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HtrA Is Essential for Efficient Secretion of Recombinant Proteins by *Lactococcus lactis*[▽]

Kalpana Sriraman and Guhan Jayaraman*

Department of Biotechnology, Indian Institute of Technology—Madras, Chennai, India 600036

Received 15 March 2008/Accepted 23 September 2008

HtrA is a unique protease on the extracellular surface of *Lactococcus lactis*. It is known to take part in the proteolysis of many secreted recombinant proteins, and the mutation of *htrA* can lead to the complete stabilization of recombinant proteins. In this work, we have shown that *htrA* mutation also leads to significant reduction of the efficiency of recombinant-protein secretion. We also show that the level of HtrA can be lowered by the suppression of the acid tolerance response (ATR) in *L. lactis*. Instead of using an *L. lactis htrA* mutant, the reduction of the HtrA level in wild-type recombinant cultures of *L. lactis* by ATR suppression may serve as a better strategy for the production of secreted recombinant proteins.

Lactococcus lactis is a potential host for the production of therapeutic recombinant proteins. *L. lactis* strains used for recombinant protein production are known to express very few membrane-bound or secreted proteases. In such strains, HtrA is the only protease that has been characterized on the extracellular surface (15). HtrA is a key factor in the *L. lactis* response to specific stress conditions (5) and is known to be involved in the maturation of native proteins, processing of propeptides (15), and proteolysis of secreted recombinant proteins (12). Hence, *L. lactis htrA* mutants have been considered as potential hosts for the production of stable recombinant proteins (13, 15). The objectives of this work were to compare the efficacy of an *L. lactis htrA* mutant with that of wild-type *L. lactis* for the production of secreted recombinant proteins and to investigate the role of HtrA in the processing of recombinant proteins.

We analyzed the secretion efficiencies of two recombinant proteins, streptokinase (a therapeutic protein) and staphylococcal nuclease (a reporter protein), in wild-type *L. lactis* and an *L. lactis htrA* mutant. The efficiency of secretion of both proteins was found to be severely affected in the *L. lactis htrA* mutant compared to their secretion in wild-type *L. lactis.* In an earlier report, we showed that the suppression of the acid tolerance response (ATR) in *L. lactis* leads to a decrease in the degradation of recombinant streptokinase (18). In this paper, we correlate our earlier observations with the observed downregulation of *htrA* expression during ATR suppression. We also demonstrate an enhancement in the accumulation of recombinant staphylococcal nuclease during ATR suppression. The results indicate that ATR suppression in wild-type *L. lactis* may serve as a better strategy than the use of an *L. lactis htrA* mutant for the production of secreted recombinant proteins.

Secretion efficiencies of recombinant proteins for wild-type *L. lactis* and an *L. lactis htrA* mutant. The wild-type *L. lactis* strains used in this work were *L. lactis* MG1363 (6) and *L. lactis* NZ9000 (MG1363 with chromosomally integrated *nisRK*) (8), which are suitable for use with a nisin-inducible expression plasmid (1). The *htrA* mutant used was NZ9000 *htrA* (16). All strains were cultured in G5-M17 medium (standard M17 medium with 5 g/liter of glucose). The two proteins under study were expressed using two different expression systems. Streptokinase was expressed by using a P170 expression system. P170 is a derivative of a native *L. lactis* promoter identified during screening for environmentally regulated promoters (7). The P170 promoter is an autoinducible promoter which is strongly upregulated at pH values below 6.5 during the transition to stationary phase (9). The plasmid pSK99 (18) was used for expressing the streptokinase gene under the control of the strongest promoter derivative, P170gx, which was 150- to 200-fold more active than the native promoter (2), in the wild-type *L. lactis* MG1363 and in NZ9000 *htrA*.

