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

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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 EmR 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 EmR NZ9000) strain (data not shown) and it is hereafter called in this study *L. lactis* wt.

Abbreviation: NICE, nisin-controlled expression.
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⁺) clpP-NZ9000 (clpP gene inactivated by insertion of the erm gene; Fig. 1(a); Bermúdez-Humaran et al., 2002) as donor strain and a chloramphenicol-resistant (CmR) derivative of the marker-free 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 EmR/CmR 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 CmR at 35°C: this temperature was chosen to allow loss of the plasmid and

![Table 1. Bacterial strains used in this study](image)

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

![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).](image)
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 \textit{clpP}-\textit{htrA} double mutant strain which had lost pGhost3 were Cm sensitive (Cm\textsuperscript{S}), but they were Em\textsuperscript{S} and Em addition was needed to maintain the \textit{clpP} mutation (Fig. 1a). These candidates were also sensitive to both streptomycin (St) and rifampicin (Rif), confirming that they were not transconjugants derived from the Str\textsuperscript{P}/Rif\textsuperscript{P} donor strain. One of these plasmid-cured Cm\textsuperscript{S} transconjugants was chosen as the \textit{clpP}\textsuperscript{−}/\textit{htrA}\textsuperscript{−} strain.

**Confirmation of \textit{clpP} and \textit{htrA} inactivation in the \textit{clpP}-\textit{htrA} strain.** In the \textit{clpP}-\textit{htrA} double mutant, inactivation of chromosomal \textit{clpP} and \textit{htrA} genes was confirmed by PCR analyses. Primers used were 5C (CCGATGCATGTTAGATGACTCCCG) and 3C (GGACTAGTCCTTATTTTAATGAATTATTTTCC) for \textit{clpP} and 5H (GGATGCCAAAAGCTAATAGGAAAATTG) and 3H (GGTTAATGGAGAAGATGACATTGGTTT) for \textit{htrA}. For Western blot analysis, we used antibodies raised against \textit{Staphylococcus aureus} HtrA protease (its highly conserved catalytic site), which specifically recognize \textit{L. lactis} HtrA (Rigoulay et al., 2004). Protein extracts were obtained from either exponential-phase (OD\textsubscript{600} 0–4.0–6) or stationary-phase (OD\textsubscript{600} 2–3) cultures.

**Production of heterologous proteins in the \textit{clpP}-\textit{htrA} strain.** Production and secretion of two heterologous proteins, the staphylococcal nuclease (Nuc) and the human papillomavirus E7 protein expressed in our mutant by single- or double-crossover inactivation. We therefore performed a conjugation between a \textit{clpP} donor strain and an \textit{htrA} recipient strain. \textit{clpP} inactivation in the \textit{clpP}-\textit{htrA} strain was confirmed by PCR analysis with primers 5C and 3C, designed to hybridize with upstream and downstream regions of the \textit{clpP} gene, respectively (Fig. 1a). The presence of a 618 bp PCR product (containing the intact \textit{clpP} gene: 600 bp) from genomic DNA of the wt (\textit{his}) and \textit{htrA}\textsuperscript{−}/\textit{NZ9000} strains but not in strains \textit{clpP}\textsuperscript{−}/\textit{NZ9000} (used as positive control) and \textit{clpP}\textsuperscript{−}/\textit{htrA} indicates the disruption of the \textit{clpP} gene in our mutant strain (Fig. 1b). This genotype was confirmed by a second PCR analysis with primers 5E (CATGCCATGGCATGAAAAGAAAAACGAAATGATA- CACC) and 3B (GAGGATTAGAAATTACGAAAG). These primers were designed to hybridize with the integrated plasmid (\textit{erm} gene, primer 5E) and with the target \textit{clpP} gene (primer 3B) (Fig. 1a). As shown in Fig. 1(c), the presence of a ~1500 bp amplified band from the \textit{clpP}\textsuperscript{−}/\textit{NZ9000} (used as positive control) and \textit{clpP}\textsuperscript{−}/\textit{htrA} strains again confirms the disruption of the \textit{clpP} gene of our mutant strain.

**RESULTS AND DISCUSSION**

**Characterization of the \textit{clpP}-\textit{htrA} strain.** Despite several preliminary efforts, we could not obtain a \textit{L. lactis} strain deficient in both \textit{ClpP} and \textit{HtrA} proteases...
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 25 mg l⁻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: ~10⁵ 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 ~10⁷ c.f.u. ml⁻¹ for the clpP-NZ9000 mutant (Fig. 4b).
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 OD\textsubscript{600} 0.10 was reached, a temperature shift from 30 °C to 39 °C was performed and growth was measured by OD\textsubscript{600} for 10 h (Fig. 5a). After the temperature upshift, the wt strain grew to OD\textsubscript{600} 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 (OD\textsubscript{600} 1.2), and the clpP-htrA strain even better (OD\textsubscript{600} 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 puromycyl peptides (the protease involved remained unknown, although

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**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 OD\textsubscript{600} 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.

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**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 OD\textsubscript{600} 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.
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: 16 kDa) and NucB protein (product of preNuc signal peptide maturation: 32 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 ± 9·9 μg ml⁻¹ and 85·5 ± 5·1 μg ml⁻¹ respectively; means ± SD, n = 3), than in wt(pSEC:Nuc) and clpP-NZ9000(pSEC:Nuc) supernatant fractions (73 ± 4·4 μg ml⁻¹ and 62·9 ± 7·3 μg 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

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**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.

**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, thermostability 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 foodstuffs 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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