Stress Response in *Lactococcus lactis*: Cloning, Expression Analysis, and Mutation of the Lactococcal Superoxide Dismutase Gene

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In an analysis of the stress response of *Lactococcus lactis*, three proteins that were induced under low pH culture conditions were detected. One of these was identified as the lactococcal superoxide dismutase (SodA) by N-terminal amino acid sequence analysis. The gene encoding this protein, designated *sodA*, was cloned by the complementation of a *sodA* *sodB* *Escherichia coli* strain. The deduced amino acid sequence of *L. lactis* SodA showed the highest degree of similarity to the manganese-containing Sod (MnSod) of *Bacillus stearothermophilus*. A promoter upstream of the *sodA* gene was identified by primer extension analysis, and an inverted repeat surrounding the −35 hexanucleotide of this promoter is possibly involved in the regulation of the expression of *sodA*. The expression of *sodA* was analyzed by transcriptional fusions with a promoterless *lacZ* gene. The induction of β-galactosidase activity occurred in aerated cultures. Deletion experiments revealed that a DNA fragment of more than 130 bp surrounding the promoter was needed for the induction of *lacZ* expression by aeration. The growth rate of an insertion mutant of *sodA* did not differ from that of the wild type in standing cultures but was decreased in aerated cultures.

Lactic acid bacteria (LAB) are widely used in fermented food production. Normal growth of LAB does not require strictly anaerobic environments. A number of stirring steps in the various production processes in which LAB are involved provide ample contact with oxygen, without obvious deleterious effects to these organisms. A number of LAB can use molecular oxygen or hydrogen peroxide to regenerate NAD⁺, by the action of NADH oxidase and NADH peroxidase (14). With the stepwise reduction of O₂ to H₂O the toxic intermediates O₂⁻ and H₂O₂ are generated. Hydrogen peroxide was found to inhibit growth of lactococci (1), and exposure of lactococci to a sublethal dose of H₂O₂ induced an oxidative stress response (14), characterized by an increased survival after exposure to a lethal level of H₂O₂ compared with cells that were not pretreated.

Most LAB can deal with oxygen radicals by either a superoxide dismutase (Sod) or a high internal Mn²⁺ concentration (2). Sod dismutates oxygen radicals by catalyzing the reaction 2 O₂⁻ + 2 H⁺ → H₂O₂ + O₂ (20).

Sods are found in a wide variety of prokaryotic and eukaryotic organisms, and in a number of instances the genes encoding these enzymes have been cloned and characterized (5, 9, 12, 33). In prokaryotes three types of Sods can be distinguished depending on the metal cofactor contained in these enzymes (Cu-Zn, Fe, or Mn) (36, 40). A single organism can have two sod genes; the corresponding enzymes differ in their metal cofactor and in their expression pattern in response to oxygen.

The regulation of Sod expression in *Escherichia coli* has been studied extensively (13, 18, 25). Inactivation of sod genes can be growth inhibiting (in *E. coli* [10]) or even lethal (in *Legionella pneumophila* [40]).

All streptococci tested (including *Lactococcus lactis* subsp. *lactis*, formerly *Streptococcus lactis*) appear to carry a manganese-containing Sod (MnSod) (52). The MnSod of *L. lactis* is active under anaerobic conditions. Higher enzyme activity has been observed with increasing O₂ concentrations in the medium (24, 43). Although most organisms use catalase for the breakdown of H₂O₂, streptococci lack this activity; instead, they have NADH-peroxidase activity to decompose this compound (1).

One of the typical properties of LAB is their ability to produce large amounts of lactic acid, thereby causing a rapid acidification of their environment. Several microorganisms are known to adapt to medium with a low pH (19, 35). In the present paper we studied the effect of a pH downshift on the expression of proteins in *L. lactis*. An acid-induced protein was isolated, and by N-terminal amino acid sequence analysis as well as by activity assays, this protein appeared to be a Sod. The lactococcal sodA gene was cloned and sequenced, and its expression as well as the growth characteristics of a sodA mutant were studied.

