Controlled Autolysis and Enzyme Release in a Recombinant Lactococcal Strain Expressing the Metalloendopeptidase Enterolysin A

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This study concerns the exploitation of the lytic enzyme enterolysin A (EntL), produced by Enterococcus faecalis strain DPC5280, to elicit the controlled autolysis of starter lactococci. EntL, a cell wall metalloendopeptidase secreted by some E. faecalis strains, can kill a wide range of gram-positive bacteria, including lactococci. The controlled expression of entL, which encodes EntL, was achieved using a nisin-inducible expression system in a lactococcal host. Zymographic analysis of EntL activity demonstrated that active enzyme is produced by the recombinant lactococcal host. Indeed, expression of EntL resulted in almost complete autolysis of the host strain 2 h after induction with nisin. Model cheese experiments using a starter strain in addition to the inducible enterolysin-producing strain showed a 27-fold increase in activity with respect to the release of lactate dehydrogenase in the strain overexpressing EntL, demonstrating the potential of EntL production in large-scale cheese production systems. Indeed, the observation that a wide range of lactic bacteria are sensitive to EntL suggests that EntL-induced autolysis has potential applications with a variety of lactic acid bacteria and could be a basis for probiotic delivery systems.

The lysis of starter cells during dairy fermentations and throughout the ripening process is thought to play a pivotal role in subsequent flavor development of cheese. In this respect, the intracellular proteolytic, peptidolytic, and esterolytic enzymes released from starter cells via lysis contribute to the initiation of the flavor development process. Moreover, the lysis of lactococcal starters has also been linked to the debittering which occurs during cheese ripening (2). The rate and level of autolysis and concomitant enzyme release are essentially uncontrolled, and individual starter strains lyse at different rates beyond the influence of the cheese manufacturer. Since the ripening of cheese can be both a slow and a costly process, controlling the rate and level of lysis would be extremely beneficial from the point of view of cheese manufacturers.

Peptidoglycan hydrolases degrade the bacterial cell wall and result in cell lysis and therefore represent a potential tool that could be exploited to override the intrinsic rate and level of autolysis of a given strain. In general, lytic enzymes may be autolysins which act on the cell wall of the producing cell or secreted enzymes which attack the wall of other cells. Five types of enzymes with lytic activity against gram-positive bacteria exist and are classified on the basis of their cleavage specificities as follows: N-acetylmuramidases, N-acetylglucoaminidases, N-acetylmuramyl-L-alanine amidases, endopeptidases, and transglycosylases (2). The construction of gene expression systems for lactic acid bacteria has allowed the production of lytic proteins in a range of starter cultures. Shearman et al. (15) expressed the gene for a lytic enzyme in Lactococcus by cloning the φML3 lysin gene on a plasmid under the control of its own promoter. In this case, lysis of the host occurred during the stationary phase of growth. However, externally stimulated inducible lysis systems would provide a more attractive approach for fine control of starter cell lysis during the cheese-ripening process. For example, acmA, the gene encoding the main lactococcal autolysin, was cloned under the control of two regulated promoters, the chloride-inducible promoter (14) and the promoter-operator region of the temperate lactococcal phage r1t (1, 11). Lysis was monitored according to the release of the cytoplasmic marker enzyme PepX and the decrease in optical density at 600 nm (OD₆₀₀). The results from these studies revealed that the producing strains lyse upon induction (although very gradually), thereby releasing their intracellular contents into the cheese matrix. In another example, de Ruyter et al. (4) used the nisin-inducible system (3) to clone the holin and lysin genes of phage φUS3 into Lactococcus lactis under the control of the nisin-inducible promoter. The addition of nisin resulted in cell lysis in this case, as evidenced by a fourfold increase in l-lactate dehydrogenase (LDH) release into the curd relative to the results seen with control strains.

