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A Chloride-Inducible Gene Expression Cassette and Its Use in Induced Lysis of Lactococcus lactis

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A chloride-inducible promoter previously isolated from the chromosome of Lactococcus lactis (J. W. Sanders, G. Venema, J. Kok, and K. Leenhouts, Mol. Gen. Genet., in press) was exploited for the inducible expression of homologous and heterologous genes. An expression cassette consisting of the positive-regulator gene gadR, the chloride-inducible promoter P\textsubscript{cl} and the translation initiation signals of gadC was amplified by PCR. The cassette was cloned upstream of Escherichia coli lacZ, the holin-lysin cassette (lytPR) of the lactococcal bacteriophage r1t, and the autolysin gene of L. lactis, acmA. Basal activity of P\textsubscript{cl} resulted in a low level of expression of all three proteins. Growth in the presence of 0.5 M NaCl of a strain containing the gadC: lacZ fusion resulted in a 1,500-fold increase of β-galactosidase activity. The background activity levels of LytPR and AcmA had no deleterious effects on cell growth, but induction of lysis expression by addition of 0.5 M NaCl resulted in inhibition of growth. Lysis was monitored by following the release of the cytoplasmic marker enzyme PepX. Released PepX activity was maximal at 1 day after induction of lytPR expression with 0.1 M NaCl. Induction of acmA expression resulted in slower release of PepX from the cells. The presence of the inducing agent NaCl resulted in the stabilization of osmotically fragile cells.

Lactococcus lactis has a history of use in human consumption for thousands of years. The organism is widely used in the dairy industry and has GRAS (generally regarded as safe) status. Therefore, L. lactis has potential as a host for recombinant protein expression in food systems. In the past decade, genetic systems have been developed for the expression in L. lactis of homologous and heterologous genes (7, 16). Recently, emphasis has been put on the design of food-grade expression systems. These include lactococcal plasmids with selection markers that do not rely on the use of antibiotics, such as sacb (11, 15), the lacF gene in combination with a lacF strain (18, 26), and an ochre suppressor allele, supB, combined with a strain with a nonsense mutation in one of the purine biosynthesis genes (8). Efficient lactococcal expression signals have been isolated, and their applicability in the constitutive expression of various prokaryotic and eukaryotic genes has been demonstrated (for a review, see reference 7). However, for certain applications, such as expression of lethal gene products, the availability of inducible gene expression systems is of paramount importance. Also, the induction of certain activities in L. lactis during an industrial process requires food-grade inducible (lactococcal) expression signals that allow tight control. The inducing signal should be a safe food additive or a physical change in environmental conditions that occurs normally or that can easily be incorporated into the process.

Only a very few inducible gene expression systems for lactococci that fulfill these requirements have been described up to now. The lacA promoter, in combination with lacR, allows limited induction by switching of the fermentable sugar from glucose to lactose (25, 33). The combination of these elements with the Escherichia coli phage T7 RNA polymerase resulted in high levels of expression of tetanus toxin fragment C upon a sugar switch, but then, of course, the system contains heterologous elements (34). The nisA promoter was shown to be induced by subinhibitory amounts of nisin (12), and its applicability was shown by the induced expression of high levels of β-glucuronidase and PepN (6). A heat-inducible homologous gene expression system has recently been developed on the basis of the repressor and genetic-switch element of the lactococcal temperate bacteriophage r1t (22).

Inducible lysis systems could be a valuable addition to the concept of inducible gene expression in L. lactis, since they would allow for the production of cell lysates containing recombinant proteins to be used as a food additive without further purification. Moreover, there is an industrial interest in controllable lysis of lactococcal cells in situ during cheese making. Current insights into the process of cheese maturation attribute an important role in the rate of amino-nitrogen formation to starter-cell lysis (4). Consequently, the amount of free amino acids in cheese was improved by phage-induced lysis (20). The need for balanced lysis of cells in the cheese matrix for optimal cheese maturation was stressed by Crow et al. (5). Intact cells are necessary for lactose fermentation and for some of the reactions involved in flavor formation, whereas release of cytoplasmic peptidases is important for the acceleration of peptide breakdown. Controlled cell wall degradation may be a feasible approach to stimulating cheese ripening.

