Construction and characterization of a *Lactococcus lactis* strain deficient in intracellular ClpP and extracellular HtrA proteases

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A *Lactococcus lactis* strain deficient in both its major proteases, intracellular (ClpP) and extracellular (HtrA), was constructed and characterized. This strain, hereafter called *clpP-htrA*, could be obtained only by conjugation between a *clpP* donor strain and an *htrA* recipient strain in the NZ9000 context, allowing heterologous gene expression under the control of the NICE (nisin-controlled expression) system. The *clpP-htrA* double mutant showed both higher stress tolerance (e.g. high temperature and ethanol resistance) and higher viability than single *clpP* or *htrA* mutant strains. In addition, the secretion rate of two heterologous proteins (staphylococcal nuclease Nuc and Nuc-E7) was also higher in *clpP-htrA* than in the wild-type strain. This strain should be a useful host for high-level production and quality of stable heterologous proteins.

INTRODUCTION

Lactic acid bacteria, as food-grade Gram-positive bacteria, are good or attractive host candidates for the production of heterologous proteins of medical and/or technological interest. *Lactococcus lactis*, the most extensively characterized lactic acid bacterium, has successfully been used for heterologous protein production and secretion (Le Loir *et al.*, 2005). As only a single secreted protein, Usp45 (van Asseldonk *et al.*, 1990), can be detected by Coomassie blue staining, purification of heterologous secreted proteins could be simplified compared to other Gram-positive bacteria such as *Bacillus subtilis*. Nevertheless, degradation by host proteases remains a recurrent problem.

Up to now, only two major proteases, ClpP and HtrA, have been identified in plasmid-free strains of *L. lactis*. ClpP is an ATP-dependent enzyme reported as the major intracellular housekeeping protease in *L. lactis* (Frees & Ingmer, 1999) whereas HtrA is a trypsin-like serine protease essential for growth at high temperatures (39 °C for *L. lactis*) and which degrades misfolded proteins at the cell surface (Poquet *et al.*, 2000; Foucaud-Scheunemann & Poquet, 2003). In this study, we constructed a *L. lactis* NZ9000 strain

deficient in its both major proteases, ClpP and HtrA (hereafter called *clpP-htrA*), and evaluated its physiology and heterologous protein production potential under the control of the NICE (nisin-controlled expression) system (Kuipers *et al.*, 1998). Comparison between the *clpP-htrA* double mutant and the wild-type control strain revealed, as expected, reduced proteolytic activities and increased stability of two heterologous proteins. However, growth thermosensitivity and lethality were found to be partly alleviated compared to either *clpP* or *htrA* single mutants, suggesting the occurrence of a secondary suppressive mutation event.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this work are listed in Table 1. As controls for growth studies, we used three strains where the erythromycin resistance gene (*erm*) is inserted by single crossover at various loci (resulting in an Em^R phenotype): the previously described *clpP* (Bermúdez-Humarán *et al.*, 2002) and *htrA* (Miyoshi *et al.*, 2002) single mutant strains, hereafter called *clpP*- and *htrA*-NZ9000, respectively, as well as a *his* strain which was constructed by insertion of the *erm* gene into the non-essential histidine (*his*) locus (Delorme *et al.*, 1993). The *his* strain has the same growth and viability behaviour as the wild-type (wt) (native Em^R NZ9000) strain (data not shown) and it is hereafter called in this study *L. lactis* wt.

Abbreviation: NICE, nisin-controlled expression.

