

Key words; *Rhizobium phage 16-3/structural proteins/two-dimensional electrophoresis*

Identification of Structural Proteins of *Rhizobium meliloti* Temperate Phage 16-3

By SÁRA ERDEI,^{1*} BRIGITTA DUDÁS,² LÁSZLÓ OROSZ²
AND ERNŐ DUDA¹

¹*Institute of Biochemistry, Biological Research Center of the Hungarian Academy of Sciences, Szeged, H-6726, Odesszai krt. 62, Hungary and* ²*Department of Genetics of Attila József University, Szeged, H-6726, Középfasor 52, Hungary*

(Accepted 19 April 1982)

SUMMARY

The structural proteins of *Rhizobium meliloti* temperate phage 16-3 have been analysed by means of polyacrylamide gel electrophoresis, isoelectric focusing and agarose gel electrophoresis. Five major and five minor proteins were identified and characterized with respect to their size, isoelectric point and their distribution between the head and tail of the phage particle. The synthesis of structural proteins was studied by one- and two-dimensional gel electrophoresis.

INTRODUCTION

The genetics of temperate phage 16-3 of *Rhizobium meliloti*, a nitrogen-fixing bacterium, is well established. Cistrons coding for the phage repressor, early and late proteins, and lysozyme have been identified and mapped (Orosz & Sik, 1970; Orosz *et al.*, 1980*a,b*; Dudás & Orosz, 1980; Dallmann *et al.*, 1980). This comprehensive genetic characterization made phage 16-3 amenable to direct biochemical investigations. A restriction map of phage 16-3 DNA has been published recently (Dallmann *et al.*, 1979; Dorgai *et al.*, 1981). In this paper we describe the characterization of the structural proteins encoded by the late cistrons of the phage and their detection in phage 16-3-infected bacteria during the course of the phage infection cycle.

METHODS

Bacteria and bacteriophage. Wild-type phage 16-3 and the host *R. meliloti* strain 41 have been described previously (Orosz *et al.*, 1973).

Growth conditions and media. The media used and the preparation of high titre phage lysates have been described previously by Orosz *et al.* (1973).

Phage purification. Phage lysates were concentrated four to five times by polyethylene glycol precipitation (Yamamoto *et al.*, 1970). The phage particles were purified by two consecutive bandings on a CsCl step-gradient (layers: 1.7, 1.5 and 1.3 g/ml). Centrifugations were performed in an SW27 rotor at 25 000 rev./min for 2.5 h. The opalescent band at the border of 1.3 g/ml and 1.5 g/ml CsCl layer was sucked off with a syringe and exhaustively dialysed against phage buffer (0.01 M-Tris-HCl pH 7.4, 0.1 M-NaCl, 0.01 M-MgCl₂). The identity of the phage band was checked by plaque assay.

Separation of the head and tail. The CsCl-purified phage suspension was kept at 40 °C for 24 h in phage buffer to dissociate the phage particles and then layered on to a 5 to 20% linear sucrose gradient. The gradient was centrifuged in an SW41 rotor at 22 000 rev/min for 60 min. Fractions (500 µl) were collected and subjected to electrophoretic and electron

microscopic analysis. From each fraction, 100 μ l was dialysed against distilled water before processing for electron microscopy. The remaining part of each of the fractions was precipitated with 9 vol. acetone. The precipitate was dissolved in SDS sample buffer and electrophoresed. In some experiments the purified phages were dialysed against phage buffer containing 0.05 M-EDTA instead of 0.01 M-MgCl₂. After the addition of bromophenol blue and saccharose [final concn. 0.01% (w/v) and 10% (w/v) respectively] the EDTA-dissociated phages were subjected to electrophoresis in a 0.8% horizontal agarose gel in 89 mM-Tris, 89 mM-boric acid, 2.5 mM-EDTA pH 8.2 buffer. The gels were run at 100 V until the bromophenol blue reached the bottom of the gel.

Electron microscopy. A drop of the sample was placed on a carbon-coated grid. The grid was washed with two drops of distilled water and stained with one drop of 2% (w/v) uranyl acetate. The grid was then washed with one drop of distilled water and the residue blotted with filter paper.