**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 M17 medium, with 0.5% glucose; solidified M17 medium contained 1.5% agar. Erythromycin (Em) and chloramphenicol (Cm) were used at final concentrations of 5 μg/ml. MRS broth (13) containing 0.5% glucose with or without 2% β-glycerophosphate was used for acid-induction experiments. *E. coli* was grown in TY broth (39) at 37°C with vigorous agitation or on TY medium supplemented with 1.5% agar. Ampicillin (Ap) and paraquat were used at 100 μg/ml and 10 μg/ml, respectively.

**Preparation of cell extracts, PAGE, and Sod activity detection.** Cultures of *L. lactis* were harvested by centrifugation, and the pellets were resuspended in 10 mM Tris-1 mM EDTA (pH 7.4) and disrupted according to the method of Van de Gucht et al. (49). The cell extract was used for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to...
the protocol of Laemmli (27). Nondenaturing PAGE was carried out similarly, except that SDS and mercaptoethanol were omitted. Prior to loading, samples were incubated at 37°C for 15 min. Polyacrylamide (PA) gels were stained with Coomassie brilliant blue for total protein detection. Sod activity in nondenaturing gels was determined according to the protocol of Beauchamp and Fridovich (4).

N-terminal amino acid analysis. Cells of acid-stressed cells were separated on SDS–16% PA-piperazine diacrylamide gels (3). Proteins were transferred to polyvinylidene difluoride membrane (Millipore Corporation, Bedford, Mass.) which was subsequently stained with Coomassie brilliant blue, according to protocols of Eurosequence b.v. (Groningen, The Netherlands). After destaining with 50% methanol, the desired protein band was cut from the gel and subjected to Edman degradation with an Applied Biosystems 477A sequencer (Applied Biosystems, Inc., Foster City, Calif.). Phenylthiohydantoin amino acids were identified with an on-line high-pressure liquid chromatograph (model 120A; Applied Biosystems, Inc.).

Molecular cloning techniques. Molecular cloning techniques were performed essentially as described by Sambrook et al. (41). DNA was introduced in E. coli by electroporation (51). A genome bank of L. lactis in pUC19 (8) was used to clone the sod gene. DNA sequencing was done on double-stranded plasmid DNA by the dideoxy chain-termination method (42) and the T7 sequencing kit (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) according to the manufacturer’s instructions. Oligonucleotides were synthesized with an Applied Biosystems 381A DNA synthesizer.

DNA sequences were analyzed with the PC/Genesequence analysis program (IntelliGenetics Inc., Geneva, Switzerland). Protein homology searches in the PIR bank (release 40) were carried out with the FASTA program (38). Protein sequence alignments were carried out with the PALIGN program of PC/Gen by using the structure genetic matrix or with the Clustal program, both with standard settings.

Primer extension analysis. RNA was isolated from an exponentially growing culture of L. lactis culture at an optical density of 600 nm of 0.5 as previously described (48). A synthetic oligonucleotide complementary to the mRNA from positions 90 to 127 (corresponding to coordinates 565 to 601 in Fig. 3) was used for primer extension. Ten picomoles of primer was annealed to 10 μg of RNA, and then cDNA synthesis was performed as previously described (41). The product was analyzed on a sequencing gel next to a sequencing reaction with the same primer, providing a size marker.

β-Galactosidase assays. Cell extracts were prepared from exponentially growing cultures, either in completely filled standing bottles or by shaking in 100-ml bottles with 15 ml of medium. β-Galactosidase activity was determined essentially as described by Miller (32). Protein concentrations of the cell extracts were determined by the method of Bradford (6) with bovine serum albumin as a standard.

Construction of a Sod” mutant. An internal NdeI-EcoRI fragment of the sodA gene was cloned in the integration insertion vector pORI19, using a rep” E. coli helper strain, EC101 (EC101 [28]). This plasmid, pSOD7, was used to disrupt the sodA gene in L. lactis MG1363, as described before (22). The proper chromosomal location of the integrated plasmid was confirmed by Southern hybridization (not shown), and the strain was named MSOD7.

