Phage Induction of Lysogenic *Rhizobium leguminosarum* biovar *trifolii* in both the Free-living and the Symbiotic Form

By TOSHIKI UCHIUMI,* YASUHIRO ONO, MIKIKO ABE AND SHIRO HIGASHI

Department of Biology, Faculty of Science, Kagoshima University, Kagoshima 890, Japan

(Received 17 April 1989; revised 10 July 1989; accepted 10 August 1989)

A lysogenic strain, *Rhizobium leguminosarum* biovar *trifolii* UK-1: ϕ U, was isolated from a wild white clover nodule. It was symbiotically effective on white clover. A lysogenic phage (U-mole) was induced from this strain by treatment with either UV irradiation or mitomycin C. Phage U-mole had an icosahedral head (40 nm wide), a short tail (9 nm long) and tail fibres (15 nm long). Phage U-mole was induced with mitomycin C both from bacterial cells in infection threads and from bacteroids in nodules. Southern hybridization using *Eco*RI fragments of phage U-mole as probe indicated that phage U-mole DNA was integrated into the chromosome of strain UK-1: ϕ U at a site involving the 6.0 kb *Eco*RI fragment of phage U-mole. Phage U-mole also lysogenized the wild-type strain *R. leguminosarum* biovar *trifolii* 4S. The resulting lysogenized strain, 4S: ϕ U, had lost its 315 kb Sym plasmid and its nodulation ability.

INTRODUCTION

Bacteriophages of *Rhizobium* (rhizobiophages) have been isolated from soils and root nodules (Demolon & Dunez, 1935; Kowalski *et al.*, 1974). Kleczkowska (1950, 1965) and Gupta & Kleczkowska (1962) suggested a role for rhizobiophages in the evolution of ineffective rhizobial strains. Barnet (1979) and Raleigh & Signer (1982) reported that symbiotic-defective mutants were isolated as surviving cells after exposure to specific virulent phages. They also referred to changes in other morphological and physiological properties but the mechanism of loss of symbiotic properties was unknown.

Transduction systems using lysogenic phages of *R. meliloti* or *R. leguminosarum* have been established and used to analyse *Rhizobium* genes (Kowalski, 1967; Finan *et al.*, 1984; Martin & Long, 1984; Sik *et al.*, 1980; Buchanan-Wollaston, 1979). In addition, genetic analyses of phage 16-3, the lysogenic phage of *R. meliloti*, have been done in detail and its possibility as a useful cloning vector has been discussed (Orosz, 1973; Dorgai *et al.*, 1981, 1986). In view of the symbiotic nitrogen fixation ability of *Rhizobium*, it is important to study phage gene expression in members of this genus under both free-living and symbiotic conditions.

We report here the isolation and properties of a lysogenic phage of *R. leguminosarum* biovar *trifolii*. Its role in symbiotic effectiveness was also assessed.

METHODS

Bacterial strains and media. *Rhizobium leguminosarum* biovar *trifolii* UK-1: ϕ U (original host strain of phage U-mole) was isolated from a nodule of a wild white clover plant (*Trifolium repens*) in the campus field and lysogeny detected according to Ördögh & Szende (1960). Wild-type *Rhizobium leguminosarum* biovar *trifolii* strain 4S (Higashi & Abe, 1980) and its derived mutants, A1 (Higashi & Abe, 1980) and H1 (Higashi *et al.*, 1983), were used as indicators or hosts of phage U-mole. These strains were maintained on mannitol/yeast agar slants (Keele *et al.*, 1969). For phage induction and plasmid isolation, TY medium (Beringer, 1974) was used to avoid excess production of exopolysaccharides. *Escherichia coli* RR1 carrying plasmid pRt032, which has *nod* genes of *R. leguminosarum* biovar *trifolii* (Schofield *et al.*, 1984) was cultured in LB medium.

