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Molecular genetics of bacteriophages of lactic acid bacteria

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Summary – Bacteriophage infection of lactic acid bacteria starter cultures can result in serious disruption or even failure of fermentation processes. With the help of newly developed techniques, progress has been made in the molecular characterisation of phages of lactic acid bacteria (LAB) and also in the elucidation of the nature of their interaction with host cultures. A number of receptors involved in phage adsorption have been indentified and DNA penetration and injection, as well as the intracellular development of the LAB phages have been investigated. The structural organisation of *Lactobacillus* and lactococcal phage genomes have also been determined and a number of phage genes has been cloned and sequenced. These include determinants for phage lysin and phage structural proteins. Specific loci, attachment sites *(att),* involved in the integration of temperate phage genomes, the cohesive ends *(cos),* involved in phage genome circularisation and the packaging sites *(pac)* of circularly permuted phages have been localised and in many cases characterised at a molecular level.

lactic acid bacteria / bacteriophages / molecular characterisation / lysogeny

Résumé — Génétique moléculaire des bactériophages des bactéries lactiques. *Les infections des cultures starter de bactéries lactiques par des bactériophages peuvent provoquer de sérieuses perturbations ou même l'arrêt du processus de fermentation. Grâce aux techniques nouvellement développées, des progrès ont été faits dans la caractérisation moléculaire des phages des bactéries lactiques, ainsi que dans la compréhension de la nature de leur interaction avec les cultures hôtes. Plusieurs récepteurs impliqués dans l'adsorption des phages ont été identifiés et l'on a étudié la pénétration et l'injection de l'ADN, ainsi que le développement intracellulaire des phages des bactéries lactiques. On a déterminé l'organisation structurale des génomes de phages de* Lactobacillus *et de* Lactococcus, *et de nombreux gènes phagiques ont été clonés et séquencés. Cela inclut des déterminants de la lysine et de protéines structurales phagiques. Des loci spécifiques, sites d'attachement* (att), *impliqués dans l'intégration des génomes des phages tempérés, les extrémités cohésives* (cos), *impliqués dans la circularisation des génomes phagiques et les sites d'empaquetage* (pac) *des phages à permutations circulaires, ont été localisés et, dans de nombreux cas, caractérisés au niveau moléculaire.*

bactéries lactiques / bactériophages / caractérisation moléculaire / lysogénie

INTRODUCTION

Bacteriophage infection is known as the major cause of the inhibition of LAB starter cultures used in food fermentations. This is particularly true in the dairy industry for the following reasons: the fermentation is carried out in a non sterile-medium (pasteurised milk); batch culture fermentations are conducted under increasingly stringent manufacturing schedules; the amount of specialized cultures available on the market is limited; and the continuous use of defined cultures provides an ever-present host for bacteriophage attack. Through the years, efforts have been made to identify the major species of bacteriophages of LAB, to elucidate the specific nature of their interactions with their hosts and to define their physiological and genetic traits as an aid to determining their molecular characteristics. The information obtained will provide novel approaches for the construction of phage resistant starter cultures and will assist in the identification of factors controlling phage gene expression.

PHAGE ADSORPTION

The first step in the interaction between a lytic phage and its host occurs when the phage particle adsorbs to the cell surface. Electron microscopic studies with lactococcal phages have shown that these either adsorb to their homologous hosts in small groups (5–30 phages) at evenly distributed spots or they adsorb uniformly over the cell surface (Budde-Niekiel and Teuber, 1987). Phage receptors are generally cell wall located; an overview of phage receptors identified to date is presented in table I. Recently, Valyasevi *et al* (1991) reported that the reversible binding to the cell wall polysaccharide is followed by an irreversible interaction with a cell membrane located protein.

Table I. Phage receptors of LAB.

Receptor	*Phage*	*Reference*
Lactococcus		
Lipoprotein of the plasma membrane	Øm13	Oram, 1971
L-Rhamnose, D-galactosamine D-glucosamine (cell-wall associated)	1b7	Koegh and Pettingill, 1983
Rhamnose, extracellular polysaccharide	Økh	Valysevi *et al*, 1990
Carbohydrate component of the peptidoglycan	P008, P127	Schäfer *et al*, 1991
Lactobacillus		
Rhamnose (cell-wall), D-galactosamine (cytoplasmic membrane)	PI-1	Yokokura, 1977
L-Rhamnosyl (peptidoglycan)	PL-1	Ishibashi *et al*, 1982

DNA PENETRATION AND INJECTION

For *Lactobacillus casei* phage PL-1, it has been shown that Ca^{2+} and ATP were essential for penetration of the phage DNA (Watanabe and Takesue, 1972; Watanabe *et al,* 1979). Watanabe *et al* (1991) have examined injection of *Lb casei* phage PL-1 by electronmicroscopy and have demonstrated that the process was inhibited by chloramphenicol and erythromycin, both of which inhibit protein synthesis. Thus it is possible that protein synthesis is needed in the early stage of the phage infection and is necessary for complete DNA transfer and injection.

