New Thermosensitive Delivery Vector and Its Use To Enable Nisin-Controlled Gene Expression in *Lactobacillus gasseri*

T. Neu and B. Henrich*

Abteilung Mikrobiologie, Fachbereich Biologie, Universität Kaiserslautern, D-67653 Kaiserslautern, Germany

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Derivatives of a cryptic plasmid from *Lactobacillus curvatus* showed temperature-sensitive replication in thermophilic lactobacilli. The thermosensitive replicon was used to construct the new delivery vector pTN1, which allows site-specific replacement of chromosomal DNA sequences. pTN1 carries an erythromycin resistance marker suitable for selection of single-copy integrants and replicates readily at 35°C, whereas replication is efficiently shut down at 42°C. To demonstrate the functionality of pTN1, the signal transduction genes (*nisRK*) of the nisin-controlled expression system were integrated downstream of the *pepN* gene into the chromosome of *Lactobacillus gasseri*. In the resulting strain, UKLbg1, expression of *nisRK* was likely driven by cotranscription with *pepN* and enabled nisin-dependent induction of a fusion of a reporter gene (*pepI*) to the *nisA* promoter. The induction rates were correlated with the amount of nisin used, and maximum *pepI* expression was achieved with nisin concentrations (above 25 ng/ml) at which growth of the bacteria was already inhibited.

Thermophilic lactobacilli, such as *Lactobacillus delbrueckii* and *Lactobacillus helveticus*, are well-known for their biotechnological importance in the production of cheeses and fermented milks. In addition, thermophilic species of *Lactobacillus* (e.g., *L. acidophilus* and *L. gasseri*) are commonly resident in the human gastrointestinal tract, where they can contribute to stabilizing the indigenous microflora (19, 28). Therefore, preparations of viable lactobacilli are currently used as health-promoting food adjuncts and probiotics. Some strains are capable of adhering to cells of the intestinal epithelium. This seems to be favorable for probiotic activities of the bacteria and may also contribute to their applicability as live vaccines. One example of a *Lactobacillus* strain that is able to survive in the intestinal tract and to attach to epithelial cell lines better than other lactic acid bacteria is the human isolate *L. gasseri* ADH (4). In a clinical study, oral administration of this strain had clear effects on the numbers and activities of intestinal and fecal bacteria (23).

Due to the technological and medical importance of thermophilic lactobacilli, there is considerable scientific and economic interest in the analysis and engineering of relevant genetic properties. This requires appropriate molecular tools for the introduction, maintenance, and controlled expression of desired functions, which, in the case of practical applications, should allow the construction of food-grade recombinants.

The use of recombinant plasmids for the expression of new or modified genes is often affected by problems related to incompatibilities, structural instabilities, variable copy numbers, and the limited range of suitable selection markers. A more reliable alternative is stable and site-specific integration of desired expression cassettes into the bacterial chromosome by using conditionally nonreplicative delivery vectors. They

allow temporal separation of the transformation and integration steps and therefore are effective even with poorly transformable strains. Most of the available temperature-sensitive vectors, however, have permissive temperatures of about 28°C, at which thermophilic bacteria do not grow effectively. Russell and Klaenhammer (26) recently adapted the lactococcal two-plasmid pORI system (20) for thermophilic lactobacilli. This system relies on the use of a helper plasmid, derived from the *Lactococcus lactis* plasmid pWV01, for temperature-sensitive complementation of a *repA* defect in the integration vector. The pWV01 replicon, however, was found to be only moderately unstable above 42°C and therefore was not suitable for performing ordinary single-plasmid integration experiments.

Here, we describe the new delivery vector pTN1, whose replication is efficiently shut down at 42°C. pTN1 was derived from the rolling-circle replicon of the cryptic plasmid pLC2 from *L. curvatus* (17) and used for directed integration of the lactococcal genes *nisK* and *nisR* in *L. gasseri* NCK102. These genes specify the signal transduction components (histidine protein kinase, transcriptional regulator) of the nisin-controlled expression system (7), which has been used for transcription control in various gram-positive bacteria (10, 15, 22).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this work are listed in Table 1. *L. gasseri* NCK102 was grown in MRS medium (Difco) (5) at 35°C or 42°C, *Lactococcus lactis* was cultivated at 30°C in M17 medium (Difco) supplemented with 0.5% (wt/vol) glucose (GM17), and *Escherichia coli* was grown at 37°C in Luria-Bertani medium (27). When appropriate, ampicillin (200 µg/ml for *E. coli*), chloramphenicol (10 µg/ml for *Lactococcus lactis*, 6.5 µg/ml for *L. gasseri*), or erythromycin (5 µg/ml for *Lactococcus lactis*, 3 µg/ml for *L. gasseri*) was added to the medium.