The gene encoding the major peptidoglycan hydrolase of L. lactis has been cloned and sequenced (2). An acmA mutant did not lyse during stationary phase (3), indicating the importance of AcmA in cell lysis. acmA was expressed under the control of the repressor-operator system of the lactococcal phage r1t, and induction with mitomycin C led to lysis of cells (3). A system to produce lysates with high levels of recombinant protein was based on the combination of a phage-inducible middle promoter with a phage-derived origin of replication. Infection with phage ds3 led to plasmid amplification and expression of β-galactosidase at very high levels (24).

In a previous study, we have described a chloride-inducible promoter from L. lactis MG1363 (28). The applicability of this
promoter for the controlled overexpression of homologous and heterologous genes is shown here. NaCl-induced expression of L. lactis acmA and of the holin and lysin genes (lytPR) of phage r1t resulted in cell lysis and release of cellular proteins.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. L. lactis was grown at 30°C in twofold-diluted M17 broth (Difco Laboratories, Detroit, Mich.) with 0.5% glucose and a final concentration of 1.9% β-glycerophosphate (1/2M17). Solidified 1/2M17 medium contained 1.5% agar. Ethrythromycin and chloramphenicol were used at final concentrations of 5 μg/ml; 5-Bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal) was used at a final concentration of 0.008%. E. coli was grown in tryptone-yeast broth at 37°C with vigorous agitation or on tryptone-yeast agar plates. Ampicillin and erythromycin were used at 100 μg/ml.

Molecular cloning techniques. DNA manipulations were performed essentially as described by Sambrook et al. (27). DNA was introduced by electroporation by using the T7 sequencing kit and protocol (Pharmacia LKB Biotechnology AB, Uppsala, Sweden).

Construction of transcriptional fusions of the gad promoter with lysin genes. A chloride-inducible gene expression vector pWV01 and the pAL10 promoter were amplified as a 1,280-bp DNA fragment (bp 821 to 2071 of the published sequence; GenBank accession no. AF005098) by PCR with primers pNS3d and pNS3f fused to the lacZ promoter and the P2 promoter, respectively. The PCR-amplified fragment was digested with SacI and ScaI and was cloned upstream of L. lactis LL108, and the resulting plasmid was designated pNS3Δ.

Plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M. lactic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1363</td>
<td>Plasmid-free derivative of NCDO712</td>
<td>9</td>
</tr>
<tr>
<td>MG1363acmAΔ1</td>
<td>AcmA derivative of MG1363</td>
<td>2</td>
</tr>
<tr>
<td>LL108</td>
<td>Em'::repA derivative of MG1363</td>
<td>15</td>
</tr>
<tr>
<td>LL302</td>
<td>repA derivative of MG1363</td>
<td>15</td>
</tr>
<tr>
<td>NS3</td>
<td>Em'::gadC::lacZ derivative of MG1363</td>
<td>28</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC1000</td>
<td>Km'::repA derivative of MC1000, carrying a single copy of the pWV01 repA gene in glgB</td>
<td>17</td>
</tr>
<tr>
<td>EC101</td>
<td>Km'::repA derivative of JM101, carrying a single copy of the pWV01 repA gene in glgB</td>
<td>14</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pORI28</td>
<td>Em'; Ori' of pWV01; Rep'</td>
<td>17</td>
</tr>
<tr>
<td>pORI13</td>
<td>Em'; promoterless lacZ; Ori' of pWV01; Rep'</td>
<td>28</td>
</tr>
<tr>
<td>pWV0007</td>
<td>Em'::lacZ, encoding a temperature-sensitive RepA protein</td>
<td>19</td>
</tr>
<tr>
<td>pNS3</td>
<td>Em'; gadC::lacZ pORI13 derivative with a 10-kb Sau3AI chromosomal DNA fragment</td>
<td>28</td>
</tr>
<tr>
<td>pNS3d</td>
<td>Em'; gadC::lacZ pORI13 derivative with a 2.5-kb PstI-Sau3AI chromosomal DNA fragment</td>
<td>28</td>
</tr>
<tr>
<td>pNS3Z</td>
<td>Em'; gadC::lacZ pORI13 derivative with a 1,280-bp PCR fragment amplified with NS3-7 and NS3-8</td>
<td>This study</td>
</tr>
<tr>
<td>pIR1PR</td>
<td>Em'; phage r1t lytPR fused to r1t regulatory region; derivative of pMG36e</td>
<td>22</td>
</tr>
<tr>
<td>pNS3PR</td>
<td>Em'; gadC::lytPR pIR1PR derivative with a 1,280-bp PCR fragment amplified with NS3-7 and NS3-8</td>
<td>This study</td>
</tr>
<tr>
<td>pAL10</td>
<td>Ap'; acmA interrupted by a SacI fragment; derivative of pAL102 with a 1,280-bp PCR fragment amplified with NS3-7 and NS3-8</td>
<td>This study</td>
</tr>
<tr>
<td>pAL102</td>
<td>Ap'; Em' Ori'; derivative of pAL101</td>
<td>This study</td>
</tr>
<tr>
<td>pNS3AL3S</td>
<td>Em' Ori' gadC::acmA; acmA interrupted by a SacI fragment; derivative of pAL102 with a 1,280-bp PCR fragment amplified with NS3-7 and NS3-8</td>
<td>This study</td>
</tr>
<tr>
<td>pNS3AL</td>
<td>Em' Ori' gadC::acmA</td>
<td>This study</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

