Sequence Analysis of the Lactococcal Plasmid pNP40: a Mobile Replicon for Coping with Environmental Hazards†

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The conjugative lactococcal plasmid pNP40, identified in Lactococcus lactis subsp. diacetylactis DRC3, possesses a potent complement of bacteriophage resistance systems, which has stimulated its application as a fitness-improving, food-grade genetic element for industrial starter cultures. The complete sequence of this plasmid allowed the mapping of previously known functions including replication, conjugation, bacteriocin resistance, heavy metal tolerance, and bacteriophage resistance. In addition, functions for cold shock adaptation and DNA damage repair were identified, further confirming pNP40’s contribution to environmental stress protection. A plasmid cointegration event appears to have been part of the evolution of pNP40, resulting in a “stockpiling” of bacteriophage resistance systems.

Lactococcus lactis, a gram-positive lactic acid bacterium, has been extensively exploited for the production of a variety of fermented dairy products. L. lactis strains exhibit biotechnologically important activities, which contribute to the character of the final food product, e.g., lactose utilization and protease production, and in addition encode properties that specifically provide a selective advantage to the bacterium itself, e.g., heavy metal resistance, bacteriocin production and/or immunity, and bacteriophage resistance (47). Many of these industrially significant traits have been found to be encoded by plasmids, which are omnipresent among this species, with most isolates containing multiple plasmids ranging in size from 2 to 80 kb (11).

In recent decades, extensive research has established the molecular mechanisms governing many of these activities, in particular with respect to bacteriophage resistance (47). Lactococcal strains used for many food fermentations are known to be persistently challenged by phages and probably as a consequence have evolved numerous bacteriophage resistance strategies (48, 67).

Presently, there are 30 completely sequenced lactococcal plasmids, the largest being pSK11P, a 75.8-kb plasmid isolated from L. lactis subsp. cremoris SK11 (47, 62). This plasmid encodes a variety of functions, including copper resistance, proteolytic activity, cold shock proteins, and cation transport activities, and displays clear “markings” of multiple recombination events that may have contributed to its evolution (62).

Previous studies of a similarly sized plasmid, pNP40, originally identified in L. lactis subsp. diacetylactis DRC3 (45), revealed that this molecule, besides its encoded nisin and cadmium resistance determinants, is responsible for an impressive bacteriophage resistance profile (16, 19, 20, 50, 65). Two such systems, AbiE and AbiF, were found to provide significant resistance that correlates to an abortive infection phenotype (19).

In addition, on the basis of phenotypic evidence, the presence of a third mechanism active at the stage of phage DNA injection was proposed (20). Most recently, a fourth resistance system, the LiaJ restriction-modification system, was identified (50).

In the present study, we report the complete sequence of pNP40. Analysis of the sequence revealed the genetic determinants involved in replication and conjugation, in addition to genes responsible for previously uncharacterized functions. Furthermore, evidence is offered which attests to pNP40’s full bacteriophage resistance potential.

MATERIALS AND METHODS

Bacteria, bacteriophage, plasmids, media and growth conditions. Details of the bacterial strains, bacteriophages, and plasmids used in the present study are summarized in Table 1. All L. lactis strains were grown in M17 broth (Oxoid Ltd., Hampshire, United Kingdom) containing 0.5% glucose at 30°C. Escherichia coli was grown at 37°C in Luria-Bertani (LB) medium (58). Where appropriate, antibiotics were added as follows: for L. lactis, tetracycline at 5 μg ml−1, chloramphenicol at 10 μg ml−1, and erythromycin at 1 μg ml−1; for E. coli, ampicillin at 100 μg ml−1, kanamycin at 25 μg of ml−1, chloramphenicol at 10 μg ml−1, and erythromycin at 100 μg ml−1. LB medium was supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg ml−1) and IPTG (isopropyl-β-D-thiogalactopyranoside; 1 mM) where appropriate. Recombinant L. lactis cells containing pAK80 and derivatives were selected on GM17 agar (containing 0.5 M sucrose) with erythromycin, supplemented with X-Gal (40 μg ml−1). Lactococcal bacteriophage were propagated as described previously (50). Plaque assays were conducted as described elsewhere (41) and the efficiency of plaquing (EOP) was calculated as the ratio of the number of plaques formed on a tested host to those formed on a sensitive host.

