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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_{gad}, 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_{gad} 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 lysin 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 *scrAB* (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 ϕ31 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

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TABLE 1. Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
L. lactis		
MG1363	Plasmid-free derivative of NCDO712	9
MG1363*acmA*Δ1	AcmA$^-$ derivative of MG1363	2
LL108	Cm^r *repA*$^+$ derivative of MG1363	15
LL302	*repA*$^+$ derivative of MG1363	15
NS3	Em^r *gadC*::*lacZ* derivative of MG1363	28
E. coli		
EC1000	Km^r *repA*$^+$ derivative of MC1000, carrying a single copy of the pWV01 *repA* gene in *glgB*	17
EC101	Km^r *repA*$^+$ derivative of JM101, carrying a single copy of the pWV01 *repA* gene in *glgB*	14
Plasmids		
pORI28	Em^r; Ori$^+$ of pWV01; Rep$^-$	17
pORI13	Em^r; promoterless *lacZ*; Ori$^+$ of pWV01; Rep$^-$	28
pVE6007	Cm^r pWV01 derivative encoding a temperature-sensitive RepA protein	19
pNS3	Em^r; *gadC*::*lacZ* pORI13 derivative with a 10-kb *Sau*3A chromosomal DNA fragment	28
pNS3d	Em^r; *gadC*::*lacZ* pORI13 derivative with a 2.5-kb *Pst*I-*Sau*3A chromosomal DNA fragment	28
pNS3Z	Em^r; *gadC*::*lacZ* pORI13 derivative with a 1,280-bp PCR fragment amplified with NS3-7 and NS3-8	This study
pIR1PR	Em^r; phage r1t *lytPR* fused to r1t regulatory region; derivative of pMG36e	22
pNS3PR	Em^r; *gadC*::*lytPR* pIR1PR derivative with a 1,280-bp PCR fragment amplified with NS3-7 and NS3-8	This study
pAL10	Ap^r; *acmA* interrupted by a *Sac*I fragment	3
pAL101	Ap^r; derivative of pAL10; smaller *Sac*I fragment; free of *Bgl*II sites	This study
pAL102	Ap^r Em^r Ori$^+$; derivative of pAL101	This study
pNS3AL3S	Em^r Ori$^+$ *gadC*::*acmA*; *acmA* interrupted by a *Sac*I fragment; derivative of pAL102 with a 1,280-bp PCR fragment amplified with NS3-7 and NS3-8	This study
pNS3AL	Em^r Ori$^+$ *gadC*::*acmA*	This study

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. Erythromycin 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 electrotransformation in *E. coli* (36) and *L. lactis* (10). DNA sequencing was carried out on double-stranded plasmid DNA by the dideoxy chain termination method 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 cassette encoding *gadR* and the P_{gad} promoter was amplified as a 1,251-bp DNA fragment (bp 821 to 2071 of the published sequence; GenBank accession no. AF005098) by PCR with primers NS3-7 (5′-GCGATATCCAGTACTTCATCATACCTCCTTATATTTATGATTG) and NS3-8 (5′-GCGAGCTCAGATCTGAGCGTTGTATAAGCTTTTATGTCTTTC) and with pNS3 as the template. Four restriction enzyme sites (*Eco*RV and *Sca*I in NS3-7 and *Sac*I and *Bgl*II in NS3-8, all underlined) were introduced to aid in cloning. This PCR fragment was digested with *Sac*I and *Eco*RV and was then ligated to pIR1PR linearized with *Sac*I and *Sca*I. The ligation mixture was used to transform *L. lactis* LL108, and the resulting plasmid was called pNS3PR. The same PCR fragment was cut with *Bgl*II and *Eco*RV and was cloned upstream of *lacZ* in pORI13 by ligation to *Bgl*II- and *Sma*I-digested vector DNA. The resulting plasmid, pNS3Z, was recovered from strain LL108. To construct a transcriptional fusion of P_{gad} with *acmA*, pAL10 was used. The *Bgl*II sites in pAL10 were deleted by cutting with *Bgl*II, flushing the ends with Klenow enzyme, and self-ligation. The resulting plasmid, pAL101, was isolated from *E. coli* EC1000 cut with *Bam*HI and *Xba*I and was ligated to *Bam*HI- and *Xba*I-digested pORI28. The proper construct, pAL102, was obtained in *E. coli* EC1000. The PCR-amplified expression cassette was cut with *Bgl*II and *Sca*I and then ligated to pAL102 linearized with the same restriction enzymes. The ligation mixture was used to transform *L. lactis* LL302, and pNS3AL3S was recovered. This plasmid was digested with *Sac*I to remove the *Sac*I insert in *acmA*. After self-ligation the mixture was used to transform *L. lactis* LL302. The resulting construct, carrying an intact copy of *acmA* fused to P_{gad}, was designated pNS3AL.

