 colonies were obtained from the control cultures (frequency lower than 10^{-9}) and no bacteria were detected in either of the phage preparations (fewer than 1.2 cfu ml^{-1}). The gene encoding neomycin resistance in RSM2004 is part of a Tn*5* insert located on the pSym, whilst the rifampicin-resistance determinant is chromosomally located. Thus, tranduction of a plasmid-located gene was demonstrated with no co-transfer of the unlinked rif^r gene. It is likely that RL2RES mobilizes genes by generalised transduction, i.e. a small proportion of phage heads enclose fragments of the host genome. In experiments using isogenic derivatives of a different *R. leguminosarum* host strain, RL38JI was shown to be capable of transduction at frequecies of 10^{-6}– 10^{-7} per recipient, and also to be able to transduce entire plasmid genomes of more than 120 kb (Buchanan-Wollaston, 1979a, b). It is possible that the transduction frequency of RL2RES would be higher in different hosts. The capacity of RL2RES is likely to be similar to that of RL38JI but the maximum length of host DNA that can be packaged for generalized transduction has not yet been tested.

Lysogens

A lysogenic derivative was isolated from a colony growing on an RSM2004 lawn treated with a high titre of RL1RES to obtain confluent lysis. The isolate was resistant to subsequent RL1RES infection, its lawns contained extremely small plaques and cell-free extracts contained infectious phage only after UV irradiation. No resistant colonies grew on RSM2004 lawns confluently-lysed with RL2RES. Plasmid profiles showed no difference in the banding patterns of RSM2004 and the putative lysogen, indicating that the phage DNA had integrated into the bacterial chromosome.

Table 2. Bacteriophage host ranges

R. leguminosarum strain	RL1RES	RL2RES
Field isolates 1-42	31 R, 11 N, 0 S	0 R, 20 N, 22 S
RSM2004, RSM2001, JI248	S	S
VF39	R	R
CT0370, LRS39401	N	R
CT0370(pRL1JI::Tn*5*)	N	R
P262Sp^r, T2	R	S
JI307	R	R
B10Str^r	R	S

R – resistant, no clearing on indicator plates, even at high concentrations N – non-replicating, no plaques but able to lyse indicator strain at high concentrations S – sensitive, plaques demonstrate replication of phage in indicator strain.

Table 3. Properties of restriction enzymes and their effect on RL2RES DNA

Enzyme	Recognition Site	Sites Cut	Sites not cut	Effect on RL2RES
*Sau*3A	GATC	G^{m6}ATC	GAT^{m5}C	No digestion
		GA^{hm5}UC	GAT^{m4}C	
			GAT^{hm5}C	
*Rsa*I	GTAC	GTA^{m5}C	GT^{m6}AC	No digestion
			GTA^{m4}C	
*Mbo*I	GATC	GAT^{m4}C	G^{m6}ATC	Discrete bands
		GAT^{m5}C	GAT^{hm5}C	
			GA^{hm5}UC	

^{m4}C= N4-methylcytosine; ^{m5}C= N5-methylcytosine; ^{hm5}C= hydroxymethylcytosine; ^{hm5}U= hydroxymethyluracil; ^{m6}A= N6-methyladenine – (McClelland et al. 1994)

DNA isolation, characterisation relationship to indigenous rhizobia

Preparations of RL1RES nucleic acids were susceptible to non-specific degradation, even after phenol treatment. Only *Tru*1I which requires an incubation temperature of 65 °C gave a distinct band pattern (data not shown), whereas other restriction enzymes (*Sau*3A, *Bam*H1, *Hin*dIII, *Eco*R1, *Mbo*1, *Pst*1) generated a non-specific smear. Despite this, several clones were obtained from *Sau*3A digests. One clone, when blotted against digests of RSM2004, RSM2004 lysogen and RL1RES DNA, gave identical bands with both RSM2004 and the lysogen, but did not hybridise to the phage DNA. This indicated that it was host RSM2004 DNA, which must have contaminated the original phage DNA preparation. However, it also demonstrates that the phage DNA preparation did not contain significant amounts of RSM2004 DNA as it did not give a positive signal with the RSM2004-derived probe. A second clone, 1-9fl, hybridised to both the phage and RSM2004 DNA digests, indicating that this clone was derived from the phage. The sequence had little similarity to those in the GenEmbl database. Both RSM2004 and its lysogenic derivative gave a PCR product of 607 bp (the size predicted for RL1RES), when screened with the nested primers designed to amplify RL1RES. This indicates that RSM2004 contains a sequence closely related to part of RL1RES and explains the homology observed on genomic DNA blots, although since it is sensitive to RL1RES, it is unlikely that the entire genome is present in RSM2004. Similarly, when the *R. leguminosarum* biovar *viciae* field isolates were screened, 14% (six out of 42 isolates tested) gave a product of 607 bp, although only five of these were resistant to RL1RES. These results indicate that rhizobia lysogenic for RL1RES, or a closely related phage, are quite common in the field. In contrast, neither the susceptible derivatves of Vf39, nor the resistant parental strain, gave a PCR product with the RL1RES-specific primers.