Table 1. Bacterial strains used in this study

Strain or plasmid	Characteristic(s)*	Reference
<i>L. lactis</i> strains		
MG1363	Wild-type, plasmid free	Gasson (1983)
NZ9000	MG1363 containing <i>nisRK</i> genes integrated into <i>pepN</i> locus, plasmid free	Kuipers <i>et al.</i> (1998)
NZ9000 <i>his</i>	Em ^r , NZ9000 containing <i>erm</i> gene integrated into <i>his</i> locus (single-crossover recombination), plasmid free	This work
<i>clpP</i> -NZ9000	Str ^r Rif ^r Em ^r , NZ9000 transconjugant carrying <i>clpP</i> disruption (single-crossover recombination), plasmid free	Bermúdez-Humarán <i>et al.</i> (2002)
<i>htrA</i> -NZ9000	Em ^r , NZ9000 carrying <i>htrA</i> disruption (single-crossover recombination)	Miyoshi <i>et al.</i> (2002)
NZ9000 <i>htrA</i>	NZ9000 carrying <i>htrA</i> disruption (double-crossover recombination)	Rigoulay <i>et al.</i> (2004)
NZ9000 <i>clpP</i> - <i>htrA</i>	Str ^s Rif ^s Em ^r , NZ9000 transconjugant carrying <i>clpP</i> and <i>htrA</i> disruption, plasmid free	This work
Plasmids		
pGhost3	Cm ^r , replicon Ts derivative of pWV01	Maguin <i>et al.</i> (1992)
pSEC:Nuc	Cm ^r , replicon pWV01, <i>usp-nuc</i> fusion expressed under P _{nisA} encoding the precursor SP _{Usp} -Nuc (preNuc)	Bermúdez-Humarán <i>et al.</i> (2003a)
pSEC:Nuc-E7	Cm ^r , replicon pWV01, <i>usp-nuc-E7</i> fusion expressed under P _{nisA} encoding the precursor SP _{Usp} -Nuc-E7 (preNuc-E7)	Bermúdez-Humarán <i>et al.</i> (2002)

*For strains, genotypic and phenotypic characteristics are given; for plasmids, plasmid and cloned-cassette characteristics are given.

L. lactis was grown in M17 medium supplemented with 1 % glucose (GM17) at 30 °C without agitation. Plasmid constructions were established in *L. lactis* by electrotransformation (Langella *et al.*, 1993). Antibiotics were added when required as follows: erythromycin (2·5 µg ml⁻¹) and chloramphenicol (5 µg ml⁻¹).

Construction of *L. lactis clpP-htrA*. The *L. lactis* strain deficient in both its major proteases, ClpP and HtrA, was obtained by conjugation between the erythromycin-resistant (Em^r) *clpP*-NZ9000 (*clpP* gene inactivated by insertion of the *erm* gene; Fig. 1(a); Bermúdez-Humarán *et al.*, 2002) as donor strain and a chloramphenicol-resistant (Cm^R) derivative of the marker-free *L. lactis* NZ9000 *htrA* as recipient strain (Fig. 1c; Rigoulay *et al.*, 2004). This latter strain was obtained by the introduction of pGhost3 plasmid (kindly provided by E. Maguin, INRA, Jouy en Josas) in the marker-free

NZ9000 *htrA* strain: pGhost3 is a thermosensitive (Ts) plasmid (isolated after mutagenesis of pGK12; Maguin *et al.*, 1992) that replicates in *L. lactis* at 28 °C but is lost above 37 °C. For conjugation, solid surface mating was carried out as described previously (Langella & Chopin, 1989) with the following modifications: 1 ml of saturated culture of the donor strain was mixed with 1 ml saturated culture of the recipient strain and cells were harvested, suspended in 200 µl and spread on non-selective GM17 medium. After 5 h of mating at 30 °C, cells were collected and plated at appropriate dilutions on GM17 medium containing antibiotics. Transconjugants were selected as double Em^R/Cm^R clones and pGhost3 plasmid was then cured by a temperature shift, as follows. One transconjugant was grown in GM17 medium containing antibiotics at 30 °C until saturation. The culture was then diluted and plated without Cm^R at 35 °C: this temperature was chosen to allow loss of the plasmid and

Fig. 1. Confirmation of *clpP* disruption in the *clpP-htrA* strain. (a) Scheme showing the construction of the single-crossover *L. lactis clpP*-NZ9000 (for more details about the construction see Frees & Ingmer, 1999). (b, c) PCR identification of the *clpP*-NZ9000 and *clpP-htrA* strains using primers 5C and 3C for identification of *clpP* disruption (b) and 5E and 3B for confirmation of integrated plasmid (c).

to limit thermal stress, as both ClpP and HtrA are heat-shock proteins (Frees & Ingmer, 1999; Poquet *et al.*, 2000; Foucaud-Scheunemann & Poquet, 2003). After 48 h incubation, some colonies were screened on selective and non-selective GM17 media incubated for 24 h. Colonies of the *clpP-htrA* double mutant strain which had lost pGhost3 were Cm sensitive (Cm^S), but they were Em^R, and Em addition was needed to maintain the *clpP* mutation (Fig. 1a). These candidates were also sensitive to both streptomycin (Str) and rifampicin (Rif), confirming that they were not transconjugants derived from the Str^R/Rif^R donor strain. One of these plasmid-cured Cm^S transconjugants was chosen as the *clpP-htrA* strain.