β-Galactosidase assay. Cell extracts were prepared by vigorous shaking of cells in the presence of glass beads (32). β-Galactosidase activity was determined as described by Miller (21). Protein concentrations were determined by the method of Bradford (1) with bovine serum albumin as the standard.

Detection of cell wall-lytic activity and quantification of cell lysis. Sodium dodecyl sulfate–(12.5%) polyacrylamide (SDS-PAA) gel electrophoresis was carried out as described previously (13). Gels were stained with Coomassie brilliant blue. For detection of cell wall-lytic activity, 0.2% autoclaved, lyophilized *Micrococcus lysodeikticus* cells were included in the gel, which was further processed as described by Buist et al. (2). Cell lysis was quantified by measuring release of the X-prolyl dipeptidyl aminopeptidase (PepX) from cells lacking the chromosomal copy of the autolysin gene *acmA*. PepX activity in a standard volume of supernatant, diluted twofold in 50 mM HEPES (pH 7.0), was determined by monitoring hydrolysis of the synthetic substrate Ala-Pro-*p*-nitroanilid (BACHEM Feinchemicalien AG, Bubendorf, Switzerland) at 405 nm for 20 min at 37°C in a 96-well microtiter plate with a Thermomax microplate reader (Molecular Devices Co., Menlo Park, Calif.). PepX activities are rates of substrate hydrolysis expressed in arbitrary units. PepX activity was not affected by the NaCl concentrations used. The presence of osmotically fragile cells in induced cultures was tested by washing the cells in 1 ml of culture with fresh medium containing NaCl at a concentration identical to that in the original culture. Subsequently, half of the cells were resuspended in 0.5 ml of (hypoosmotic) 1/2M17, and the other half were resuspended in 0.5 ml of 1/2M17 containing the original NaCl concentration. Samples were incubated at 37°C for 30 min, and PepX activity in the supernatants was measured in order to determine PepX activity in the hypoosmotic extractable cell fraction.

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

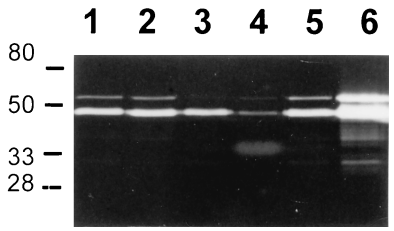

FIG. 1. Zymographic analysis of cell wall hydrolase activity in cell extracts prepared from samples taken 6 h after addition of 0.5 M NaCl to cultures at an OD_{600} of 0.5 (see arrow in Fig. 2). Lanes: 1 and 2, LL108(pNS3Z); 3 and 4, LL108(pNS3PR); 5 and 6, LL108(pNS3AL); 1, 3, and 5, no NaCl; 2, 4,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

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 at 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 30 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(pNS3PR) 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

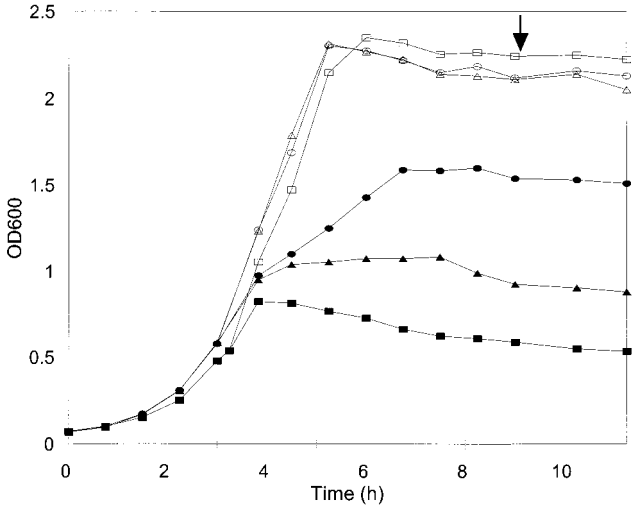

FIG. 2. Growth of LL108(pNS3PR) (squares), LL108(pNS3AL) (triangles), and LL108(pNS3Z) (circles) in 1/2M17. At an OD_{600} of 0.5, the cultures were split. To one half of each culture, NaCl (end concentration, 0.5 M) was added (solid symbols), after which all cultures were further incubated. The arrow 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).