Preparation of radioactive lysates. *R. meliloti* 41 cells were grown to 5×10^8 cells/ml in GTS medium at 28 °C. The cells were infected with phage 16-3 at a multiplicity of infection of 10. After 30 min adsorption at 4 °C, the suspension was transferred to 28 °C and incubated at that temperature. At a given time 0.18 MBq [³⁵S]methionine (52.3 TBq/mmol; Amersham International) was added to 1 ml samples. After a 10 min pulse the suspensions were quickly chilled in ice with simultaneous addition of cold methionine at 50 μ g/ml concentration and the cells were processed in two alternative ways. Either they were directly lysed in SDS sample buffer or were prepared for two-dimensional electrophoresis as described by O'Farrell (1975).

Preparation of the phage proteins for electrophoresis in polyacrylamide gels. Phage proteins were prepared essentially according to Murialdo & Siminovitch (1971).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out as described by Laemmli (1970) using 10% separating gels and 5% stacking gels. For gradient gels the acrylamide concentration ranged from 5 to 20%. The gels were stained in 0.2% Coomassie Brilliant Blue R250 in methanol and acetic acid. Gels containing radioactive proteins were dried under vacuum and exposed to Kodak X-Omat X-ray film. The mol. wt. of proteins was estimated using mol. wt. standards (Pharmacia) and *Escherichia coli* RNA polymerase (a gift of Dr A. Udvardy) as reference proteins. The protein standards were: phosphorylase b (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), lactalbumin (14 000), β' subunit (165 000), β subunit (155 000), σ subunit (90 000), α subunit (40 000).

Two-dimensional gel electrophoresis of proteins. Preparation of cell extracts and the electrophoresis of proteins were carried out exactly as described by O'Farrell (1975). The total concentration of ampholines was 2%, consisting of 1.6% pH 3.5 to 10 and 0.4% pH 4 to 6, or 1.6% pH 3.5 to 10, 0.2% pH 7 to 9 and 0.2% pH 8 to 9.5. In all cases the second dimension SDS separating gel consisted of 10% acrylamide.

Iodination of proteins. Phage 16-3 proteins were ¹²⁵I-labelled by the chloramine T procedure described by Hunter & Greenwood (1962).

RESULTS

Structural proteins of phage 16-3

Phage 16-3 was purified by CsCl step-gradient ultracentrifugation and six polypeptides were detected on electrophoresis of proteins from purified phage. Their mol. wt. were $86 \times$