Confirmation of *clpP* and *htrA* inactivation in the *clpP-htrA* strain. In the *clpP-htrA* double mutant, inactivation of chromosomal *clpP* and *htrA* genes was confirmed by PCR analyses. Primers used were 5C (CCAATGCATCAGGTTATTTAGTACCTACCG) and 3C (GGACTAGTCCTTATTTTAATGAATTATTTCC) for *clpP* and 5H (GGATGGCAAAAGCTAATATAGGAAAATTGC) and 3H (GGTTAATTAGAAGAAGATGAAGTATTTGTTTC) for *htrA*. For Western blot analysis, we used antibodies raised against *Staphylococcus aureus* HtrA1 protease (its highly conserved catalytic site), which specifically recognize *L. lactis* HtrA (Rigoulay *et al.*, 2004). Protein extracts were prepared from either exponential-phase (OD₆₀₀ 0.4–0.6) or stationary-phase (OD₆₀₀ 2–3) cultures.

Production of heterologous proteins in the *clpP-htrA* strain. Production and secretion of two heterologous proteins, the staphylococcal nuclease (Nuc) and the human papillomavirus E7 protein fused to Nuc (Nuc-E7), were analysed in the *clpP-htrA* strain. For this, pSEC:Nuc or pSEC:Nuc-E7 plasmids, encoding Nuc and Nuc-E7, respectively (Bermúdez-Humarán *et al.*, 2002, 2003a), were established in the four following *L. lactis* strains: wt, *clpP*-NZ9000, *htrA*-NZ9000 and *clpP-htrA*. Nuc and Nuc-E7 production was then examined in these strains by Western blotting essentially as described previously (Le Loir *et al.*, 1998), using anti-Nuc or anti-E7 antibodies. Briefly, for cell fractionation, 2 ml of induced *L. lactis* cultures at a given OD₆₀₀ was harvested by centrifugation at 4 °C and 10 000 r.p.m. The supernatant and cells were processed separately. To compare the amounts of secreted and cell-associated proteins, both cell and supernatant fractions were concentrated. Sample concentration was calculated as follows. The equivalent of 1 ml of 1 OD₆₀₀ unit of culture (cell or supernatant) was concentrated in a 100 µl final volume as described below, and 10 µl was loaded for SDS-PAGE. Supernatants were precipitated by the addition of 10% trichloroacetic acid, harvesting by centrifugation at 4 °C and 13 000 r.p.m. The precipitate was dissolved in 1:20 volume of 50 mM sodium hydroxide. Cell pellets were resuspended in 70 µl TES containing lysozyme (10 mg ml⁻¹). After 30 min of incubation at 37 °C, cells were lysed with 30 µl 20% SDS. Equal volumes of 2 × loading buffer were added to all samples.

Determination of nuclease activity yields. Nuclease (Nuc) activity in the supernatants of induced cultures was determined spectrophotometrically, essentially as described previously (Heins *et al.*, 1967). Briefly, DNA added as substrate is degraded by Nuc to mono- and oligonucleotides, which are non-precipitable by perchloric acid. The products of the degradation are then quantified by measuring DNA as A₂₆₀. Nuc concentration is estimated by comparison to a Nuc standard of known concentration.

RESULTS AND DISCUSSION

Characterization of the *clpP-htrA* strain

Despite several preliminary efforts, we could not obtain a *L. lactis* strain deficient in both ClpP and HtrA proteases

by single- or double-crossover inactivation. We therefore performed a conjugation between a *clpP* donor strain and an *htrA* recipient strain. *clpP* inactivation in the *clpP-htrA* strain was confirmed by PCR analysis with primers 5C and 3C, designed to hybridize with upstream and downstream regions of the *clpP* gene, respectively (Fig. 1a). The presence of a 618 bp PCR product (containing the intact *clpP* gene: 600 bp) from genomic DNA of the wt (*his*) and *htrA*-NZ9000 strains but not in strains *clpP*-NZ9000 (used as positive control) and *clpP-htrA* indicates the disruption of the *clpP* gene in our mutant strain (Fig. 1b). This genotype was confirmed by a second PCR analysis with primers 5E (CATGCCATGGCATGCAAAAGAAAAACGAAATGATACACC) and 3B (GAGCGATTAGAATTATCAGCAAGG). These primers were designed to hybridize with the integrated plasmid (*erm* gene, primer 5E) and with the target *clpP* gene (primer 3B) (Fig. 1a). As shown in Fig. 1(c), the presence of a ~1500 bp amplified band from the *clpP*-NZ9000 (used as positive control) and *clpP-htrA* strains again confirms the disruption of the *clpP* gene of our mutant strain.