Construction of chloride-inducible transcriptional gene fusions. The chloride-inducible expression signals of the L. lactis gad operon (29) were exploited for the expression of homologous and heterologous genes in L. lactis. A gene expression
cassette was designed that included the activator gene gadR, the chloride-inducible promoter P_{gad}, and the ribosome binding site and start codon of gadC, the first gene of the gadCB operon that is under the control of P_{gad}. The DNA fragment encoding these elements was amplified, and four restriction enzyme sites were introduced by PCR. To assess its suitability for driving gene expression, the cassette was inserted upstream of two lysin genes, namely, lytPR (22) of the lactococcal bacteriophage r1t (yielding plasmid pNS3PR) and acmA, encoding the lactococcal autolysin (2) (pNS3AL). For quantitative analysis and as a negative control in lysis experiments, the expression cassette was placed upstream of E. coli lacZ.

**Sodium chloride-induced expression of the gene fusions.** β-Galactosidase activity in *L. lactis* LL108(pNS3Z) grown to an optical density of 600 nm (OD_{600}) of 0.5 in the presence of 0.5 M NaCl reached 1,500 U/mg. This amount of protein was visible in a Coomassie brilliant blue-stained SDS-PAA gel (data not shown). In contrast, in the absence of NaCl, 1 U of β-galactosidase activity per mg of protein was present, which is slightly above the detection level (0.25 U/mg). Control of gene expression by P_{gad} is, thus, very tight. The expression level of lacZ was positively correlated to the NaCl concentration from 50 to 750 mM (reference 28 and data not shown). Lysin expression in cultures of *L. lactis* LL108 carrying either pNS3PR or pNS3AL was induced at an OD_{600} of 0.5 by the addition of 0.5 M NaCl. A comparison of lanes 3 and 4 in Fig. 1 shows that 6 h after induction, in addition to autolysin activity specified by the chromosomal acmA gene, a band of phage lysin activity of approximately 50 kDa is present in the induced cells of LL108(pNS3PR) only. This was also seen as an extra band in a Coomassie brilliant blue-stained SDS-PAA gel (data not shown). A higher level of AcmA activity was detected in LL108(pNS3AL) when expression of the plasmid-located acmA gene was induced with NaCl than was detected in the uninduced culture. The level of AcmA activity derived from the chromosomal copy of acmA is not affected by the presence of NaCl, as can be seen in cultures of LL108(pNS3Z) (Fig. 1; compare lanes 1 and 2).

**Induction of cell wall-degrading activities inhibits growth.** Growth in 1/2M17 of LL108(pNS3Z) and LL108(pNS3AL) did not differ from that of the control strain LL108(pNS3Z) (Fig. 2). The background activity level of P_{gad} under noninduced conditions apparently did not harm the cells. Induction of expression of lytPR or acmA had a clear effect on the cultures. Addition of NaCl led to a rapid stop of the increase of the optical density of LL108(pNS3PR), followed by a decrease in optical density 3 h after induction (Fig. 2), whereas addition of NaCl to LL108(pNS3AL) resulted in slower growth and stabilization of the optical density after approximately 3 h. Also, lower levels of chromosomally encoded AcmA were found in the culture expressing LytPR (Fig. 1, lane 4) than in the uninduced culture. These differences in the lysis behaviors of LL108(pNS3PR) and LL108(pNS3AL) are probably due to the rapid loss of viability of the former upon induction of lytP, as LytP is proposed to introduce holes in the cell membrane without affecting the cell wall (22, 35). On the contrary, LL108(pNS3AL) can continue to grow and produce AcmA upon the addition of NaCl. The lacZ-expressing strain continued to grow after NaCl addition, albeit at an approximately threefold-reduced rate (Fig. 2) and to a 1.4-fold-reduced maximum OD_{600}. Similar reductions in growth rate and maximum OD_{600} were observed for plasmid-free wild-type cells and for LL108 carrying the cloning vector pORI13 (data not shown), indicating that NaCl itself also affected the growth rates of the strains independently of lysin expression. Limited regrowth of LL108(pNS3PR) cultures was observed 2 days after induction (data not shown), as was also observed by Shearman et al. (30).