Nuclease expression in *L. lactis* NZ9000 and in NZ9000 *htrA* was induced with 2 ng/ml of nisin, added during the exponential phase of growth (at an optical density at 600 nm [OD_{600}] of ~0.5). For exploitation of the autoinduction mechanism of nisin for gene expression, the genes for the signal transduction system *nisK* and *nisR* have been inserted into the chromosome of *L. lactis* MG1363 (nisin negative), creating strain NZ9000 (8). When a gene of interest is placed under the control of promoter P*nisA* in a plasmid, the expression of that gene can be induced by the addition of a subinhibitory concentration of nisin to the growth medium (11). The plasmid used for expression was pSEC:Nuc (1). To assess the secretion efficiency, the distribution of the recombinant protein in the cytoplasmic, extracellular, and membrane fractions was analyzed. Cell fractionation was carried out according to the method of Obis et al. (14). Enzymatic assays were used to determine the activities of streptokinase (18) and nuclease (4). The activity of the protein obtained in each fraction was normalized to the activity obtained per unit of dry cell weight (DCW). The activity in the membrane fraction (<1.5%) was found to be negligible for both proteins and is not reported. The results of activity analysis of the various fractions showed that the secretion efficiency of streptokinase in MG1363 was nearly 100%, while in NZ9000 *htrA*, the efficiency was reduced to about 20%, with most of the streptokinase accumulating in the cytoplasm (Table 1). The

* Corresponding author. Mailing address: Department of Biotechnology, Indian Institute of Technology—Madras, Chennai, India 600036. Phone: 91 44 22574108. Fax: 91 44 22574102. E-mail: guhanj@iitm.ac.in.

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TABLE 1. Activities of recombinant streptokinase and nuclease in the extracellular and cytoplasmic fractions of (wild type) *L. lactis* MG1363 and NZ9000 and mutant NZ9000 *htrA* during early stationary phase culture in G5-M17 medium

Strain and cellular fraction	Streptokinase in MG1363 or *htrA* mutant		Nuclease in NZ9000 or *htrA* mutant	
	Sp act (IU/g of DCW)	% Secretion efficiency (mean ± SD)	Sp act (IU/g of DCW)	% Secretion efficiency (mean ± SD)
Wild type				
Extracellular	14,500	~100	5,141	71 ± 2
Cytoplasmic	ND[a]		2,007	
NZ9000 *htrA*				
Extracellular	13,895	20.7 ± 1.2	563	33 ± 2
Cytoplasmic	55,680		1,167	

[a] ND, not detectable by assay.

results of Western blot analysis showed that streptokinase bands appear in all three fractions of the NZ9000 *htrA* culture (Fig. 1). The bands corresponding to the cytoplasm and membrane fractions were congruent with those for the extracellular fraction, as well as the standard streptokinase (without the signal sequence), thus indicating the inability of processed streptokinase to be secreted. On the other hand, the streptokinase bands appear only in the extracellular fraction of the wild-type strain *L. lactis* MG1363, thus indicating complete secretion of the processed protein (Fig. 1). One can also clearly observe bands corresponding to degraded streptokinase in the extracellular fraction of the *L. lactis* MG1363 culture. The absence of these streptokinase degradation bands in the NZ9000 *htrA* culture clearly shows that HtrA is primarily responsible for streptokinase degradation in *L. lactis*.

Similar results were obtained with recombinant staphylococcal nuclease using the nisin-inducible expression system. The secretion efficiency of staphylococcal nuclease is high in NZ9000 (71%) and low in NZ9000 *htrA* (33%) at 6 h after induction (Table 1). A temporal analysis of the nuclease secretion efficiency confirmed that the percentage of nuclease in the extracellular fraction was lower at all times in NZ9000 *htrA* than in NZ9000. Since HtrA is involved in the processing of NucB to NucA (15), the above data also correlated well with a higher intracellular accumulation of NucB (data not shown). These results suggest that the secretion of recombinant proteins is impaired in *L. lactis* NZ9000 *htrA*, irrespective of the protein or expression system used. Some reports in the literature have concluded that the secretion of recombinant proteins (and not secretion efficiency) is better in *L. lactis htrA* mutants (3, 12, 13). These conclusions were based on estimations made from Western blot analyses of the extracellular fraction only. Based on quantitative analysis of all cellular fractions, we have shown that secretion efficiency is lower in an *L. lactis htrA* mutant.

