Further biological and molecular characterization of actinophage VWB

JOZEF ANNÉ,^{1*} LIEVE VAN MELLAERT,¹ BENNY DECOCK,² JO VAN DAMME,^{2†}
ARTHUR VAN AERSCHOT,³ PIET HERDEWIJN^{3†} and HENDRIK EYSEN¹

¹Laboratory of Microbiology, ²Laboratory of Immunobiology, and ³Laboratory of Pharmaceutical Chemistry, Rega Institute, University of Leuven, B-3000 Leuven, Belgium

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The development cycle of the temperate actinophage VWB was investigated. Adsorption of most phage particles occurred within 30 min and the adsorption constant was $0.6 \times 10^{-8} \text{ ml min}^{-1}$. The latent and rise periods were 140 and 100 min, respectively, and the burst size was estimated to be 130–250 p.f.u. Although phage VWB could infect only *Streptomyces venezuelae* ETH 14630 (ATCC 40755), of six different *S. venezuelae* strains tested, phage DNA could be introduced by transfection into most non-infectible strains. Upon transfection, phage DNA was propagated in these non-infectible strains and phage particles were released. In addition, the transfected strains could be lysogenized. By comparison of restriction fragments of VWB DNA, either free or integrated in the chromosomal DNA of the *S. venezuelae* ETH 14630 lysogen, the attachment site was localized. PAGE of the phage proteins revealed at least 17 different proteins with three major bands estimated as 16.5, 27.2 and 43 kDa in size. The N-terminal amino acid sequence of these supposed major head and tail proteins was determined. The corresponding DNA sequences on the phage genome were localized using oligonucleotides synthesized on the basis of the N-terminal amino acid sequences. The genes coding for the major structural proteins were shown to be clustered, as has been observed for other bacteriophages.

Introduction

Several articles have mentioned the isolation of actinophages infecting *Streptomyces* strains (e.g. Ackermann *et al.*, 1985). Most phages have been isolated from soil in which they are widespread, but some isolates were obtained from lysogenic wild-type strains. Among the reasons for the interest in phages are the fact that they can be used as a tool for the genetic analysis of host strains (Stuttard, 1983; Chung & Thompson, 1985), and that some phages are suitable for the development of a phage-based cloning vector (Chater, 1986). With a few exceptions, however, actinophages have not been studied in much detail. Their characterization has been limited to the description of some morphological properties and the construction of a restriction map.

To obtain a better understanding of actinophage biology and the regulatory processes involved in phage replication, more fundamental studies are required. This knowledge could be useful in the isolation and identi-

fication of interesting regulatory sequences including repressor proteins (Sinclair & Bibb, 1988) or sequences involved in transduction (McHenney & Baltz, 1988). It is also of importance to obtain a better insight into phage taxonomy and evolution.

In a previous article we reported the isolation of actinophage VWB (Anné *et al.*, 1984). This temperate phage, with a genome size of 47.3 kb and cohesive ends (Anné *et al.*, 1985), is infectious to *Streptomyces venezuelae* ETH 14630. The phage is rather unique in its resistance to chelating agents and it is able to package stably at least 4 kb of additional DNA (Van Mellaert *et al.*, 1987). Consequently, it is of potential use for development as a phage-based cloning vector. Therefore, we were interested in studying this phage in more detail and we have determined both the characteristics of the development cycle of VWB and the narrow host specificity. Furthermore, the attachment site (*att*) as well as the start of the three most prominent structural phage proteins were localized on the phage genome.

Methods

Organisms, phages and plasmids. *Streptomyces* phage VWB and VW3 (Anné *et al.*, 1984) were propagated on *S. venezuelae* ETH 14630

† Research Associate of the Belgian Nationaal Fonds voor Wetenschappelijk Onderzoek.

