Isolation and characterization of two bacteriophages of a stem-nodulating *Rhizobium* strain from *Sesbania rostrata*

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Two rhizobiophages, RS1 and RS2, were isolated in Senegal from a soil sample and dry stem nodules of *Sesbania rostrata*, a tropical legume that is infected by two categories of *Rhizobium* strains: "stem strains," which nodulate both roots and stems (type strain, ORS571), and "root strains," which induce effective nodules only on roots. Both phages were found to have a host range restricted to ORS571; all root strains were found to be resistant. By electron microscopy, phage RS1 showed an hexagonal head 63 nm wide and a tail 87 nm long; phage RS2 revealed an hexagonal head 60 nm wide. Characterization of phage growth cycle by one-step growth experiments showed that the latent period was ca. 75 min for RS1 and ca. 4 h for RS2, that the rise period lasted ca. 2 h for both RS1 and RS2, and that the average burst size was ca. 100 for RS1 and 130 for RS2. Temperature denaturation occurred at 60–65°C (RS1) and 45–50°C (RS2). Serum neutralization tests revealed that the phages were not serologically related. In contrast to RS1, RS2 appeared to be temperate, since stable lysogens were isolated.

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Deux rhizobiophages, RS1 et RS2, ont été isolés au Sénégal d'un échantillon de sol et de nodules de tiges séchées de *Sesbania rostrata*, une légumineuse tropicale qui est infectée par deux catégories de souches de *Rhizobium*: les "souches de tige" qui causent la nodulation à la fois des racines et des tiges (souche type, ORS571) et les "souches de racine" qui n'induisent efficacement des nodules que chez les racines. Les deux phages se sont avérés avoir une gamme d'hôtes restreinte à l'ORS571; toutes les souches de racine se sont révélées résistantes. En microscopie électronique, le phage RS1 présentait une tête hexagonale de 63 nm de largeur et une queue de 87 nm de longueur; le phage RS2 possédait quant à lui une tête hexagonale de 60 nm de largeur. La caractérisation du cycle de croissance des phages par des expériences de croissance conduites en une seule étape s'est traduite par une période de latence pour le RS1 de ca. 75 min et pour le RS2 de ca. 4 h, par une période d'accroissement d'une durée de ca. 2 h tant pour RS1 que pour RS2, et par un rendement moyen en phages de ca. 100 pour RS1 et 130 pour RS2. La température de dénaturation a été de 60–65°C (RS1) et 45–50°C (RS2). Les tests de neutralisation du sérum ont révélé que les phages ne sont pas sérologiquement reliés. Contrairement au phage RS1, celle de RS2 semble tempéré, puisque des lysogènes ont été isolées.

[Traduit par le journal]

The tropical legume *Sesbania rostrata* forms both root and stem nodules in symbiotic association with *Rhizobium* (Dreyfus and Dommergues 1981). Two categories of strains can nodulate *Sesbania rostrata*: stem strains, which nodulate both roots and stems (type strain ORS571), and root strains, which induce effective nodules only on roots (B. Dreyfus. 1982. Thèse de doctorat d'état, Université Paris VII). A genetic analysis of strain ORS571 has already been initiated (Elmerich *et al.* 1982).

Bacteriophages have been isolated in most *Rhizobium* species: *R. lupini* (Lotz and Mayer 1972), *R. trifolii* (Atkin 1973), and *R. leguminosarum* (Ley *et al.* 1972). Some rhizobiophages have been reported to be transducing, e.g., *R. melilioti* (Svab *et al.* 1978; Casadesus and Olivares 1979), *R. leguminosarum* (Buchanan–Wollaston 1979), and *R. japonicum* (Shah *et al.* 1981). This paper reports the isolation of two distinct bacteriophages, termed RS1 and RS2, specific for *Rhizobium* from *Sesbania rostrata*. We also describe some of their properties, including morphology, host range, adsorption, growth characteristics, and temperature sensitivity. Some lysogens for RS2 were isolated and studied for their stability.