Molecular techniques and shotgun cloning. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Hertfordshire, United Kingdom) and used according to the manufacturer’s instructions. E. coli plasmid DNA was isolated by using the SV Wizard plasmid miniprep kit (Promega, Madison, WI). Routine lactococcal plasmid DNA isolations were performed as described previously (52). Isolation of pNP40 plasmid DNA for shotgun cloning was performed as described previously (2), and the resulting DNA preparation was purified by cesium chloride-ethidium bromide density gradient ultracentrifugation using standard techniques (55). Electroporation of plasmid DNA into E. coli was performed by using standard techniques (58) and in L. lactis as described previously (69). Purified pNP40 DNA was digested with HindIII, EcoRI, and XbaI separately and shotgun cloned into the E. coli plasmid pBluescript KS(−). Large restriction fragments (>5 kb) were further digested with DraI, Sau3A, or...
Plasmids

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| MG1614(pNP40) | MG1614 transconjugant containing pNP40 | | }

Bacteriophages

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TABLE 1. Bacteria, bacteriophages, and plasmids used in this study

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<td>RepA⁺ integration vector; Em⁺</td>
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<td>pSoc1</td>
<td>pORI280 derivative containing SOEing PCR fragments from pNP40 (see Materials and Methods)</td>
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a Em⁺, erythromycin resistance; Cm⁰, chloramphenicol resistance; Te⁺, tetracycline resistance; Kan⁺, kanamycin resistance.

b MFRC, Moorpark Food Research Centre, Moorpark, Fermoy, Cork, Ireland.
using the ECL direct nucleic acid labeling and detection system (Amersham) according to the manufacturer’s instructions. Control phage DNA was isolated from lysates as described previously (49).

Freeze-thaw challenge and DNA damage assays. Freeze-thaw challenge experiments were performed essentially as described elsewhere (71, 72). Briefly, cells were grown in GM17 medium at 30°C to an OD600 of 0.5 and subjected to a cold shock by rapid temperature downshift from 30 to 10°C for 0, 2, or 4 h, after which 1 ml of these cultures was frozen at −20°C. After a 24-h freezing period, the cells were allowed to thaw at 30°C for 4 min with subsequent survival of strains assayed by determining viable counts. This freeze-thaw cycle was performed four times. Sensitivity to chemically induced DNA damage was assayed by inclusion of mitomycin C (MMC; 2.5 μg ml⁻¹) in growth media. Cells were grown to early log phase (OD 600 = 0.2), at which point the mutagen was added, followed by monitoring of growth by means of OD 600 measurement. Viable counts were performed at selected time points to corroborate OD 600 measurements.

β-Galactosidase assays. β-Galactosidase assays were performed essentially as described previously (30). For analysis of cold shock-induced transcription, cells containing various lacZ-transcriptional fusions were grown to an OD600 of 0.5 and subjected to a cold shock at 10°C for 0, 2, and 4 h, after which 1-ml samples were harvested and analyzed for β-galactosidase activity.

For analysis of DNA damage-induced gene expression, cells containing various lacZ-transcriptional fusions were grown to an OD600 of 0.2 to 0.3, followed by the addition of MMC (2.5 μg ml⁻¹), after which 1-ml samples were harvested at 0, 15, 30, 60, and 90 min and analyzed for β-galactosidase activity.

Construction of a pNP40 deletion derivative. The deletion derivative of pNP40 was constructed by targeted deletion mutagenesis as described previously (10, 37, 38). Briefly, two PCR products, A and B, flanking the region of pNP40 to be deleted, were generated. Product A was amplified by using primers M1Fa and M1Rb, whereas product B was amplified by using primers M1Fc and M1Rd, and the two resulting DNA fragments were joined by SOEing PCR as described previously (29). This SOEing product was then inserted into the Ncol-BamHI sites of the integration vector pOri280 (RepA⁺) in the E. coli cloning host EC101 (RepA⁺), to generate plasmid pSoe1. Plasmid pSoe1 was subsequently established in the MG1614/pNP40 background and selected for first and second crossover events. The integrity of the pNP40 deletion derivative, pNP40△soe1, was verified by PCR, Southern hybridization, and sequencing.