RL2RES DNA was highly resistant to restriction digestion, and, among the four-base recognition site

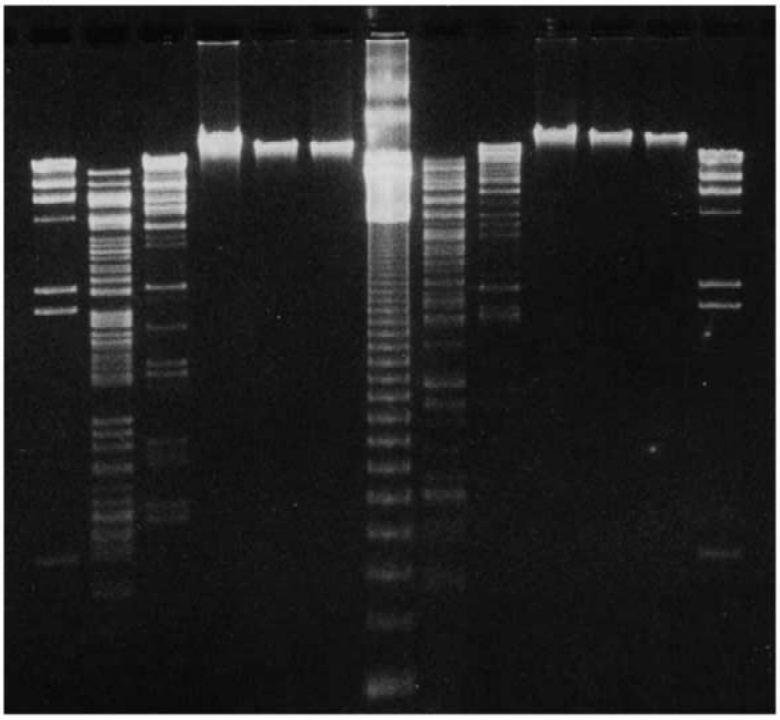

Figure 3. Restriction enzyme profiles of phage DNA Tracks: 1, 13, size marker λ *Hin*dIII; 7, 123 bp ladder (Life Technologies Ltd, UK); 2 – 5, RL38JI; 8 – 12, RL2RES. Digests in track: 2, 8, *Tru*1I; 3, 9, *Mbo*I; 4, 10, *Rsa*I; 5, 11, *Sau*3A; 6, 12, undigested.

enzymes tested, it was cleaved by *Tru*1I and *Mbo*I but not by *Rsa*1, *Sau*3A, or *Hae*III. Since *Tru*1I recognises TTAA and, unlike the other enzymes tested, *Mbo*I is not affected by cytosine methylation (Table 3; McClelland et al., 1994), it is highly likely that RL2RES DNA has a modification on the cytosine ring. The restriction sensitivity profile indicates that this could be methylation at the N4 position, however atypical modification patterns have been described in other phages: RL38JI has an unusual cytosine modification and has the same resistances to restriction digestion (Swinton et al., 1985). Like RL2RES, RL38JI was not cut by *Rsa*1 or *Sau*3A but digested with *Mbo*I and *Tru*1I to give banding patterns distinct from those of RL2RES, demonstrating that they are different phages (Figure 3). Similar findings have been reported in virulent phages of *R. leguminosarum* biovar *trifolii*, which have a similar morphology to RL2RES and RL38JI, and are resistant to a range of restriction endonucleases, all of which have cytosine in their recognition sites (Kankila & Lindstrom, 1994). In *E. coli* infected with bacteriophage T4, all cytosine residues are replaced by 5-hydroxymethyl cytosine that is glycosylated after incorporation into DNA (Kornberg & Baker, 1992). Phage modification systems probably evolved to overcome destruction by the restriction enzymes of potential host bacteria, thus it is unsurprising that they occur in rhizobial phages, especially those with broad host ranges.