Preparation of phage U-mole lysates. *R. leguminosarum* biovar *trifolii* UK-1: ϕ U was grown at 28 °C in 100 ml TY liquid medium to about 5×10^8 cells ml⁻¹ and mitomycin C was added to a final concentration of 1 μ g ml⁻¹. After 6–18 h incubation at 28 °C with vigorous shaking, 0.5–1.0 ml chloroform was added and incubation continued for 30 min. Bacterial cells and cell debris were removed by centrifugation at 10000 g for 10 min. The supernatant obtained was dialysed against phage buffer (Sik *et al.*, 1980) overnight to remove chloroform.

Phage sensitivity test (spot test). Phage lysates of phage U-mole or virulent phages 4S-phage (Higashi & Abe, 1978), and kp1 and kp2, isolated from soil samples using *R. leguminosarum* biovar *trifolii* 4S as a host, were spotted on test strains. The plates were examined for bacterial lysis after 3 d incubation at 28 °C.

Lysogenization of *R. leguminosarum* biovar *trifolii* 4S with phage U-mole. Phage U-mole was spotted on plates of strain 4S, and incubated for 7 d at 28 °C. Colonies that had grown on the lytic area were isolated and purified by clonal isolation. Phage induction by UV irradiation (15 W at 50 cm height for 30 s) and immunity against phage U-mole were demonstrated with each isolate.

Isolation of phage U-mole DNA and plasmid DNAs. Phage U-mole DNA was prepared as described by Yamamoto *et al.* (1970) and Dallmann *et al.* (1979), using polyethylene glycol 6000 and Actinase E (Kaken Seiyaku Co., Japan) followed by phenol extraction. For plasmid isolation from *Rhizobium*, clear lysates were prepared as described by Casse *et al.* (1979) except that the SDS concentration was 4% (w/v). Plasmid pRt032 was purified according to Birnboim & Doly (1979). Prepared DNAs were digested with restriction endonucleases according to standard methods.

Agarose gel electrophoresis. Phage U-mole DNA and plasmid DNAs were analysed on 0.7% agarose gels. Electrophoresis was performed at 100 V for 4 h for restriction fragments and at 50 V for 14 h for plasmids, using TB buffer (89 mM-Tris base, 2 mM-EDTA, 89 mM-boric acid, pH 8.4).

Southern hybridization. After agarose gel electrophoresis, the gels were treated with hydrochloric acid solution followed by sodium hydroxide solution. Denatured DNAs in the gels were electrophoretically transferred to Millipore GVHP304F0 filters employing a Trans-Blot cell (Bio-Rad). Phage U-mole DNA restriction fragments were labelled with ³²P using the Multiprime DNA labelling system (Amersham) and used as probes. Hybridization was performed at 60 °C for 18 h. Then the filters were washed with SSC solution as described by Southern (1975) and autoradiographed. Southern hybridization using pRt032 as the probe was done in the same way.

Nodulation test. Nodulation and nitrogen fixation tests of strains UK-1: ϕ U and 4S: ϕ U were done as described elsewhere in detail (Higashi *et al.*, 1983).

Electron microscopy. Phage specimens obtained by glycerol stepwise density-gradient centrifugation (Vande Woude *et al.*, 1979) were negatively stained with 2% (w/v) uranyl acetate. Nodules inoculated with strain UK-1: ϕ U or strain 4S: ϕ U were observed by scanning electron microscopy and transmission electron microscopy (Higashi *et al.*, 1986).

RESULTS AND DISCUSSION

Characteristics of Rhizobium leguminosarum biovar trifolii UK-1: ϕ U

To obtain lysogenic strains, 15 strains of *R. leguminosarum* biovar *trifolii* were isolated from wild white clover nodules and tested for lysogeny using *R. leguminosarum* biovar *trifolii* 4S as an indicator. One strain, UK-1: ϕ U, indicated lysogeny. This strain also formed effective nodules on white clover (*Trifolium repens* cv. Ladino). A cell homogenate of UK-1: ϕ U was prepared and examined by polyacrylamide gel electrophoresis. The activity staining patterns of esterase, acid phosphatase and basic phosphatase of strain UK-1: ϕ U were very similar to those of strain 4S (data not shown). A clear lysate of strain UK-1: ϕ U was prepared and separated on 0.7% agarose gel. Three mega-plasmids, of 525, 420 and 315 kb were detected (see Fig. 5a, lane 1, and Fig. 7a, lane 2).