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(= ATCC 40755). SV1 (Stuttard, 1983) and SVL1 (see Results) were cultured on *S. venezuelae* ATCC 10595. The host ranges of different actinophages were checked on the following strains: *S. venezuelae* ATCC 10712, ATCC 14583, ATCC 21113 and DSM 40727; and *Streptomyces exfoliatus* ATCC 12672. Plasmid pBR322 (Bolívar *et al.*, 1977), used to clone restriction fragments of VWB, was propagated in *Escherichia coli* JM83 [*ara* Δ (*lac proAB*) *rpsL* Φ 80, *lacI^q* Δ (*lacZ*) M15]. VWB04 is a derivative of VWB. It contains a thiostrepton resistance gene as selection marker and is 48.5 kb in size (Van Mellaert *et al.*, 1987).

Media and buffers. *Streptomyces* strains were cultured on R2YE (Hopwood *et al.*, 1985) or ISP-2 medium (Difco). Phage particles were maintained in phage buffer (Anné *et al.* 1984). Protoplasts were prepared in P medium (Okanishi *et al.*, 1977) from *S. venezuelae* mycelia grown in S medium (Hopwood *et al.*, 1985) supplemented with 0.6% glycine, and they were regenerated on R2YE medium. S medium supplemented with 0.6% glycine was also the medium to culture *Streptomyces* for chromosomal DNA isolation. *E. coli* was cultivated in liquid LB medium (Miller, 1972) and transformants, obtained as described by Maniatis *et al.* (1982), were selected on solid LB. When applicable, antibiotics were added to the medium at final concentrations of 100 and 50 μ g ml⁻¹ for ampicillin and thiostrepton, respectively. DNA was stored in TE (0.01 M-Tris/HCl, 0.001 M-EDTA, pH 7.5). Buffers required for restriction enzyme digestions were obtained from Boehringer Mannheim.

Propagation of *S. venezuelae* phages. All platings for phage assays were carried out by the agar layer technique (Adams, 1959). High-titre lysates (10¹¹ p.f.u. ml⁻¹) were obtained on solid phage medium and, in the case of VWB, occasionally in liquid phage medium. On solid medium, lysates could be harvested after 20 h of cultivation, whereas in liquid cultures at least 32 h of incubation was required. For liquid cultures, mycelial fragments (OD₅₉₀ = 0.1) were infected with phage particles at an m.o.i. between 0.001 and 1.0, depending on the type of experiment, and cultures were incubated on a rotary shaker (27 °C, 350 r.p.m.). Determination of the host range was done on solid phage medium by addition of 50–100 μ l phage suspension (10¹⁰–10¹¹ p.f.u. ml⁻¹) on top of the soft agar medium inoculated with mycelial fragments of the strain to be investigated. Infection could be scored by the presence of plaques in a lawn of *Streptomyces* mycelium after 20 h incubation (27 °C).

Purification of phages and denaturation of phage proteins. Phages from high-titre lysates were purified by CsCl gradient centrifugation as described previously (Anné *et al.*, 1984). Gradients were prepared with 0.785 g CsCl ml⁻¹ for VWB and 0.715 g CsCl ml⁻¹ for SV1 and other *S. venezuelae* phages. Phage proteins used for immunization or gel electrophoresis were denatured by treatment of CsCl-gradient-banded phage particles with 2.3% (w/v) SDS in the presence of 5% (v/v) β -mercaptoethanol at 95 °C for 3 min. Denatured phages were dialysed (3 $\times$ 2 h) against 0.01 M-Tris/HCl, pH 7.5.

Antiserum production. Serum containing VWB antibodies was prepared in rabbits as described by Adams (1959). The amount of phage antibodies, expressed as *K*, was calculated from the formula $K = 2.3D/t \times \log(p_0/p)$, where *p*₀ = phage assay at zero time, *p* = phage assay at time *t* min and *D* = final dilution of serum in the phage-serum mixture (Adams, 1959).