RESULTS AND DISCUSSION

Plasmid sequence and genetic organization. The conjugative lactococcal plasmid pNP40, originally identified in L. lactis subsp. diacetylactis DRC3 (45), was established in the plasmid-free lactococcal strain MG1614, from which plasmid DNA was subsequently purified and sequenced (as outlined in the Materials and Methods). Assembly of the resulting sequences resulted in a single contiguous contig representative of a circular 64,980-bp DNA molecule with a G+C content of 32.33%. This is somewhat lower than that of L. lactis chromosomal DNA (35.4% for IL1403 [4], 35.8% for MG1363 [34], and 35% for SK11 [Joint Genome Institute]) but is within the
range exhibited by most sequenced lactococcal plasmids to date (30 to 40%) (47). A total of 62 ORFs were identified in the pNP40 sequence, which were analyzed in detail below (see Materials and Methods, Fig. 1, and Table S1 in the supplemental material).

pNP40 replication functions. Previous work had localized the minimal replicon for pNP40 to a cloned 7.6-kb EcoRI fragment, which also expressed a nisin resistance activity (see below) (16). Further subcloning suggested that the replication functions were localized on a 5.0-kb EcoRI-XbaI fragment, corresponding to coordinates 59625 to 64637 of the pNP40 sequence. This region was found to contain orf59 (designated repA), orf60 (designated repB), and orf61 (see Table S1 in the supplemental material and Fig. 2A).

Analysis of the amino acid sequence of RepA revealed similarity to a number of pLS32-type theta replication proteins, including that of pCI2000 (AAF27326) from Lactococcus (73% identity). Consistent with this was the presence of a replication initiation N-terminal domain (pfam06970), which was noted to contain a helix-turn-helix motif (amino acid [aa] 68 to 89). Analysis of the coding region of RepA revealed the presence of a 40-bp sequence directly repeated two and three quarter times (Fig. 2B; additional features are as indicated). Such repeats, also termed iterons, are common elements of the origin of replication (ori) for many theta replicating plasmids (12, 25, 33, 35). Interestingly, the pLS32 ori is believed to be similarly located within the coding sequence for its replication initiation protein, RepN (63). This intragenic iteron locality was also noted for the pNP40 RepA homologue present on pCI2000 (31).

Located upstream of repA and transcribed divergently, repB is predicted to encode a gene product, which exhibits significant similarity to a number of replication-associated proteins from gram-positive plasmids (see Table S1 in the supplemental material), while containing SoJ (COG1192), Mrp (COG0489), and COG0455 domains, thus making it a member of the ParA ATPase family (pfam00991) involved in plasmid and chromosome partitioning. An equivalent
ParA-type protein has previously been associated with an active plasmid partitioning system, and RepB likely mediates an equivalent stability function (31). Immediately downstream of repB, a small coding region, orf61, is present. Homologous RepB-linked coding regions have been noted in many theta replicons which, due to their proximity and apparent transcriptional and/or translational coupling to RepB, are thought to be involved in the replication-partitioning process (3, 5, 25).

The genetic organization, similarities, and iteron structure of the pNP40 replication region strongly suggests that this large plasmid replicates via a theta-type mechanism. Furthermore, and typical of theta replicating plasmids, pNP40 appears to contain determinants that contribute to its high segregational stability (45).