Nucleotide sequence accession number. The sequence presented in Fig. 3 has been assigned the GenBank nucleotide sequence accession number U17388.

RESULTS

A 24-kDa acid-induced protein of L. lactis is a Sod. L. lactis MG1363 was subjected to acid stress by growth in MRS medium with or without 2% β-glycerophosphate as the buffering agent. After overnight incubation the medium had acquired a pH of 6.2 in the buffered and a pH of 4.8 in the unbuffered medium. Cell extracts were analyzed by SDS-PAGE (Fig. 1A). At least three proteins with estimated molecular sizes of 72, 64, and 24 kDa were expressed at a higher level in a medium with a low pH, although the effect, probably due to slight variations in the growth conditions, was not invariably observed. Proteins of 72 and 64 kDa were also expressed at a higher level in exponentially growing cells subjected to a heat shock of 42°C for 1 h (Fig. 1). Therefore, and on the basis of their sizes (16, 26), we speculate that these proteins represent the lactococcal DnaK and GroEL proteins, respectively. The 24-kDa protein was partially purified by SDS-PAGE, and the N-terminal amino acid sequence was determined to be (Thr or Ala)-Phe-Thr-Leu-Pro-Glu-Leu-Pro-Tyr-Ala-Pro-Asn-Ala-Leu-Glu-

FIG. 1. (A) SDS–12.5% PAGE analysis of cell extracts of acid-stressed L. lactis cells. Lanes: 1, cells grown overnight in buffered MRS; 2, cells grown overnight in unbuffered MRS; 3, cells taken from exponential growth phase and incubated 1 h at 42°C. (B) Nondenaturing (12.5%) PA gel assayed for Sod activity. Culture conditions for lanes 1 and 2 were the same as those for lanes 1 and 2 in panel A. The arrows indicate three acid-induced proteins. The pH values of the culture media after growth and the growth temperatures are indicated below the lanes. Molecular masses (in kilodaltons) are indicated at the left. Equal amounts of protein (30 μg) were applied per lane.

TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. lactis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1363</td>
<td>Plasmid-free derivative of NCDO712</td>
<td>21</td>
</tr>
<tr>
<td>LL108</td>
<td>repA⁺ derivative of MG1363, Km’</td>
<td>28a</td>
</tr>
<tr>
<td>MGSO4D2</td>
<td>sodA::lacZ Em’</td>
<td>This study</td>
</tr>
<tr>
<td>MGSO4D7</td>
<td>Sod⁺ derivative of MG1363, Em’</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
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<tr>
<td>NM522</td>
<td>supE Δ(lac-proAB) Δ(lsdS) (r’ m’-r’)</td>
<td>23</td>
</tr>
<tr>
<td>OX326A</td>
<td>ΔsodA sodB</td>
<td>45</td>
</tr>
<tr>
<td>ECI101</td>
<td>repA⁺ derivative of MJ101, Km’</td>
<td>28</td>
</tr>
<tr>
<td>ECI1000</td>
<td>repA⁺ derivative of MCI01, Km’</td>
<td>27a</td>
</tr>
<tr>
<td>Other strains</td>
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<td></td>
</tr>
<tr>
<td>EC101</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC19</td>
<td>lacZ‘ Ap’</td>
<td>50</td>
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<td>pORI13</td>
<td>Promoterless lacZ, Em’, Ori’ of pWV01, Rep’</td>
<td>41a</td>
</tr>
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<td>pORI19</td>
<td>lacZ Em’, Ori’ of pWV01, Rep’</td>
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<tr>
<td>pVE6007</td>
<td>pWV01 derivative encoding a temperature-sensitive Rep protein, Km’</td>
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<td>pSOD1</td>
<td>Ap’, pUC19 with 7.6-kb Sau3A fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pSOD2</td>
<td>Ap’, pUC19 with 0.8-kb + 0.9-kb PvuII fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pSOD3</td>
<td>sodA::lacZ Em’</td>
<td>This study</td>
</tr>
<tr>
<td>pSOD4</td>
<td>sodA::lacZ Em’</td>
<td>This study</td>
</tr>
<tr>
<td>pSOD5</td>
<td>sodA::lacZ Em’</td>
<td>This study</td>
</tr>
<tr>
<td>pSOD6</td>
<td>sodA::lacZ Em’</td>
<td>This study</td>
</tr>
<tr>
<td>pSOD7</td>
<td>Internal NdeI-EcoRI fragment of sodA in pORI19</td>
<td>This study</td>
</tr>
</tbody>
</table>

