Phage Acml-mediated transduction in the facultatively methanol-utilizing Acetobacter methanolicus MB 58/4

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Phage Acml, generally virulent for the acidophilic facultatively methanol-utilizing strain of Acetobacter methanolicus MB 58/4, is also capable of lysogenizing its host strain at a low rate. Using amino acid-auxotrophic mutants of A. methanolicus MB 58/4 as recipient strains, transduction of His, Leu and Tyr markers could be demonstrated in this system. The ability to prepare transducing lysates by propagation of phage Acml on the prototrophic donor strain A. methanolicus MB 58/4, the transduction of three different markers as well as the efficiency of transduction, and the occurrence of permutations in the phage genome indicate that phage Acml mediates generalized transduction. Phage Acml might be a useful tool in genetic studies of methylotrophic A. methanolicus.

Introduction

Methylotrophic bacteria are characterized by the ability to use organic substances with only one carbon atom (e.g. methane, methanol or formaldehyde) as the sole carbon and energy source, i.e. they are capable of forming numerous chemically complex compounds of high molecular mass (including biologically active substances such as enzymes) by assimilation of very simple organic molecules. Therefore, the different groups of methylotrophic bacteria have been very intensively investigated with regard to their biology and possible applications in biotechnology. At the beginning, genetic investigation of methylotrophic bacteria was considerably hindered by the lack of suitable methods for the isolation of mutants and, especially, for gene transfer. Both conjugative gene transfer and transformation have since been demonstrated. Transduction, however, has not yet been shown in this bacterial group.

The absence of transducing systems has been regarded as an important limiting factor in the genetic analysis of methylotrophs (Lidstrom-O'Connor, 1984; Holloway, 1984) and is largely due to the small number of phages of these bacteria which have been isolated and sufficiently characterized (Table 1). In our laboratory, five very closely related phages (Acml1, 2, 5, 6 and 7) of the acidophilic, facultatively methanol-utilizing Acetobacter methanolicus have been isolated and characterized. These phages are morphologically identical, but they can be easily and clearly distinguished by their strict host strain specificity (Kiesel et al., 1989a, b). The most intensely investigated phage of this group, phage Acml1, can only be propagated on the strain A. methanolicus MB 58/4. Measured under optimal conditions for growth of the host strain, the latent period is 120 min and the burst size is 68 phage particles (Wünsche et al., 1983a).

Phage Acml contains linear dsDNA. The phage genome size is calculated to be approximately 60 kbp according to direct measurements with the electron microscope, using plasmid pBR322 and phage M13 as standards, and as 52 to 56 kbp from the sum of restriction fragments (Kiesel et al., 1989b). In restriction patterns some DNA fragments arise in substochiometric amounts, indicating that permutations and terminal redundancy occur within the genome (Mamat, 1988). Phage Acml is temperate; Mamat (1988) has shown that after lysogenization, the prophage genome exists as a separate extrachromosomal element within the host cell. The lysogenization frequency in the system A. methanolicus MB 58/4-phae Acml was estimated to be 2.6% using a method described by Schrenk & Weisberg (1975).

In this paper, we demonstrate transduction processes in methylotrophic bacteria through experiments with the facultatively methanol-utilizing strain A. methanolicus MB 58/4 and its phage Acml.

Methods

Strains. All experiments were carried out with the bacterial strain A. methanolicus MB 58/4 and its phage Acml1, both isolated and characterized in the Institute of Biotechnology, Leipzig (Wünsche et al., 1983a; Uhlig et al., 1986). Amino acid-auxotrophic mutants were obtained by a combination of u.v.-irradiation and N-methyl-N'-nitro-N-nitrosoguanidine (MNNNG) treatment of bacterial suspensions under standardized conditions. This method has also been applied successfully to the production of mutants of other methylotrophic bacteria (Quayle, 1972; Bamford & O' Connor, 1979; Warner et al., 1980; Holloway, 1981; Marrison & Attwood, 1982).
Media. The basic composition of the nutrient media used, standard and soft agar for the cultivation of bacterial strains, and the propagation and determination of phage titre were described previously (Wünsche & Kiesel, 1981). Both basic liquid and solid media contained methanol (0.5%, v/v) and yeast extract (0.05%, w/v) or pantothentic acid (4 μg/ml). For the cultivation of the auxotrophic mutants, the required amino acids were added in concentrations of 20 μg/ml.