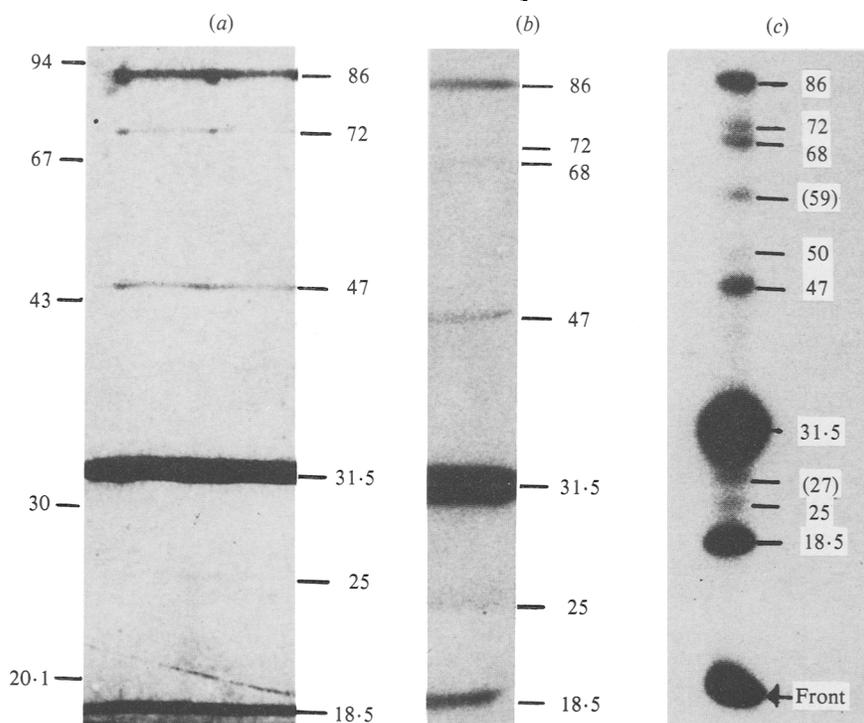


Fig. 1. SDS-PAGE of the purified phage 16-3. (a) The proteins from purified phage were prepared as described in Methods, analysed on a 10% SDS-polyacrylamide gel and stained by Coomassie Brilliant Blue. The mol. wt. ($\times 10^{-3}$) of the phage proteins are shown on the right-hand side, while the positions of the mol. wt. markers are indicated on the left-hand side. (b) The proteins of the [^{35}S]-methionine-labelled phage were run on a 5 to 20% polyacrylamide gel. (c) The phage proteins were labelled with ^{125}I and run on a 10% gel.

10³ (86K), 72K, 47K, 31.5K and 18.5K (Fig. 1a). The authenticity of the structural proteins was confirmed by the fact that further purification of the phage by equilibrium CsCl gradient did not result in any change in the protein pattern.

To increase the sensitivity of protein detection we also analysed the proteins of phage labelled *in vivo* with [^{35}S]methionine or *in vitro* with ^{125}I . To extend the separation range the labelled proteins were run in a 5 to 20% gradient gel. The proteins detected on the autoradiogram of the [^{35}S]methionine-labelled phage (Fig. 1b) were essentially identical to those seen in Fig. 1(a) except for two minor differences. Firstly, one additional faint band corresponding to a 68K protein appeared on the autoradiogram. Secondly, the 72K protein was not seen on the autoradiogram, and this probably reflects its low methionine content.

The gel electrophoresis of ^{125}I -labelled phage (Fig. 1c) revealed a further three additional protein bands with mol. wt. 59K, 50K and 27K respectively. In some cases the 50K protein could also be seen in the overexposed gels of [^{35}S]methionine-labelled proteins, but the 59K and 27K proteins were found exclusively in ^{125}I -labelled samples. We cannot exclude the possibility that these proteins are artefacts of the iodination procedure. Consequently, we refer to them as tentative minor proteins of phage 16-3, and this is indicated in the figures by their inclusion in parentheses. The 5 to 20% gradient gel did not reveal any additional small mol. wt. polypeptide, and 10% uniform SDS-polyacrylamide gels were used in subsequent experiments.