Characteristics of phage U-mole

Table 1 shows the host range of phage U-mole determined by spot tests using a U-mole lysate (see Methods). Phage U-mole indicated lysogeny on *Rhizobium leguminosarum* biovar *trifolii* 4S and its derived mutants A1 and H1.

Electron microscopy of phage U-mole (Fig. 1) showed it to have an icosahedral head (diameter, 40 nm) and a short tail (length, 9 nm) resembling that of Salmonella phage P22 (Bradley, 1967) and rhizobiophage MM1H reported by Werquin *et al.* (1988). The tail has three parts, collar (diameter, 10 nm), rigid short tail, and thin fibres (length, 15 nm). The thin fibres, which have a spherical molecule at the end (Fig. 1b), may extend from the neck-collar region.

Fig. 1. Electron micrographs of lysogenic phage U-mole, negatively stained with uranyl acetate. (a) Bar, 100 nm. (b) The arrows indicate the spherical molecule of tail fibre. Bar, 50 nm.

Fig. 2. Restriction endonuclease patterns of phage U-mole DNA on 0.7% agarose gel. Lane 1, λ phage DNA digested with *Hind*III; lanes 2-7, phage U-mole DNA digested with *Hind*III, *Eco*RI, *Pst*I, *Sma*I, *Bam*HI and *Kpn*I, respectively.

Fig. 3. Transmission electron micrographs of phage U-mole in host plant cells. Nodules formed by *R. leguminosarum* biovar *trifolii* UK-1: ϕ U were treated overnight with mitomycin C before sectioning. (a) *R. leguminosarum* biovar *trifolii* UK-1: ϕ U in an infection thread (IT). The arrow indicates phage U-mole particles. Bar, 0.5 μ m. (b) phage U-mole particles (arrowed) in the host plant cell. Bar, 1 μ m. CW, host plant cell wall; Bd, bacteroid; PBM, peribacteroidal membrane.

According to the morphological classification of Bradley (1967), phage U-mole is a member of group C, *Podoviridae*.

DNA isolated from phage U-mole particles was digested with restriction endonucleases *Hind*III, *Eco*RI, *Pst*I, *Sma*I, *Bam*HI and *Kpn*I (Fig. 2). *Hind*III, *Eco*RI and *Pst*I gave at least ten fragments (lanes 2–4). *Sma*I and *Bam*HI gave six and four fragments, respectively (lanes 5 and 6). *Kpn*I did not digest the DNA (lane 7). The genome size of phage U-mole was estimated from these digests to be about 40 kb.

Fig. 4. Root protrusions induced by *R. leguminosarum* biovar *trifolii* 4S:φU. (a) White clover roots inoculated with *R. leguminosarum* biovar *trifolii* 4S:φU after 2 months incubation. The arrows indicate root protrusions. (b) Scanning electron micrograph of a longitudinal section of the root protrusion in (a). Bar, 400 μm. VB, vascular bundles.

Table 1. Characteristics of *R. leguminosarum* biovar *trifolii* strains against *U*-mole and the other virulent phages

Strain	Lysogenic phage U-mole	4S-phage	Virulent phage kpl	kp2	Symbiotic ability
UK-1:φU	R	S	S	R	+
4S:φU	R	S	S	S	—
4S	L	S	S	S	+
H1	L	S	S	S	—
A1	L	S	S	S	—

* R, Resistant; L, lysogeny; s, sensitive.

† +, Effective nodule formation; —, no nodule formation.