htrA inactivation was then confirmed in the *clpP-htrA* strain by PCR analysis with primers 5H and 3H, designed to hybridize with upstream and downstream regions, respectively, of the *htrA* gene (Fig. 2a). The presence of a 902 bp PCR product (containing a fragment of the *htrA* gene with a 322 bp internal deletion) from genomic DNA of strains *htrA*-NZ9000 (used as positive control) and *clpP-htrA* indicates the disruption of the *htrA* gene in our mutant strain (Fig. 2b). PCR amplification from genomic DNA of wt and *clpP*-NZ9000 strains results in a 1228 bp product (containing an intact *htrA* gene: 1224 bp) (Fig. 2b). In addition, we confirmed the absence of HtrA protein in strain *clpP*-NZ9000 by Western blot analysis using antibodies raised against *S. aureus* HtrA1 protease (its highly conserved catalytic site) and found to specifically recognize *L. lactis* HtrA (Rigoulay *et al.*, 2004). As shown in Fig. 2(c), protein extracts prepared from exponential-phase wt cultures revealed a band corresponding to the lactococcal HtrA protein (42 kDa; Rigoulay *et al.*, 2004). Interestingly, no HtrA signal was detected in extracts prepared from stationary-phase cultures, suggesting that HtrA is mainly produced in the exponential phase (data not shown). *htrA* inactivation either by single crossover in *htrA*-NZ9000 (Miyoshi *et al.*, 2002) or by double crossover in *clpP-htrA* (Fig. 2a) leads to the absence of HtrA (Fig. 2c) in exponential-phase protein extracts.

Influence of *clpP* and *htrA* inactivation on *L. lactis* physiology

We first determined the impact of both *clpP* and *htrA* inactivations on the growth and viability of the *clpP-htrA* strain under optimal culture conditions, on rich medium and at 30 °C. Strains were cultured on GM17 liquid medium plus antibiotic (Em 2.5 µg ml⁻¹) and growth was monitored by OD₆₀₀ measurements for 10 h. At 30 °C, growth of both the *htrA*- and *clpP*-NZ9000 strains was somewhat

Fig. 2. Confirmation of *htrA* mutation in the *clpP*-*htrA* strain. (a) Scheme showing the construction of the double-crossover *L. lactis* NZ9000 *htrA* (for details of the construction see Rigoulay *et al.*, 2004). A fragment of the *htrA* gene from *L. lactis* MG1363 with a 322 bp internal deletion was PCR amplified and cloned into pSMA500 vector (Madsen *et al.*, 1996), resulting in pAMJ411. *L. lactis* NZ9000 was then transformed with this plasmid and selection with Em allows single-crossover inactivation by insertion of the *erm* gene into the *htrA* gene (*htrA*-NZ9000 Em^R strain). The second crossover event was performed by cloning an ectopic non-identical *htrA* copy from *L. lactis* IL1403 downstream of the nisin-inducible promoter in pNZ8048 (Kuipers *et al.*, 1998), resulting in pAMJ167. This vector, in which IL-1403-*htrA* gene expression is controlled by the nisin promoter, was introduced into the *htrA*-NZ9000 Em^R strain and recombinant clones were selected as Em^R/Cm^R and by the addition of nisin. To obtain the second crossover event, one recombinant colony was grown in the presence of Cm and nisin. The Em^S strain, containing the expected *htrA* disruption, was isolated and grown in non-selective medium to induce the loss of pAMJ167. Finally, one Cm^S strain clone (*htrA* double crossover) was isolated and *htrA* deletion was confirmed by PCR. The resulting HtrA product is truncated at position 155. (b) PCR identification of *htrA*-NZ9000 and *clpP*-*htrA* strains using primers 5H and 3H for identification of *htrA* disruption. (c) Confirmation of the absence of HtrA in *htrA*-NZ9000 and *clpP*-*htrA* strains by Western blotting using anti-HtrA antibodies in protein extracts prepared from exponential-phase (OD₆₀₀ 0.4–0.6) cultures.