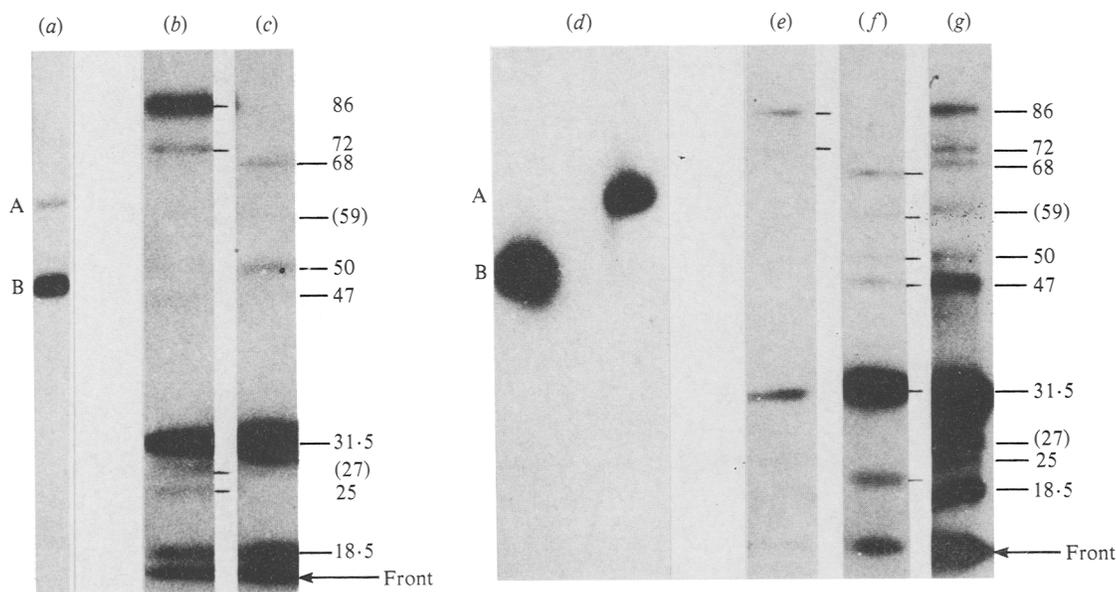


Fig. 2. Separation of the phage head and tail. The 0.05 M-EDTA-treated ^{125}I -labelled phage 16-3 was electrophoresed in a 0.8% horizontal agarose gel as described in Methods. The proteins of band A from the agarose gel (a) are shown in (b) while the proteins of band B of the same gel (a) can be seen in (c). The bands from gel (a) were cut out and re-electrophoresed separately in a second 0.8% agarose gel (d). The protein composition of band A (e) and band B (f) from the second agarose gel (d) are compared with that of the intact phage (g).

Head and tail proteins

Long incubation of phage 16-3 at 40 °C causes a 10^2 to 10^3 -fold decrease of the phage titre and the process is reversible at 4 °C. We presumed that this was due to the reversible dissociation of the head and tail. On the basis of this assumption, we incubated the phage suspension at 40 °C for 24 h and then layered it on to a 5 to 20% sucrose gradient. The separation was followed by electron microscope analysis of the individual fractions. The upper part of the gradient contained mainly structures resembling phage tails, while the middle part was enriched in phage heads. SDS-PAGE analysis showed that the 86K and 72K proteins were in the upper fractions of the gradient, while the 47K, 31.5K and 18.5K proteins were mainly in the middle fractions.

Although we could achieve a certain separation of the phage head and tail, both the dissociation and the separation procedure were incomplete. In attempts to get complete dissociation of the phage we tried other agents (high salt, increasing detergent concentrations, EDTA). Only the 0.05 M-EDTA treatment proved to be efficient but we had to increase the resolution power of the separation as well by using 0.8% horizontal agarose gels to separate the heads and tails. Fig. 2 (a) presents the result of the agarose electrophoresis of ^{125}I -labelled, EDTA-treated phage 16-3. Only two bands can be seen, and these were further analysed by SDS-PAGE. Fig. 2 (b) shows the proteins found in band A, while Fig. 2 (c) presents the protein composition of band B. In accordance with our previous results we conclude that during the electrophoresis in 0.8% agarose gel, the separation of the head and the tail took place. The upper band, A, corresponds to the phage tail and the lower band, B, to the phage head. Comparison of the protein composition of these two bands suggests some apparent cross-contamination (see especially 31.5K protein). To exclude contamination of band A material with band B material, we re-electrophoresed the separate bands in a second

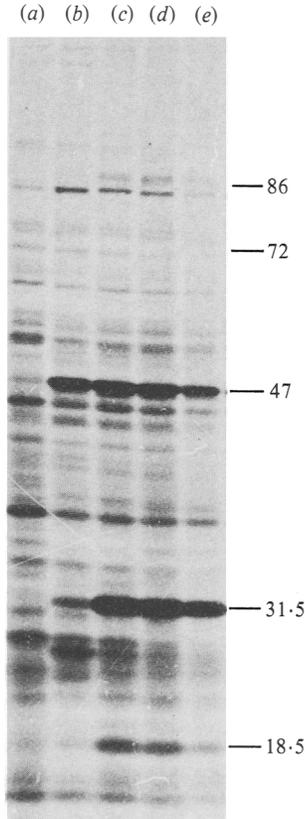


Fig. 3. SDS-PAGE of the proteins of phage 16-3-infected cells labelled with [^{35}S]methionine. A 0.18 MBq [^{35}S]methionine pulse was given to infected cells at (b) 30, (c) 60, (d) 90 and (e) 120 min after infection, and also to uninfected cells (a). After 10 min the cells were collected, processed for electrophoresis and run on a 10% SDS-polyacrylamide gel. The positions of the stained proteins of the purified phage are indicated on the right-hand side.

