Differentiation of Two Abortive Mechanisms by Using Monoclonal Antibodies Directed toward Lactococcal Bacteriophage Capsid Proteins†

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Monoclonal antibodies were used to monitor the accumulation of the major capsid protein of the lactococcal small isometric bacteriophage u136 (P335 species) over the course of a one-step growth curve. A sandwich enzyme-linked immunosorbent assay was then used to distinguish two abortive phage resistance mechanisms, Hsp and Prf. Capsid protein production of u136 was almost totally inhibited by the Hsp-induced abortive mechanism, supporting previous data that this mechanism blocks phage DNA replication. Prf-induced abortive infection only partially (50%) inhibited capsid protein production, suggesting that this mechanism targets some other point, perhaps within transcription or translation processes. The results confirmed that Hsp and Prf act at different targets in the phage lytic cycle. Use of monoclonal antibodies also demonstrated that production of the major capsid protein is a nonlimiting step in the lytic cycle of lactococcal phage u136.

Recurrent milk fermentation failures due to bacteriophage attack still account for substantial economic losses in the cheese industry (1). Studies on this problem have been the focus of research programs worldwide (17). Naturally occurring lactococcal phage-insensitive bacterial strains have been isolated and found to harbor plasmids encoding for multiple defense systems (16). The characterization of a number of phage resistance plasmids has enabled researchers to use genetic approaches in the construction of new phage-insensitive strains (16, 23). Three major classes of plasmid-encoded phage defenses exist in lactococci which include mechanisms that interfere with phage adsorption, restrict and modify phage DNA (R/M system), and abort the phage infection (17).

Abortive infection appears to be one of the most important classes of phage resistance identified thus far in lactococci. Unfortunately, the mechanisms through which phage infections are aborted are the least understood at the present (16). During an abortive infection, phage development is inhibited while adsorption, DNA injection, and the early stages of viral gene expression are normal. In aborted phage infections, the host is killed while halting intracellular phage development (7). Therefore, abortive mechanisms are potentially very powerful because they enable cells to trap phages and prevent the release of viable progeny phages (25). A novel starter rotation system was recently designed based on these trapping abilities of natural abortive mechanisms (18).

Long-term phage resistance will be best achieved if defense systems that act on different targets of the phage lytic cycle are combined (17). However, our knowledge about the development processes of lactococcal phages is limited, and thus, it is difficult to assess the point at which an abortive process impacts the lytic cycle (25). Proper selection of complementary abortive mechanisms would be greatly enhanced by a more thorough understanding of their mechanisms and point of action.

Many naturally occurring abortive systems have been identified in lactococci (for a review, see reference 5). However, only three genes relating to abortive mechanisms have been cloned and sequenced to date (4, 9, 10, 12, 14). Two of these have been recently characterized in our laboratory (9, 12). They were cloned from the naturally occurring self-transmissible phage resistance plasmids pTR2030 and pTN20, respectively (10, 14). These two plasmids were isolated from the prototype phage-insensitive strain Lactococcus lactis ME2 (16). The plasmids harbor different R/M systems as well as the abortive systems designated Hsp (pTR2030 [12]) and Prf (pTN20 [9]). The essential DNA regions of these abortive mechanisms have been cloned and sequenced, and the genes have been designated abIA and abIC, respectively. Each gene encodes for a single protein of unknown function. Using a method to evaluate intracellular phage DNA replication (11), it was shown that after phage DNA injection, DNA replication of small isometric-headed phages (P335 species) is inhibited by Hsp (abIA). In contrast, Prf does not block that step of phage development (9). These results suggest that Hsp and Prf act at grossly different stages of the phage lytic cycle. Additional information on the mechanism of action of these two systems, or any other abortive system, is not available (5, 25).

We have developed monoclonal antibodies (MAbs) against the denatured major capsid protein (35 kDa) of the small isometric-headed lactococcal phage u136 (20), a member of the P335 species (15). The MAbs were developed initially to detect phages in milk and cheese whey (19). We present in this report the development of a sandwich enzyme-linked immunosorbent assay (ELISA) using MAbs for the quantitative assessment of phage protein accumulation during phage infection in lactococci. We investigated the effects of the abortive mechanisms Hsp and Prf on the accumulation of the major capsid proteins of u136 after infection of L. lactis NCK203. To our knowledge, this is the
first report quantifying the intracellular effect of an abortive defense mechanism on phage protein production.