Downloaded from pnas.org at university library on March 26, 2007
the sodA and sodB genes were deleted (OX326A). This strain lacks all Sod activity and is sensitive to 10 μg of paraquat per ml, in contrast to E. coli strains carrying one or both sod genes (45).

A genome bank of L. lactis MG1363 in pUC19 was used to transform E. coli OX326A, and transformants were selected for resistance to ampicillin and paraquat. Eight paraquat-resistant colonies were assayed for Sod activity on a nondenaturing PA gel. Six of these showed a band of Sod activity with a mobility similar to that of L. lactis Sod. The mobility pattern of Sod activity of one of these clones is shown in Fig. 2, lane 4. The Sod activity was obviously derived from lactococcal DNA, as it was clearly different from the two E. coli Sod activities (Fig. 2, lane 2) which are lacking in the cloning host (Fig. 2, lane 3).

Plasmid DNA of the Sod-expressing clones was isolated. Restriction enzyme analysis of three clones showed that all contained a 7.6-kb DNA fragment (data not shown). A partial PvuII digest of one of these clones (pSOD1) was ligated into the dephosphorylated SmaI site of pUC19. The ligation mixture was used to transform OX326A to paraquat resistance. Plasmid DNA of paraquat-resistant transformants was isolated. The smallest plasmid expressing active Sod, pSOD2, consisted of two adjacent PvuII fragments of 0.8 and 0.9 kb. The 0.8-kb fragment hybridized with a degenerate oligonucleotide probe designed from the N-terminal amino acid sequence of the isolated protein (data not shown).

Part of the nucleotide sequence of both strands of the insert in pSOD2 is presented in Fig. 3. The cloned fragment contained an open reading frame (ORF) of 618 bp that could encode a protein of 206 amino acids with a predicted molecular weight of 23,254 and a calculated pI of 4.8. The translation product of the first 18 codons of this ORF (except for Met) was identical to the N terminus of the 24-kDa acid-induced protein, indicating that the L. lactis sod gene had indeed been cloned. Accordingly, the ORF was designated sodA. The gene is preceded by a putative ribosome binding site with complementarity to the 3′ end of the ribosomal 16S rRNA of L. lactis (11) with a ΔG° of −14.4 kcal/mol (−60.2 kJ/mol) (47). Upstream of the ribosome binding site a promoter-like structure was present, consisting of the −35 hexanucleotide TTGACA, a spacing of 17 bp and the −10 hexanucleotide TATAAT. The −35 hexanucleotide is surrounded by an 8-bp inverted repeat (ΔG[25°C] = −4.6 kcal/mol [−19.2 kJ/mol]). To examine whether the putative promoter was active in vivo, primer extension was carried out. The results are presented in Fig. 4 and show that transcription started at an adenine 7 bp downstream of the −10 hexanucleotide. Downstream of sodA, a 13-bp inverted repeat (ΔG[25°C] = −14.4 kcal/mol [−60.2 kJ/mol]) followed by a stretch of T's could function as a transcription terminator.

FIG. 2. Nondenaturing (12.5%) PAGE of cell extracts; the gel was stained for Sod activity. Lanes: 1, L. lactis MG1363; 2, E. coli NM522; 3, E. coli OX326A; 4, E. coli OX326A(pSOD2). Thirty micrograms of protein was applied per lane.

One hundred fifty-three base pairs upstream of the sodA gene the stop codon of a second ORF is present. This ORF starts beyond the 5′ end of the cloned fragment in pSOD2. The inverted repeat surrounding the −35 sequence of the sodA promoter may function as a terminator for this second ORF, although its level of free energy is low. The PIR protein database did not contain proteins homologous to the amino acid sequence deduced from the incomplete ORF.

