SHORT COMMUNICATIONS

Isolation and Characterization of Phage-resistant Strains of *Azotobacter vinelandii*

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(Received 8 August 1974; revised 24 February 1975)

Several bacteriophages which infect *Azotobacter* have been described and divided into four serological groups (Duff & Wyss, 1961). Plaques of the azotophages are distinctive in that they exhibit a halo, composed of cells that have had their capsules stripped away, which surrounds the central area of lysis. The halo is the result of the action of capsule-depolymerizing enzymes associated with azotophage infection which have been the subject of several studies (Eklund & Wyss, 1962; Barker, Eklund & Wyss, 1968; Humphrey & Wyss, 1972). We used azotophage infection to select acapsulated strains from cultures of *Azotobacter*, and characterized these strains as lysogenic, pseudolysogenic or phage-resistant acapsulated mutants. Lysogeny of *Azotobacter* by azotophage A22 was reported by Nimeck & Wyss (1962). Pseudolysogeny by A23 was reported by Humphrey & Wyss (1971) wherein the affected cells were phage-resistant mutants with a rate of back mutation to wild-type sufficient to yield a continuous supply of susceptible cells in which phage multiplication occurred. Acapsulated mutants of *Azotobacter* have also been isolated without prior exposure of the cells to an azotophage and these mutants, easily recognized by colonial morphology, were resistant to infection by any of the azotophages.

METHODS

The azotophages used in this study were those reported by Duff & Wyss (1961) and further described by Konvicka, Pope & Wyss (1972). All were propagated on *Azotobacter vinelandii* ATCC12518 (AVO), and the same strain was used as the source of acapsulated variants. Cultures were grown on a nitrogen free (NF) medium (Duff & Wyss 1961), with 0.5 % sucrose, sterilized separately, as the carbon source. For solid media, agar was added to give a final concentration of 1.8 % (w/v), and for soft agar overlays used to demonstrate azotophage plaques, the NF salts solution was used in 2.0 ml amounts with 0.5 % agar added.

A young culture of AVO was inoculated with an azotophage. After lysis the culture was incubated for an additional 24 h to allow survivors of the phage infection to divide and to facilitate the isolation of strains with heritable phage-resistance. Cultures were streaked on agar plates and one colonial variant showing loss of capsule was selected from each culture for further examination. The strains isolated in this manner were designated with three digits. The first two digits were taken from the azotophage used to select the strain, the third digit was unique to the strain (e.g. a strain isolated from AVO infected by A31 was designated 310). Confluent lawns of the colonial variants were prepared by the soft agar overlay method, and immediately spotted with an azotophage suspension. Lysis indicated phage

susceptibility, and all positive results were confirmed by plating the variant with a dilution of phage to give individual plaques.

The enzyme was detected by spotting a mature lawn of AVO with the enzyme preparation and observing crater formation (Eklund & Wyss, 1962). It was quantitated by measuring the rate of increase in the fluidity of a capsular polysaccharide solution with a Wells-Brookfield LVT microviscometer as described by Humphrey & Wyss (1972). The size of the enzyme was estimated by gel filtration using a Sephadex G-75 column (Andrews, 1964), which had been standardized with bovine serum albumin (fraction V) and pancreatic ribonuclease A. Antiphage serum was prepared by immunizing rabbits against the purified phage. Broth was inoculated with approximately 10^6 AVO cells/ml and simultaneously infected with equivalent numbers of azotophage isolated from the lysogenic culture. To one of these suspensions was added antiphage A31 serum at a final dilution of 1:100. Replicate suspensions were treated similarly, but inoculated with a lysogenic strain. After 24 h of growth all cultures were titrated for bacteria and phage, measured by plate counts for colony-forming units (c.f.u.) and plaque-forming units (p.f.u.) respectively. The identity of the phage types was confirmed by incorporating specific antiphage serum in the soft agar overlay and observing inhibition of plaque formation.

Young broth cultures of the variants were exposed to a germicidal ultraviolet lamp and samples were taken after various intervals of post-irradiation incubation for assay of both c.f.u. and p.f.u. The relative lethal effects of acridine orange and mitomycin C were determined in broth cultures of wild-type and colonial variants by exposing the cells to the chemicals and titrating for c.f.u. at various time intervals. To measure induction by mitomycin C, the lysogenic cultures were exposed to $2.0 \mu\text{g/ml}$ for 30 min and washed to remove both chemical and free phage, after which the cell-to-phage ratio was monitored for 6 h.