agarose gel. The bands separated in the first gel proved to be completely homogeneous in the second run (Fig. 2*d*). The protein composition of the bands was again compared. The 86K and 72K proteins could be detected only in band A (Fig. 2*e*), while the 68K, 59K, 50K, 47K, 31.5K and 18.5K proteins were characteristic of band B (Fig. 2*f*). Despite the fact that the re-electrophoresis of bands A and B showed them to be homogeneous, 31.5K protein was still detectable in both bands although its enrichment in band B was significant. The localization of two minor proteins (27K and 25K) is uncertain.

Appearance of head and tail proteins after phage infection

From temperature shift-up experiments with temperature-sensitive (*ts*) mutants it was known that the late genes of phage 16-3 are expressed after 50 min of infection at 28 °C (Orosz' *et al.*, 1973). Having identified the structural proteins of the purified phage it became possible to study the expression of late genes by monitoring the corresponding proteins in infected bacteria. *R. meliloti* was infected with phage 16-3 (m.o.i. of 10), and a 10 min pulse of [^{35}S]methionine was given at 30, 60, 90 and 120 min after infection. The samples were then subjected to SDS-PAGE (Fig. 3). Three bands were present only in the infected bacteria. These proteins co-migrated with the 18.5K, 31.5K and 47K proteins of the purified phage.

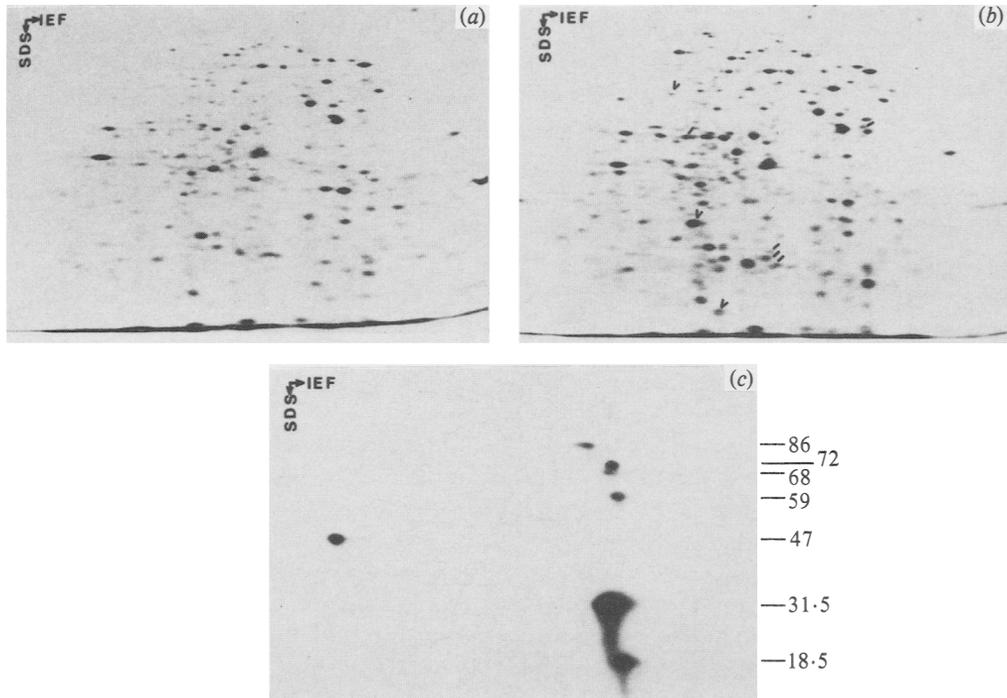


Fig. 4. Two-dimensional gel electrophoresis of the proteins of (a) uninfected cells, (b) phage 16-3-infected cells and (c) purified phage 16-3 (^{125}I -labelled). The cells were labelled with [^{35}S]methionine (0.18 MBq/ml) for 30 min. <, Spots which co-migrated with the 18.5K, 31.5K and 72K proteins of the purified phage, which had been run and stained in a parallel gel. —, Additional spots appearing after infection. The pH gradient in gels (a) and (b) was from 4.5 to 7; the pH gradient in gel (c) was from 5 to 8.5. The numbers on the right-hand side show the mol. wt. ($\times 10^{-3}$) of the spots.