Cultures were grown both on solid and in liquid media under optimal conditions (pH 4.0, temperature 32 °C) for A. methanolicus.

Production of phage lysates. The general principles of phage manipulation techniques were as described by Adams (1959). Phage lysates were obtained by mixing suspensions of exponentially growing bacteria and phage suspensions in a ratio corresponding to an m.o.i. of 0.1. After incubation for 15 h the suspension was centrifuged and filtered through a G5 glass filter.

Phage lysates contained approximately 5 x 10⁸ p.f.u. per ml. Phages were further concentrated by precipitation with polyethylene glycol (PEG) 6000 (Maniatis et al., 1982).

Preparation of antiphage serum. Antiphage serum was kindly produced by Professor W. Erler (Institut für bakterielle Tierseuchenforschung, Jena, Germany) according to the procedure described by Hudson & Hay (1978).

Electron microscopy. Phage lysates concentrated by precipitation with PEG 6000 were purified by cesium chloride density gradient centrifugation. A drop of the phage concentrate was deposited on a copper grid with a carbon-coated Formvar film, stained with 2% phosphotungstate at pH 6.8 and studied using a Hitachi electron microscope. These investigations were kindly performed by A.E. Khenrova (All Union Research Institute of Genetics and Selection of Industrial Microorganisms, Moscow, Russia).

Transduction experiments. The first steps of the standard procedure consisted of the preparation of transducing lysates by propagation of phage Acml on the donor strain A. methanolicus MB 58/4 (prototrophic wild-type) and the overnight precultivation of the recipient strains (auxotrophic mutants of A. methanolicus MB 58/4). Recipient cells were washed and resuspended in solutions of the mineral components of the standard nutrient medium yielding cell densities of approximately 2 x 10⁶ c.f.u./ml. Transduction mixtures were prepared by addition of phage lysate to recipient cell suspensions. In all cases, the m.o.i. was determined exactly by counting the cell number (c.f.u./ml) and estimating the phage titre (p.f.u./ml) at the beginning of transduction experiments. All experiments were performed with several phage-host ratios (0.1, 0.25, 0.5, 1.0, 2.5, 5.0). Transduction mixtures were exposed to a temperature of 32 °C for 10 min, then antiphage serum was added and, finally, the mixture was plated on minimum agar containing mineral salts, methanol and pantothentic acid. In parallel experiments, frequency of reversion and the occurrence of possible transformation events were determined as controls and for the calculation of the true transduction frequency. The efficiency of transduction (e.o.t.) was calculated as described by Bendig & Drexler (1977) according to the equation e.o.t. = (N - R)/p.f.u., where N denotes the total number of cells growing after plating of the transduction mixture, R denotes the number of prototrophic revertants, and p.f.u. is the phage titre employed in the transduction experiment.

Results

Characterization of phage Acml

Table 1 gives a survey of the phages of obligately and facultatively methanol-utilizing bacteria known at present. The majority of these phages have been neither illustrated nor sufficiently described. Phages have also been isolated for a few obligately methanotrophic bacteria (Tyutikov et al., 1976, 1980; Wünsche et al., 1977; Tikhonenko et al., 1982). A first description of phage Acml was published using the designation 'MO1' in 1983 (Wünsche et al., 1983a). After the final taxonomic classification of its bacterial host strain as A. methanolicus sp. nov. (Uhlig et al., 1986), we renamed the phage according to the recommendations of the International Committee on Taxonomy of Viruses for the nomenclature of phage species (Matthews, 1982; Ackermann, 1987). During our studies of this phage, we obtained results that complete our first description and make it more precise. According to its morphology, phage Acml belongs to the Myoviridae family (Matthews, 1982). Phage heads show regular hexagonal outlines, the head diameter is 67 ± 2 nm. The sheathed tail is contractile, 73 ± 2 nm long, and is provided with a base plate which carries short spikes (Fig. 1). A special morphological feature of phage Acml is the presence of a collar with collar filaments, similar to phage A-1 of Gluconobacter oxydans described by Schocher et al. (1979).