Adsorption. Adsorption experiments were carried out with VWB suspensions added to mycelial fragments at an m.o.i. of 0.01. Suspensions were incubated with gentle shaking at 27 °C. Samples were withdrawn at regular intervals after contact. The mycelial fragments were removed by centrifugation (5 min, 5000 *g*) and the number of phages remaining in the supernatant was counted as p.f.u.

ml⁻¹ on the tester strain. The rate of adsorption was determined as described by Adams (1959).

One-step growth experiment. Mycelial fragments were infected with VWB particles at an m.o.i. of 0.001. Following adsorption for 15 min, the suspension was treated with diluted (1:20) rabbit antiserum (*K* = 10) for 20 min at 27 °C and subsequently diluted 500-fold before incubation (27 °C, 350 r.p.m.). At regular intervals 1 ml samples were withdrawn, centrifuged (5 min, 5000 *g*) and the number of phage particles in the supernatant estimated as p.f.u. ml⁻¹ on a lawn of *S. venezuelae*. Burst size was calculated according to Adams (1959), the number of infectious centres being estimated from the adsorption efficiency.

Separation of phage proteins by PAGE or by hydroxylapatite chromatography and detection of protein bands. Phage proteins (10 to 100 μ g, depending on the staining method) along with a calibration kit for molecular mass determination (Pharmacia) were subjected to SDS-PAGE under reducing conditions in 12.5% (w/v) gels (1 mm thick) according to Laemmli (1970). After electrophoresis, proteins were either visualized in the gel by staining with Coomassie Blue R-250 (Chrambach *et al.*, 1967) or with silver stain (Guevara *et al.*, 1982), or they were electroblotted onto nitrocellulose membranes (BioRad) for immunological detection. In those cases where samples were used for amino acid sequence analysis, they were blotted onto PVDF membranes (Millipore). Electroblotting with a semi-dry electroblotter (JC Biotechnical Instruments) was done according to the manufacturer's instructions. Immunological detection of the membrane-bound proteins was done according to the method of Towbin *et al.* (1979) using rabbit antiserum against VWB and anti-rabbit IgG alkaline phosphatase conjugate (Sigma). Proteins electroblotted onto PVDF membranes were visualized by staining with Coomassie Blue R-250. After electroblotting the membrane was first washed with water for 5 min and then stained with 0.1% (w/v) Coomassie Blue R-250 in 50% (v/v) methanol, 10% (v/v) acetic acid for 5 min. Finally the membrane was washed again in water for 5 min and air-dried.

Separation of proteins by hydroxylapatite chromatography was carried out in the presence of SDS essentially as described by Moss & Rosenblum (1972). After adding 0.1 vol. 10% (w/v) SDS and 0.01 vol. of β -mercaptoethanol to the protein solution (1 mg protein ml⁻¹), the sample was boiled for 2 min. Meanwhile, a 1 ml column of hydrated Bio-Gel HTP hydroxylapatite (BioRad) was prepared and equilibrated with 0.01 M-sodium phosphate buffer, pH 6.4 and 0.1% (w/v) SDS. The denatured protein sample was diluted with 20 vols column equilibrating buffer and then applied (1 ml min⁻¹) to the hydroxylapatite column. After washing with equilibrating buffer the proteins were eluted with a linear (0.01–0.5 M) sodium phosphate buffer (pH 6.4) gradient in 0.1% (w/v) SDS. Flow was maintained at 1 ml min⁻¹ and 2 min fractions were collected. The proteins in the fractions were analysed by SDS-PAGE on a 8–20% gradient gel and silver-stained.

Amino acid sequence analysis of phage proteins. Protein N-terminal amino acid sequences were determined with an automated 477A-120A protein sequencing system (Applied Biosystems). Cysteines were not alkylated and therefore not detectable.

Electrophoretically separated proteins electroblotted onto PVDF membranes were excised after staining. The excised protein bands were cut into small pieces (2 $\times$ 4 mm) and placed on top of a polybrene-conditioned glass fibre filter in the 477A cartridge block.