**MATERIALS AND METHODS**

**Bacterial strains, phages, and plasmids.** Lactococcal strains and phages, described in Table 1, were propagated at 30°C in M17 broth (27) supplemented with glucose (M17G) and CaCl₂ as described previously (20). Erythromycin was also added at a concentration of 5 μg/ml to ensure plasmid retention.

**Bacteriophage assays.** Preparation of bacteriophage lysates and determination of titers (PFU/per milliliter) were conducted as described previously (27) except that 0.3 ml of log-phase cells (optical density at 600 nm = 0.5) was used in titration assays. The efficiency of plaque formation (EOP) was calculated by dividing the phage titer on the test strain by the titer on the phage-sensitive host NCK203.

Center of infection assays (COI) were conducted on log-phase cells (optical density at 600 nm = 0.8) essentially as described by Sing and Klaenhammer (26). Bacterial culture (2 ml) was centrifuged and resuspended in 900 μl of M17G–5 mM CaCl₂. Phage lysates were used to achieve a final multiplicity of infection of 0.1 or less. Phage were allowed to adsorb for 5 min. Cells and adsorbed phage were washed twice in 1 ml of M17-Ca²⁺ to remove free phage and then diluted 10,000-fold to a final volume of 10 ml. One milliliter of the infected cells was withdrawn immediately for initial COI determinations (time zero), and the remainder was incubated at 30°C for one-step growth curve experiments. Samples were taken periodically over 120 min, diluted, and plated onto a lawn of the phage-sensitive host (NCK203). Enumeration of the plaques defined the number of COI formed on the test strain. The efficiency at which COI formed (ECOI) was obtained by dividing the number of COI from the test strain by the number of COI from the sensitive host NCK203. The burst size for the phage on a test strain was determined as (phage titer at the end of the single step growth curve, time 75 min – initial titer, time zero)/initial titer. The latent period was estimated at the midpoint of the exponential phase of the one-step growth curve (21).

**Sample preparation for ELISA.** Strains were grown until the optical density at 600 nm reached 0.1. Phage u136 was added to a multiplicity of infection of 10. At intervals of 5 min, 1-ml samples were mixed with sodium dodecyl sulfate and β-mercaptoethanol to achieve final concentrations of 0.01 and 0.03%, respectively. The samples were then heated in boiling water for 10 min to promote rapid disruption of the cells and to partially denature proteins. Samples were left at room temperature until the experiment was completed, and then the sandwich ELISA was performed.

**ELISA method to detect major capsid protein of phage u136.** The production and characterization of the MAbs to the capsid protein of u136 (7H5 and 6C1) were described previously (19). Flat-bottom polystyrene 96-well plates (Immulon-4; Dynatech Industries, Inc., McLean, Va.) were coated overnight at 4°C with the anti-u136 MAb (7H5, 200 ng per well) in carbonate buffer (100 mM, pH 9.6). After three washes with 200 μl of TBS (50 mM Tris [pH 7.5], 200 mM NaCl, 0.1% Tween 20), wells were saturated with 200 μl of 1% nonfat dry milk for 2 h at 37°C, washed twice, and stored at 4°C until use (up to 1 week). Lower background was obtained with nonfat dry milk than with 1 or 3% bovine serum albumin (BSA). After two additional washes with TBS, the denatured phage samples (200 μl in triplicate) were added and incubated for 2 h at 37°C. Following three washes, a biotinylated anti-u136 MAb (6C1), recognizing a different epitope than 7H5, was added (200 μl of 6C1 diluted 1:1,000 in TBS–1% BSA per well) and incubated for 2 h at 37°C. After washes, the conjugate streptavidin-alkaline phosphatase (GIBCO BRL, Gaithersburg, Md.) was added (50 μl of a 1:1,000 dilution), and incubation continued for 30 min at 37°C. Finally, after the unbound conjugate was washed, 100 μl of the substrate pNPP (2 mg/ml in 10% ethanolamine–0.5 mM MgCl₂ [pH 9.8]; GIBCO BRL) was added, and the plates were incubated at room temperature for 15 min. The reaction was stopped with 50 μl of 3 N NaCl. The A₄₀₅ was read with a Dynatech MR5000 automatic ELISA reader.

