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The Conjugative Plasmid pTR2030 Encodes Two Bacteriophage Defense Mechanisms in Lactococci, Restriction Modification (R^+/M^+) and Abortive Infection (Hsp^+)†

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pTR2030 is a conjugative plasmid which encodes resistance to bacteriophage in lactococci by a mechanism that aborts the phage infection (Hsp^+). Subcloning and in vivo deletion events showed that two independent mechanisms of resistance are located on a 13.6-kilobase *BglII* fragment cloned in pSA3; one mechanism is responsible for the abortive infection, and the other encodes a restriction modification system. The introduction of pTR2030 or the recombinant plasmid pTK6 resulted in the loss of a resident restriction modification plasmid in *Lactococcus lactis* NCK202 which was not previously identified.

A number of plasmids which encode resistance to bacteriophage in lactococci have been identified (4, 13, 17). The resistance mechanisms encoded by these plasmids include interference with phage adsorption (6, 19), restriction modification (R/M) (3, 7–9, 18, 24), and abortive infection (7, 8, 10, 11, 14–16). In general, these systems have been studied independently, and little is known regarding the cooperative or counteractive effects of combining defense mechanisms. Naturally occurring systems of multiple-resistance mechanisms which confer a strong resistance phenotype on the host have been described elsewhere (2, 3, 7, 8, 19). We have previously described one such phage-insensitive host, *Lactococcus lactis* ME2, which contains plasmid-encoded resistance via adsorption inhibition (pME0030; 19) and R/M (pTN20; 9) and which can conjugally transfer a 46.2-kilobase (kb) plasmid, pTR2030, associated with an abortive-infection mechanism defined as Hsp^+ (12, 14, 17, 21, 23). The Hsp region of pTR2030 was localized to a 13.6-kb *BglII* fragment and cloned in the *Streptococcus-Escherichia coli* shuttle vector pSA3 (5). The Hsp^+ phenotype directed by the recombinant plasmid pTK6 in *L. lactis* MG1363 has been described previously (10) as a reduction in plaque size accompanied by a 1-log decrease in the efficiency of plaquing (EOP). The *hsp* locus was defined by Tn*5* mutagenesis to an essential 3-kb region within the cloned fragment (10).

In this report, we demonstrate the presence of a second, independent resistance mechanism, defined as R^+/M^+, encoded within the 13.6-kb region of pTR2030. We also report the presence of a previously undetected native R/M plasmid in *L. lactis* NCK202 which appears to be incompatible with both pTR2030 and pTK6.

L. lactis strains were grown at 30°C in M17 medium supplemented with 0.5% glucose (25). Transformation of *L. lactis* protoplasts was performed as previously described (10). Plasmid species were confirmed in transformants by the lysis procedure of Anderson and McKay (1). Plaque assays were conducted as described previously (25) at 30°C on cells grown to an optical density at 600 nm of 0.5. Construction of pTK6 and isolation and characterization of pTRK18 were described previously (10). Plasmid curing was induced by successive transfers at 37°C under nonselective conditions.

pTR2030 was introduced previously to NCK202 (originally designated L2FA; 12) by solid-surface conjugation (12). In this study, the pTR2030 transconjugant NCK209 was challenged with two small isometric phages, nck202.31 (ϕ31) and nck202.48 (ϕ48), homologous for *L. lactis* NCK202. Phage ϕ31 was described previously (12). Phage ϕ48 was isolated from whey samples following the extended use of an NCK202-related pTR2030 transconjugant as an industrial starter culture. The morphological and genetic characteristics of ϕ48 will be described in a subsequent report (T. Alatossova and T. R. Klaenhammer, manuscript in preparation). The response of pTR2030 to both phages is presented in Table 1. The reactions of ϕ31 are typical of those described previously (12) for pTR2030 in this and other lactococcal backgrounds; no plaques were recovered, even when a high-titer phage preparation ($>10^9$) was employed. However, plaques of normal size were detected for ϕ48, albeit at a reduced efficiency (EOP, 10^{-3}). The absence of the typical Hsp^+ response (small plaques) indicated that ϕ48 was capable of circumventing, in part, the resistance mechanism(s) encoded by pTR2030. ϕ48.NCK209 was unrestricted by strain NCK209(pTR2030), while ϕ48.NCK 209.NCK202 suffered restriction at the original level. These data demonstrate host-dependent phage replication, suggesting the presence of an R/M system. The Hsp response encoded by pTR2030 has been investigated in some detail and does not display characteristics typical of R/M (12, 14). Therefore, the operation of an R/M system was probably revealed because ϕ48 eludes the abortive-infection mechanism (Hsp).