Nodules formed by UK-1:φU

The nodulation process of *R. leguminosarum* biovar *trifolii* UK-1:φU was followed by light microscopy. As with the other *R. leguminosarum* biovar *trifolii* wild-type strains, root hair curling and infection thread formation were observed. Several nodules were visible on clover roots 5 d after inoculation. The nodulation ability of strain UK-1:φU was the same as that of strain 4S as to the numbers of nodules formed. These nodules were functional, as indicated by acetylene reduction ability and growth of host plants.

The nodule exudate prepared from a surface-sterilized nodule formed a large number of plaques on indicator plates. This indicates that phage induction should occur both in bacteria and in bacteroids within host plant cells. Nodules were incubated overnight at 28 °C in sterilized water containing mitomycin C (10 μg ml⁻¹), then sectioned and examined by transmission

Fig. 5. (a) Crude plasmids from *R. leguminosarum* biovar *trifolii* strains prepared according to Casse (1979) and separated on 0.7% agarose gel; (b) Southern hybridization under stringent conditions using a *Hind*III digest of pRt032 as a *nod* probe. Lanes 1, UK-1: ϕ U; lanes 2, 4S; lanes 3, 4S: ϕ U.

Fig. 6. (a) Restriction endonuclease patterns of *R. leguminosarum* biovar *trifolii* strains digested with *Hind*III; (b) Southern hybridization using 32 P-labelled *Hind*III digest of pRt032 as a *nod* probe. Lane λ , *Hind*III fragments of λ DNA; lanes 1, UK-1: ϕ U; lanes 2, 4S: ϕ U; lanes 3, 4S. A 7.2 kb fragment in UK-1: ϕ U and 4S hybridized with the *nod* probe. No hybridization was observed with 4S: ϕ U.

electron microscopy. *Rhizobium* cells invade the host plant cells using infection threads, and then change their form to a bacteroid. Fig. 3(a) shows a bacterium in an infection thread and Fig. 3(b) shows a bacteroid surrounded by a peribacteroidal membrane. Highly electron-dense particles (arrowed in Fig. 3) were observed in both cases. In Fig. 3(b), the particles are apparently free in the host cell cytoplasm. Observation of these particles at higher magnification revealed them to be the same size and shape as phage U-mole heads. These particles appear to be phage U-mole. Although phage U-mole is expected to be induced spontaneously in host plant cells, the frequency of such spontaneous induction was not determined.

As phage U-mole gene expression is induced in strain UK-1: ϕ U under both symbiotic and free-living conditions, this phage may become a useful tool for gene transfer in a rhizobial symbiosis system.

Fig. 7. (a) Crude plasmids of *R. leguminosarum* biovar *trifolii* strains separated on 0.7% agarose gel. The arrows indicate additional bands (see Results and Discussion); chr, fragmented chromosomal DNA. (b) An *EcoRI* digest of phage U-mole DNA was used as a probe for Southern hybridization. Lanes 1, 4S; lanes 2, UK-1:φU; lanes 3, UK-1:φU incubated with mitomycin C ($1 \mu\text{g ml}^{-1}$) for 4 h; lanes 4, 4S:φU.

Lysogeny and symbiotic ability

To lysogenize strain 4S, phage U-mole lysates were spotted on spread plates of strain 4S (see Methods). Bacteria isolated from turbid plaques were purified and examined for lysogeny. One of these isolates, strain 4S:φU, was used for further investigation.

The phage sensitivity of strain 4S:φU is summarized in Table 1. Strain 4S:φU, like its parent strain, was sensitive to all three virulent phages tested, but resistant to phage U-mole. The morphology of phages induced in strain 4S:φU by either UV irradiation or mitomycin C treatment was identical to that of phage U-mole. The growth rates of 4S:φU in YM medium and TY medium were the same as those of 4S.

Strain 4S:φU was inoculated onto 200 clover seedlings and incubated for about 2 months. These inoculated seedlings did not form nodules, and only two root protrusions were induced on one plant (Fig. 4a). These protrusions were very similar in shape to those described by Hirsch *et al.* (1985). Fig. 4(b) shows a scanning electron micrograph of a cross-section of one of the protrusions. The vascular bundles are at the centre and the cells are empty. It appeared that normal nodulation processes might have been inhibited by lysogenization, through an unknown mechanism.