Proteins in hydroxylapatite chromatography fractions were concentrated by ultrafiltration in Ultrafree-MC filter units (Millipore) and directly applied to polybrene-conditioned glass fibre filters.

Formation and transfection of *Streptomyces* protoplasts. After growth of the strains for 20 h at 27 °C, 350 r.p.m., mycelium was harvested by centrifugation and washed in saline followed by P medium. Protoplasts

were produced by lysozyme treatment (10 mg ml^{-1}) at 27°C for 30 min. They were then separated from mycelial remnants by centrifugation (700 g , 5 min) and harvested (3000 g , 10 min). After washing twice with P medium, protoplasts were transfected using a modification of the method described by Suarez & Chater (1980). Phage DNA ($0.5 \mu\text{g}$ in $15 \mu\text{l}$ TE buffer) was added to $200 \mu\text{l}$ protoplast suspension ($\text{OD}_{590} = 0.1$) and immediately mixed with $500 \mu\text{l}$ 40% (w/v) polyethylene glycol (molecular mass 6000 Da, Koch Light) dissolved in P medium. This suspension was spread at different dilutions on R2YE. Transfection was assayed after 5 d of incubation at 27°C . For VWB transfectants, plates were soaked with phage buffer and the presence of phages in the buffer was investigated by drop test on *S. venezuelae* ETH 14630. VWB04 transfectants were selected by replica plating on selective R2YE medium. Thiostrepton-resistant clones were investigated for the presence of VWB04. The procedure used gave 10^5 – 10^6 transformants (μg phage DNA) $^{-1}$.

DNA manipulations and restriction enzyme digestion. Phage DNA was obtained from CsCl-purified phages after treatment with phenol (Maniatis *et al.*, 1982) and following dialysis ($3 \times 2 \text{ h}$) against TE buffer. Chromosomal DNA of *S. venezuelae* was isolated after SDS- and pronase-treatment of lysozyme-digested cells (Womble *et al.*, 1977). *E. coli* plasmid DNA was obtained from cells grown in LB (300 r.p.m. , 37°C , 16 h) by the alkaline lysis method (Birnboim & Doly, 1979). DNA was purified by CsCl/ethidium bromide gradient centrifugation (Maniatis *et al.*, 1982). Restriction enzymes and T4 DNA ligase were supplied by Boehringer Mannheim or Pharmacia and they were used according to the manufacturer's instructions. Digested DNA was separated by electrophoresis on agarose gels using 40 mM-Tris/10 mM-sodium acetate/1 mM-EDTA, pH 7.8 (Maniatis *et al.*, 1982) as running buffer.

Oligonucleotide synthesis. Oligonucleotides were synthesized on a Gene Assembler Plus (Pharmacia). After deprotection, the oligonucleotides were desalted on an NAP-10 column and subsequently lyophilized; or, alternatively, they were purified on a Mono Q ion exchanger (Pharmacia) and desalted prior to lyophilization.