Materials and methods

Bacterial strains

The strains used are listed in Table 1.

Growth conditions and media

Strain ORS571 was grown on YL medium (Dreyfus *et al.* 1983) and all other strains in YMA medium (Vincent 1977). Bacteria were grown in shake culture at 30°C.

Isolation of rhizobiophages

RS1 was isolated from dry *Sesbania rostrata* stem nodules (B. Dreyfus, thèse de doctorat d'état) and RS2 from a sample of *Sesbania rostrata* rhizosphere soil. Crushed nodules or soil sample were suspended in YL medium, homogenized with a magnetic stirrer, and centrifuged (10000 *g*, 10 min). Super-

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TABLE 1. Bacterial strains used

Strains	References
Rhizobium isolated from nodules of *Sesbania rostrata*	
Stem strain	
ORS571	Dreyfus and Dommergues 1981
Root strains	
ORS51	Dreyfus and Dommergues 1981
ORS52	Dreyfus and Dommergues 1981
ORS53	Dreyfus and Dommergues 1981
ORS56	Dreyfus and Dommergues 1981
ORS57	Dreyfus and Dommergues 1981
Rhizobium isolated from root nodules of *Sesbania pubescens*	
ORS502	Dreyfus and Dommergues 1981
Rhizobium isolated from root nodules of *Sesbania pachycarpa*	
ORS507	Dreyfus and Dommergues 1981
"Cowpea" *Rhizobium*	
CB756	Bergersen *et al.* 1976
Rhizobium meliloti	
L5-30	Casse *et al.* 1979
Agrobacterium tumefaciens	
C58	Van Larebeke *et al.* 1974

natant was filtered on membrane filters (0.45 μm pore size) (Millipore HAWP-45) and assayed for lysis plaque formation on strain ORS571 using the standard agar overlay method (Adams 1959) which was performed as follows. YL medium with 1.6% Bacto-agar (Difco) was used as basal agar layer, while YL medium with an agar concentration of 0.6% was used for the top layer. Each phage was purified through three successive platings.

Constitution of phage stocks

Phage stocks were obtained, either by using the confluent lysis method described by Eisenstark (1967) or by infecting an exponentially growing liquid culture of strain ORS571 (optical density of 1 at 570 nm) with a suspension of phages at a multiplicity of infection of 10^{-2} plaque-forming units (pfu)/bacterium. Using this procedure, we generally obtained titers of 10^8 and 10^{12} pfu/mL for RS2 and RS1, respectively. The phages were diluted and stored in a solution of 5 m*M* $CaCl_2$ and 10 m*M* $MgCl_2$. Phage suspensions stored at 4°C were stable.

Electron microscopy of phage particles

A drop of phage suspension was applied to a 200-mesh copper grid coated with carbon and stained with 2% uranyl acetate. The grid was examined using a Siemens Elmiskop 101 electron microscope.

One-step growth curves and burst-size experiments

The principle of the technique used has been described by Adams (1959). Five millilitres of an exponentially growing culture of ORS571 were centrifuged and the resulting pellet was resuspended in a solution of 5 m*M* $CaCl_2$ and 10 m*M* $MgCl_2$. This suspension was infected with phage (multiplicity of infection, 10^{-3} pfu/bacterium). The phage–bacteria mixture was left for adsorption for 30 min at 30°C, then diluted to 10^{-4}–10^{-5}, and incubated at the same temperature. Phage release was followed by titrations of the suspensions at intervals of 15 to 30 min for 6–8 h.

Host specificity

Samples (0.1 mL) of exponentially growing cultures of the bacterial strains were suspended in 3 mL of melted agar and overlayered onto YL or YMA plates. Shortly after the agar was solidified, 50 μL of a phage suspension (10^9 pfu/mL) was spotted onto the overlay. Plates were examined for lysis plaque formation following overnight incubation (fast-growing bacteria) or after 72 h (slow-growing bacteria).