The enterococcal metalloendopeptidase enterolysin A (EntL) exhibits cell wall-degrading activity and was characterized in two separate studies (6, 12). One of the producing strains, E. faecalis DPC5280, was also found to produce the lantibiotic cytolysin and displayed a broad spectrum of inhibition which can be attributed to production of both antimicrobials (enterolysin and cytolysin). Partially purified EntL was found to have a broad spectrum of inhibition that included most gram-positive strains tested but, unlike cytolysin, had no associated hemolytic activity. Lactococcal strains were particularly sensitive to EntL, as evidenced by their complete lysis within 90 min of exposure. The purpose of this study was to...
to exploit EntL for the controlled lysis of lactococci by using a tightly controlled system whereby the rate and level of auto-
tolysis of lactococcal starters could be predictably pro-
grammed. The possibility of exploiting EntL in applications
involving lactococcal starter cultures was investigated. A re-
combinant lactococcal strain was generated which expresses
EntL under the control of the nisin-inducible promoter and
consequently allows nisin-induced autolysis. This was success-
fully demonstrated both in broth and in model cheese experi-
ments.

### MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The bacterial strains and plasmids used in the study are listed in Table 1. Lactococcal strains were propagated in M17 (Difco Laboratories, Detroit, Mich.) broth and/or agar (1% [wt/vol]) containing glucose (0.5% [vol/vol]).

**TABLE 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant property(ies)</th>
<th>Reference or source</th>
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<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactococcus lactis subsp. lactis HP NZ9800</td>
<td>Lac⁺, Pst⁺, MG1363 derivative, nisRK EntL⁺</td>
<td>This study</td>
</tr>
<tr>
<td>Enterococcus faecalis DPC5280</td>
<td>EntL⁺, Hem⁺</td>
<td>9</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pNZ8048</td>
<td>Cm⁺, nisin-inducible expression vector</td>
<td>6</td>
</tr>
<tr>
<td>prR0H7</td>
<td>EntL⁺, Cm⁺</td>
<td>This study</td>
</tr>
</tbody>
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* Lac, lactose; Pst, proteinase; Hem, hemolysin; Cm, chloramphenicol; nisRK, nisin signal transduction genes.

Plasmid construction and analysis. PCR primers were obtained from Genosys (Cambridge, United Kingdom). The entL structural gene from Enterococcus faecalis DPC5280 was amplified using primers RH114 (5' GGAAGATGGATCT TAATTAGGAGGG-3') and RH115 (5’-CCAAGCTTGGCAATACTAC TGTGTC3'), and the resultant 868-bp product was introduced into the pREP4 (Promega, Madison, Wis.) vector by the SpeI/HindIII sites (indicated with italic characters in the sequences) in the multiple cloning site of the pNZ8048 vector (3), resulting in the plasmid pR0H7. These steps were performed using E. coli as a host.

**Zymogram analysis of EntL induction.** The L. lactis NZ9800/prR0H7 strain was grown to an OD₆₀₀ of approximately 0.5. Cells were then induced with 10 ng of nisin/ml or left untreated. As a control, the same conditions were used for L. lactis NZ9800. At intervals of 20 min, 10-ml volumes of culture were centrifuged and the supernatant was retained. EntL was concentrated from the lactococcal supernatants as described by Hickey et al. (6). The semipurified EntL was analyzed by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using a Mini Protein II cell unit (Bio-Rad) and a 10% acrylamide resolving gel, by the method of Laemmli (8). A prestained standard (Sigma) (wide range [6,500 to 205,000 Da]) was used as a molecular mass marker. To visualize EntL, activity gels called zymograms were employed. This initially involved incorporating an autoclaved overnight culture of L. lactis HP in the resolving gel. A total of 100 ml of the culture was centrifuged, and the resulting pellet was added to the gel. This was used to view lytic activity upon renaturing as described by Lederc and Asselin (10) and Potvin et al. (13). The gel was examined for the presence of a lytic zone after 2 h of incubation at 37°C. The levels of intensity of the lytic zones were compared using image analysis software according to the instructions of the manufacturer (Kodak, Rochester, N.Y.).

**Investigation of the lytic ability of L. lactis prR0H7.** The effect of the autolytic activity was monitored by measuring the decrease in turbidity of a cell suspension of L. lactis NZ9800/prR0H7 was grown to an OD₆₀₀ of approximately 0.5. Cells were then induced with 10 ng of nisin/ml or not treated. As a control, the same conditions were used with L. lactis NZ9800. The turbidity of the cultures was measured spectrophotometrically at 600 nm at time intervals of 15 min over a 2-h period. The experiments were performed in triplicate.