RESULTS

Thirty variants with an unusual colonial morphology were isolated after exposure of AVO to the azotophages. Each was resistant to the azotophage used to select it, and appeared to be acapsulated when examined microscopically. While AVO colonies were raised, mucoid and light grey in colour, the variant colonies were flatter, darker and smaller in diameter. Colonies of two of the variants retained a mucoid surface texture, but the others had a rough, dry appearance. Of the 30 strains, five showed an association with one or another of the azotophages. Despite repeated colony isolations, the two mucoid strains, 310 and 314, isolated from A31 lysates, retained an azotophage serologically identical to phage A31. Three others, two isolated from A21 lysates (216 and 217) and one from a lysate of A23 (235), showed an azotophage association which did not persist in the face of multiple single-colony isolations. With these strains, a nearly constant ratio of phage to cells resulted when transferred serially in broth culture. The remaining 25 strains showed no phage association.

Table 1 reports the phage susceptibility of representatives of the 30 colonial variants. Strains 310 and 314 were susceptible to all azotophages tested except A31, supporting the supposition that they had been lysogenized by that phage. Strain 319 was resistant to all the phages; 23 of the other 24 acapsulated mutants with no phage association were similarly resistant and were not included in the Table. Strain 241, although acapsulated, was nevertheless susceptible to A31. Attachment studies indicated that 241 had lost the receptor sites for the other azotophages. Strains having an unstable phage association (216, 217 and 235) were resistant to all the azotophages. Because 241 had little or no capsule, and 310 and 314 produced capsule depolymerase, plaques on lawns of these cultures were devoid of haloes.

Table 1. *Susceptibility to the azotophages*

Azotobacter strain	Phages					
	A14	A21	A22	A23	A24	A31
AVO* (wild-type)	+	+	+	+	+	+
216†	—	—	—	—	—	—
217†	—	—	—	—	—	—
235†	—	—	—	—	—	—
241*	—	—	—	—	—	+
310‡	+	+	+	+	+	—
314‡	+	+	+	+	+	—
319*	—	—	—	—	—	—

+, Phage lysis; —, no phage lysis.

* No phage association.

† Unstable phage association.

‡ Stable phage A31 association.

Table 2. *Comparison of the effect of anti-A31 serum on the growth of strain 310 and release of phage A31, and on the growth of strain AVO infected with phage A31*

Strain 310 was grown in the presence and absence of anti-A31 serum. As a control, strain AVO with added azotophage A31 was grown in the presence and absence of the antiserum. Initial inoculum for all cultures was 1.0×10^6 c.f.u./ml, and when phage was added it was at a multiplicity of infection of one. Cultures were titrated after 24 h of incubation.

Contents of broth	C.f.u./ml	P.f.u./ml
AVO + A31	3.5×10^8	1.7×10^8
AVO + A31 + antiserum	4.5×10^8	0
310	3.6×10^8	9.5×10^7
310 + antiserum	8.5×10^7	1.5×10^7

Filtrates of cultures of 310 and 314 contained a capsule depolymerase whose molecular weight, estimated by Sephadex chromatography, was in the range 35000 to 42000, which is similar to the depolymerase induced in AVO by azotophage A31. Cultures showing an unstable phage association also possessed a depolymerase, but phage-free variants isolated from such cultures by repeated clonings had lost the enzyme. After these 30 strains were characterized, additional acapsulated mutants were selected from AVO cultures that had not been exposed to azotophage. All were similar to 319 in that they showed no phage association, were resistant to all azotophages and did not revert to wild-type. When strains 310 and 314 were plated to obtain isolated colonies, a few were found to have reverted to the AVO colonial character. One such revertant was isolated and showed other wild-type characteristics in that it was susceptible to phage A31 and lacked capsule depolymerase and azotophage. An estimate of the frequency of reversion, made by comparing the number of revertants with the total number of colonies, showed that not more than one in one hundred bacteria had lost the azotophage. No revertants to wild-type were found with any of the other strains when tested by this method.