slower than that of the wt. This phenotype has not been studied previously, and the original *clpP* mutant strain was only described to grow at similar rates on plates at 30 °C (colonies of equal size and frequency; Frees & Ingmer, 1999). Surprisingly, the growth of the *clpP*-*htrA* double mutant strain was improved compared to the *clpP*-NZ9000 single mutant (Fig. 3a). All strains reached the same growth levels at 10 h (Fig. 3a). Viability was then analysed by monitoring strains at 30 °C for 3 days (72 h). Bacterial populations were estimated every 24 h using a spiral plater (Spiral System DS, Interscience) on GM17 agar (Em 2.5 µg ml⁻¹) and viability was measured as c.f.u. ml⁻¹. At 30 °C, viability of all the strains was comparable (Fig. 3b).

Stress resistance of the *clpP*-*htrA* strain

It was previously demonstrated that the *clpP*- and *htrA*-NZ9000 strains are thermosensitive: the original *clpP* mutant strain was found to be unable to form colonies at

37 °C (Frees & Ingmer, 1999), whereas the *htrA* mutant was severely affected at 37 °C in liquid culture medium but completely stopped growing at 39 °C (Poquet *et al.*, 2000). We thus checked the growth phenotype of *clpP*-*htrA* at 37 °C.

At 37 °C, *clpP*-NZ9000 was unexpectedly found to grow almost normally in liquid culture medium. A significant improvement in growth of the *clpP*-*htrA* strain was observed when compared to both single *clpP*- and *htrA*-NZ9000 mutant strains (Fig. 4a). Furthermore, the *clpP*-*htrA* strain was able to form colonies at 37 °C (~20-fold lower frequency than at 30 °C, data not shown). Thus, unexpectedly high growth rates at both 30 °C and 37 °C were observed for the *clpP*-*htrA* strain. Survival analyses showed a marked defect at 37 °C for all mutant strains: ~10⁷ c.f.u. ml⁻¹ were measured for the wt strain after 72 h versus ~10⁶ c.f.u. ml⁻¹ for the *clpP*-*htrA* and *htrA*-NZ9000 mutants and ~10⁵ c.f.u. ml⁻¹ for the *clpP*-NZ9000 mutant (Fig. 4b).

Fig. 3. Influence of *clpP* and *htrA* inactivation on growth (a) and survival (b) of *L. lactis*. Growth of the *clpP-htrA* strain (●) was monitored by OD₆₀₀ measurements for 10 h at 30 °C and compared to that of the wt (◆), *clpP*-NZ9000 (■) and *htrA*-NZ9000 (▲) strains. Viability was measured for all strains by estimating bacterial populations every 24 h for 3 days.

The results observed for growth and viability of the *clpP-htrA* double mutant strain suggest that in this strain, growth thermosensitivity and lethality are partially alleviated compared to both the *clpP*- and *htrA*-NZ9000 single mutant strains, suggesting the occurrence of a secondary suppressive mutation event.

We then evaluated the response of the *clpP-htrA* mutant to higher temperature, ethanol and oxidative stress conditions as described previously (Foucaud-Scheunemann & Poquet, 2003). We first tested temperature stress: briefly, overnight cultures were diluted 100-fold and after ~2 h, once OD₆₀₀ 0.10 was reached, a temperature shift from 30 °C to 39 °C was performed and growth was measured by OD₆₀₀ for 10 h (Fig. 5a). After the temperature upshift, the wt strain grew to OD₆₀₀ 2 whereas the growth of the *htrA*-NZ9000 strain was rapidly arrested as previously described (Poquet *et al.*, 2000; Foucaud-Scheunemann & Poquet, 2003). Unexpectedly, but in agreement with the growth characteristics at 37 °C, the *clpP*-NZ9000 strain grew better than *htrA*-NZ9000 (OD₆₀₀ 1.2), and the *clpP-htrA* strain even better (OD₆₀₀ 1.5) (Fig. 5a). Again, our data are in

agreement with the presence of a secondary suppressor mutation.

We then evaluated the growth of the wt, *clpP-htrA* and *clpP*- and *htrA*-NZ9000 strains in the presence of 5% (v/v) ethanol: growth of the wt and *clpP-htrA* remained unaffected, in contrast to *clpP*- and *htrA*-NZ9000, for which a significant reduction in growth was observed (Fig. 5b). Finally, we tested the effect of different concentrations (5, 10 and 15 mM) of hydrogen peroxide (data not shown): growth of all strains was affected to similar extent, as previously observed (Foucaud-Scheunemann & Poquet, 2003).