DNA preparation and cloning. Standard techniques were used for DNA cloning (27) and for the isolation of plasmids from *E. coli* (2) and *Lactococcus lactis* (8). To prepare total DNA from *L. gasseri*, a single colony was picked from an agar plate and resuspended in 50 µl of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA–25% (wt/vol) sucrose. After addition of lysozyme (10 mg/ml final concentration) and incubation for 15 min at 37°C, the suspension was diluted with 32.5 µl of 10 mM Tris-HCl (pH 8.0)–50 mM EDTA. Cell lysis was induced by the addition of

* Corresponding author. Mailing address: Abteilung Mikrobiologie, Fachbereich Biologie, Universität Kaiserslautern, Postfach 3049, D-67653 Kaiserslautern, Germany. Phone: 49 631 205 2347. Fax: 49 631 205 3799. E-mail: henrich@rhrk.uni-kl.de.

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant properties	Reference or source
<i>L. gasseri</i>		
NCK102	φadh-cured derivative of human isolate ADH	25
UKLbg1	NCK102, <i>pepN</i> :: <i>'nisP-nisR-nisK</i>	This work
<i>Lactococcus</i>		
<i>lactis</i> MG1363	Derivative of NCDO712, plasmid free, prophage cured	12
<i>E. coli</i> ER1562	<i>F⁻ endA1 hsdR17 supE44 thi-1 mcrA1272::Tn10 hsdR2 mcrB1</i>	New England Biolabs
Plasmids		
pUH89	Ap ^r , lysis gene of φX174, ColE1 replicon	13
pUK200	Cm ^r , <i>P_{nisA}</i> , terminator of <i>brnQ</i> , pSH71 replicon	32
pUK200I	pUK200, <i>P_{nisA}</i> :: <i>pepI</i>	32
pLN1363	Ap ^r , <i>leuB</i> :: <i>'nisP-nisR-nisK</i> , ColE1 replicon	14
pIL253	Er ^r , derivative of pAMβ1	29
pJK355	Cm ^r , <i>ori⁺</i> and <i>rep</i> from cryptic plasmid of <i>L. curvatus</i>	17
pTN1	pJK355, Cm ^r replaced by Er ^r from pIL253	This work
pTN1int	pTN1 with <i>'pepN</i> and 3'-flanking region from <i>L. gasseri</i> NCK102, interrupted by multiple cloning site	This work
pTN1intRK	pTN1int, <i>'pepN</i> :: <i>'nisP-nisR-nisK</i>	This work

35 µl of 2% (wt/vol) sodium dodecyl sulfate–5 mM Tris (pH 8.0)–2 mM EDTA and 1 µl of proteinase K (0.5 mg/ml). After incubation at 55°C for 1 h, the sample was diluted with 50 µl of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA and extracted several times with phenol-CHCl₃ (24:1) until a clean interphase was formed and once with CHCl₃. The DNA was then precipitated with ethanol and redissolved in 0.1 ml of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA. Restriction endonucleases, DNA polymerases, and T4 DNA ligase (Roche, New England Biolabs, MBI Fermentas, and Invitrogen) were used as recommended by the suppliers. *E. coli*, *Lactococcus lactis*, and *L. gasseri* were transformed by electroporation (9, 21, 33) with a Gene Pulser (Bio-Rad). Oligonucleotide primers were purchased from MWG-Biotech.