INTRACELLULAR DEVELOPMENT OF LAB PHAGES

The burst sizes and the latent periods of the LAB phages are summarised in table II. Generally, the optimum temperature for phage replication is similar to that of the host strain. Phage multiplication can also be dependent on the nutritional status of the host and on electrolyte availability, both of which can be active in promoting phage replication and cell lysis (Keogh, 1980; Klaenhammer, 1984).

Very little is known about the intracellular development of LAB phages at a molecular level. Hill *et al* (1991a) have reported that the DNA molecules of the cohesive ended phage 31 are synthesized in a concatameric form between 20–40 min after infection of *L lactis* NCK203. Sixty min after infection, there was a decrease in the detectable amount of phage DNA, which was possibly due to packaging and release of the phage particles. Powell *et al* (1992) have analysed the replication of the broad host range prolate-headed phage c6A and found that infection of *L lactis* C6 caused inhibition of culture growth within 10 min and cell lysis after 25 min. DNA synthesis was detected after 6-8 min and stayed constant until cell lysis. Using ^{3}H-thymidine-labelled host DNA, it could be shown that degradation of the host DNA occurred after 4-6 min. The breakdown products were incorporated into the phage DNA. Molecular studies with the *Lb casei* phage PI-1 showed that it used unmodified host RNA polymerase to transcribe its entire genome. Early genes were expressed within the first 20 min, whereas the late genes were expressed after 40 min (Stetter *et al,* 1978).

Following the synthesis of the phage macromolecular components, the phage

Table II. Intracellular development of LAB phages.

Organism	*Latent period (min)*	*Burst size*	*Reference*
Lactococcus	10–140	10–400	Keogh, 1973 Klaenhammer, 1984
Lactobacillus	40–75	80–300	Sechaud *et al,* 1988
Leuconostoc mesenteroides	28	41	Neve and Teuber, 1991
Leuconostoc oenos	60	16–20	Arendt *et al,* 1990

particles are assembled by a mechanism which has not yet been elucidated. At the end of the latent period the peptidoglycan layer of the host cell is lysed by phage encoded lysozyme like lysin. Lysins of LAB phages have been isolated and characterised in detail (see table III).

CLASSIFICATION OF LAB PHAGES

Much effort has been focussed on the development of a coherent classification system for LAB phages. These taxonomic studies are very important for the following reasons: they provide an insight into the relationship between phages; they may allow identification of the origin of the phages disturbing fermentation processes; they provide knowledge of the genetic characteristics of phages, which can then be useful in predicting the potential utility of phage resistance mechanisms. Taxonomic characterisations of phage are based on morphology, DNA homology, protein composition and serological analysis. Since it is beyond the scope of this article to review the characterisation and classification of LAB phages, the reader is directed to a number of comprehensive publications dealing with this topic (Lahbib-Mansais *et al*, 1988; Neve *et al*, 1989; Jarvis *et al*, 1991; Boizet *et al*, 1992).

BACTERIOPHAGE GENOME CHARACTERISATION

All LAB phages examined to date possess double-stranded DNA. The majority of these appear to have cohesive ends although a few phages show a circularly permuted structure with terminal redundancy. This is based on the following criteria : the presence of submolar fragments after restriction analysis; evidence for the presence of a *pac* site at which packaging of the phage genome is initiated; the absence of cohesive ends; and homology between the submolar fragment containing the terminal redundancy and the area of the genome close to the *pac* site. The G+C

Table III. Genes/elements cloned from LAB phages.

Gene/Element	*Phage*	*Reference*
Lactococcus		
Lysin	ØvML3	Shearman *et al*, 1989
Minor structural proteins	F4-1	Kim and Batt, 1991a,b
Major capsid protein	F4-1	Kim and Batt, 1991a,b
Undefined ORF	Ø7-9	Kim and Batt, 1991c
LlaI methylase	Ø50	Hill *et al*, 1991b
BK5-T promoter inhibitor	BK5-T	Lakshmidevi *et al*, 1990
Phage encoded resistance and phage origin of replication	Ø50	Hill *et al*, 1990
Lactobacillus		
Lysin	LL-H	Trautwetter et al, 1986
Phage structural proteins	LL-H	Trautwetter *et al*, 1986 Alatassova *et al*, 1987
Lysin	mv1	Boizet *et al*, 1990
Insertion sequence ISL1	ØFSV	Shimizu-Kadota *et al*, 1985

contents of the LAB phages are as follows : *Lactococcus* (37–39 %); *Lb plantarum* (37%) and *Lb casei* (45–48%). The genome lengths of LAB phages are summarised in table IV. Genetic determinants which have been cloned from phages of LAB are summarised in table III.