HtrA is known to be involved in the alteration of cell surface properties; *L. lactis htrA* mutants have increased bacterial sedimentation in liquid cultures and increased bacterial chain length compared to these characteristics in wild-type strains (5). This increased aggregation of cells may reduce the exposure of secretion machinery to the outside environment, potentially reducing secretion efficiency. Apart from inefficient secretion, *L. lactis htrA* mutant strains also pose other problems. We observed that the overall amount of recombinant nuclease produced in *L. lactis* NZ9000 *htrA* was very low compared to the level in the wild-type strain (Table 1). This has also been observed in other reports on recombinant protein production in an *L. lactis htrA* mutant (12). Out of five proteins studied by using nisin-inducible expression systems, the overall production of four proteins was low in the *L. lactis htrA* mutant. Such low productivity was not observed in our work with streptokinase in the P170 expression system. This may be attributed to the mechanism of induction. P170 promoter activity depends on the cytoplasmic regulator molecule, RcfB, which gets activated in acidic conditions and binds to the 14-bp regulatory region of P170 (10). Thus, the promoter activity will not be affected by cellular aggregation. On the other hand,

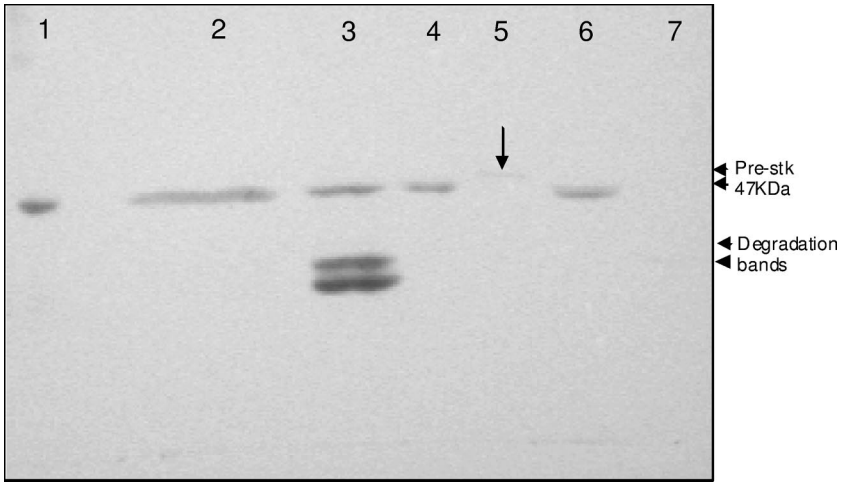

FIG. 1. Expression of streptokinase in MG1363 and NZ9000 *htrA*: Western blot analysis of different cellular fractions. Lanes: 1, standard streptokinase; 2, extracellular fraction from NZ9000 *htrA*; 3, extracellular fraction from MG1363; 4, membrane fraction from NZ9000 *htrA*; 5, membrane fraction from MG1363; 6, cytoplasmic fraction from NZ9000 *htrA*; 7, cytoplasmic fraction from MG1363. Pre-stk, prestreptokinase (streptokinase with signal sequence).

TABLE 2. Comparison of recombinant streptokinase and nuclease activities in the extracellular fraction during ATR development and suppression, and effects of ATR on protein processing by HtrA[a]

Protein and medium	ATR level (% survival of cells after acid shock [mean ± SD])	Sp act (IU/g of DCW)	% Degradation of streptokinase (mean ± SD)	% NucB converted to NucA (mean ± SD)
Streptokinase[b]				
G5-M17	94 ± 6	15,000	71 ± 4.5	
G5-3xM17	<0.01	51,100	33 ± 3	
Nuclease				
G5-M17	97 ± 3	3,621		26 ± 4.2
G5-3xM17	<0.01	9,947		18 ± 3.7

[a] Recombinant proteins streptokinase and nuclease were assayed in strains MG1363 and NZ9000, respectively. The effects of ATR on protein processing by HtrA are shown as percentage of degradation of streptokinase and proportion of NucB converted to NucA.
[b] Streptokinase data are from reference 18.

nisin induction depends on the activation of NisK, a membrane-bound histidine kinase (11). Nisin binds to the receptor NisK. Subsequently, NisK activates NisR by phosphorylation and the activated NisR induces the transcription of the P*nisA*-based genes. Thus, the increased aggregation of cells in NZ9000 *htrA* cultures reduces the membrane exposure and may affect the activation of NisK. We also observed that the *htrA* mutant cultures have a longer lag phase (2 to 3 h as against <1 h for wild-type *L. lactis* for similar inoculation conditions) and can often grow very slowly.