Conjugal transfer determinants. In other microorganisms the structure and function of the conjugative apparatus has been examined in detail (18, 26, 36, 39). The conjugal capacity of pNP40 has previously been phenotypically demonstrated (27, 65). On the basis of similarity searches of the pNP40 sequence, we predict the presence of a conjugal transfer gene cluster on an approximately 17-kb section (coordinates 44565 to 61395), which contains 19 ORFs (orf40 to orf58) arranged in an operon structure (Fig. 3A and Table S1 in the supplemental material). The protein specified by orf58 (designated MobD) exhibits homology to the nickase-relaxase family of proteins (pfam03432), which introduce a nick at the origin of transfer (oriT) to initiate single-stranded plasmid DNA transmission from the donor to a recipient cell. A number of inverted repeat structures, reminiscent of an oriT, were identified downstream of the MobD coding region which was noted to be significantly AT-rich (~70%) (Fig. 3B). However, no obvious candidate consensus nick site (26) is present within this putative oriT.

The pNP40 conjugation region appears to consist of modules each of which displaying sequence similarity to discrete sections from assumed conjugation regions from enterococcal plasmids (Fig. 3A). The notable exceptions include orf44, orf45, and orf53, which appear to represent components unique to pNP40. The product encoded by orf54 (designated TraF) is a membrane-spanning protein likely to be involved in mating channel formation, whereas ORF55 was noted to contain the “antirestriction” domain COG4227, in addition to a conserved H-E-X-X-K catalytic active site, and likely provides a temporal protection to the transferred plasmid DNA against restriction endonucleases, allowing establishment in the recipient cell (70).

The orf43 and orf48 gene products correspond to the conserved TraG (cd01126) (pfam02534) and TraE (COG3451) conjugation proteins, respectively, whose conserved domains are suggestive of a type IV secretion function (9, 23, 24, 61). Both ORF49 and ORF50 exhibit homology to distinct regions within the protein product of ep0036 on pAM373 (AAG40447). ORF49 contains a conserved FigJ muramidase domain (COG1705), whereas ORF50 contains a CHAP amidase domain (pfam05257), in contrast to EP0036, which contains both. These conserved domains are present in cell wall-
metabolizing proteins; therefore, it can be speculated that ORF49 and ORF50 participate in facilitating the passage of DNA and/or proteins across the cell envelope by virtue of their peptidoglycan-degrading activity.

Cadmium resistance. Previously, the cadmium resistance encoded by pNP40 has been demonstrated to be a selectable marker for pNP40 dissemination to an industrial starter culture (65). Furthermore, the presence of a CadA homologue was confirmed by PCR using primers specific for the previously published CadA homologue of pAH82 (51). The cadmium resistance region of pNP40 was found to reside in a section encompassed by coordinates 40808–43281, where two similarly oriented overlapping coding regions were distinguished (ORF36 and ORF37). The first ORF, ORF36, corresponds to the previously identified cadA homologue (65), whose product contained conserved domains consistent with proteins involved with Cu, Cd, Co, and Zn transport and detoxification (cdd00371), inorganic ion transport (COG2608), and cation transport (COG2217, pfam00122, pfam00702, COG0474, COG2216, and COG4087). The presence of these conserved regions indicates that the CadA activity spectrum is not solely restricted to cadmium efflux, as has previously been reported for other homologues (60).

The protein encoded by orf37 was found to be 100% identical to an abundance of CadC proteins, all of which were encoded by genes adjacent to CadA homologues. CadC was noted to possess a number of helix-turn-helix containing conserved domains (cdd00090, smart00418, and CAG0640) typical of homodimeric repressors, which dissociate from their target DNA in the presence of metal ions.

Nisin resistance region. As with cadmium resistance, the nisin resistance capabilities of pNP40 have been documented (16, 45). The sequenced nisin resistance gene, designated nisR, was located on a 1.8-kb EcoRI-KpnI fragment (coordinates 803 to 2584) (17). Analysis of the amino acid sequence of NisR confirmed the presence of an N-terminal membrane-spanning domain (aa 7 to 29) (but no signal peptide sequence) as suggested by Froseth and McKay (17) and further revealed the presence of conserved protease-peptidase domains (smart00245 and pfam03572) spanning approximately 200 aa at the C terminus, which was predicted to reside outside the cell membrane. From this it can be inferred that the mechanism of

![Graph A](image1.png)

![Graph B](image2.png)