Sequencing of *pepN*. A fragment of 1,468 bp covering the distal part of the *pepN* gene was PCR amplified from chromosomal DNA of *L. gasseri* NCK102 with the Platinum *Taq* DNA polymerase (Invitrogen) by using the degenerate primers 5'-GAATGCGCYACCAATGGTTCGG and 5'-TAGCRAATTCCA TGTCRCRCC, where Y stands for C or T and R stands for A or G. After filling in the ends with T4 DNA polymerase, the PCR product was inserted into the unique *Ecl*136II site of the positive selection vector pUH89 and cloned in *E. coli* ER1562. The resulting plasmid was purified on a Nucleobond AX (Machery-Nagel) column, and the insert was sequenced on a LI-COR model 4000L sequencer (MWG-Biotech). The 3'-flanking sequence of *pepN* was acquired by using a GenomeWalker kit as described by the supplier (Clontech). Nucleotide sequences were analyzed with the ClustalW (30) and Blast (1) programs.

Plasmid constructions. *Lactococcus lactis* MG1363 was used as the host during cloning in pJK355 and pTN1.

To construct the delivery vector pTN1, a 1,053-bp DNA fragment carrying the erythromycin resistance marker was PCR amplified from pIL253 with ULTma DNA polymerase (Perkin-Elmer) by using the primers 5'-ATAGTCGACGTG TTCTGTGCTGACTGCACC and 5'-ATATGTCGACCTCTTTAGTCTCCTTG GAAGC, and the product was cloned into the *Pvu*II site of the vector pJK355. A recombinant plasmid containing the erythromycin resistance (*erm*) and chloramphenicol acetyltransferase (*cat*) genes in divergent orientations was denoted pJK355E. Finally, the *cat* gene was excised as a 3,297-bp *Nar*I-*Sma*I fragment, and the remaining plasmid was recircularized to give pTN1 after the *Nar*I ends were filled in with T4 DNA polymerase.

To construct a pTN1 derivative suitable for targeted integration of the *nisRK* genes into *L. gasseri*, two adjacent sections of the *pepN* region were PCR amplified from chromosomal DNA of strain NCK102 by using the primer pairs 1a (5'-atactgagTCAGAAGTGCTGTTTGTAGTTAAGC) and 1b (5'-atatcatgcagct gTTAAGCAACTGCTTTAGC) and 2a (5'-atatcatgcagGATTAATAAATAAA AAAGATGCGC) and 2b (5'-atatcatgcagCACCGTGATATTACCAACTGG) (nucleotides shown in lowercase letters were added in order to introduce the *Xho*I, *Pvu*II, *Sph*I, and *Pst*I sites shown in italics). After both PCR products were cut with *Sph*I, their ends were joined with T4 DNA ligase. The desired product (1,052 bp), covering the two fragments in the same order as in the chromosome, was PCR amplified from the ligation mixture by using primers 1a and 2b, cut with *Xho*I and *Pst*I at its ends, and inserted between the unique *Xho*I and *Pst*I sites of the vector pTN1. The resulting plasmid, pTN1int, was linearized at its unique *Pvu*II site and ligated with a 2,833-bp *Stu*I-*Nru*I fragment from plasmid pLN1363, containing *nisR*, *nisK*, and the 3' end of *nisP*. One recombinant plasmid carrying *nisRK* in the direction of *pepN* was named pTNintRK.

Delivery of *nisRK* to the chromosome. Plasmid pTN1intRK was used in *L. gasseri* essentially as described previously for the pGhost/*Lactococcus lactis* system (3, 14). To establish the plasmid in *L. gasseri* NCK102, transformants were initially selected at the permissive temperature (35°C) in the presence of erythromycin. Overnight cultures grown under the same conditions were then diluted and plated at the nonpermissive temperature (42°C) with antibiotic to obtain single-crossover integrants. To calculate the frequency of plasmid integration, the cultures were also plated at 42°C in the absence of antibiotic. Integration of the plasmid was verified by PCR with appropriate primers. Excision of the vector by a second single-crossover event was subsequently stimulated by growing individual integrants in liquid medium at 35°C without antibiotic. This incubation was extended for a number of cell generations by daily redilution of the cultures. At intervals, dilutions of the cultures were plated at 42°C without erythromycin to eliminate the excised vector and in the presence of erythromycin to determine the frequency of plasmid excision (percentage of erythromycin-sensitive clones). Erythromycin-sensitive clones were identified by replica-plating single colonies from plates without antibiotic on plates with and without erythromycin. Erythromycin-sensitive clones were checked for the presence of the integrated DNA fragment by PCR with appropriate primer pairs and DNA sequencing. One recombinant clone carrying *nisRK* integrated at the 3' end of the chromosomal *pepN* gene was denoted UKLbg1.