To explain our data, we propose that a secondary suppressor mutation occurred allowing improved growth of the double mutant strain; such an event might have been favoured during the temperature shift necessary to eliminate pGhost from the potential transconjugant candidates. It has previously been shown that in both wt and *clpP* mutant strains of *L. lactis*, *trmA* inactivation increased thermal stress tolerance and proteolysis of puromycin peptides (the protease involved remained unknown, although

Fig. 4. Influence of *clpP* and *htrA* inactivation on growth (a) and survival (b) of *L. lactis* at 37 °C. Growth of the *clpP-htrA* strain (●) was monitored by OD₆₀₀ measurements for 10 h at 37 °C and compared to that of the wt (◆), *clpP*-NZ9000 (■) and *htrA*-NZ9000 (▲) strains. Viability was measured for all strains by estimating bacterial populations every 24 h for 3 days.

Fig. 5. Stress tolerance of the *clpP-htrA* strain. Growth of the *clpP-htrA* strain (●) at 39 °C (a) or in the presence of 5% ethanol (b) was monitored by OD₆₀₀ measurements for 10 h and compared to that of the wt (◆), *clpP*-NZ9000 (■) and *htrA*-NZ9000 (▲) strains. Cultures were grown to OD₆₀₀ 0.10 and temperature shift or ethanol challenge was performed. Growth was monitored by OD₆₀₀ measurements for 10 h.

different from FtsH or HtrA), without having any effect of oxidative stress tolerance, and TrmA was proposed to be a negative regulator of stress tolerance and proteolysis (Frees *et al.*, 2001). Comparing the thermosensitive phenotype of our double mutant strain with a *trmA-clpP* mutant strain (kindly provided by Karin Hammer, Technical University of Denmark, DK-2800 Lyngby, Denmark) which is known to grow at normal rates in stress conditions (e.g. at 37 °C or in the presence of puromycin), we observed that our double mutant has a lower growth rate than the *trmA-clpP* mutant (data not shown). Alternatively, *htrA* suppressors might have arisen, as previously shown in *Escherichia coli*, where *sohA* and *sohB* are multi-copy suppressors of *htrA* (Baird & Georgopoulos, 1990; Baird *et al.*, 1991).

Stable production of heterologous proteins in *L. lactis clpP-htrA*

To determine the capacities of the *clpP-htrA* strain to produce and secrete heterologous proteins, production of Nuc and Nuc-E7 was compared in wt, *clpP*-NZ9000, *htrA*-NZ9000 and *clpP-htrA* strains. For Nuc protein, Western blot analysis with anti-Nuc antibodies revealed two bands in the cell fractions of wt(pSEC:Nuc) and *clpP*-NZ9000(pSEC:Nuc) strains that correspond to Nuc precursor (preNuc, synthesized as a preproprotein: ~22 kDa) and to NucB protein (product of preNuc signal peptide maturation: ~19 kDa). Note that separation of preNuc and NucB bands could not be well achieved, perhaps due to the high concentration of SDS-PAGE (15%) used to separate NucA from NucB. In the supernatant of these strains, a third form of Nuc was detected and it corresponds to NucA protein [product of a secondary cleavage of a 21 aa NucB propeptide: ~19 kDa (Le Loir *et al.*, 1998) performed by HtrA in *L. lactis* (Poquet *et al.*, 2000)] plus NucB protein (Fig. 6). In contrast, in *htrA*-NZ9000(pSEC:Nuc) and *clpP-htrA*(pSEC:Nuc) extracts, no NucA in supernatant fractions was observed, in agreement with the absence of HtrA protease (responsible for NucB-to-NucA processing)