**FIG. 4.** (A) β-Galactosidase assays of the cspC promoter transcriptional fusion (present on pAKC) after 2 and 4 h of cold shock at 10°C. The graph depicts the fold increase in promoter activity after cold shock treatment relative to the promoter activity under non-cold-shock conditions (i.e., 30°C). Bars: 1, MG1614/pAKC (2 h at 10°C); 2, MG1614/pAKC (4 h at 10°C); 3, MG1614/pNP40/pAKC (2 h at 10°C); 4, MG1614/pNP40/pAKC (4 h at 10°C). Absolute values are listed beneath the graph. (B) β-Galactosidase assays of the cspD promoter transcriptional fusion (present on pAKD) after 2 and 4 h of cold shock at 10°C. The graph depicts the fold increase in promoter activity after cold shock treatment relative to the promoter activity under non-cold-shock conditions (i.e., 30°C). Bars: 1, MG1614/pAKD (2 h at 10°C); 2, MG1614/pAKD (4 h at 10°C); 3, MG1614/pNP40/pAKD (2 h at 10°C); 4, MG1614/pNP40/pAKD (4 h at 10°C). Absolute values are listed beneath the graph. (C) Survival of MG1614 and MG1614/pNP40 frozen at −20°C after successive freeze-thaw cycles after exposure to a cold shock at 10°C for 0, 2, and 4 h as indicated.

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<td>58±1</td>
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NisR-mediated nisin resistance occurs via proteolytic degradation of nisin. Located immediately downstream and convergently oriented to the nisR gene, orf1 is predicted to encode an integral membrane protein containing a conserved, C-terminally located Abi, CAAX amino-terminal protease domain (pfam02517), which is typical of this diverse family of metal-dependent membrane proteases that are involved in protein and peptide modification and secretion (53). It has been suggested that these proteins may also play a role in bacteriocin maturation and transport and in resistance (14, 53).

**Cold shock determinants.** The protein products of two similarly oriented open reading frames, ORF38 and ORF39, are identical or nearly identical to the cold shock proteins CspD and CspC, respectively, of lactococcal chromosomal or plasmid origin. In addition, both conserved “cold shock” DNA- and RNA-binding domains (pfam00313 and smart00357, respectively) were observed within the pNP40-encoded CspC and CspD amino acid sequences. Previous studies on the chromosomal CspC and CspD homologues have illustrated the physiological response mediated by these cold shock proteins; expression of CspD was enhanced by cold shock and resulted in increased survival at low temperatures, whereas only a modest increase in CspC expression accompanied cold shock, which was found to directly alter the levels of other cold shock proteins (71–73).

Transcriptional fusions of both the cspC and the cspD promoter regions of pNP40 showed that both promoters were inducible by cold shock in a manner that was independent of the presence of pNP40 (Fig. 4A and B). The presence of these cold shock-related determinants on pNP40 suggests that this plasmid mediates an enhancement of the cold shock response. An analysis of the freeze-thaw survival capacity of MG1614 containing pNP40 compared to the plasmid-free strain illustrated a small but appreciable increase in survival (Fig. 4C), which was also observed with a construct containing the cspC and cspD genes cloned in tandem (pNZ-CD; data not shown).

The full phenotypic potential associated with these pNP40-encoded determinants, while clearly inducible, is likely only to be significantly detectable in a host (perhaps the original pNP40-containing host [45]) that does not encode chromosomal or plasmid cspC and cspD copies (or possibly under alternative stress or cold shock conditions).

**DNA damage repair.** pNP40 was found to possess three ORFs—ORF17, ORF18, and ORF25—which were predicted to encode proteins involved in DNA repair. The RecA (ORF18) and UmuC-like (ORF17) homologues, designated RecA_{L,P} and OrfU, respectively, have been described previously (19, 21) (see Table S1 in the supplemental material and Fig. 1).