L. lactis SodA is a MnSod. A homology comparison with other Sod proteins revealed that lactococcal SodA is similar to Sods of various organisms of both prokaryotic and eukaryotic origin (Fig. 5). The size of 206 amino acid residues of the lactococcal SodA is in agreement with the sizes of other Sods.

FIG. 3. Nucleotide sequence and deduced amino acid sequence of the sodA gene of L. lactis MG1363 and its surrounding regions. Facing arrows, inverted repeats; rbs, ribosome binding site (with the bases in lowercase and boldface type); strategic sites, possible transcription start site. Stop codons are given in boldface type and indicated with asterisks. A number of relevant restriction enzyme sites are indicated.

FIG. 4. Primer extension analysis of the transcription start site of the sodA gene of L. lactis MG1363. The primer used was synthesized from the nucleotide sequence presented in Fig. 3.
which range from 191 to 240 residues. The amino acids involved in the binding of metal ligands are present in the lactococcal SodA (His-27, His-82, Asp-168, and His-172 [Fig. 5]), as are the postulated active-site residues (36, 37). Since the lactococcal SodA contains the residues typical of MnSods and none of the residues specific for iron-containing Sods (FeSods) (36) (Fig. 4), we conclude that the \textit{L. lactis} SodA belongs to the class of MnSods.

**Analysis of sodA::\textit{lacZ} fusions.** Since in \textit{L. lactis} higher levels of Sod activities have been reported at the higher \textit{O$_2$} concentrations (24), we examined the expression of sodA under aerobic conditions. For this purpose transcriptional fusions of sodA to the \textit{E. coli lacZ} gene were constructed. A 0.8-kb \textit{PvuII} fragment carrying the 5' end of sodA, the promoter, and part of the upstream ORF was cloned in the multiple cloning site of the integration vector pORI13 (29). In \textit{L. lactis} cells carrying this construct, pSOD3, an unusually high level of \textit{\beta}-galactosidase activity was observed (Table 2). To examine whether this could be the result of translational coupling, the efficient translation of a gene depending on the translation of an immediately preceding or even overlapping gene (49), pSOD4 was constructed by filling in a unique \textit{BamHI} site (Fig. 6). In this way a frameshift was introduced in the truncated sodA, resulting in a stop in translation 21 bp upstream of \textit{lacZ}. As a consequence, the overlap by 1 bp of the sodA stop codon and the \textit{lacZ} start codon, as in pSOD3, was eliminated in pSOD4. Both plasmids were introduced in the \textit{Rep}1 lactococcal helper strain LL108, which provides the plasmid replication protein in trans (28a). The 100-fold-higher level of \textit{\beta}-galactosidase activity observed in strain LL108 (pSOD3) as compared with that of LL108 (pSOD4) (Table 2) strongly suggests that the high \textit{\beta}-galactosidase expression in the former is indeed caused by translational coupling.

To avoid the effect of the titration of possible regulatory proteins on the expression of sodA in multiple-copy situations, a single copy of pSOD4 was inserted in the chromosome of \textit{L. lactis} MG1363 by Campbell-type integration. The level of \textit{\beta}-galactosidase activity in aerated cells of the resulting strain, MGSOD4, was twofold higher than in cells grown as standing culture (Table 2). After 1 h of aeration no further increase of \textit{\beta}-galactosidase activity was measured (data not shown). An overall higher level of \textit{\beta}-galactosidase activity was observed when the fusion was located on the replicating form of pSOD4 in strain LL108, most probably as a result of a gene dose effect.

**FIG. 4.** Determination of the transcriptional start point of sodA. Lane P, primer extension product. The sequence ladder was obtained with the same primer on sodA template DNA. At the left is indicated the sequence of the both the transcribed strand and the sequenced strand. The -10 region of the promoter and the transcription start point are indicated in boldface type.