**Intracellular proteins are released upon induction of lysin genes.** The effect of overexpression of cell wall-degrading activities on cell integrity was determined. Cytoplasmic proteins could be detected in the supernatants of both the lytPR- and the acmA-expressing strains 6 h after NaCl addition (Fig. 3, lanes 4 and 6), whereas in the supernatants of the uninduced strains only the secreted protein Usp45 (31) was visible (Fig. 3, lanes 3 and 5). Addition of NaCl to the control strain carrying the lacZ fusion did not result in the release of cytoplasmic proteins (Fig. 3; compare lanes 1 and 2).

**Moderate induction of cell wall-lytic activity results in optimal cell lysis.** PepX was chosen as an intracellular marker enzyme to quantify release of intracellular proteins into the supernatant. PepX activity is stable for at least 40 h in a cell extract in 1/2M17, either with or without NaCl (data not shown). Experiments were carried out with the AcmA-negative mutant MG1363acmA1 (2) in order to exclusively study the effect of the induced cell wall hydrolases. Plasmids pNS3AL and pNS3Z are ORI vectors and are able to replicate only when the plasmid replication protein RepA is provided in trans (16). Therefore, all constructs were introduced in MG1363acmA1(pVE6007), in which pVE6007 sup-

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**FIG. 1.** Zymographic analysis of cell wall hydrolase activity in cell extracts prepared from samples taken 6 h after addition of 0.5 M NaCl to cultures at an OD_{600} of 0.5 (see arrow in Fig. 2). Lanes: 1 and 2, LL108(pNS3Z); 3 and 4, LL108(pNS3PR); 5 and 6, LL108(pNS3AL); 1, 3, and 5, no NaCl; 2, 4, and 6, plus NaCl. Molecular masses (in kilodaltons) of marker proteins are indicated at the left.

**FIG. 2.** Growth of LL108(pNS3PR) (squares), LL108(pNS3AL) (triangles), and LL108(pNS3Z) (circles) in 1/2M17. At an OD_{600} of 0.5, the cultures were split. To one half of each culture, NaCl (end concentration, 0.5 M) was added (solid symbols), after which all cultures were further incubated. The arrow indicates the time point at which samples were taken from the culture for analysis of cell wall-degrading activity and protein release (see Fig. 1 and 3).
of the strain carrying the slower, and the highest levels were reached 30 h after induction (NaCl). This may be explained by the fact that a fraction of the

drolase expression at a moderate level (induction with 0.1 M concentration. Optimal cell lysis was obtained with cell wall hy-

lysis can be modulated by manipulation of the chloride con-

centration. Molecular masses (in kilodaltons) of marker proteins are shown on the left. Equal volumes of supernatant were applied to all lanes.

plies RepA. PepX activity in culture supernatants was moni-
tored after induction of the culture with 0.1 or 0.5 M NaCl (in cheese, a natural environment for L. lactis, the NaCl concentration is approximately 0.5 M) at an OD_{600} of 0.5. During the first few hours after induction, little PepX activity was released into the culture supernatants (Fig. 4). The highest PepX levels at 0.5 M NaCl were obtained 8 h after induction of acmA and 30 h after induction of lytPR. At 0.1 M NaCl, PepX release is slower, and the highest levels were reached 30 h after induction of the strain carrying the gadC::lytPR fusion and 70 h after induction of the gadC::acmA strain (Fig. 4). The ultimate levels of liberated PepX are higher with 0.1 M NaCl than with 0.5 M NaCl. These data show that the extent and the progression of lysis can be modulated by manipulation of the chloride concentration. Optimal cell lysis was obtained with cell wall hydrolase expression at a moderate level (induction with 0.1 M NaCl). This may be explained by the fact that a fraction of the

cells can continue to divide upon lysis expression at a moderate level, whereas cells producing a high level of lysis are rapidly killed. Alternatively, the released proteins (including the lysins and peptidases) may be inactivated upon exposure to the supernatant environment. Degradation of PepX has been reported to occur in cheese after an initial increase in the free PepX level during the first week of ripening (4).