We have previously described (10, 20) a spontaneously deleted derivative of pTR2030, designated pTR2023, which had lost approximately 11.5 kb and was Hsp^-. When strain NCK210(pTR2023) was challenged with ϕ48, no reduction in plaque size or EOP was evident (Table 1), indicating that genetic determinants for both Hsp and R/M are associated with the deleted region. The deletion is contained within a 13.6-kb *BglII* fragment, which had been cloned in the shuttle vector pSA3 (pTK6). This recombinant plasmid was introduced to *L. lactis* NCK202 by protoplast transformation to create NCK211. This derivative was subsequently chal-

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TABLE 1. Phage reactions against *L. lactis* NCK202 and derivatives

Phage[a]	Strain	Relevant plasmid	EOP[b]
φ48	NCK202	NC[c]	1.0
φ48	NCK209	pTR2030	2×10^{-3}
φ48.NCK209	NCK202	NC	1.0
φ48.NCK209	NCK209	pTR2030	1.0
φ48.NCK209.NCK202	NCK209	pTR2030	1×10^{-3}
φ48	NCK210	pTR2023	1.0
φ31	NCK202	NC	1.0
φ31	NCK209	pTR2030	$<1 \times 10^{-9}$

[a] Unless otherwise stated, phage were propagated through NCK202. Modified phage are designated by suffixes denoting the last host used for propagation.
[b] Plaques were 1.5 mm wide. No plaques were formed when NCK209 was challenged with φ31.
[c] NC, Native plasmid complement.

lenged with φ48 and φ31 (Table 2). Phage φ48 gave plaques of normal size but was restricted to a level of 10^{-2}. A cycle of host-dependent phage replication similar to that observed for φ48 on pTR2030 was obtained with φ48 propagated through NCK211 (pTK6) (Table 2). These data confirmed that pTR2030 R/M determinants are present on the fragment cloned on pTK6. It is notable that the R/M activity displayed by pTK6 against φ48 was slightly less than that of pTR2030 (Table 2), even though all the relevant structural information is apparently contained within the cloned fragment.

The plaques of φ31 on NCK211(pTK6) were smaller than those obtained on the parental NCK202, and the EOP was reduced to 10^{-4}. Phage φ31.NCK211 formed plaques at an EOP of 10^{-1} on NCK211(pTK6), and the plaque size remained small. This phenotypic reaction is identical to the Hsp response of small isometric phages (φp2 and φsk1) on *L. lactis* MG1363 containing pTK6 (10), i.e., small plaques and

TABLE 2. Phage reactions against *L. lactis* NCK202 and derivatives

Phage[a]	Strain	Relevant plasmid	EOP	Plaque size (mm)
φ48	NCK202	NC[b]	1.0	1.5
φ48	NCK211	pTK6	1.6×10^{-2}	1.5
φ48.NCK211	NCK211	pTK6	1.0	1.5
φ48.NCK211	NCK203[c]	NC	1.0	1.5
φ48.NCK211.NCK203	NCK211	pTK6	1.2×10^{-2}	1.5
φ31	NCK202	NC	1.0	1.5
φ31	NCK211	pTK6	1.1×10^{-4}	0.2
φ31.NCK211	NCK211	pTK6	1.3×10^{-1}	0.2
φ31.NCK211	NCK203	NC	1.0	1.5
φ31.NCK211.NCK203	NCK211	pTK6	1.0×10^{-4}	0.2
φ48	NCK218	pTRK18	1.0	1.5
φ31	NCK218	pTRK18	1.2×10^{-1}	0.2
φ31.NCK218	NCK211	pTK6	2.0×10^{-4}	0.2
φ31	NCK216	pTRK70	1.0	1.5
φ31.NCK216	NCK211	pTK6	1.0×10^{-1}	0.2
φ48.NCK211	NCK202	NC	1.1×10^{-3}	1.5
φ48.NCK203	NCK211	pTK6	1.0×10^{-4}	1.5
φ48.NCK203	NCK202	NC	1.0×10^{-3}	1.5
φ48.NCK203	NCK218	pTRK18	1.1×10^{-3}	1.5