**RESULTS**

**EOP.** Traditional plaque assays were conducted to assess phage u136 development in NCK203 infected cells. Reduced EOPs for phage u136 were obtained from NCK203 derivatives containing either of the recombinant plasmids pTRK18 and pTRK99 bearing the abortive genes abiA and abiC, respectively (Table 2). Phage u136 plated at an EOP of 0.025 on NCK203 harboring pTRK18 (abiA Hsp⁺ Prf⁻) and at an EOP of 0.002 on NCK203 harboring pTRK99 (abiC Hsp⁺ Prf⁻). In both cases, when plaques formed on Hsp⁺ or Prf⁺ hosts, they were reduced in size. These results showed that u136, like other small isometric-headed phages, was sensitive to Hsp and Prf, with Prf eliciting a stronger effect.

**ECOI.** The ECOI for phage u136 on Hsp⁺ NCK203 (pTRK18) was 0.044, indicating that only 4 to 5% of the infected cells release progeny, whereas 48% of infected Prf⁺ NCK203(pTRK99) cells released viable phages. The Hsp phage defense system therefore aborted more u136 phage infections than did Prf.

**Latent period.** A latent period of approximately 45 min was observed for all three strains infected with u136 (Fig. 1). This indicates that phages escaping either abortive mechanism are not delayed in their ability to provoke cell lysis.

**Burst size.** When lactococcal phage u136 was propagated on NCK203, each infected cell released an average of 326 progeny phage. Of the Hsp⁺ NCK203 cells, which are successfully infected and release progeny (5%), each released an average of 244 viable phages. This illustrates that when u136 escapes the Hsp abortive mechanism, the phage...
PROPAGATES ALMOST NORMALLY. ON THE OTHER HAND, PRF+ NCK203 CELLS, WHICH ARE SUCCESSFULLY INFECTED AND RELEASE PROGENY (48%),produced only an average of 21 PHAGES. THESE RESULTS INDICATE THAT INFECTIVE CENTERS FORMED ON PRF+ CELLS ARE NOT COMPLETING A NORMAL CYCLE, AND, THEREFORE, THE NUMBER OF PROGENY GENERATED IS SEVERELY LIMITED.

PRODUCTION OF MAJOR CAPSID PROTEIN. USING AN ELISA, PRODUCTION OF THE MAJOR CAPSID PROTEIN OF u136 WAS MONITORED DURING INFECTION OF NCK203 CELLS (FIG. 2). THE MAJOR CAPSID PROTEIN WAS FIRST DETECTED 30 MIN AFTER U136 INFECTION OF NCK203 CELLS, AND PRODUCTION PEAKED AT 60 MIN. THE RELATIVELY LATE Expression OF THE MAJOR CAPSID PROTEIN AGREES WITH STUDIES OF ESCHERICHIA COLI PHAGES IN WHICH CAPSID PROTEIN MRNAs ARE TRANSCRIBED FROM LATE PROMOTERS (2, 8). FOR PRF+ NCK203(pTRK99) INFECTED CELLS, THE ACCUMULATION OF MAJOR CAPSID PROTEIN STARTED AT 30 MIN AND PEAKED AT 65 MIN. HOWEVER, ONLY 50% OF THE PROTEIN WAS DETECTED RELATIVE TO THAT PRODUCED BY INFECTED NCK203 CONTROL CELLS. FINALLY, FOR HSP+ NCK203(pTRK18) INFECTED CELLS, NO MAJOR CAPSID PROTEIN WAS DETECTED BEFORE 75 MIN, AND THEN ONLY 10% OF THE PROTEIN WAS PRODUCED AT 80 MIN RELATIVE TO THE CONTROL. THE RESULTS DEMONSTRATED THAT BOTH HSP AND PRF ABDUCTIVE MECHANISMS SIGNIFICANTLY REDUCED MAJOR CAPSID PROTEIN PRODUCTION.