This suggests that they represent the intracellular form of the main structural phage proteins. Their synthesis became dominant between 30 and 60 min after infection, an observation which is in good agreement with the results of shift-up experiments.

Two-dimensional gel separation of proteins of phage-infected bacteria

The resolving power of one-dimensional SDS-PAGE can be improved by two-dimensional analysis of cellular proteins. The same migration of two proteins by two criteria (isoelectric point and mol. wt.) is strong evidence for their identity. Labelled proteins from infected and uninfected bacteria were compared in two-dimensional gels. Fig. 4 shows the autoradiograms from a typical experiment. It compares the labelled proteins of the bacterial cells (Fig. 4a) with those of phage-infected cells at 60 min after infection (Fig. 4b). In a parallel gel the proteins from CsCl-purified phage were also run and stained. The spots 18.5K, 31.5K and 86K of the purified phage coincided with spots in Fig. 4(b). These spots were found in the infected cell extract and were absent from the uninfected extract. As we could detect proteins of the same size in the phage-infected cells in our previous experiment (Fig. 3) it is probable that they correspond to the intracellular form of the main phage structural proteins. Other spots characteristic of the phage-infected cells could represent the phage-encoded non-structural proteins, whose identification is in progress.

We could not find spots corresponding to the 47K protein, although our previous result (Fig. 3) suggested that it is present in the infected bacteria in fairly large amounts. We also

failed to detect it in the two-dimensional gels of the purified phage. As the actual pH gradient formed in the experiment shown in Fig. 4 (*a, b*) was between 4.5 and 7, we extended the pH gradient of the focusing gel from 5 to 8.5. The autoradiogram of the two-dimensional run of the ^{125}I -labelled purified phage using a pH gradient from 5 to 8.5 is shown in Fig. 4 (*c*). It gives the explanation for our failure to detect the 47K protein on the gel shown in Fig. 4 (*b*). The 47K spot is easily recognizable in the upper, basic part of the focusing gel. While the pI of the majority of the structural proteins falls between 6 and 6.5, the 47K protein is a basic protein with a pI around pH 8.

DISCUSSION

We have presented evidence that purified phage 16-3 consists of five major (18.5K, 31.5K, 47K, 72K and 86K) and five minor (25K, 27K, 50K, 59K and 68K) proteins. The 27K and 59K proteins were detected only in ^{125}I -labelled phages. The difference in the number of proteins between the [^{35}S]methionine and ^{125}I -labelled phage can be explained in two ways. Either the 27K and 59K proteins do not contain methionine residues or they are the artefacts of the *in vitro* iodination procedure. As we have not seen any breakdown products on the gels of the ^{125}I -labelled proteins we favour the first explanation and refer to these proteins as minor components of phage 16-3. The 72K protein could be clearly seen in the stained (Fig. 1 *a*) and in the iodinated samples (Fig. 1 *b*), while it was missing from the autoradiogram of the [^{35}S]methionine-labelled phage proteins. This difference can be easily explained by the assumption that the 72K protein has no methionine residue.