Southern blotting and DNA labelling reactions. After separation of DNA restriction fragments by agarose gel electrophoresis the DNA was blotted onto Hybond-N membranes (Amersham) according to the manufacturer's instructions. Blotting was obtained either through capillary action (Southern, 1975) or using a Vacugene system (Pharmacia). The DNA of interest bound to the membrane was detected by hybridization. When VWB DNA was used as probe, it was labelled with [α - ^{32}P]dCTP ($3000 \text{ Ci mmol}^{-1} = 111 \text{ TBq mmol}^{-1}$) by the method of random primed labelling (Feinberg & Vogelstein, 1983) applying the DNA labelling kit supplied by Boehringer Mannheim or using Boehringer Mannheim's non-radioactive DNA labelling and detection kit. When applicable, hybridization was done with $1.5 \times 10^4 \text{ c.p.m. cm}^{-2}$ at 65°C overnight and washing was done under stringent conditions according to the manufacturer's instructions for Hybond-N. Oligonucleotides used as DNA probe were end-labelled as follows. Oligonucleotides ($2\text{--}4 \text{ nmol}$) were dissolved in $40 \mu\text{l}$ water and mixed with $5 \mu\text{l}$ kinase buffer (700 mM-Tris/HCl , pH 7.6, 100 mM-MgCl_2 , 50 mM-DTT), $3 \mu\text{l}$ [γ - ^{32}P]dATP ($3000 \text{ Ci mmol}^{-1}$) and 20 units T4 polynucleotide kinase. The reaction was carried out at 37°C for 30 min. The oligonucleotides were next precipitated by adding 0.1 vol. sodium acetate (3 M , pH 6.0), $20 \mu\text{g}$ tRNA and 1 vol. of propan-2-ol. After 30 min at -70°C , the precipitated DNA was centrifuged in an Eppendorf centrifuge. The DNA pellet was dried in a vacuum rotor (Speed Vac Concentrator) and redissolved in $200 \mu\text{l}$ TE. Hybridization was done with about $1.5 \times 10^4 \text{ c.p.m. cm}^{-2}$ at 65°C overnight. Washing was under non-stringent conditions (twice with $2 \times \text{SSC}$ at 65°C) ($1 \times \text{SSC}$ is 0.15 M-NaCl , $0.015 \text{ M-trisodium citrate}$, pH 7.0).

Both hybridization and washing were as described in the protocol for Hybond-N.

Results and Discussion

Adsorption and growth characteristics of phage VWB

Adsorption of VWB was determined using *S. venezuelae* ETH 14630 cells grown in phage medium to the early exponential growth phase, i.e. 15 h cultures. Most phages quickly adsorbed to the cells. About 70% of all infective VWB particles were adsorbed within 20 min after contact. The adsorption constant ($= K$) was $0.6 \times 10^{-8} \text{ ml min}^{-1}$ as determined by the formula $K = 2.3/(B)t \times \log(p_0/p)$ (Adams, 1959), where p_0 = phage assay at zero time, p = phage not adsorbed at time t min, (B) = concentration of bacteria as number of cells ml^{-1} and K = velocity constant expressed as ml min^{-1} .

One-step growth experiments indicated that VWB has a latent period of 140 min, followed by a rise period of 100 min (Fig. 1). Although the length of these periods is not exceptional, they are rather long compared to those of many other actinophages (Lomovskaya *et al.*, 1980). The mean burst size was estimated to be 130–250 p.f.u.

Host-range determination of VWB to different *S. venezuelae* strains

It has previously been observed that VWB has a very narrow host range (Anné *et al.*, 1984). Further investigations on the host range versus different *S. venezuelae* strains showed that VWB infects *S. venezuelae* ETH 14630 but no other *S. venezuelae* strain (Table 1). This observation raised the question about the ETH 14630 strain being *S. venezuelae*. Reinvestigation of the culture by numerical cluster analysis indicated that the ETH 14630 strain resembles the *S. venezuelae* type strain ATCC 10712 (W. Wohlleben, personal communication), but it also shows a close relationship to *S. exfoliatus* ATCC 12672. Moreover, *S. exfoliatus* could be infected by VWB and when comparing the host range of three other *S. venezuelae* phages, *S. venezuelae* ETH 14630 and *S. exfoliatus* indeed showed a close relationship (Table 1).

In order to explain the narrow host range of VWB we also investigated the adsorption of this phage to other *S. venezuelae* strains. From these experiments it could be concluded that under the test conditions VWB efficiently adsorbed to ETH 14630 and to *S. exfoliatus*, but not to any of the other *S. venezuelae* strains tested (data not shown). Adsorption was, therefore, one of the restricting factors. The limited adsorption (1–10%) of VWB to the different *S. venezuelae* strains could be due to non-

Fig. 1. One-step growth curve of phage VWB development on *S. venezuelae* ETH 14630.