in these strains (Poquet *et al.*, 2000). Interestingly, supernatant samples of strains *htrA*-NZ9000(pSEC:Nuc) and *clpP-htrA*(pSEC:Nuc) showed a slightly higher concentration of NucB as determined by comparison with the signals of known amounts of Nuc by quantitative scanning of blots after immunodetection (Image-Quant; Bermúdez-Humarán *et al.*, 2002; data not shown). To confirm these observations, Nuc yields were determined by a Nuc activity test (see Methods). The results revealed that Nuc activity was significantly higher in *htrA*-NZ9000(pSEC:Nuc) and *clpP-htrA*(pSEC:Nuc) ($94.4 \pm 9.9 \mu\text{g ml}^{-1}$ and $85.5 \pm 5.1 \mu\text{g ml}^{-1}$ respectively; means $\pm$ SD, $n=3$), than in wt(pSEC:Nuc) and *clpP*-NZ9000(pSEC:Nuc) supernatant fractions ($73 \pm 4.4 \mu\text{g ml}^{-1}$ and $62.9 \pm 7.3 \mu\text{g ml}^{-1}$, respectively), confirming higher Nuc secretion in our double mutant strain than in wt and *clpP* strains.

In the case of Nuc-E7, Western blot analysis with anti-E7 antibodies revealed three bands in the cell fractions of the wt(pSEC:Nuc-E7) strain that correspond to: (i) Nuc-E7 precursor (preNuc-E7); (ii) Nuc-E7 mature form; and (iii) a putative product resulting from secondary proteolytic

Fig. 6. Production and secretion of staphylococcal nuclease (Nuc) by the *clpP-htrA* strain: Western blot analysis of induced exponential-phase cultures (10 ng nisin ml⁻¹) of wt, *clpP-htrA*, *clpP*-NZ9000 and *htrA*-NZ9000 strains containing pSEC:Nuc. Arrows indicate the positions of NucB, NucA and precursor form. C, cell lysates; S, supernatant fraction.

cleavage of Nuc-E7 as previously observed (Bermúdez-Humarán *et al.*, 2002, 2003b). In the supernatant, one major band corresponding to the secreted Nuc-E7 fusion was observed (Fig. 7). In the cell fractions of the *clpP*-NZ9000(pSEC:Nuc-E7) strain, preNuc-E7 and Nuc-E7 forms were detected. Surprisingly, no Nuc-E7 signal was observed in the supernatant, suggesting that secretion of this protein in a single *clpP* mutant cannot be achieved. This observation could be explained by the lower metabolism of the *clpP* mutant once induced with nisin. Indeed, in the induced *L. lactis* cultures for the preparation of proteins, the OD₆₀₀ reached by the *clpP* mutant was ~15-fold lower than other strains, suggesting significant decreases both of growth rate and of protein synthesis. We can conclude that the *clpP* strain is not a good candidate for heterologous protein production with the NICE system. In the cell fractions of *htrA*-NZ9000(pSEC:Nuc-E7) and *clpP-htrA*(pSEC:Nuc-E7) strains, Western blotting revealed the same three bands observed in the wt strain (Fig. 7). Strikingly, the amounts of Nuc-E7 in the supernatant fractions of these two strains were about threefold higher in *htrA*-NZ9000(pSEC:Nuc-E7) and *clpP-htrA*(pSEC:Nuc-E7) strains compared to the wt(pSEC:Nuc-E7) strain.

Altogether, these results revealed that *htrA*-NZ9000 and *clpP-htrA* strains could be better hosts for stable production and secretion of heterologous proteins than wt and *clpP*-NZ9000 strains. Indeed, we previously reported that *htrA* inactivation in *L. lactis* allows high-level production of heterologous proteins. However, thermosensitivity of the *htrA*-NZ9000 mutant limits its use to overproduce heterologous proteins. Strikingly, the *clpP-htrA* strain has better resistance to both temperature and ethanol stresses than the *htrA*-NZ9000 and even the *clpP*-NZ9000 strain. This feature makes the *clpP-htrA* strain a more suitable vector for heterologous protein production and delivery in food-stuffs or in the digestive tract. Furthermore, as the study of

new protein degradation pathways generally requires the inactivation of known proteases, the *clpP-htrA* strain described in this work could be a good tool for the identification of new proteolytic enzymes.

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Fig. 7. Production and secretion of the human papillomavirus E7 protein fused to Nuc (Nuc-E7) by the *clpP-htrA* strain: Western blot analysis of induced exponential-phase cultures (10 ng nisin ml⁻¹) of wt, *clpP*-NZ9000 and *htrA*-NZ9000 strains containing pSEC:Nuc-E7. Arrows indicate positions of Nuc-E7 and precursor form as well as a putative product resulting from secondary proteolytic cleavage. C, cell lysates; S, supernatant fraction.

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