HtrA activity and suppression of ATR. We have shown in an earlier report (18) that the suppression of ATR in *L. lactis* leads to at least a 2.5-fold increase in productivity for recombinant streptokinase and a significant decrease in the degradation of streptokinase (a process mediated by HtrA, as seen in this study). We also showed that the suppression of ATR could be achieved by increasing the phosphate level and the buffering capacity of the medium (18). In this study, we investigated the effect of ATR suppression on the production of recombinant staphylococcal nuclease and the processing by HtrA of the NucB form to the NucA form (both of which are active). The ATR level, nuclease activity, and the extent of processing by HtrA (as given by the proportion of the NucA form of nuclease) were analyzed for nuclease production in two different media, G5-M17 (standard M17 medium with 5 g/liter of glucose) and a medium with a higher buffering capacity, G5-3xM17. The latter had three times the concentration of all M17 components with the exception of glucose. The buffering capacity of G5-3xM17 is increased due to the additional levels of glycerophosphate in the medium, and therefore, ATR is suppressed during the growth of *L. lactis* in this medium (18). The ATR level was measured as follows. The cells were given an acid shock by exposure to G5-M17 medium (without sodium β-glycerophosphate) at pH 4 (adjusted using acetic acid). The cell numbers were estimated as CFU by plating shocked cells immediately after resuspension and after 2 h on G5-M17 plates containing the appropriate antibiotic. The percentage of organisms surviving was taken as the measure of ATR (18). The proportion of NucA was measured by densitometric analysis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, using bovine serum albumin as the internal standard.

TABLE 3. Primers used for real-time PCR

Primer	Sequence
htrA Forward	5′ ACGAATACCTAAGGCAGGAC3′
htrA Reverse	5′ ATCACAACGACTGAAGATGG3′
16S rRNA Forward	5′ GGTAGACTTGAGTGCAGGAG3′
16S rRNA Reverse	5′ GTGTCAGTTACAGGCCAGAG3′

The activity of nuclease increased by 2.7-fold in the G5-3xM17 medium, and this correlated with the suppression of ATR. It was also observed that the proportion of NucA decreased with suppression of ATR (Table 2). The results with nuclease were similar to the ones obtained in the earlier work with streptokinase (18), thus indicating that the effect of ATR on recombinant protein production in *L. lactis* cultures may be a general phenomenon. The reductions in streptokinase degradation and nuclease processing suggest that HtrA levels may decrease with suppression of ATR, leading to decreased processing of recombinant proteins by HtrA. To confirm this hypothesis, the mRNA level of *htrA* was measured by using real-time PCR during the growth of streptokinase-expressing MG1363 cultures in both the media mentioned above.

The mRNA level was measured from cDNA (obtained from the reverse transcription of total RNA [Qiagen-GmBH]) by using a Quantitect Sybr green real-time PCR kit (Qiagen-GmBH) in Applied Biosystems 7500 real-time PCR equipment (Applied Biosystems, United States) according to the manufacturer's recommendations. The kit has a Hotstart *Taq* DNA polymerase. The primers used for the experiment were designed by using web-based Primer3 software (17). The primers used for amplifying the *htrA* and 16S rRNA transcripts, along with the conditions for the real-time PCR experiment, are given in Tables 3 and 4. The mRNA level was computed by using relative quantification (threshold cycle method) with 16S rRNA as the internal reference gene. The mRNA level at early exponential phase (OD_{600}, ~0.3) was taken as the basal level and was used to calibrate samples for the relative quantification of mRNA. The changes in *htrA* mRNA levels during growth were expressed as multiples of the basal level of mRNA.