specific adsorption phenomena. Efficient adsorption (more than 90% under the test conditions) of VWB to *S. venezuelae* ETH 14630 and to *S. exfoliatus* was probably due to particular phage receptor proteins at the cell surface of these strains. However, VWB or its derivative VWB04 could be introduced in most *S. venezuelae* strains by transfection (Table 1). Moreover, they could also lysogenize these strains, as observed with VWB04. In this case the phage DNA became inserted in the host chromosome, rendering the strain thiostrepton-resistant. From these lysogens free phage particles could be isolated. After multiplication on ETH 14630 analysis of their DNA proved that the phage particles consisted of VWB04. It is evident that no plaques could be observed on *S. venezuelae* strains different from ETH 14630 when transfected with VWB DNA, because these phages could not infect the surrounding cells.

Fig. 2. (a) Mapping of prophage VWB DNA by comparison between restriction endonuclease digests of phage VWB and of chromosomal DNA isolated from *S. venezuelae* ETH 14630 lysogenized with VWB. Mapping was done using Southern blots following hybridization with VWB DNA labelled either with digoxigenin (*SphI* digest) or radioactively labelled (*XmnI* and *BglII* digests). Identification of restriction fragments containing the *att* site was achieved by scoring the bands with changed mobility (arrowed). The size of the original bands are indicated. Lanes 1 and 2, chromosomal DNA of lysogens; V, VWB; C, control *S. venezuelae* chromosomal DNA. (b) Restriction map of phage VWB showing the fragments that contain the *att* site (hatched). In this manner the *att* site was localized on a 2.5 kb *BglII*-*XmnI* fragment.

Table 1. Host range of several *S. venezuelae* phages on different *S. venezuelae* strains and on *S. exfoliatus*

Host	After infection				After transfection	
	VWB	SV1	SVL1	VW3	VWB	VWB04
<i>S. venezuelae</i> ETH 14630	+	+	-	+	+	+
<i>S. venezuelae</i> ATCC 10595	-	+	+	+	+	+
<i>S. venezuelae</i> ATCC 10712	-	+	-	+	+	+
<i>S. venezuelae</i> ATCC 14583	-	-	-	-	-	-
<i>S. venezuelae</i> ATCC 21113	-	-	-	-	+	+
<i>S. venezuelae</i> DSM 40727	-	-	-	-	-*	-*
<i>S. exfoliatus</i> ATCC 12672	+	+	-	+	+	+

* No protoplasts obtained.

During the determination of the VWB host range to other *S. venezuelae* strains it was noticed that the type strain ATCC 10712 is a lysogenic strain. In the supernatant of liquid cultures or after soaking plates containing mycelia of strain ATCC 10712, a phage could be detected that produced clear plaques on ATCC 10595 (Table 1). This phage, designated SVL1, was spontaneously released from ATCC 10712. Since the latter strain is a lysogen, it could not be infected by this phage probably as a result of phage homoimmune superinfection. The observation of the ATCC 10712 strain harbouring an actinophage was independently observed by Hahn *et al.* (1988). The occurrence of wild-type actinomycetes being lysogens is not totally unusual. It has been described earlier, e.g. for *Streptomyces hygroscopicus* (Rautenstein *et al.*, 1976), *Streptomyces rimosus* (Hranueli *et al.*, 1979), *Streptomyces lividans* 803 (Lomovskaya *et al.*, 1980), *Streptomyces galilaeus* (Kuhn *et al.*, 1987) and *Faenia rectivirgula* (Schneider *et al.*, 1987).

Localization of the att site on the VWB phage genome

In order to locate the att site on the phage genome, we isolated chromosomal DNA from several lysogens of *S. venezuelae* ETH 14630, digested it with restriction endonucleases and compared the restriction pattern to those of ligated VWB DNA. After digestion and electrophoresis the restriction fragments were blotted onto a membrane by Southern blotting and visualized by probing with ³²P-labelled or digoxigenin-labelled VWB DNA. The restriction bands containing the att site could be detected because in the DNA of the lysogens this restriction band is lost as a result of the addition of bacterial chromosomal DNA at the att site. Instead, two new bands representing the right and left phage DNA-bacterial DNA junction bands appear.