LYSOGENIC PHAGES OF LACTIC ACID BACTERIA

Lytic phages are of major concern in dairy fermentations, since they are primarily responsible for the disruption of fermentation processes. However, there is also significant interest in phages which can enter an alternative relationship with their host, the lysogenic cycle. In this case, the phage genome integrates into the chromosome after injection into the target cell, usually by recombination between 2 sites termed *attP* (phage attachment site) and *attB* (bacterial attachment site) on their respective genomes. Little information is available regarding the molecular or genetic basis for control and maintenance of the lysogenic relationship in LAB hosts.

Table IV. Genome length of LAB phages.

Organism	*Genome length (kb)*
Lactococcal phage	
Small isometric	29–40
Large isometric	53–55
Prolate-headed	18–22
Lactobacillus phage	34–41
L delbrüeckii	75
Streptococcus thermophilus phage	33.8–44.2
Leuconostoc mesenteroides phage	27.4
Leuconostoc oenos phage	25.3

The study of lysogeny in LAB is very worthwhile for a number of reasons. Lysogenic hosts can serve as a reservoir of lytic phage in dairy plants. One classical example demonstrating this was presented by Shimizu-Kadota *et al* (1983), who showed that a phage which was derived from a lysogenic *Lb casei* host, was able to infect a *Lb casei* strain used in a yakult fermentation.

Lysogeny can also serve as a model system for the analysis of the regulation and control of phage gene expression. The information obtained from such a study could be used, for example, in the construction of integration vectors. Chopin *et al* (1989) were able to generate a chromosomal integration vector harboring a fragment of lactococcal prophage DNA, which was homologous to a resident prophage in the host chromosome. Integration was achieved by homologous recombination. Temperate phages are also capable of mediating transduction which can be exploited to transfer plasmid- and chromosomally-encoded traits and which may also prove useful in developing chromosomal maps for various members of the LAB (Fitzgerald and Gasson, 1988; Davidson *et al,* 1990). Transduction of lactose-fermentation ability and proteolytic activity has already been reported (McKay and Baldwin, 1974; Gasson, 1983; Fitzgerald and Gasson, 1988).

CHARACTERISATION OF TEMPERATE PHAGES AT THE MOLECULAR LEVEL

Lakshmidevi *et al* (1988) have characterised the temperate *L lactis* subsp *cremoris* phage BK5-T in detail. The unit genome size of this phage is 37.6 kb, and it was shown to be circularly permuted with terminal redundancy and therefore DNA packaging is carried out by a headfull mechanism, giving rise to considerable variation

in genome size (39.7–46 kb). Its lysogenic host is *L lactis* subsp *cremoris* BK5 but the phage is also capable of propagating lytically on *L lactis* subsp *cremoris* H2. When phage BK5-T was grown on this lytic host, it lost its ability to lysogenize its lysogenic host, a feature which was linked to a deletion of 0.6–2.5 kb in a specific region of the genome, at a location separate from the *attP* site. Lakshmidevi *et al* (1990) also isolated 5 promoters from phage BK5-T and they could also identify a region (621 bp) on the phage genome which was responsible for the inactivation of 3 of these.

The temperate lactococcal phage Tuc2009 isolated from *Lactococccus lactis* subsp *cremoris* UC509 by induction with mitomycin C has been analysed in some detail. The phage has a small isometric-head (52 nm), possesses a non-contractile tail (152 nm) and a base plate (16 nm across). Electronmicroscopic examination of *Lactococccus lactis* subsp *cremoris* UC526 host cells mixed with phage Tuc2009 revealed that the phage particles attached evenly over the cell surface. When the structural proteins of the phage were analysed by SDS-PAGE 2 major proteins with a molecular weight of 30 and 21.5 kDa were identified. The first 15 and 8 amino acids, respectively, were identified by N-terminal sequencing (Arendt, unpublished results). Restriction analysis showed that phage Tuc2009 has a genome size of ≈ 40 kb. No evidence could be obtained for the presence of cohesive sites *(cos)*. More-over, the presence of the submolar fragments in restriction enzyme digests of the phage DNA suggested that packaging occurs though a headfull mechanism yielding circularly permuted genomes. The site at which packaging starts *(pac)*, and the attachment site *(attP)*, have been localised to particular restriction fragments. Based on DNA–RNA and DNA–DNA hybridization studies, early and late gene expression functions could be attributed to specific regions of the phage genome. Phage-specific DNA could be detected 40 min after infection and there was a consistent increase up to 80 min, and thereafter the concentration of detectable DNA decreased presumably due to packaging. While low levels of transcription products were detected 28 min after infection of the host, there was not a significant increase in the level of RNA until 48 min. Using various restriction fragments spanning the entire Tuc2009 genome to probe the RNA samples, it was possible to identify early and late functions on the phage restriction map (Fitzgerald, unpublished results).

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