[a] Unless otherwise stated, phage were propagated through NCK202. Modified phage are designated by suffixes denoting the last host used for propagation.
[b] NC, Native plasmid complement.
[c] NCK203 is a pTK6-cured derivative of NCK211.

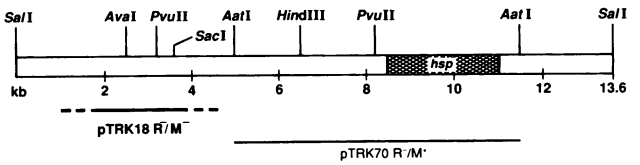

FIG. 1. Linear restriction map of the 13.6-kb pTR2030 fragment cloned in pTK6. The location of the essential *hsp* region is indicated by the shaded area (10). The approximate location of the 3.5-kb deletion leading to the formation of pTRK18 (R^-/M^-) is indicated by the heavy boldface horizontal line. The dashed lines approximate the endpoints of the 3.5-kb deletion. The location of the deletion leading to the formation of pTRK70 (R^-/M^+) (bottom) is also indicated.

a slight reduction in EOP. Phage φ31.NCK211.NCK203 (NCK203 is NCK211 cured of pTK6) gave the same reactions as φ31 did. The reduced Hsp phenotype encoded by pTK6, unlike that encoded by pTR2030, allowed detection of R/M activity against φ31.

A spontaneous in vivo deletion derivative of pTK6, designated pTRK18, which retains Hsp^+ activity in *L. lactis* MG1363 has been described elsewhere (10). pTRK18 lost approximately 3.5 kb of the original 13.6-kb insert (Fig. 1). pTRK18 was introduced to NCK202 by protoplast transformation to determine whether the deletion affected the R/M phenotype. pTRK18 was reisolated from the NCK202 transformant (NCK218) and subjected to restriction enzyme analysis. No additional deletions or rearrangements occurred (data not shown). Phage reactions for φ48 and φ31 on strain NCK218(pTRK18) are shown in Table 2. pTRK18 did not restrict φ48 and continued to provide the Hsp^+ response to φ31, but restriction of φ31 was reduced 3 logs below that of NCK211(pTK6). These data indicate that *r/m* genes were lost during the deletion event that generated pTRK18. Phage φ31.NCK218 was again restricted by NCK211(pTK6) (Table 2), confirming that modification ability was lost concurrently with restriction activity. An in vitro deletion derivative of pTK6 was constructed by an *Aat*I digestion and religation (Fig. 1). This recombinant plasmid, designated pTRK70, was introduced to *L. lactis* NCK203. The transformant NCK216 (pTRK70) did not exhibit Hsp or restriction activity against φ31 (Table 2). However, φ31.NCK216 was no longer restricted by NCK211(pTK6) (Table 2), suggesting that φ31 was modified by pTRK70. These data segregated the restriction and modification activities encoded by pTK6 and thereby confirm that two independent genotypes (*res* and *mod*) are responsible for the host-dependent phage replication. In addition, separation of the *r/m* and *hsp* loci (Fig. 1) demonstrates that the R/M phenotype exhibited against φ48 and φ31 is not a secondary effect of the *hsp* gene(s).