Fig. 3. SDS-PAGE of phage VWB proteins on a 12.5% gel. The amount of proteins applied per sample was 10 µg (lane 1) and 40 µg (lane 3). Proteins were visualized by Coomassie staining. Lane 4 contained 2.5 µg phage proteins made visible following immunoblotting using an anti-rabbit IgG alkaline phosphatase conjugate. The calibration kit (Pharmacia) used for molecular mass determinations (lane 2) consisted of phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor 20.1 kDa and α-lactalbumin (14.4 kDa).

In this manner the att site could be located on a 2.5 kb *Bgl*II-*Xmn*I restriction fragment (Fig. 2b). The fragments *Sph*I/B, *Bgl*II/C, *Kpn*I/A (data not shown) and *Xmn*I/A disappeared and new bands became visible (Fig. 2a). Since it was observed that in different lysogens the same restriction fragments always contained the att site and the newly formed bands were always identical, it could be concluded that the phage usually integrated into a preferred site.

Protein composition of VWB

Electron microscopic investigations of VWB have shown that this phage has an icosahedral head, a long tail and a base-plate like structure (Anné *et al.*, 1984). Electrophoresis of CsCl gradient-purified phage particles on 12.5 or 15% SDS-PAGE revealed the presence of at least 17 protein bands, a similarly high number of protein bands as observed with φC31 (Suarez *et al.*, 1984). Molecular masses ranged between 12 and 83 kDa (Fig. 3). Coomassie and immunoblotting staining clearly showed that three bands with molecular masses of 16.5 kDa, 27.2 kDa and 43 kDa are the most prominent proteins.

	1				5					10						15				20
16.5 kDa	M	E	L	S	I	K	T	Q	T	F	G	G	D	D	Q	S (L)	L	G	X	
27.2 kDa	A	G	D	T	D	N	P	R	L	W	E	G	A	D	F	Y	A	X	P	V
43 kDa	S	W	T	L	D	T	E	F	I	E	P	T	Q	L	T	G	L	I (R)	E	
							E	F	I	E	P	T	Q	L	T	G	L	I (R)	E	
							↑													

Fig. 4. N-terminal amino acid sequences of VWB proteins. The sequence of the 16.5 kDa protein was determined using both material purified by SDS-PAGE and electroblotting to PVDF (85 pmol, repetitive yield 87%) and material purified by hydroxylapatite chromatography (40 pmol, repetitive yield 83%); the sequences of the 27.2 kDa (141 pmol, repetitive yield 88%) and the 43 kDa (67 pmol, repetitive yield 90%) proteins were derived from hydroxylapatite-chromatography-purified material. Amino acids are represented by their one-letter symbols. Positions where no amino acids could be identified are indicated by an X. Amino acids that are likely to occur in a sequence are shown in parentheses. The arrow in the 43 kDa protein sequence indicates the N-terminus of the truncated form of this molecule.

They account, respectively, for 24, 10 and 20% of the total amount of proteins as estimated by Gelscan XL (Pharmacia). The proteins in these bands are thought to be capsid proteins. In most bacteriophage proteins analysed by SDS-PAGE, three to four structural proteins are similarly present in much higher amounts than the other proteins (Murialdo & Siminovitch, 1971; Relano *et al.*, 1987; Suarez *et al.*, 1984; Trautwetter *et al.*, 1987). In all instances these major proteins are proven or thought to be major head and tail proteins. Some minor bands could be contaminating host proteins or precursor polypeptides of the phage structural proteins that have been incorporated into the capsids or remained absorbed to the phage particles.