**Laboratory-scale cheddar cheese manufacture.** Laboratory-scale cheesemaking trials were performed using 500 ml of pasteurized whole milk (preincubated at 32°C for 30 min). A 2% inoculum of both L. lactis HP and L. lactis NZ9800/prR0H7 was added. Rennet (0.018%) was added 60 min after inoculation, followed by the addition of 50 ng of nisin/ml after 75 min for induction of EntL production. Controls with no added nisin were also included. The milk was allowed to set for 25 min, after which the curd was cut and stirred. At 10 min after cutting, the incubation temperature was increased by 1°C every 5 min until a temperature of 38.5°C was reached. The whey was drained when a pH of 6.2 was reached, and the incubation temperature was decreased to 32°C. The pH of the curd was monitored until it reached 5.3. LDH activity was assayed in the curd juice directly before the addition of nisin and at hourly intervals for 4 h. The average of triplicate LDH measurements was determined.

**Measurement of intracellular enzyme release.** LDH was determined using sodium pyruvate as a substrate) by the procedure described by Wittenberg and Angelo (20). The oxidation of NADH was followed by monitoring the decrease in OD at 340 nm of the assay mixture in a Milton Roy Spectronic R Genesi spectrophotometer. Activity was expressed as units per milliliter of supernatant, where 1 U is the amount of enzyme that catalyzes the oxidation of 1 μmol of NADH/min/ml of supernatant.

**RESULTS**

**Use of the nisin-inducible system for controlled expression of EntL.** Although it has been established that lactococcal cells exposed to extracellular EntL undergo rapid lysis, the fate of cells producing the enzyme intracellularly was unknown. EntL was expressed (using the nisin-inducible expression [NICE] system) (3) in lactococci. This system gives maximal expression at sublethal concentrations of nisin and also has been reported to give negligible expression in the absence of the inducer (4). The gene entL (encoding EntL from E. faecalis DPC5280) was cloned into the expression vector pNZ8048 under the control of the inducible nisA promoter, resulting in the plasmid pR0H7 (Fig. 1). This plasmid then was introduced into the L. lactis NZ9800 strain, an MG1363 derivative containing the
signal transduction genes integrated on the chromosome. To investigate the amount of EntL expression that could be achieved upon induction, zymographic analysis was performed (Fig. 1). Mid-log-phase cells (as determined when the OD\textsubscript{600} reached 0.5) were induced with 10 ng of nisin/ml. At time intervals of 20 min, supernatant was taken from the culture and partial purification of EntL was performed. Controls included NZ9800 cells and the test strain with no added nisin. Expression was evident as a band of lysis at approximately 34 kDa, corresponding to the molecular mass of mature EntL. The zymogram shows that the level of EntL in the supernatant was 14-fold higher after 80 min of induction compared to the results seen with the uninduced control. In addition, a low level of EntL was produced even without induction by nisin. Interestingly, a cell wall active protein of approximately 16 kDa was also produced in response to nisin induction.

**Targeted lysis by EntL in a model cheese system.** To determine whether enterolysin overexpression could result in autolysis and potentially influence flavor development in a food system, laboratory-scale cheesemaking experiments were performed using *L. lactis* HP as the starter strain and *L. lactis* NZ9800.pRH07 as a source of intracellular enzymes. The pH profiles are presented in Fig. 3. The reporter enzyme (LDH) was used to quantify the level of cell lysis and subsequent intracellular enzyme release. The level of LDH released into the curd in the experimental cheese increased dramatically

**FIG. 1.** Construction scheme for the expression vector containing the inducible *nisA* promoter. Boxed white arrows represent chloramphenicol acetyltransferase, the marker gene. The boxed grey arrow represents *entL*, the EntL structural gene. T represents the terminator, and MCS represents the multiple cloning site of pNZ8048. The photo is a zymogram containing autoclaved *L. lactis* HP cells. The sample was EntL partially purified from the induced lactococcal host, which appears as a dark zone of clearance upon renaturing. Lane 1, the control NZ9800 strain plus nisin after 80 min; lane 2, strain NZ9800.pRH07 without nisin after 80 min; lane 3, NZ9800.pRH07 plus nisin at time 0; lane 4, NZ9800.pRH07 plus nisin after 20 min; lane 5, NZ9800.pRH07 plus nisin after 40 min; lane 6, NZ9800.pRH07 plus nisin after 60 min; lane 7, NZ9800.pRH07 plus nisin after 80 min.
during the first 4 h after induction with nisin (unlike the results seen with the control strain, which exhibited low levels of the enzyme even where nisin was added) (Fig. 3). Indeed, the enzyme release obtained in the experimental cheese was approximately 27-fold higher than the release from the control cheese. These results demonstrate that extensive lysis occurred in the model cheese and that controlled production of EntL in lactococci has potential for cheese applications. The observation that the pH profiles of both model cheeses were similar suggested that the EntL-producing strain itself was autolysing but that the expressed enzyme was not acting externally on the companion HP strain to any significant degree. This was confirmed in that the \textit{L. lactis} HP cell numbers were comparable in both the experimental and control cheeses (unlike the results seen with the EntL-containing strain, which rapidly decreased in viability following nisin induction) (Fig. 3).