Hybridization analyses

Plasmid DNAs of strains UK-1:φU, 4S:φU and 4S were compared on agarose gel (Fig. 5). Strains UK-1:φU and 4S both carried three plasmids, of 525, 420 and 315 kb. The 315 kb plasmids in each strain were identified as Sym plasmids (Higashi *et al.*, 1983). This 315 kb plasmid could not be detected in strain 4S:φU, although the two larger plasmids were present (Fig. 5a, lane 3; Fig. 7a, lane 4).

It was not clear whether the Sym plasmid of 4S:φU had been cured or whether phage U-mole DNA had integrated into the *nod* region of the Sym plasmid. If phage U-mole DNA was integrated into Sym plasmid, the mobility of the plasmid should be decreased on agarose gel. Crude plasmid DNAs (Fig. 5) and *HindIII* digests of total DNA (Fig. 6) from each strain were hybridized with pRt032, as a *nod* gene probe. In strains UK-1:φU and 4S, the 315 kb plasmid (Fig. 5b, lanes 1 and 2) and a 7.2 kb *HindIII* fragment (Fig. 6b, lanes 1 and 3) hybridized with the

Fig. 8. (a) Restriction endonuclease patterns of phage U-mole and of *R. leguminosarum* biovar *trifolii* strains digested with *Eco*RI or *Hind*III. (b) Southern hybridization using 32 P-labelled *Eco*RI or *Hind*III digests of phage U-mole as probe. The symbols $\blacktriangleleft$, $\triangleleft$, and $\blacktriangleleft\blacktriangleleft$ are explained in Results and Discussion. Lane λ , *Hind*III fragments of λ DNA; lanes 1, *Eco*RI fragments of U-mole DNA; lanes 2, *Eco*RI digests of strain UK-1: ϕ U; lanes 3, *Eco*RI digests of strain 4S: ϕ U; lanes 4, *Hind*III fragments of U-mole DNA; lanes 5, *Hind*III digests of strain UK-1: ϕ U; lanes 6, *Hind*III digests of strain 4S: ϕ U.

nod probe. However, no hybridization was detected in strain 4S: ϕ U (Fig. 5b, lane 3; Fig. 6b, lane 2). These results support the hypothesis that the Sym plasmid was cured in strain 4S: ϕ U.

Additional broad bands were, however, detected in strain 4S: ϕ U. These new bands (arrowed in Fig. 7a) were always detected, independent of mitomycin C treatment. These additional bands also appeared in UK-1: ϕ U, but only when the cells were incubated with mitomycin C ($1 \mu\text{g ml}^{-1}$). The bands were first detected after 20 min incubation with mitomycin C. Their staining intensity on agarose gel increased with increasing length of incubation time with mitomycin C (data not shown). These bands were presumed to be phage DNA. An *Eco*RI digest of phage U-mole DNA was labelled and hybridized with plasmid DNA of the lysogenic strains treated or not treated with mitomycin C (Fig. 7b). The additional bands in UK-1: ϕ U treated with mitomycin C and in 4S: ϕ U with or without mitomycin C treatment all hybridized with phage U-mole DNA. In UK-1: ϕ U not treated with mitomycin C (Fig. 7, lanes 2), the phage U-mole probe hybridized with fragmented chromosomal DNA. These results suggest integration of phage U-mole into the chromosome. To investigate this further, *Eco*RI or *Hind*III digests of total DNA of UK-1: ϕ U and 4S: ϕ U were hybridized and compared with *Eco*RI or *Hind*III fragments of phage U-mole DNA isolated from phage U-mole particles (Fig. 8). The 7.4 kb and 5.0 kb fragments in the *Eco*RI digest of strain UK-1: ϕ U (Fig. 8b, lane 2, indicated by $\blacktriangleleft$) could not be detected in the *Eco*RI digest of U-mole DNA (Fig. 8a,b, lanes 1) and the 6.0 kb fragment in the UK-1: ϕ U digest (Fig. 8b, lane 2, indicated by $\triangleleft$) was very faint. These results indicate that phage U-mole DNA integrated in strain UK-1: ϕ U chromosome at the 6.0 kb *Eco*RI fragment. All three fragments were detected in the *Eco*RI digest of strain 4S: ϕ U (Fig. 8b, lane 3), but the 6.0 kb fragment (indicated by $\triangleleft$) was more strongly labelled than that in the UK-1: ϕ U digest, suggesting that phage U-mole DNA might replicate autonomously without integration into the chromosome in strain 4S: ϕ U. Furthermore, fragments characteristic to strain 4S: ϕ U were detected (Fig. 8b, lanes 3 and 6, indicated by $\blacktriangleleft\blacktriangleleft$). The origin of these fragments is not clear, but they may relate to Sym plasmid curing.