When φ48.NCK211 formed plaques on NCK202, the EOP was 10^{-3} and plaque sizes were normal (Table 2). This result was unexpected, because NCK211 was constructed by introducing pTK6 to NCK202. Phage propagated on NCK211 should not have been restricted by the parental NCK202 strain, unless a second R/M system was functioning in NCK202. When φ48 was propagated through NCK203 (the pTK6-cured derivative of NCK211), φ48.NCK203 was restricted by both NCK211(pTK6) and NCK202 (Table 2). These data indicate that a second R/M system was present in NCK202 and was cured upon introduction of pTK6 to form NCK211. Phage φ48.NCK203 was also restricted by NCK218 (Table 2), which does not possess the pTK6 R/M system. Therefore, the introduction of pTRK18 did not cause the loss of the native system. The plasmid profiles of

the strains described above were examined to determine whether a specific plasmid could be linked to the native R/M system in NCK202. Of the four plasmids present in NCK202, one (pTRK68, 46 kb) was absent in NCK211 (pTK6) but present in NCK218(pTRK18) (data not shown). Since the introduction of pTRK18 did not result in loss of the native pTRK68, it may be that the 3.5-kb region on pTK6, which is absent in pTRK18, contains an origin of replication from pTR2030. The introduction of pTR2030 by conjugation also resulted in the loss of pTRK68, providing additional evidence that the 3.5-kb region is responsible for the observed incompatibility (data not shown).

No R/M system against the small isometric-headed phages φsk1 or φp2 was evident in *L. lactis* MG1363(pTK6) (10), even though the reduced Hsp response should facilitate detection. It is not clear why the expression of the *r/m* genes cloned on pTK6 should vary between MG1363 and NCK202, since the level of Hsp resistance is essentially identical in both hosts. The difference may reflect a variation between phages rather than between hosts. A possible explanation would be that φ48 and φ31 possess more target sites for the pTR2030-encoded *r/m* genes that do the phages φsk1 and φp2 tested in MG1363.

It is difficult to assess the contribution of the *r/m* genes to the overall resistance encoded by pTR2030 against small isometric-headed phages. The effect on φ48 is clear, since this phage is able to circumvent the Hsp mechanism. It is also evident that the *r/m* genes contribute to the resistance of strain NCK211(pTK6) against φ31, although this was presumably detected only because of the relatively weak expression of Hsp from the recombinant plasmid pTK6. The role of R/M is relatively minor compared with that of Hsp: in situations in which the R/M system is not effective (i.e., against both φp2 and φsk1 in *L. lactis* MG1363), the resistance encoded by pTR2030 remains total; however, in situations in which Hsp is ineffective (i.e., against φ48 in *L. lactis* NCK209), resistance is at least 6 orders of magnitude lower. It is important to consider that during any study of phage-plasmid-host interactions, all three components will influence the outcome of each experiment; caution must be excercised in interpreting the data.

Froseth et al. (7) have described a 26-megadalton plasmid, pBF61, which encodes resistance to phage via an abortive-infection mechanism which is similar in many respects to the Hsp phenotype of pTR2030. This plasmid also exhibits host-dependent phage replication, suggesting the presence of two independent resistance mechanisms, as described here for pTR2030. Some differences exist between pTR2030 and pBF61. pTR2030 is heat sensitive and shows a restriction pattern different from that published for pBF61. In addition, unlike pTR2030, pBF61 has not been shown to be conjugative. However, in view of their overall phenotypic similarities, pTR2030 and pBF61 may represent a general class of multiple-resistance plasmids disseminated among lactococci.

When different resistance systems are combined in a single strain, it is important to consider the individual mechanisms involved. The abortive-infection mechanism encoded by *hsp* can work simultaneously with at least three different R/M systems: one coresident on pTR2030, a second on pTN20 in *L. lactis* ME2 (9) and, in the case of pTRK18 in NCK218, the native pTRK68. This additive effect presumably reflects the fact that Hsp and R/M act at different points of the phage lytic cycle. A third additive mechanism would be inhibition of adsorption (19), which acts at yet another stage of infection. This arrangement of phage defense mechanisms occurs naturally in the prototype phage-insensitive strain *L. lactis* ME2 and provides an extremely effective barrier to phage attack (20).

The presence of (at least) two independent phage resistance mechanisms on a single conjugative plasmid could be expected to confer a distinct advantage upon a population which is subject to phage attack. This appears to be true for pTR2030 transconjugants, which exhibit prolonged resistance under industrial conditions in which parental strains have failed because of phage attack (13, 17, 22).

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