**DISCUSSION**

The purpose of this study was to utilize the NICE system (3) to examine the potential of the use of EntL to achieve controlled autolysis of lactococcal cells. EntL was previously isolated in two separate studies from two individual enterococcal strains and was shown to lyse a broad spectrum of gram-positive bacteria (6, 12). Inducing EntL expression in the lactococcal cells results in extremely efficient autolysis, as evidenced by the decrease in OD. Indeed, a 90% reduction in \( \text{OD}_{600} \) values is seen after the first hour of induction. In contrast, the use of the inducible promoter/operator region of the temperate lactococcal phage \textit{r1t} for overexpression of AcmA in lactococci resulted in a much slower decrease in \( \text{OD}_{600} \) values 2 to 3 h after induction (1). Similarly, the use of the NICE system for expression of the lytic genes \textit{lytA} and \textit{lytH} from the lactococcal phage \( \phi \text{US3} \) required 2 h before the turbidity of the culture reduced 10-fold (4).

Model cheeses made with the EntL-producing lactococcal strain CPC5280 exhibited a 27-fold higher release of LDH into the curd. The level of LDH release from the model cheeses in this study was unusually high. For instance, de Ruyter et al. (4) found in a similar study that bacteriocin-induced lysis generally gave no more than a fourfold increase in the release of LDH in the experimental cheese. Therefore, exploiting the controlled overexpression of EntL in such cheese systems has potential for accelerating ripening and thereby reducing the cost of the process. In addition, \textit{L. lactis} HP, the starter strain, is not significantly lysed, as cell numbers at the end of cheesemaking are similar for the experimental and the control cheeses. EntL causes lysis of lactococci when present in the medium, but neighboring bacteria do not appear to lyse significantly, most probably because the enzyme does not reach high enough levels or is bound to the producer cell walls. Given that nonstarter lactic acid bacteria are thought to be important in the development of the final texture (e.g., the holes in Swiss cheese) and flavor in many cheese types (5) and that lysis has been shown to influence the growth rates and types of non-starter lactic acid bacteria (2), production of an enzyme which only lyzes the producing strain may be advantageous.

The potential application of EntL, however, may not be simply limited to increasing autolysis during cheesemaking. EntL is known to inhibit a range of lactic acid bacteria, and programmed lysis of cells through the use of EntL may aid in the development of recombinant probiotic strains which would release nutraceuticals in either in vivo or in vivo situations,
facilitating the delivery of bioactive compounds which are produced intracellularly.

In conclusion, we have constructed a lactococcal strain capable of producing EntL in a controlled manner which may have a number of potential applications in cheese manufacture. The delivery of intracellular enzymes into the cheese matrix by EntL-producing strains may facilitate the acceleration of ripening. In addition, such a system may be broadly applicable as the basis for delivery of bioactive compounds by a range of lactic acid bacteria, given their sensitivity to EntL.

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REFERENCES


FIG. 3. pH profile of laboratory-scale Cheddar cheese production with 2% L. lactis HP plus 2% L. lactis NZ9800.pRH07. ▲ pH without nisin induction; ◯ pH with nisin induction. The bar chart indicates levels of activity (AU/ml of culture) of LDH released into the curd of model cheese after 0, 1, 2, 3, and 4 h of induction with nisin. Open columns, LDH without nisin; columns shaded in gray, LDH plus nisin. Error bars represent standard deviations of triplicate readings. (Table inset) Cell numbers of L. lactis HP and NZ9800.pRH07 after cheesemaking in the absence (−) and presence (+) of nisin.