The mRNA level of *htrA* increased severalfold during the transition to stationary phase of *L. lactis* MG1363 cultured in G5-M17 medium (Fig. 2). This result correlated well with the high ATR level (which also develops during late exponential phase) and the high degradation of streptokinase observed in

TABLE 4. Conditions used for PCR

Stage	Temperature (°C)	Time
Initial activation step	95	15 min
Cycles (45)		
Denaturation	94	15 s
Annealing	55	30 s
Extension	72	30 s

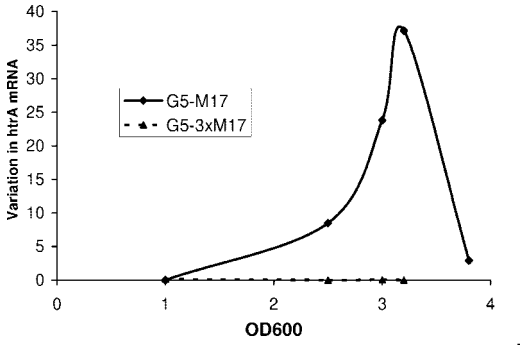

FIG. 2. Variations in *htrA* mRNA relative to basal level during growth in G5-M17 or G5-3xM17 medium. Basal level was mRNA level at an OD_{600} of 0.3, corresponding to early exponential phase. Correspondence of OD_{600} values to growth phases: 1, mid-exponential phase; 2.5, late exponential phase; above 3.0, transition phase and early stationary phase (growth phases at corresponding OD_{600} values are comparable in the two media).

this medium (Table 2). The cell densities for the two cultures (in G5-M17 and G5-3xM17) are mainly dependent on the initial glucose concentration, and the growth phases of the two cultures are comparable at a given OD (data not shown). Therefore, the *htrA* mRNA levels at corresponding growth phases can be suitably compared by taking measurements of the culture at a particular OD (Fig. 2). In the G5-3xM17 medium, the *htrA* mRNA level did not change significantly from the basal level throughout the culture, correlating well with the reduced ATR level and lower level of streptokinase degradation (Fig. 2 and Table 2). These results confirm that suppression of ATR leads to lowering of the HtrA level and thus reduces the processing of recombinant proteins in *L. lactis*. It was also seen that the lower level of HtrA resulting from ATR suppression does not result in the changes in bacterial chain length generally associated with *htrA* mutation (4). Microscopic examination of cells grown in G5-3xM17 showed chain lengths similar to those of cells grown in G5-M17 (data not shown).

It can be easily discerned from the results in Table 2 that the reduction in HtrA processing is not enough to account for the increase in recombinant protein activity obtained due to ATR suppression. First, the increase in activity was more than proportionate to the reduction in degradation of streptokinase by HtrA; the increase in activity was more than threefold, while the reduction in degradation was only around 40% (Table 2). Second, nuclease does not get degraded by HtrA; HtrA only processes one form of nuclease (NucB) to another (NucA), both of which are active. Hence, the increase in activity per unit of cell mass for streptokinase, as well as nuclease, is due to the increase in the accumulation of the recombinant protein. It is not clear what factors contribute to this increase in accumulation. We have observed that the mRNA level of streptokinase during ATR suppression (obtained from real-time PCR experiments) is actually lower than the mRNA level during fully developed ATR (data not shown). Therefore, the increase in the accumulation of the recombinant protein may be due to enhanced translation rates or a reduction in proteolysis by other undetermined proteases.

In conclusion, the results presented in this paper highlight the limitations of using *L. lactis htrA* mutants for the production of recombinant proteins. It was observed that *htrA* mutation reduces the efficiency of secretion of the recombinant protein produced, although the protein produced was completely stabilized. We have also shown that suppression of ATR leads to improved levels of the active recombinant protein and also correlates well with lower HtrA levels in wild-type *L. lactis* cultures. These results suggest that the suppression of ATR may serve as a better alternative strategy (to using *L. lactis htrA* mutants), as it improves both the productivity and stability of proteolytically sensitive recombinant proteins.

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