The molecular mechanism of Sym plasmid curing caused by lysogenization is incompletely understood. Incompatibility may occur between unintegrated phage U-mole DNA and the Sym plasmid of strain 4S: ϕ U. Lysogenic phage may also effect the symbiotic properties of *Rhizobium* in a manner unlike that of virulent phages.

REFERENCES

- BARNET, Y. M. (1979). Properties of *Rhizobium trifolii* isolates surviving exposure to specific bacteriophage. *Canadian Journal of Microbiology* **25**, 979-986.
- BERINGER, J. E. (1974). R-factor transfer in *Rhizobium leguminosarum*. *Journal of General Microbiology* **84**, 188-198.
- BIRNBOIM, H. C. & DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513-1523.
- BRADLEY, D. E. (1967). Ultrastructure of bacteriophages and bacteriocins. *Bacteriological Reviews* **31**, 230-314.
- BUCHANAN-WOLLASTON, A. V. (1979). Generalized transduction in *Rhizobium leguminosarum*. *Journal of General Microbiology* **112**, 135-142.
- CASSE, F., BOUCHER, C., JULLIOT, J. S., MICHEL, H. & DÉNARIÉ, J. (1979). Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. *Journal of General Microbiology* **113**, 229-242.
- DALLMANN, G., OROSZ, L. & SAIN, B. (1979). Restriction mapping of DNA of temperate *Rhizobium meliloti* phage 16-3: comparison of genetic and physical maps indicates a long, genetically silent chromosomal arm. *Molecular and General Genetics* **176**, 439-448.
- DEMOLON, A. & DUNEZ, A. (1935). Recherches sur le rôle du bactériophage dans la fatigue des luzernières. *Annales agronomiques* **5**, 86-111.
- DORGAI, L., OLASZ, F., BERÉNYI, M., DALLMANN, G., PÁY, A. & OROSZ, L. (1981). Orientation of the genetic and physical map of *Rhizobium meliloti* temperate phage 16-3. *Molecular and General Genetics* **182**, 321-325.
- DORGAI, L., OLASZ, F. & NÉMETH, K. (1986). Lyso-genic control of temperate phage 16-3 of *Rhizobium meliloti* 41 is governed by two distinct regions. *Molecular and General Genetics* **205**, 568-571.
- FINAN, T. M., HARTWIG, E., LEMIEUX, K., BERGMAN, K., WALKER, G. C. & SIGNER, E. R. (1984). General transduction in *Rhizobium meliloti*. *Journal of Bacteriology* **159**, 120-124.
- GUPTA, B. M. & KLECZKOWSKA, J. (1962). A study of some mutations in a strain of *Rhizobium trifolii*. *Journal of General Microbiology* **27**, 473-476.
- HIGASHI, S. & ABE, M. (1978). Phage induced depolymerase for exopolysaccharide of *Rhizobiaceae*. *Journal of General and Applied Microbiology* **24**, 143-153.
- HIGASHI, S. & ABE, M. (1980). Promotion of infection thread formation by substances from *Rhizobium trifolii*. *Journal of Applied Environmental Microbiology* **39**, 297-301.
- HIGASHI, S., UCHIUMI, T. & ABE, M. (1983). Elimination of *Rhizobium* infectivity by temperature treatment. *Journal of General and Applied Microbiology* **29**, 281-285.