The lack of amber mutants of phage 16-3 makes difficult the functional characterization of the various structural proteins. We have tried different biochemical approaches to gain some information about the localization of these proteins within the phage particle. Heat dissociation of the phage followed by sucrose gradient centrifugation led to partial dissociation and separation of the head and the tail. The electron microscopic analysis and SDS-PAGE of the individual fractions suggested that the 86K and 72K proteins are part of the tail while the other proteins belong to the head of the phage. This was further confirmed by the agarose gel electrophoresis of the EDTA-dissociated phage which apparently resulted in a complete separation of the head and tail (Fig. 2 *d*). The 86K and 72K proteins proved to belong to the tail, while the 68K, 59K, 50K, 47K, 31.5K and 18.5K proteins are the constituents of the phage head. The localization of two minor proteins (27K and 25K) is uncertain. The 31.5K protein could be detected in both fractions, although its enrichment in the head band is clear cut. This could reflect some inherent feature of this protein, or the contamination of the tail fraction simply could be due to its large quantity since it is the most abundant structural protein.

Despite the fact that the powerful u.v. irradiation technique for reducing host protein synthesis (Ptashne, 1967) is not available in the *Rhizobium* system, we could identify a number of phage proteins in phage-infected cells. The synthesis of the structural proteins during the infectious cycle was followed by one- and two-dimensional PAGE of the [^{35}S]methionine pulse-labelled proteins. Considering the 31.5K protein as a representative of the phage structural proteins, it was possible to conclude that the structural proteins appear between 30 and 60 min after infection. This result agrees well with that of previous temperature shift-up experiments with *ts* mutants (Orosz *et al.*, 1973). The two-dimensional gel electrophoresis of the infected cell extracts confirmed that the heavily labelled bands at the positions of 18.5K and 31.5K proteins represent the intracellular forms of the corresponding phage 16-3 structural proteins. The other phage-specific proteins detected in the autoradiogram of the two-dimensional gel should represent the phage-encoded non-structural proteins whose characterization is presently under investigation. The isoelectric points of the

structural proteins are between 6 and 6.5 except for the 47K protein which has a pI of about 8. The basic 47K protein is a good candidate for a protein playing a crucial role in the assembly of the DNA with the proteins of the phage head.

The identification and characterization of the structural proteins of phage 16-3 is an important step in the understanding of genome organization of this temperate phage.

REFERENCES

- 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.
- DALLMANN, G., OLASZ, F. & OROSZ, L. (1980). Virulent mutants of temperate *Rhizobium meliloti* phage 16-3: loci avirC and avirT, and increased recombination. *Molecular and General Genetics* **178**, 443–446.
- 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.
- DUDÁS, B. & OROSZ, L. (1980). Correlation between map position and phenotype of *Cti* mutants in the *C* cistron of *Rhizobium meliloti* phage 16-3. *Genetics* **96**, 321–329.
- HUNTER, W. M. & GREENWOOD, F. C. (1962). Preparation of Iodine-131-labelled human growth hormone of high specific activity. *Nature, London* **194**, 495–496.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- MURIALDO, H. & SIMINOVITCH, L. (1971). The morphogenesis of bacteriophage lambda. III. Identification of genes specifying morphogenetic protein. In *The Bacteriophage Lambda*, pp. 711–723. Edited by A. D. Hershey. New York: Cold Spring Harbor Laboratory.
- O'FARRELL, P. H. (1975). High resolution two-dimensional electrophoresis of protein. *Journal of Biological Chemistry* **250**, 4007–4025.
- OROSZ, L. & SIK, T. (1970). Genetic mapping of Rhizobiophage 16-3. *Acta microbiologica Academiae scientiarum hungaricae* **17**, 185–194.
- 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.
- OROSZ, L., ROSTÁS, K. & HOTCHKISS, R. D. (1980a). A comparison of two point, three point and deletion mapping in the *C* cistron of Rhizobiophage 16-3, with an explanation for the recombination pattern. *Genetics* **94**, 265–276.
- OROSZ, L., PÁY, A. & DALLMANN, G. (1980b). Heterozygosis of phage 16-3 of *Rhizobium meliloti*: moderate level of mismatch repair or gene conversion. *Molecular and General Genetics* **179**, 163–167.
- PTASHNE, M. (1967). Specific binding of the phage repressor to DNA. *Nature, London* **222**, 541–544.
- YAMAMOTO, K. R., ALBERTS, B. M., BENZINGER, R., LAWTHORNE, 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.

(Received 14 December 1981)