Preparation and analysis of cell extracts. Transformants of strain UKLbg1 carrying pUK200I were grown at 42°C, induced with nisin (Sigma-Aldrich) at the mid-exponential growth phase (optical density at 600 nm [OD₆₀₀], 0.5 U) and incubated further. Cell extracts were prepared from culture aliquots removed at different times after induction and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described for *Lactococcus lactis* (32).

To determine the activity of *PepI* in cell extracts, 1,000-fold dilutions of the samples were incubated with the chromogenic substrate Pro-*p*-nitroanilide as described previously (18). The specific *PepI* activity was calculated as nanomoles of nitrophenol released per milligram of protein per minute.

Nucleotide sequence accession number. The complete nucleotide sequence of pTN1 has been deposited under GenBank accession no. AJ518839.

RESULTS AND DISCUSSION

Temperature sensitivity of pLC2 derivatives. The cloning vector pJK355 (3.2 kb) was previously described as a derivative of the small *L. curvatus* plasmid pLC2, which lacks the potential minus origin of replication, confers resistance to chloramphenicol, and replicates in *Lactobacillus casei*, *Lactococcus lactis*, and *Bacillus subtilis* (17). In the course of experiments in which pJK355 was used for gene expression in *L. casei* at 37°C (16), it appeared that replication of the vector was less efficient at elevated temperatures. The thermosensitivity of pJK355 was subsequently verified in the thermophilic species *L. gasseri* and *L. helveticus* and exploited to knock out the peptidase genes of

FIG. 1. Features of delivery vector pTN1. (A) Plasmid map, derived from the known sequences of pJK355 (17) and the erythromycin resistance (*Er*^r) gene of pIL253 (29). Unique recognition sites for some commonly used restriction enzymes are indicated. *mcs*, multiple cloning site; *ori*⁺, predicted origin of double-strand replication; *rep*, reading frame encoding a member of bacterial plasmid replication proteins; *orf*, reading frame encoding a protein with 64% identity to *AbiN* of *Lactococcus lactis* (24). (B) Temperature-dependent growth of *L. gasseri* NCK102 and replication of pTN1. Growth rates (●) were derived from growth curves obtained with NCK102 in MRS medium at different temperatures. Percentages of erythromycin-resistant colonies of NCK102(pTN1) (bars) refer to the total numbers of colonies obtained on MRS agar plates in the absence of erythromycin.

L. gasseri NCK102 by insertion of an erythromycin resistance marker (11; C. Aichinger, personal communication). The presence of resistance genes in the recombinants, however, is often undesirable, and the range of alternative selection markers is limited.

We therefore modified the pJK355 vector to enable proper insertions or deletions by directed exchange of a chromosomal target sequence with a plasmid copy of this sequence carrying the desired mutation. This exchange proceeds through two successive single crossovers, by which the recombinant vector is first integrated into the chromosome and subsequently excised together with the original chromosomal target sequence (3). The *cat* gene present in pJK355, however, appeared to be unsuitable for selection of the single-copy plasmid integrants resulting from the first crossover, because *L. gasseri* (without the *cat* gene) produced a massive background of small colonies on plates with chloramphenicol concentrations below 4 μ g/ml. Selection on erythromycin proved to be more reliable for this purpose, since almost no background was observed when this antibiotic was used at a concentration (3 μ g/ml) low enough to select single-copy integrants (14). We therefore removed the *cat* gene from pJK355 and replaced it with the erythromycin resistance marker from pIL253 (29). The resulting vector was named pTN1 (Fig. 1A).