- HIGASHI, S., KUSHIYAMA, K. & ABE, M. (1986). Electron microscopic observations of infection threads in driselase treated nodules of *Astragalus sinicus*. *Canadian Journal of Microbiology* **32**, 947-952.
- HIRSCH, A. M., DRAKE, D., JACOBS, T. W. & LONG, S. R. (1985). Nodules are induced on alfalfa roots by *Agrobacterium tumefaciens* and *Rhizobium trifolii* containing small segments of the *Rhizobium meliloti* nodulation region. *Journal of Bacteriology* **161**, 223-230.
- KEELE, B. B., HAMILTON, P. B. & ELKAN, G. H. (1969). Glucose catabolism in *Rhizobium japonicum*. *Journal of Bacteriology* **97**, 1184-1191.
- KLECZKOWSKA, J. (1950). A study of phage-resistant mutants of *Rhizobium trifolii*. *Journal of General Microbiology* **4**, 298-310.
- KLECZKOWSKA, J. (1965). Mutations in symbiotic effectiveness in *Rhizobium trifolii* caused by transforming DNA and other agents. *Journal of General Microbiology* **40**, 377-383.
- KOWALSKI, M. (1967). Transduction in *Rhizobium meliloti*. *Acta microbiologica polonica* **16**, 7-12.
- KOWALSKI, M., HAM, G. E., FREDERICK, L. R. & ANDERSON, I. C. (1974). Relationship between strains of *Rhizobium japonicum* and their bacteriophages from soil and nodules of field-grown soybeans. *Soil Science* **118**, 221-228.
- MARTIN, M. O. & LONG, S. R. (1984). Generalized transduction in *Rhizobium meliloti*. *Journal of Bacteriology* **159**, 125-129.
- ÖRDÖGH, F. & SZENDE, K. (1961). Temperate bacteriophages isolated from *Rhizobium meliloti*. *Acta microbiologica Academiae scientiarum hungaricae* **7**, 65-71.
- OROSZ, L., SVÁB, Z., KONDOROSI, Á. & SIK, T. (1973). Genetic studies on *Rhizobiophage* 16-3 I. Genes and functions on the chromosome. *Molecular and General Genetics* **125**, 341-350.
- RALEIGH, E. A. & SIGNER, E. R. (1982). Positive selection of nodulation-deficient *Rhizobium phaseoli*. *Journal of Bacteriology* **151**, 83-88.
- SCHOFIELD, P. R., RIDGE, R. W., ROLFE, B. G., SHINE, J. & WATSON, J. M. (1984). Host-specific nodulation is encoded on a 14 kb DNA fragment in *Rhizobium trifolii*. *Plant Molecular Biology* **3**, 3-11.
- SIK, T., HARWATH, J. & CHATTERJEE, S. (1980). Generalized transduction in *Rhizobium meliloti*. *Molecular and General Genetics* **178**, 511-516.
- SOUTHERN, E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503-517.
- VANDE WOUDE, G. F., OSKARSSON, M., ENQUIST, L. W., NOMURA, S. & FISCHINGER, P. J. (1979). Cloning of integrated Moloney sarcoma proviral DNA sequences in bacteriophage λ . *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4464-4468.
- WERQUIN, M., ACKERMANN, H.-W. & LEVESQUE, R. C. (1988). A study of 33 bacteriophages of *Rhizobium meliloti*. *Applied and Environmental Microbiology* **54**, 188-196.
- YAMAMOTO, K. R., ALBERTS, B. M., BENZINGER, R., LAWHORNE, L. & TREIBER, G. (1970). Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. *Virology* **40**, 734-744.