Induction of cell lysis by addition of NaCl to cultures in the stationary phase resulted in limited PepX release (up to 17 arbitrary units [A.U.]) (data not shown). Apparently, the exponential-growth phase is optimal for inducing gene expres-
sion, or cells are most susceptible for lysis when actively divid-
ing, as was also observed for phage-induced lysis (20).

Low levels of PepX activity were observed after prolonged incubation of both uninduced strains (Fig. 4). The control strain MG1363::acmAΔ1(pVE6007, pNS3Z) did not release detectable levels of PepX (data not shown). As in all three strains the chromosomal copy of acmA had been inactivated, these results are indicative of a very low basal level of expression of both cell wall-lytic activities from P_{gad}. This basal activity of P_{gad} may be due to the amount of chloride already present in 1/2M17 (4 mM) (27a). It may also be related to the presence in the medium of glutamate or to the pH reached by the cultures under the conditions used, as chloride-dependent P_{gad} activity was recently found to be enhanced by glutamate and at low pH (29). Low-pH conditions were not included in this study, as autolytic activity is almost completely lost below pH 5 (1a, 23).

Cell wall hydrolase activity causes the formation of osmotically fragile cells. The addition of NaCl to a culture not only induces the activity of P_{gad} but also increases the osmolarity of the medium. This could result in stabilization of protoplasts or of osmotically fragile cells possibly formed by cell wall-degrad-
ing activity in the cultures and, thus, in a limited release of the cytoplasmic content. Induction of cell wall-degrading activity in 1/2M17 containing 0.5 M sucrose led to the release of only a small but detectable amount of PepX (3 A.U. for gadC::lytPR (data not shown). When gadC::lytPR cells grown in 1/2M17 were induced with NaCl, extra PepX activity could indeed be released by incubation in a hypoosmotic medium (Fig. 5). Induction of acmA expression with NaCl resulted in much lower levels of PepX in the culture medium than did induction of lytPR (see above). However, much more PepX was extractable with hypoosmotic medium from induced gadC::acmA cells (Fig. 5). The sum of PepX in the supernatant and PepX extrac-
table from osmotically fragile cells is comparable for T.gadC::lytPR and gadC::acmA cells induced with 0.25 M NaCl and is about 95% of the amount of PepX that could be obtained from gadC::lytPR cells by mechanical disruption (data not shown). The amount of PepX extractable from induced gadC::acmA cells increased with the amount of NaCl used for induction. After induction with 0.5 M NaCl, the amount of extractable PepX was reduced. Apparently, AcmA activity results in weakening of the cell wall but not in lysis of cells, due to the inducing concentration of NaCl, which concurrently osmostabilizes the cells. Previously, prevention of cell lysis was observed with a phage lysis-expressing lactococcus strain in a sucrose-buffered medium (30). In the natural (cheese) envi-

riment, the activity of, presumably, AcmA also results in spheroplast formation, as evidenced by the fact that a small fraction of semi-intact nonviable osmostabilized cells exists in Saint Paulin-type cheeses (4).

The differences in cell lysis between the gadC::lytPR and gadC::acmA strains (the former releases more PepX whereas the latter produces more osmotically fragile cells) may also be due to the activity of LytP. LytP destabilizes the cytoplasmic membrane and could thus promote cell disruption before the
cell wall is degraded to a level that would normally cause cell lysis. Alternatively, the differences in the autolytic behaviors of these two strains may be attributed to slight differences in the expression levels of the two constructs, or to differences in the specificity and/or activity of the enzymes.

\( P_{\text{gad}} \) fulfills the essential requirements of a food-grade inducible gene expression system and is suitable for the induced expression of industrially important proteins, since it showed a 1,000-fold by NaCl, an agent normally present in, for instance, cheese. The expression cassette used here allows precise positioning of the \( \text{gadC} \) start codon to make either translational fusions to, or translational couplings with, any gene of interest. \( P_{\text{gad}} \) activity depends on the NaCl concentration, allowing precise adjustment of the desired expression level of a protein to be produced. These data show that balanced cell lysis of \( L.\ lactis \) can, in principle, be obtained with a system that is entirely based on lactococcal DNA and should thus be labeled food grade.

**ACKNOWLEDGMENTS**

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