Selection of recipient strains

For transduction experiments, we used amino acid-auxotrophic mutants of the prototrophic wild strain A. methanolicus MB 58/4, produced and characterized in our laboratory. From a large number of available auxotrophic mutants with different markers, we chose six strains (see Table 2) with the markers Met⁺, Arg⁺, His⁺, Leu⁻ and Tyr⁻, which showed a high e.o.p. (compared to the wild-type) and, especially, a stable and relatively low frequency of reversion. Most transduction experiments were performed with the His⁺ mutant MB 58/4-101, characterized by a high e.o.p. (1:1) and a stable average reversion frequency of 5 x 10⁻⁸.

Optimization of transduction

At first, possibilities of stimulating the transduction process by u.v. irradiation were examined. In most other well investigated transduction systems, u.v. irradiation led to an increase in recombinant frequency and, consequently, to enhancement of e.o.t. due to single-strand scissions in the DNA (Rubin & Rosenblum, 1971; Helling, 1973). In our phage-host system, transduction experiments using both irradiated and untreated phages and recipient strain suspensions showed a similarly strong influence of u.v. irradiation on transduction in general, in such a way that transduction could only be obtained by preparing the transduction mixture from u.v.-exposed phage suspensions and untreated recipient cells. In any other combination of unirradiated phage suspensions with irradiated and unirradiated recipient
Table 1. Phages of methanol-utilizing bacteria

<table>
<thead>
<tr>
<th>Host strain</th>
<th>Phage</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Obligately methanol-utilizing bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanomonas methylovora</td>
<td>MP1, MP2, MP3</td>
<td>Oki et al. (1972)</td>
</tr>
<tr>
<td>Methylomonas methanolis BNK-84</td>
<td>P1, P2, P3</td>
<td>Ichikawa et al. (1977)</td>
</tr>
<tr>
<td>Methylomonas methanolis PR 3102</td>
<td>P10</td>
<td></td>
</tr>
<tr>
<td>Methylomonas cereida K-81</td>
<td>P4, P5, P6</td>
<td></td>
</tr>
<tr>
<td>Methylomonas cereida BU-1</td>
<td>P7, P8</td>
<td></td>
</tr>
<tr>
<td>Methylophilus spp. KISR1-5,</td>
<td>φ KISR1</td>
<td></td>
</tr>
<tr>
<td>KISR1-5112 and KISR1-6.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylobacillus sp. MB 53</td>
<td>MO3, MO4</td>
<td></td>
</tr>
<tr>
<td>Facultatively methanol-utilizing bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetobacter methanolicus MB 58/4</td>
<td>MO1 (now Acm1)</td>
<td></td>
</tr>
<tr>
<td>Acetobacter methanolicus,</td>
<td>Acm2, Acm5, Acm6, Acm7</td>
<td></td>
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<tr>
<td>different strains</td>
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Table 2. E.o.t. of different auxotrophic markers

<table>
<thead>
<tr>
<th>Recipient strains (auxotrophic mutants of A. methanolicus MB 58/4)</th>
<th>Marker</th>
<th>E.o.t.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB 58/4-3 Met&lt;sup&gt;-&lt;/sup&gt;</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>MB 58/4-77 Arg&lt;sup&gt;-&lt;/sup&gt;</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>MB 58/4-100 His&lt;sup&gt;-&lt;/sup&gt;</td>
<td>6.4 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>MB 58/4-101 His&lt;sup&gt;-&lt;/sup&gt;</td>
<td>8.2 × 10&lt;sup&gt;-7&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>MB 58/4-116 Leu&lt;sup&gt;-&lt;/sup&gt;</td>
<td>1.4 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>MB 58/4-134 Tyr&lt;sup&gt;-&lt;/sup&gt;</td>
<td>6.6 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Phage Acml of A. methanolicus MB 58/4. Negative staining with phosphotungstic acid. Bar marker represents 100 nm.

strains, the number of growing cells on transduction plates did not exceed the corresponding number of revertants in control experiments.