The temperature sensitivity of the new plasmid was evaluated in a growth experiment in which transformants of *L. gasseri* NCK102 carrying pTN1, grown at 35°C in liquid medium, were plated and incubated at increasing temperatures in the presence and in the absence of erythromycin. Figure 1B shows the ratios of erythromycin-resistant to total colony num-

bers together with the temperature optimum for growth of *L. gasseri*. At 35°C, virtually all of the colonies were resistant, whereas at 37°C the number of resistant colonies was reduced by 20%. At 42°C, which is the optimal growth temperature for *L. gasseri*, not a single resistant colony arose from 10⁴ cells spread on an agar plate within 5 days. Thus, it appeared that pTN1 could be used as a delivery vector for thermophilic lactobacilli with a permissive temperature (35°C) high enough to support reasonable growth of the transformants and efficient replicational shutdown at the optimal growth temperature (42°C).

Selection of a site for gene integration in *L. gasseri*. The regulatory features of the well-characterized *nis* gene cluster of *Lactococcus lactis* have been exploited to establish a convenient expression system for gram-positive bacteria which can be induced with the lantibiotic nisin (7). This system (nisin-controlled expression) has been disseminated as two compatible broad-host-range plasmids, one encoding the signal transduction proteins NisR and NisK and the other suitable for cloning the gene(s) of interest under the control of the nisin-responsive *nisA* promoter (*P*_{*nisA*}) (15). To avoid problems related to the use of two-plasmid systems, we recently developed a strategy for the delivery of *nisRK* to the chromosome or the sex factor of *Lactococcus lactis* (14).

We now attempted to integrate the *nisRK* genes into the chromosome of the thermophilic *L. gasseri* by using the new vector pTN1. From previous studies (14, 15), it appeared that a potential promoter localized between *nisP* and *nisR* in the *nis* gene cluster (6) is not sufficient for effective expression of the adjacent *nisRK* genes. We therefore searched for an appropri-

FIG. 2. Construction of *nisRK* delivery vector for *L. gasseri*. Details are outlined in Materials and Methods. The distal part of the *pepN* gene and its 3'-flanking sequence were amplified from the chromosome of *L. gasseri* NCK102 (i) on two separate fragments and cloned into the pTN1 vector with unique *PvuII* and *SphI* sites between them (ii). The *PvuII* site was used to insert a *nisRK* cassette (iii) isolated from pLN1363.

ate integration site in the *L. gasseri* chromosome which would enable reliable transcription of *nisRK* from a preceding promoter without disturbing the integrity or the expression of surrounding genes. For this purpose, we eventually selected the downstream region of the gene for a general aminopeptidase (*pepN*), which was expected to be constitutively transcribed during growth of the bacteria in rich medium (31).

By using degenerate primers derived from the published nucleotide sequences of the *pepN* genes of *L. delbrueckii*, *L. helveticus*, and *Lactococcus lactis*, we amplified the distal part (1.47 kb) of *pepN* from the genome of *L. gasseri* NCK102. This DNA fragment was cloned, sequenced, and used to acquire the 3'-flanking sequence of *pepN* by a genome-walking strategy. The total sequence (GenBank accession no. AJ506050) spanned the last 555 codons of *pepN* and 574 bp of the downstream region, including a truncated open reading frame of unknown function. The amino acid sequence encoded by the *pepN* part had 65% identity with PepN of *L. helveticus* (31). At a distance of 23 bp, *pepN* was followed by a putative ρ -independent transcription terminator ($\Delta G = -16$ kcal/mol), containing two perfectly complementary regions of 15 bp and ending in a run of five T residues. We chose the region between this potential stem-loop structure and the *pepN* stop codon as a promising site for integration of the *nisRK* genes.

Delivery of *nisRK* genes to *L. gasseri*. To achieve expression of the nisin signal transduction proteins in *L. gasseri*, we integrated *nisRK* 3 bp downstream of the stop codon of the chromosomal *pepN* gene. Two DNA fragments, one covering the last 524 bp of *pepN* and the other covering 498 bp of the 3'-flanking sequence, were PCR amplified with primers allowing directed cloning of the products and the generation of unique restriction sites between them. The two fragments were cloned next to each other in their original order into the new vector pTN1, and the *nisRK* genes were inserted between them (Fig. 2). The insertion, preformed in the resulting plasmid pTNintpepN, was then transferred to the chromosome of *L. gasseri* in two steps (3). pTNintpepN was first integrated into the chromosome at 42°C, and plasmid excision was subsequently stimulated at 35°C. The frequency of the initial integration was as high as 2×10^{-2} per CFU, and plasmid excision was observed in more than 60% of the clones after about 250 generations at 35°C. Of these clones, 65% contained the *nisRK*

insert. The resulting derivative of *L. gasseri* NCK102 was designated UKLbg1.