N-terminal amino acid sequence analysis of VWB proteins

In a first approach 40 µg VWB proteins were separated by SDS-PAGE and electroblotted onto a PVDF membrane. After staining, the three major protein bands (16.5, 27.2 and 43 kDa) were excised from the PVDF membrane and subjected to 20 Edman degradation cycles. Of the 16.5 kDa protein 18 amino acids could be identified (Fig. 4). In contrast, no sequence could be obtained for the 27.2 and 43 kDa proteins due to insufficient sequenceable material.

To prepare sufficient amounts of purified proteins for sequencing, 500 µg of a VWB protein preparation was subjected to hydroxylapatite chromatography. Fractions containing only one protein were concentrated and sequenced. The sequence obtained for the 16.5 kDa protein was identical to the sequence obtained after SDS-PAGE and electroblotting. In addition, the amino acid at position 17 was likely to be a leucine residue. The 20 N-terminal amino acids of the 27.2 kDa protein (with the exception of residue 18) and of the 43 kDa protein were also determined from material recovered from

hydroxylapatite chromatography. Remarkably, the 43 kDa protein showed N-terminal heterogeneity, since about 19% of the molecules started with Glu₇. This explains the observation that a protein with a slightly lower molecular mass co-purified with the 43 kDa protein during hydroxylapatite chromatography (data not shown).

Localization of the three most prominent structural proteins on the phage genome

Certain bacteriophages, e.g. λ (Hendrix *et al.*, 1983), T7 (Hausmann, 1976), P1 (Sternberg & Hoess, 1983) have a well-characterized genetic map. These maps were constructed by painstaking genetic work using numerous different mutants. From these maps it became clear that the genes coding for the structural proteins are clustered. With respect to actinophages, a relatively detailed genetic map is available for only one phage, φC31 (Lomovskaya *et al.*, 1980).

In order to study the genetic organization of VWB, we intended to map the three most prominent structural proteins by probing with mixtures of synthetic oligonucleotides based on the results of N-terminal amino acid sequencing, instead of using classical genetic means. The choice of oligonucleotides to be synthesized was based on the observation that the codon usage in *Streptomyces* shows a marked asymmetry in favour of codons with G or C in the third position (Hopwood *et al.*, 1986). To minimize the number of possible oligonucleotides corresponding to the N-terminal amino acids of the prominent structural proteins the following amino acids were chosen for the different proteins: 16.5 kDa = QTFGGDDQ; 27.2 kDa = WEGADFYA and 43 kDa = EFIEPTQL. Taking into account the average codon usage in *Streptomyces* the oligonucleotide mixtures contained a total of 18 different 24-mers in the case of the

Fig. 5. Localization on the restriction map of VWB of the DNA region coding for the N-terminal amino acid sequence of the 16.5 (□), 27.2 (X) and 43 (*) kDa proteins. Localization was done by hybridization of restriction fragments of VWB on Southern blots using as probe mixtures of oligonucleotides synthesized on the basis of the known N-terminal amino acid sequences. The upper open bar shows the restriction map of VWB digested with *SphI*. The other line represents the *SphI*/C fragment (6.2 kb) and *ApaI* (λ) and *PvuII* (○) restriction sites.

16.5 kDa protein, and 12 and 32 different 23-mers for the 27.2 kDa and 43 kDa proteins, respectively.

The mixtures were end-labelled and hybridized to different restriction fragments of VWB DNA separated on agarose gels. By this method, it was observed that the start sites of the coding regions of the prominent proteins were all located in the *SphI*/C fragment (6.2 kb) (Anné *et al.*, 1985). Finer mapping obtained after cloning the *SphI*/C fragment in pBR322 followed by restriction enzyme analysis of the cloned fragment indicated that the 16.5 kDa and 43 kDa proteins are located very close to each other (Fig. 5).

From these results it could be concluded that in VWB the genes coding for the main structural proteins are clustered in the same way as in bacteriophages investigated, e.g. λ, T4 and T7. Sequencing of the genes coding for the prominent structural proteins is in progress. Comparison of these sequences to those of other bacteriophages is without doubt of much interest for evolutionary studies.

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