Thermostability of phages

Phage samples were diluted to 10^7 pfu/mL in a solution of 5 m*M* $CaCl_2$ and 10 m*M* $MgCl_2$ and then incubated at various temperatures. Inactivation of phages was followed by titrations of the suspension at different times for 1 h.

Specificity of antiphages serum

Antiphage serum were prepared according to Eisenstark (1967). To determine antiphage specificity, we added 0.1 mL of a phage suspension (10^7 pfu/mL) to 0.1 mL of antiserum and let it neutralize for 1 h at 37°C. Inactivation of plaque-forming units was measured by titration of the mixture.

Isolation and identification of lysogens

An exponentially growing culture of strain ORS571 was infected by a phage suspension (multiplicity of infection of

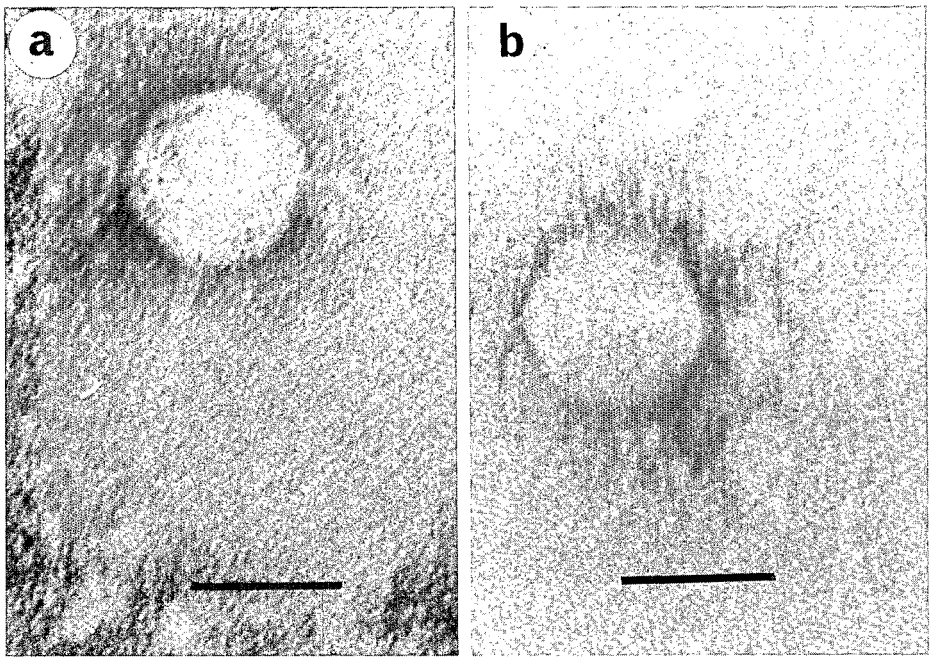

FIG. 1. Electron micrograph of strain ORS571 phages RS1(*a*) and RS2(*b*) stained with 2% uranyl acetate. Bar marker is 50 nm.

10^{-2} pfu/bacterium) and incubated for 16 h at 30°C. The culture was then centrifuged and bacteria were washed three times in YL medium to eliminate most of the free phages. The bacteria were resuspended in YL medium and plated to isolate survivors. Each colony obtained was then transferred using a toothpick onto an overlay of strain ORS571. Lysis plaque formation was examined after 16 h at 30°C..

The stability of lysogen was tested by using phage-specific antiserum. The lysogen was grown overnight in YL medium with the addition of the phage-specific antiserum at a dilution of 1:10. The culture was then diluted to an optical density of 0.1 at 570 nm in phage-specific antiserum containing YL medium and incubated overnight at 30°C. At the end of each cycle, the supernatant of the culture was checked for the absence of free phages. After four such subcultures, the lysogens were examined; colonies were isolated and picked on an overlay of strain ORS571 for assaying phage-producing ability.