Transduction under optimized conditions

Under optimized conditions of u.v. irradiation, the His<sup>-</sup> marker was transduced by phage Acml from the prototrophic wild-type A. methanolicus MB 58/4 to the histidine-auxotrophic mutant MB 58/4-101 with an average e.o.t. of 8.2 × 10<sup>-7</sup>. In addition to the phage Acml-mediated transduction of the His<sup>-</sup> marker using the corresponding auxotrophic mutants of A. methanolicus MB 58/4 as recipient strains, we showed the transduction of the same marker to another His<sup>-</sup> mutant and also the transduction of both Leu<sup>-</sup> and Tyr<sup>-</sup> markers with sufficient e.o.t. The e.o.t. differed considerably depending on the marker to be transduced (Table 2). In transduction experiments with Met<sup>-</sup> and Arg<sup>-</sup> auxotrophic mutants, the number of growing colonies on minimum agar exceeded the number of revertants determined in corresponding control experiments. In addition to the determination of the revertant frequency, we performed two additional control experiments to ensure that colonies on transduction plates were undoubtedly generated by phage-mediated gene transfer. In the first we mixed phage lysates with recipient cells and plated this mixture on minimum agar (the phage lysates were produced by propagation of phage Acml on the donor strain and inactivated by addition of phage Acml antiserum). In the second we prepared phage lysates by propagation of Acml on the recipient strain and added this phage suspension to recipient cells. In both cases, the number of colonies was significantly lower than the number of colonies on the transduction plate and equal.
to (in some cases even less than) the number of revertants that grew on the corresponding control plates.

After plating of the transduction mixture 1500 colonies of transductants carrying the His \(^+\) marker were isolated from single colonies grown on minimum agar. The colonies were investigated with respect to the transduced property, sensitivity to phage Acml and possible phage segregation. Without exception, the transduced His \(^+\) marker proved to be very stable in the investigated colonies; this could be demonstrated by continuous transfer into fresh media. All transductants were sensitive to phage Acml. Approximately 30% of the Acml-sensitive transductant clones still released phages during the first transfer to fresh medium. After addition of phage Acml antiserum, any superinfection was excluded and, consequently, no further release of phage Acml could be observed.

**Discussion**

Phage Acml is specific for the facultatively methanol-utilizing bacterial strain *A. methanolicus* MB 58/4 and belongs to the Myoviridae virus family according to its morphology. On its host strain, phage Acml generally shows a virulent multiplication cycle. However, at a relatively low rate, phage Acml is also capable of lysogenizing its host strain. It could be shown that the prophage genome is not inserted into the host chromosome but exists as an extrachromosomal element within the bacterial cell like a plasmid. Therefore, Acml must be considered to be a temperate phage.

The possibility of preparing transducing lysates by propagation of phage Acml on the prototrophic donor strain *A. methanolicus* MB 58/4, its ability to transduce at least three different bacterial markers, the data on efficiency of transduction which are very similar to those reported for generalized transduction systems (Table 2) and, finally, the permutations proved by Mamat (1988) indicate that phage Acml mediates generalized transduction in *A. methanolicus*. However, due to its host specificity, phage Acml is only capable of intraspecific transduction.

In contrast to lysogenic clones obtained by lyosenization of *A. methanolicus* MB 58/4 by phage Acml, which are basically resistant against superinfection, all of the investigated transductants proved to be sensitive to renewed infection by phage Acml. Because of this and the finding that transduced clones did not release infectious phage particles after treatment by Acml antiserum, it can be assumed that important sequences of the phage genome, at least genes coding for repression mechanisms, are not present within the genetic material of recipient cells after transduction. This could mean that transducing phages contain exclusively, or to a large extent, chromosomal DNA from the donor strain. Therefore it should be possible to transfer more than one marker simultaneously. This possible co-transduction is under investigation. Phage Acml could be a valuable tool in genetic studies of its host strain *A. methanolicus* MB 58/4.

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**References**


Transduction in methylotrophic bacteria


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