Nisin-controlled gene expression in *L. gasseri*. To test the function of nisin signal transduction in the *nisRK* integrants, we used the previously constructed plasmid pUK200I, which carries a translational fusion of *P_{nisA}* with the proline iminopeptidase gene (*pepI*) of *L. delbrueckii* (32) as a reporter. Controlled expression of PepI may be of technological importance in dairy applications due to the high proline content of several milk proteins (32). Transformants of UKLbg1 carrying pUK200I were induced with nisin, and the expression of *pepI* was monitored by electrophoretic analysis of cell extracts and determination of specific PepI activities (Fig. 3). A protein band corresponding to the molecular mass of PepI (32.9 kDa) was clearly visible 30 min after addition of nisin (10 ng/ml), and its intensity increased with time after induction. The activity of PepI was induced with a similar lag of about 30 min and increased with average rates of 85 and 375 U/min between 30 and 90 min after induction with 3 and 10 ng of nisin per ml, respectively. This demonstrated that the nisin-controlled expression system was functional in *L. gasseri* UKLbg1. As in other bacteria (15), the level of expression was dependent on the amount of nisin used for induction.

In order to describe this correlation more precisely, conditions which gave the maximum yield of PepI activity were tested. Induction was most effective when an overnight culture of UKLbg1(pUK200I) was diluted 1:50 with fresh medium, further incubated at 42°C, and supplemented with nisin at OD₆₀₀ values of between 0.2 and 0.3. Highest PepI activities were measured between 2 and 3 h after induction. As shown in Fig. 3B, the expression rates strongly increased with nisin concentrations up to 25 ng per ml and remained rather constant above 50 ng of nisin per ml. Growth of *L. gasseri* was clearly affected even by small amounts of nisin. The addition of 10 ng of nisin per ml resulted in a reduction of the growth rate from 0.64 to 0.37 per h, whereas higher nisin concentrations (up to 200 ng per ml) did not decrease the growth rate below 0.2 per h. This effect was independent of *pepI* expression, since a similar drop in growth rate (1.7-fold reduction in the presence of 10 ng of nisin per ml) was observed with transformants which carried the unmodified vector pUK200. Despite this reduction in the growth rate, nisin concentrations of up to 400

FIG. 3. Induction of *P_{nisA}::pepI* fusion in *L. gasseri*. (A) Time course of induction. Strain UKLbg1 harboring plasmid pUK200I was grown in MRS medium and induced with nisin at an OD₆₀₀ of 0.45, and cell extracts were prepared from culture aliquots at 0, 30, 60, and 90 min after induction. Extracts from a culture induced with 10 ng of nisin/ml were analyzed by SDS-PAGE on a 12% polyacrylamide gel. The arrowhead indicates the expected position of PepI (32 kDa). Molecular masses of protein markers (lane M) (in kilodaltons) are indicated on the left. Extracts from cultures induced with 3 ng of nisin/ml (■) and 10 ng of nisin/ml (●) were used to determine specific activities of PepI. (B) Correlation between nisin concentration and induction level. Strain UKLbg1 harboring plasmid pUK200I was grown in MRS medium at 42°C. At an OD₆₀₀ of 0.2, *pepI* expression was induced in culture aliquots by adding various amounts of nisin. Cell growth was further monitored by OD₆₀₀ measurement, and growth rates (●) were calculated from the exponential sections of the resulting curves. Cell extracts prepared 3 h after induction were used to determine PepI specific activities (■).

ng per ml had virtually no effect on the final optical densities of the cultures measured after overnight incubation. In the presence of more than 900 ng of nisin per ml, growth of *L. gasseri* was completely abolished.

From these results, it appears that efficient induction of the nisin-controlled expression system in *L. gasseri* can be achieved with nisin concentrations far below the bactericidal level, although these concentrations result in slowed bacterial growth. This is consistent with applications of the nisin-controlled expression system in other gram-positive bacteria (10).

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