Results

Isolation and serological relationship of bacteriophages RS1 and RS2

The two bacteriophages were easily distinguished on the basis of characteristic plaque morphologies. Phage RS1 formed 2 mm diameter clear lysis plaques surrounded by a small turbid halo. Plaques formed by RS2 were turbid, 1 mm diameter.

Differences in phage morphology were observed by electron microscopy as shown in Fig. 1. Phage RS1 was characterized by an hexagonal head 63 nm wide and a tail 87 nm long (Fig. 1*a*). Phage RS2 exhibited a smaller hexagonal head, 60 nm diameter, with no observable tail or baseplate (Fig 1*b*).

The study of antiphage specificity showed that when antiphage serum was mixed with its corresponding phage, neutralization was complete. No effect on phage activity was observed when anti-RS1 was mixed with phage RS2 and vice versa. Thus RS1 and RS2 were not serologically related.

Host range

RS1 and RS2 were tested on the different strains listed in Table 1, namely stem and root strains of *Rhizobium* isolated from *Sesbania* sp., "cowpea" *Rhizobium*, *Rhizobium meliloti*, and *Agrobacterium tumefaciens*. The host range of both phages appeared to be limited to ORS571, the strain used for their isolation.

Thermostability of phages

Phage RS1 remained stable when heated at 60°C for 60 min (Fig 2A). Phage RS2 was less stable, since 90% of the phage particles were inactivated when heated at 50°C for 30 min (Fig. 2B).

Adsorption and one-step growth experiments

For both phages, adsorption was complete in the first 20-25 min (data not shown).

Figure 3 illustrates the one-step growth experiments. The latent period lasted ca. 75 min (RS1) and ca. 4 h (RS2). For both phages, the rise period, during which

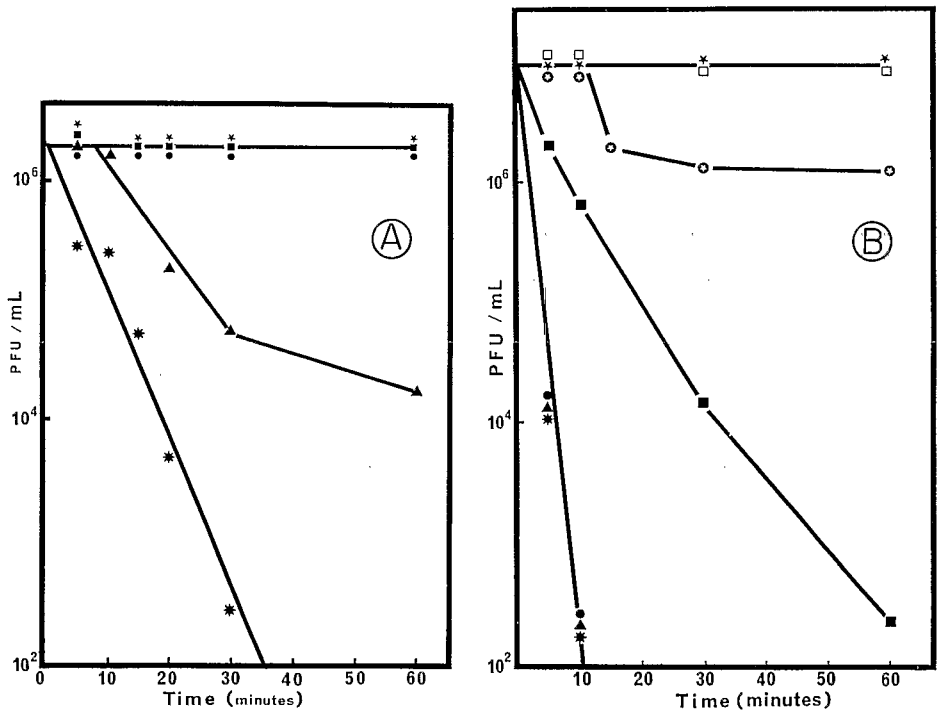

FIG. 2. Thermal inactivation of bacteriophages RS1(A) and RS2(B). ★, 30°C; □, 45°C; ✪, 50°C; ■, 55°C; ●, 60°C; ▲, 65°C; ✱, 70°C.