DISCUSSION

A SANDWICH ELISA WITH MABS TO THE MAJOR CAPSID PROTEIN OF THE SMALL ISOMERIC PHAGE u136 WAS USED IN THIS STUDY TO MONITOR THE COURSE OF PHAGE INFECTION IN SENSITIVE AND PHAGE-RESISTANT NCK203 CELLS. THE METHOD DETECTED INTRACELLULAR PHAGE PROTEIN AS WELL AS THE MAJOR CAPSID PROTEIN PRESENT IN PHAGE PARTICLES RELEASED DURING THE LYtic INFECTION. THE PHAGE-HOST COMBINATION USED IN THIS STUDY IS AN IDEAL SYSTEM FOR OBSERVING INTRACELLULAR PHAGE DEVELOPMENT BECAUSE OF THE RELATIVELY LONG LATENT PERIOD (45 MIN) AND LARGE BURST SIZE (326). LATENT PERIODS RANGING FROM 32 TO 56 MIN AND BURST SIZES FROM 2 TO 125 HAVE BEEN REPORTED FOR LACtOCOCCAL PHAGES (21, 22). THE LENGTHY LATENT PERIOD OF u136 ALLOWS MORE SAMPLES TO BE TAKEN OVER THE TIME COURSE OF THE ONE-STEP GROWTH CURVE. A LARGE BURST SIZE REQUIREs HIGH PRODUCTION OF PHAGE PROTEINS AND, THEREFORE, EASES THEIR DETECTION BY ELISA METHODOLOGIES.

L. LACTIS NCK203 TRANSFORMANTS CONTAINING EITHER OF THE TWO RECOMBINANT PLASMIDS ENCODING DISTINCT ABDUCTIVE RESISTANCE MECHANISMS WERE PARTIALLY RESISTANT TO U136. NINETY-FIVE PERCENT OF INFECTED HSP+ CELLS DID NOT RELEASE PROGENY PHAGE. IN THE REMAINING 5% OF INFECTED CELLS THAT DID RELEASE PHAGE, BURST SIZE WAS ALMOST NORMAL. IT APPEARS THAT THE INFECTION PROCEEDS NORMALLY IF THE PHAGE ESCAPES THE HSP MECHANISM. IT IS NOTeworthy THAT THESE PHAGES ESCAPING THE HSP ABDUCTIVE MECHANISM ARE STILL SENSITIVE TO THIS DEFENSE SYSTEM. PREVIOUS DATA HAVE SHOWN THAT PHAGE DNA REPLICATION IS INHIBITED IN HSP+ CELLS (11). THEREFORE, THE PHAGE TO REPLICATE ITS DNA WILL ACCORDINGLY LIMIT PHAGE PROTEIN SYNTHESIS. INDEED, THE INFECTED HSP+ CELL POPULATION PRODUCED BARELY DETECTABLE LEVELS OF MAJOR CAPSID PROTEIN (10%). WE ESTIMATE THAT THIS 10% PRODUCTION OF MAJOR CAPSID PROTEIN COMES FROM THOSE INFECTED CELLS (5%) IN WHICH U136 REPLICATION IS NOT RETARDED.

ON THE OTHER HAND, THE PRF ABDUCTIVE MECHANISM WAS LESS STRINGENT THAN HSP SINCE 48% OF U136 INFECTIONS WERE STILL SUCCESSFUL. A PREVIOUS STUDY HAS SHOWN THAT PRF DOES NOT RETARD PHAGE DNA REPLICATION (9), BUT STILL, MAJOR CAPSID PROTEIN PRODUCTION WAS REDUCED BY HALF COMPARED WITH THAT OF THE CONTROL CELLS. A POSSIBLE EXPLANATION IS THAT A NORMAL LEVEL OF MAJOR CAPSID PROTEIN PRODUCTION IS OCCURRING ONLY IN

TABLE 2. Effects of the abortive mechanisms HSP+ and PRF+ on the lactococcal phage u136