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 a 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 MG1363*acmA*Δ1 (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 MG1363*acmA*Δ1(pVE6007), in which pVE6007 sup-

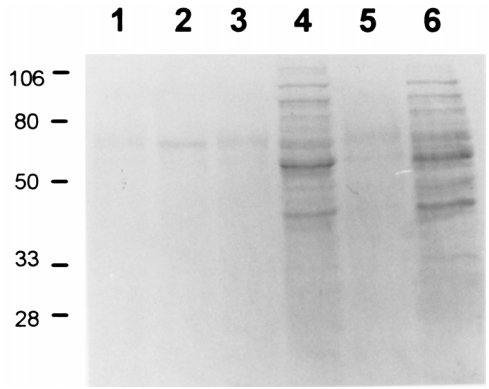

FIG. 3. SDS-PAA gel, stained with Coomassie brilliant blue, of supernatant samples of *L. lactis* cultures taken 6 h after NaCl induction (see arrow in Fig. 2). Lanes: 1 and 2, LL108(pNS3Z); 3 and 4, LL108(pNS3PR); 5 and 6, LL108(pNS3AL); 1, 3, and 5, no NaCl added; 2, 4, and 6, plus 0.5 M NaCl. Molecular 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 monitored 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

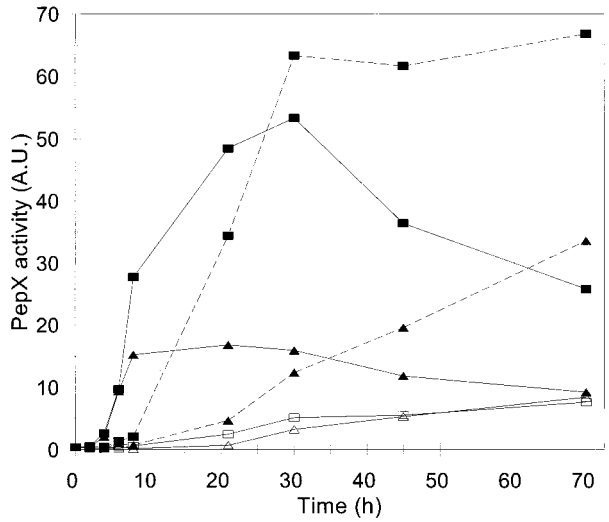

FIG. 4. PepX activity (in arbitrary units) in supernatants of cultures of MG1363*acmA*Δ1(pVE6007, pNS3PR) (squares) and MG1363*acmA*Δ1(pVE6007, pNS3AL) (triangles). At 0 h (at an OD_{600} of the cultures of 0.5), NaCl was added (solid symbols) to a final concentration of 0.5 M (solid lines) or 0.1 M (broken lines). Open symbols, cultures to which no NaCl was added.

cells can continue to divide upon lysin expression at a moderate level, whereas cells producing a high level of lysin 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 expression, or cells are most susceptible for lysis when actively dividing, 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*acmA*Δ1(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-degrading 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 extractable from osmotically fragile cells is comparable for *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 lysin-expressing lactococcus strain in a sucrose-buffered medium (30). In the natural (cheese) environment, 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

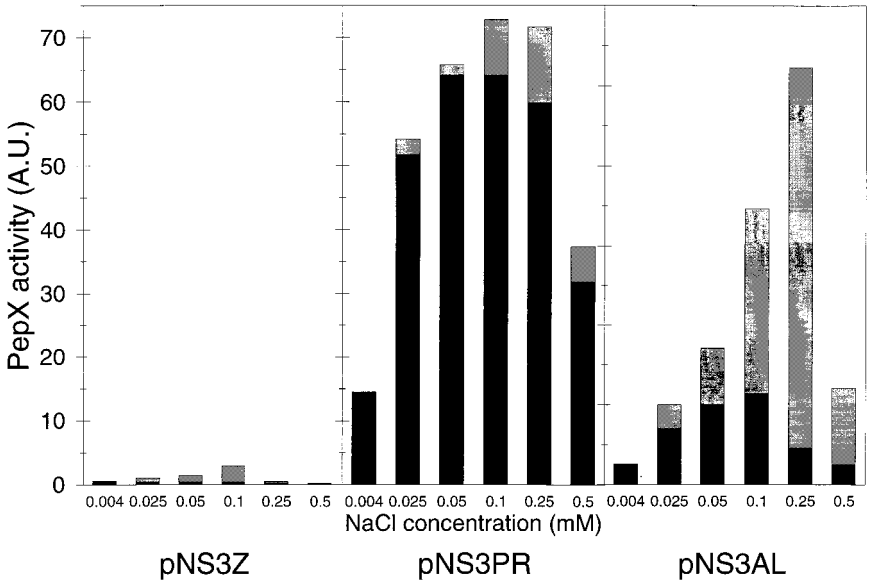

FIG. 5. PepX activities in supernatant fractions (solid bars) and in hypoosmotic wash fractions (shaded bars) of cultures of MG1363*acmA*Δ1(pVE6007) carrying pNS3Z, pNS3PR, or pNS3AL. Exponentially growing cells in 1/2M17 were induced with the indicated amounts of NaCl at a culture OD_{600} of 0.5. As a negative control, 1/2M17, which contains 0.004 M NaCl, was used. PepX levels were determined 2 days after induction and are expressed in arbitrary units.

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_{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 very low basal level of activity and could be induced more than 1,000-fold by NaCl, an agent normally present in, for instance, cheese. The expression cassette used here allows precise positioning of the *gadC* start codon to make either translational fusions to, or translational couplings with, any gene of interest. P_{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.

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