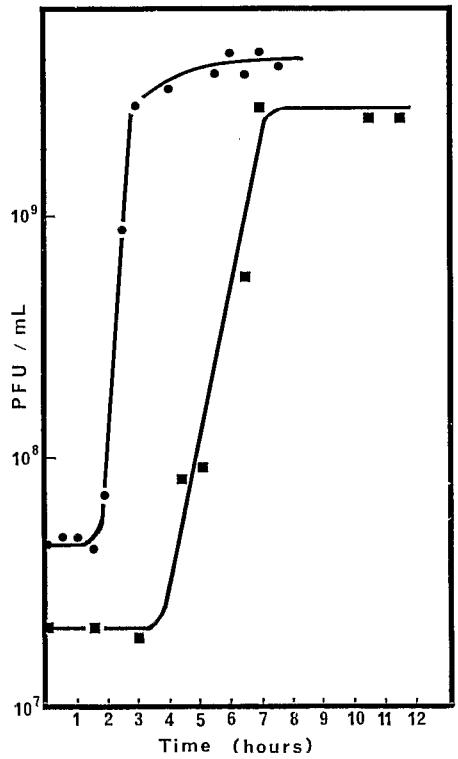

FIG. 3. One-step growth curves of phages RS1 (●) and RS2 (■) on strain ORS571.

the extracellular phage concentration increased logarithmically, lasted about 120 min. The average burst sizes, which were calculated from the pfu per millilitre values in the latent period and at the plateau, were 100 and 130 for RS1 and RS2, respectively.

Isolation and identification of lysogens

Out of the one hundred RS1-resistant colonies tested, none were found to be lysogenic. The RS2 phage formed turbid plaques on its host, suggesting the temperate nature of this phage. Among 180 survivors from a culture of strain ORS571 infected by phage RS2, 13 were found to form lysis plaques on an overlay of the same strain. The temperate nature of phage RS2 was confirmed by subculturing a lysogenic derivative of strain ORS571 in the presence of phage-specific antiserum. After four passages of the lysogen in antiserum containing YL medium, single colonies from this culture were still able to produce phages (ca. 10^5 pfu/mL) when stabbed onto the indicator strain ORS571. The phage–host association was shown to be stable for at least four generations.

Discussion

Rhizobiophages RS1 and RS2 differed in the following characteristics: plaque morphology, electron microscopy after negative staining, and antigenic properties.

In contrast to RS1, RS2 gave stable lysogens, indicating that it was temperate similar to phages of *R. meliloti*, *R. trifolii*, and *R. leguminosarum* (Vincent 1977). In preliminary experiments using both phages for transducing rifampicin resistance, no transduction occurred (data not shown), but we will conduct new trials as soon as other antibiotic resistances and auxotrophic mutations are available.

Both phages were shown to be specific for the stem strain ORS571. Neither was able to infect root strains. This finding suggests a possible relationship between phage resistance and root or stem nodulating ability. It is known that bacteriophages utilize bacterial cell surface proteins or saccharides as receptors (Lindberg 1973). On the other hand it has been put forward that *Rhizobium*–legume recognition might result from a specific interaction between a plant cell surface receptor (lectin) and a distinctive *Rhizobium* surface polysaccharide (Schmidt 1979). If the same *Rhizobium* receptors are involved in phage adsorption and stem recognition, we can attempt (*i*) to use phages RS1 and RS2 in bacterial typing and (*ii*) to isolate mutants of *Rhizobium* affected in their nodulation ability.Thus Raleigh and Signer (1982) selected and characterized mutants of *Rhizobium phaseoli* on the basis of their phage resistance. Isolation and characterization of such phage-resistant mutants should be of great interest in studying stem-nodulation specificity.

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