Analysis of the amino acid sequence of RecA_{L,P} revealed the presence of multiple conserved domains characteristic of bacterial RecA homologues involved in homologous recombination, DNA repair, and SOS response induction (cd00983, cd01393, cd01120, and pfam00154). Adjacent and similarly oriented with respect to recA_{L,P}, the orf17 gene encodes the UmuC-like protein, OrfU, which contains two conserved domains: the IMS (impB/mucB/samB) family (pfam00817; UV protection) and DinP (COG0389; DNA repair DNA polymerases). The UmuC family of proteins are an essential component of the DNA damage mutagenesis mechanism of *E. coli* and constitute the catalytic subunit of DNA polymerase V, which possesses a translesion DNA synthesis activity at the expense of normal replicative fidelity (54, 55, 64, 68).

The product of orf25 was predicted to harbor an UvrA exonuclease domain (COG0178) containing conserved, interrupted, N- and C-terminal ABC-ATPase motifs and similarly located zinc fingers. UvrA-type proteins are believed to comprise the ATPase subunit of the UvrABC nucleotide excision repair system (66). BLAST searches of the ORF25 (designated
UvrA) amino acid sequence revealed 64% identity to UvrA of Lactobacillus plantarum WCFS1 (CAD63845) and 62% identity to UvrA of L. casei ATCC 334 (ZP_00386320).

The presence of pNP40-encoded components of DNA repair systems prompted us to investigate the growth and survival of a pNP40-containing host in response to chemical-induced DNA damage. To this end, MG1614 containing pNP40 was challenged with MMC, and its growth profile was monitored. As can be seen from Fig. 5A, a significant difference in the growth profile was evident for the pNP40-containing strain compared to that of the control strain in response to MMC. The pNP40-containing strain not only reached a higher final optical density but also did not lyse to the same extent as the control strain. The optical density values described were corroborated by viable plate counts (data not shown).

To establish whether the expression of orfU, recA<sub>Lp</sub>, and uvrA was induced in response to MMC, transcriptional fusions were constructed (see Materials and Methods). Of these, only the upstream regions of orfU and uvrA were found to contain active (but relatively weak) promoters (in the absence of MMC) in a lactococcal host. The upstream recA<sub>Lp</sub> region apparently did not contain any active transcriptional signals (in the presence or absence of MMC). Given that recA<sub>Lp</sub>-specific mRNA was detectable by dot blot analysis and the apparently identical recA<sub>Lp</sub> and orfU transcriptional yields (21), it is likely that these genes constitute a dicistronic operon transcribed from the orfU promoter. This may account for the inability of a cloned recA<sub>Lp</sub> to complement a chromosomal recA mutant (21), since recA<sub>Lp</sub> expression may not be entirely synchronized with that of the chromosomal version (see below).

As can be seen from Fig. 5B, expression from the orfU promoter was induced up to threefold when monitored for 90 min after exposure to MMC (which was not influenced by the presence of pNP40 [data not shown]), whereas no such increase in transcriptional activity was observed for the uvrA promoter. The latter promoter appears to be constitutive during exponential growth and increases in activity up to two- to threefold during early stationary phase in the absence of a DNA-damaging agent (data not shown). This lack of induction of uvrA in response to DNA damage, although in contrast to that observed for E. coli (32) and B. subtilis (8), is consistent with that noted for a uvrA homologue of Pseudomonas aeruginosa (56).

Analysis of the sequence of the inducible orfU promoter region revealed the presence of a conserved “HdiR” box (39) (Fig. 5C). HdiR is a LexA-like DNA damage regulator of L.
**ISS1 is a member of the IS6 family, whose insertion sequences thus far have been noted to give rise exclusively to co-integrate replicon fusions (43).** Downstream of the abiF gene within the ISS1-flanked section of pNP40, a 23-bp sequence repeated three and a half times (reminiscent of the iteron-containing origin of replication from some theta plasmids) has previously been reported (19). In addition, the orf8-to-orf10 region (in particular orf10) appears to encode plasmid stability and maintenance determinants. The presence of these coding regions, and the iteron-like sequences mentioned above suggest that this section may have at some stage been capable of autonomous replication. These observations provide evidence which corroborates suggestions that pNP40 may have evolved as a result of a co-integration event (19).