**FIG. 5.** Alignment of the deduced amino acid sequences of \textit{L. lactis} SodA with amino acid sequences of MnSods and the \textit{E. coli} FeSod. Asterisks, identical amino acids; periods, similar amino acids; ■, putative metal ligands; □, putative active site residues; ×, discriminating residues for metal cofactor (36). The Sods shown are MnSods from \textit{Bacillus stearothermophilus} (\textit{B. stearo}) (5), \textit{S. mutans} (33), and \textit{E. coli} (\textit{E. coli} Mn) (44); the FeSod of \textit{E. coli} (\textit{E. coli} Fe) (9); and human MnSod (Human) (12).

**TABLE 2.** \textit{\beta}-Galactosidase activity of sodA::\textit{lacZ} fusions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Standing culture</th>
<th>Shaken culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL108(pSOD3)</td>
<td>3,740 ± 120</td>
<td>7,310 ± 170</td>
</tr>
<tr>
<td>LL108(pSOD4)</td>
<td>29.0 ± 6.3</td>
<td>47.6 ± 3.0</td>
</tr>
<tr>
<td>MG50D4</td>
<td>16.8 ± 1.8</td>
<td>37.3 ± 2.5</td>
</tr>
<tr>
<td>LL108(pSOD5)</td>
<td>5.7 ± 1.3</td>
<td>11.9 ± 1.6</td>
</tr>
<tr>
<td>LL108(pSOD6)</td>
<td>16.6 ± 1.7</td>
<td>15.1 ± 3.2</td>
</tr>
</tbody>
</table>

* Values are means of at least three independent experiments.

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The 24-kDa acid-induced protein was identified as SodA on the basis of its N-terminal amino acid sequence identity with the deduced amino acid sequence of the cloned sodA gene. The deduced amino acid sequence of SodA is highly similar to those of Sods from evolutionarily closely and distantly related organisms. The observed homology of lactococcal SodA with the group of MnSods rather than with FeSods is in agreement with the previous observation that all streptococci, and \textit{L. lactis}, possess MnSods (52).

The role of SodA under acid conditions is not clear. The protein does not play an obvious role in the growth of \textit{L. lactis} in standing cultures, since growth of the SodA-negative mutant under these conditions was similar to that of the parent strain. In contrast, a negative effect of the sodA mutation on growth was observed in aerated cultures and on plates. Apparently, oxygen radicals inhibit growth under these conditions. Similar effects have been observed for Sod- derivatives of \textit{E. coli} (10) and \textit{Streptococcus mutans} (33). A Sod-free \textit{E. coli} strain grew slowly in rich medium and was unable to grow in minimal medium under aerobic conditions. An intracellular Sod appeared to be essential for \textit{P. pneumophila}, a bacterium that needs oxygen for growth (40). The inactivation of Sod of the obligate anaerobe \textit{Porphyromonas gingivalis} caused a rapid loss of viability upon exposure to oxygen (34). It was concluded that the latter organism lacks efficient alternative protection or repair systems to overcome oxidative damage, whereas \textit{E. coli} and \textit{S. mutans} do have such systems. The ability of SodA-deficient \textit{L. lactis} to grow under aerated conditions indicates that also in this organism alternative protection systems operate. A candidate protection mechanism is a high intracellular glutathione concentration, both in \textit{E. coli} and in \textit{L. lactis} (17), since glutathione can also detoxify free radicals (31). The properties of Sod-deficient strains described thus far suggest that Sod is essential for obligate aerobic or anaerobic bacteria to withstand oxygen, whereas it is not essential for facultative (an) aerobic species.