Antiserum specific for A31 prevented the infection of AVO cells by A31 added to broth cultures (Table 2) thus permitting multiplication of the bacteria due to rapid inactivation of the phage. As would be expected, the phage carried by strain 310 was protected from destruction by the homologous antiphage serum. Failure of the antiserum to eliminate the phage from strain 310 is proof of lysogeny. Although the lysogenic phages could not be induced by u.v. irradiation, strain 310 was more sensitive to the lethal effects of both acridine orange and mitomycin C than were strains AVO, 241 and 319. This greater sensitivity of

Fig. 1. Induction of prophage in 310 cells by 2.0 µg mitomycin C/ml. ●, C.f.u. in control; ▲, p.f.u. in control; ○, c.f.u. induced; △, p.f.u. induced.

strain 310 to the mutagen suggested induction of the prophage. Figure 1 indicates induction of the A31 prophage in strain 310 by 2.0 µg mitomycin C/ml. During the post-treatment incubation period, while c.f.u. increased less than fivefold, p.f.u. increased 100-fold.

DISCUSSION

Populations of *A. vinelandii* that survived exposure to azotophages were either acapsulated mutants or lysogenic strains. The former had a rough colonial morphology and were resistant to all phages or to all except A31. Although three were pseudolysogenic, the associated phage was not responsible for the properties of the variants since loss of this phage did not change the colonial morphology or the phage sensitivity. Evidently the receptor sites for azotophages are associated with the capsules and the receptor site for phage A31 differs from those of the other azotophages. Phage A31 is unusual in that it has a long non-contractile tail and is the only example in its serological group. It found specific adsorption sites on strain 241 while the other phages did not. This is quite unlike the ten laboratory stock strains of *A. vinelandii* tested by Duff & Wyss (1961), which were susceptible to A31 and to all or to all but one of the other phage strains. The two isolates that were lysogenic were both lysogenized by A31. We regard it as significant that two of the four strains selected after exposure to A31 were lysogenic and none of the strains selected after exposure to the other bacteriophages was lysogenized. If natural lysogeny is similar to that observed in strain 310 the lysogenized population would seem to be at an ecological disadvantage, since the accompanying depolymerase reduces its capsule which protects it against rapid drying and is necessary to permit the normal maturation of the resistant cyst (Eklund, Pope & Wyss, 1966). On the other hand the depolymerization of the capsule may make additional carbohydrate available to the cell; capsular material must be metabolized slowly if at all by the uninfected cell, otherwise it would not accumulate and persist in cells grown on a limited concentration of carbohydrate. We have never isolated a phage from, or detected lysogeny in, any stock culture of *Azotobacter* although extensive attempts were made in our laboratories. This is not unexpected, since the atypical colonial appearance and the meagre colonial size of lysogenic cultures would lead to their rejection by anyone isolating and purifying *Azotobacter* from the soil.

This investigation was supported by Public Health Service research grant AI-02830 and training grant GM00600.

REFERENCES

- ANDREWS, P. (1964). Estimation of molecular weights of proteins by Sephadex gel-filtration. *Biochemical Journal* **91**, 222–223.
- BARKER, T., EKLUND, C. & WYSS, O. (1968). Physical differences of virus-associated depolymerases. *Biochemical and Biophysical Research Communications* **30**, 704–710.
- DUFF, J. T. & WYSS, O. (1961). Isolation and classification of a new series of *Azotobacter* bacteriophages. *Journal of General Microbiology* **24**, 273–289.
- EKLUND, C., POPE, L. & WYSS, O. (1966). Relationship of encapsulation and encystment in *Azotobacter*. *Journal of Bacteriology* **92**, 1828–1830.
- EKLUND, C. & WYSS, O. (1962). Enzyme associated with bacteriophage infection. *Journal of Bacteriology* **84**, 1209–1215.
- HUMPHREY, R. D. & WYSS, O. (1971). Phage associated with *Azotobacter vinelandii* strain o. *Bacteriological Proceedings*, 174.
- HUMPHREY, R. D. & WYSS, O. (1972). Viscosity changes in a polymerized substrate due to the action of a hydrolytically depolymerizing enzyme. *Experientia* **28**, 1433–1434.
- KONVICKA, J., POPE, L. & WYSS, O. (1972). Morphology and nucleic acid composition of the *Azotobacter* bacteriophages. *Journal of Virology* **10**, 150–152.
- NIMECK, M. & WYSS, O. (1962). Azotophage–cyst relations. *Texas Reports on Biology and Medicine* **20**, 139.