The four remaining IS elements of pNP40 (orf23, orf24, orf26, and orf62) all encode apparent truncated and/or inactivated derivatives of members of the IS3 family (whose genetic organization usually consists of two genes translated together as a single polypeptide via translational slippage (6, 43).

**The fourth bacteriophage-resistance phenotype: injection blocking versus synergy.** Two abortive infection phage resistance systems (AbiE and AbiF) (19) and a putative DNA penetration blocking system (20) have been reported to reside on pNP40. Evidence for the latter system was initially based on the enhanced pNP40-mediated resistance to \( \phi H9278 \) compared to the level of resistance afforded by AbiF alone, with the observed phenotype noted to act prior to AbiF-mediated cell killing. The discovery of an active pNP40-encoded restriction-modification system, LlaJI (50) (which fulfills most of the penetration blocking phenotypes (20), made it essential to redefine the residual pNP40-mediated phage resistance activity. A small isometric-headed phage, \( \phi H9278\) sk1, was selected for this analysis since this phage could grow with apparently equal efficiency on a host containing either AbiE or AbiF alone. Although \( \phi H9278\) sk1 was restricted by a host containing the LlaJI system, propagation of surviving phage on the same LlaJI-containing host...
yielded completely LlaII-insensitive progeny. These methylated phage ($\delta$sk1.m) were therefore expected to be insensitive to all characterized phage resistance systems present on pNP40 and ideal for detection of any remaining unidentified resistance mechanism (provided $\delta$sk1.m was sensitive to such a system).

As can be seen from Table 2, $\delta$sk1.m formed plaques with equal efficiency on all strains (although slightly tighter plaques were formed on the AbiE-containing host), with the exception of the host possessing pNP40, confirming the presence of a residual resistance phenotype against this phage. In addition, accumulation of intracellular $\delta$sk1.m DNA was considerably delayed in the pNP40-containing host compared to that of the sensitive host (Fig. 6). Here, a high intracellular $\delta$sk1.m DNA concentration was detected in the control MG1614 host after 40 min, with lysis ensuing. In the pNP40-containing host, an equivalent concentration of intracellular $\delta$sk1.m DNA was not detected until 80 to 100 min postinfection.

A “scan” of pNP40 was performed by the construction of a deletion derivative (pNP40ΔSoe1) and multiple subclones (pPORF1, pPORF13, pNZ-6.2, pNZ-7, and pNZ-10), essentially encompassing the orf11-to-orf35 region, which, when examined for any (loss of) associated phage resistance phenotype, failed to reveal the presence of an as-yet-unidentified resistance system (see Table 1 and Materials and Methods). Therefore, the residual phage resistance phenotype associated with pNP40 could not be ascribed to any of the distinctive genetic determinant(s) characterized. This resistance may be attributable to synergistic enhancement of the characterized resistance systems rather than the presence of a fourth (penetration block- ing) system, particularly since AbiE and AbiF have previously been implicated in such an enhancement phenotype (46). An examination of the phage resistance profiles of specific AbiE– and AbiF– deletion derivatives of pNP40 would be required to verify this suggestion.

Concluding remarks. Analysis of the sequence of the 64.9-kbp pNP40 plasmid has provided the genetic confirmation and localization of a number of previously described functions such as conjugation, cadmium resistance, nisin resistance, bacteriophage resistance, and replication. In addition, new determinants for cold shock resistance and DNA damage repair were identified and confirmed phenotypically.

Lactococcal plasmids such as pNP40 appear to endow their respective hosts with multiple biologically and biotechnologically important properties, many of which have been genetically characterized (47). The extent to which pNP40 is able to limit bacteriophage proliferation must surely be a reflection of the selective pressure to which this plasmid and associated host have been exposed. In conclusion, it would appear that relative to the sequenced lactococcal plasmids to date (47, 62), the magnitude of the genetic “arsenal” possessed by pNP40 to cope with environmental hazards (some of which are unique to this plasmid, e.g., recA1.1, uvrA, abiEi, abiEii, LlaI, and abiF) is particularly significant.

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REFERENCES


LACTOCOCCAL PLASMID-ENCODED SAFEGUARD