In two independent studies (24, 43), a twofold higher level of Sod activity was observed in \textit{L. lactis} upon aeration. We show here that under similar conditions sodA is induced twofold. Together with the results obtained with the sodA mutant, these results indicate that SodA is the only or, if not the only, the major Sod in \textit{L. lactis}. The results of the sodA::lacZ transcrip-

In the framework of a general interest in stress-induced protein expression in \textit{L. lactis} we have examined the induction of proteins synthesized at a low pH and observed in overnight cultures the induction of three proteins with molecular sizes of 72, 64, and 24 kDa. The induction pattern resembles that of \textit{Salmonella typhimurium} upon infection of macrophages, which have a low interior pH. In this case a 58-kDa protein was identified as GroEL, and a 68-kDa protein was identified as Dnak. In addition, an unknown 27-kDa protein was induced (7). The \textit{L. lactis} Dnak and GroEL proteins are 65 and 57 kDa, respectively (16, 26). The 72- and 64-kDa lactococcal proteins identified here may, therefore, represent the products of \textit{dnaK} and \textit{groEL} in \textit{L. lactis}.

\section*{DISCUSSION}

In the framework of a general interest in stress-induced protein expression in \textit{L. lactis} we have examined the induction of proteins synthesized at a low pH and observed in overnight cultures the induction of three proteins with molecular sizes of 72, 64, and 24 kDa. The induction pattern resembles that of \textit{Salmonella typhimurium} upon infection of macrophages, which have a low interior pH. In this case a 58-kDa protein was identified as GroEL, and a 68-kDa protein was identified as Dnak. In addition, an unknown 27-kDa protein was induced (7). The \textit{L. lactis} Dnak and GroEL proteins are 65 and 57 kDa, respectively (16, 26). The 72- and 64-kDa lactococcal proteins identified here may, therefore, represent the products of \textit{dnaK} and \textit{groEL} in \textit{L. lactis}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig7.png}
\caption{Non-denaturing PAGE of cell extracts of MG1363 (lane 1) and MG-SOD7 (lane 2). The gel was stained for Sod activity; 30 \(\mu\)g of protein was applied per lane.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig8.png}
\caption{Growth of \textit{L. lactis} MG1363 derivatives in liquid GM17 medium, either as aerated (dotted lines) or standing cultures (solid lines). Strains: filled box, MGSOD8 (Sod\textsuperscript{+} Em\textsuperscript{-}); filled triangle, MGSOD7 (Sod\textsuperscript{-} Em\textsuperscript{-}); A600, Absorbance at 600 nm.}
\end{figure}
tional fusions indicate that expression of sodA is, most likely, regulated at the transcriptional level. Primer extension revealed that the promoter upstream of sodA is active under standard growth conditions (standing culture). lacZ expression from pSOD5 indicates that the 500-bp DNA region upstream of sodA is required for inducibility under aerobic conditions. Since lacZ expression from pSOD6 was independent from aeriation, the inducibility of sodA expression is probably specified by the 370-bp PvuII-BglII fragment. The fact that lacZ expression from pSOD6 is similar to that from pSOD5 under aerated conditions suggests that sodA expression is repressed under nonaerated conditions by a mechanism acting on DNA sequences 70 to 440 bp upstream of the sodA promoter.

Another possible target site for regulation is the putative inverted repeat sequence that encompasses the 35 hexanucleotide of the sodA promoter. A stem-loop structure is also present upstream of E. coli sodA (46). Studies of the expression of E. coli sodA showed that it is regulated by six global regulators: ArcA, Fnr, Fur, SoxRS, CfxB, and IHF (13). These regulators all act on a DNA fragment of less than 120 bp, so the constitutive expression of sodB is regulated at the transcriptional level. Primer extension revealed that the constitutive expression of sodB depends on growth conditions and on the presence of O2, Fe, NO3-, or xenobiotics (18). Several differences in levels of expression exist between E. coli and L. lactis sodA. E. coli exhibits a basic level of Sod by the constitutive expression of sodB. Additional Sod is synthesized from sodA in response to environmental conditions. Under nonstress conditions the expression of sodA is repressed. In contrast, in L. lactis, sodA provides the cell with a basic level of SodA or with higher levels when required. It is conceivable that, as with E. coli, a number of regulatory systems are involved in the control of sodA expression, as Sod activity in L. lactis is not only oxygen pressure dependent, but also dependent on the carbon source (24). The cloning of the lactococcal sodA and the availability of a SodA-negative mutant form the basis for further studies to obtain more insight in the aerobic life of this fermentative organism.

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