<table>
<thead>
<tr>
<th>Strain</th>
<th>EOP±</th>
<th>ECOI±</th>
<th>Burst size±</th>
<th>Latent period (min)</th>
<th>MCP production± (%)</th>
<th>DNA replication±</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCK203</td>
<td>1.000</td>
<td>1.000</td>
<td>326±54</td>
<td>45</td>
<td>100</td>
<td>++++</td>
</tr>
<tr>
<td>NCK203(pTRK18) (HSP+)</td>
<td>0.025</td>
<td>0.044</td>
<td>244±72</td>
<td>45</td>
<td>10</td>
<td>–</td>
</tr>
<tr>
<td>NCK203(pTRK99) (PRF+)</td>
<td>0.002</td>
<td>0.480</td>
<td>21±15</td>
<td>45</td>
<td>50</td>
<td>+++.</td>
</tr>
</tbody>
</table>

* EOP, ECOI, and burst sizes are the average of three trials for NCK203 and two trials for NCK203(pTRK99) and NCK203(pTRK18).

# Estimated from four experiments.

* Normal (+++) or inhibited (−) phage DNA replication.

FIG. 1. One-step growth curves for phage u136 on L. lactis. Symbols: ▲, NCK203 (HSP+ PRF+); ●, NCK203 (HSP+ PRF−); ○, NCK203(pTRK99, HSP+ PRF+). Error bar indicates standard deviation for three trials. The first phage count (time zero) is approximately 5 min after adding the phage to the cells (see Materials and Methods).

FIG. 2. Accumulation of the major capsid protein of phage u136 during infection of L. lactis. Symbols: ▲, NCK203 (HSP+ PRF+); ●, NCK203(pTRK18, HSP+ PRF+); ○, NCK203(pTRK99, HSP+ PRF+). Error bar indicates standard deviation for four trials. A 5-min period was allowed for phage adsorption before the first sample was taken (time zero). OD, optical density.
that proportion of the population replicating the phage (48%). However, if this were the case, one would expect a burst size in Prf+ cells similar to that in the control. This is, in fact, not the case, since burst size is strongly reduced (over 90%) in Prf+ cells that release progeny.

Another possibility is that the Prf abortive system may act at some point to limit protein production in all infected cells. The reduction of the burst size (over 90%) is more pronounced than the reduction of the major capsid protein (50%), suggesting that the major capsid protein is not the limiting step in the lytic cycle of this phage. Failure to accumulate the major capsid protein may be a secondary or downstream effect due to the action of the Prf resistance mechanism. Targets for this abortive mechanism, therefore, may include any step in transcription and translation processing, modulation, or regulation.

A comparison of the growth curve (Fig. 1) and the relative amount of major capsid protein produced (Fig. 2) showed similar phage populations in Hsp+ and Prf+ samples but a significant difference in the production of major capsid protein at 75 min. The major capsid protein is definitely overproduced in Prf+ cells (and probably in the control cells as well), indicating that production of major capsid protein is a nonlimiting step. This finding may have two important consequences. First, excess production of major capsid protein suggests that this protein may be an effective target to use for phage detection by ELISA techniques. Second, because the production of major capsid protein appears as a nonlimiting step in the phage cycle, it is not a suitable target for the development of phage resistance based on antisense technologies. Recently, Chung et al. (3) reported that antisense RNA directed against the major capsid protein of L. lactis subsp. cremoris bacteriophage F-41 confers only partial (50%) resistance to the host. These data further support the ELISA data indicating that the major capsid protein is produced in excess.

Using an ELISA method and standard plaque formation techniques, we observed a lactococcal phage infection from two vantage points: one quantitively assessing intracellular production of the major capsid protein and the other assessing the extracellular release of viable progeny phage. When the two types of data were combined, a more thorough depiction of the infection process was possible. We provide additional evidence that the abortive mechanisms Hsp and Prf act at different points of the phage lytic cycle and, therefore, that they should complement one another in a manner that elicits a greater overall level of phage resistance. Use of Mabs to various phage structural proteins could be an important tool to facilitate our subsequent investigation of abortive defense mechanisms. Understanding and characterization of natural abortive mechanisms will help to define new efficient phage defense combinations that can act at multiple targets within the phage lytic cycle.

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