The sequence of the *Mbo*I fragment from RL2RES did not show significant homology to sequences in the GenEmbl database. Probes generated from the cloned fragment hybridized to RL2RES DNA and to plaque lifts but not to RSM2004 DNA. However, when the 42 field isolates were screened with nested PCR primers specific for RL2RES, a product of the predicted size was obtained with six isolates (14%). All of these six isolates were fully sensitive to RL2RES. They were not the same isolates that had sequence homology to RL1RES. Interestingly, parental Vf39 also gave a product of the predicted size whereas its derivatives lacking a pSym and transconjugants containing a new pSym from RSM2004 did not, although all of these were resistant to RL2RES. The bacteriophage-related sequences on the pSym of Vf39 might also confer immunity to RL1RES.

DNA extracted from the soil microbial community gave a product of 173 bp (the predicted size) with the first set of RL2RES-specific primers, when the soil had been seeded with at least 10^4 pfu g^{-1} phage.

Conclusions

The two bacteriophages had very different morphologies and properties. RL1RES is temperate and unable to replicate in any of the indigenous field isolates although six contained related sequences, five of which were fully resistant to the phage, indicating that lysogeny may be a common and effective survival strategy of phages. The isolates that gave PCR products with RL1RES-specific primers may contain the entire genome of RL1RES or that of a closely related phage, or retain part of the genome following abortive infection. It has not been established whether the phage exists as an autonomously-replicating prophage or integrates into the host genome: if the latter is the case, RL1RES would probably be capable of specialised transduction; however, no generalised transducing ability was detected.

In contrast, RL2RES was virulent and could either plaque or lyse all 42 field isolates tested. The french and german isolates were also sensitive with the exception of Vf39 and its derivatives. After a release in the field, CT0370 survived at relatively high levels, c. 5 x 10^4 cfu g^{-1} soil, similar to the indigenous *R. leguminosarum* population (Hirsch 1996) and it is possible that resistance to RL2RES was a contributing factor. In contrast, numbers of RSM2004 fell down to around 10^2 cfu g^{-1}soil following release (Hirsch & Spokes 1994). RL2RES could replicate in about 50% of indigenous field isolates and lyse the others, indicating that the presence of a virulent phage has not selected for a resistant host population. This suggests that, in the field, RL2RES lytic infection is not a common event. The presence of RL2RES-related sequences in six of the sensitive isolates could be explained, if phage sequences remained following abortive infection or pseudolysogeny with a closley related or identical phage.

The presence of sequences from both virulent and temperate phage in indigenous bacteria indicates that phage-bacteria interactions occur in soil (although the frequency is unknown as they may have co-existed for millenia), and also that cells can survive infection by virulent phage. Nevertheless, the majority of isolates did not show homology to the phage-specific primers although all were sensitive to, and potential hosts for, RL2RES. One reason is likely to be the heterogeneous structure of soil, which results in spatial separation of the phage and host. However, where phage and susceptible bacteria coincide, especially in regions of enhanced growth such as the rhizosphere, infection will probably occur to make gene transduction possible. Since transduction can occur in hosts where phage cannot replicate (Jun et al. 1993), all the native field isolates are potential recipients of genes by transduction, although the probability of this is very low. Nevertheless, virulent phages such as RL2RES can provide a potential reservoir for bacterial genes in conditions where the host might not survive.

It would be interesting to know the relative numbers of the two phage genomes in the soil, as phage particles and in infected host cells, as they represent different survival strategies. Temperate RL1RES probably exists mainly in lysogenic bacteria whereas virulent RL2RES will experience a rapid increase in population on infection of a host micro-colony, but then must survive in particles until another hosts appears. The possibility that these phage have other, non-rhizobial hosts, cannot be discounted. Different hosts could result in different properties and so change the way in which their ecology is interpreted. The isolation of phages by enrichment is unsatisfactory for determining numbers but quantitative PCR with phage-specific primers may be used to enumerate RL1RES and RL2RES in soil-extracted DNA, although the presence of related sequences in indigenous rhizobia could complicate the interpretation of results. Nevertheless, PCR with phage-specific primers shows considerable promise for future studies on the ecology of phages in soil.

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