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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 O_2 to H_2O the toxic intermediates O_2^- and H_2O_2 are generated. Hydrogen peroxide was found to inhibit growth of lactococci (1), and exposure of lactococci to a sublethal dose of H_2O_2 induced an oxidative stress response (14), characterized by an increased survival after exposure to a lethal level of H_2O_2 compared with cells that were not pretreated.

Most LAB can deal with oxygen radicals by either a superoxide dismutase (Sod) or a high internal Mn^{2+} concentration (2). Sod dismutes oxygen radicals by catalyzing the reaction $2\ O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$ (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 O_2 concentrations in the medium (24, 43). Although most organisms use catalase for the breakdown of H_2O_2, 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 (15) 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 Guchte et al. (49). The cell extract was used for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
L. lactis		
MG1363	Plasmid-free derivative of NCDO712	21
LL108	*repA*$^+$ derivative of MG1363, Cm^r	28a
MGSOD4	*sodA*::*lacZ* Em^r	This study
MGSOD7	Δ*sodA* derivative of MG1363, Em^r	This study
MGSOD8	Sod$^+$ derivative of MG1363, Em^r	This study
E. coli		
NM522	*supE thi* Δ(*lac-proAB*) Δ*hsd5* (r$^-$ m$^-$) [F′ *proAB lacI*^{q}*Z*Δ*M15*]	23
OX326A	Δ*sodA* Δ*sodB*	45
EC101	*repA*$^+$ derivative of JM101, Km^r	28
EC1000	*repA*$^+$ derivative of MC1000, Km^r	27a
Plasmids		
pUC19	*lacZ′* Ap^r	50
pORI13	Promoterless *lacZ*, Em^r, Ori$^+$ of pWV01, Rep$^-$	41a
pORI19	*lacZ′* Em^r, Ori$^+$ of pWV01, Rep$^-$	28
pVE6007	pWV01 derivative encoding a temperature-sensitive Rep protein, Cm^r	30
pSOD1	Ap^r, pUC19 with 7.6-kb *Sau*3A fragment	This study
pSOD2	Ap^r, pUC19 with 0.8-kb + 0.9-kb *Pvu*II fragment	This study
pSOD3	*sodA*::*lacZ* Em^r	This study
pSOD4	*sodA*::*lacZ* Em^r	This study
pSOD5	*sodA*::*lacZ* Em^r	This study
pSOD6	*sodA*::*lacZ* Em^r	This study
pSOD7	Internal *Nde*I-*Eco*RI fragment of *sodA* in pORI19	This study

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. Cell extracts of acid-shocked 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 automated 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 chromatographer (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 electrotransformation (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/Gene sequence 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/Gene 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 standing *L. lactis* culture at an optical density at 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 sequence 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 *Nde*I-*Eco*RI fragment of the *sodA* gene was cloned in the integration insertion vector pORI19, using a Rep$^+$ *E. coli* helper strain (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 MGSOD7.

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-Pro-Phe. A computer homology search revealed that this amino acid sequence is similar to the N-terminal amino acid sequences of a number of Sods (see Fig. 5). To determine whether Sod was induced by low pH conditions, cell extracts were separated on a nondenaturing PA gel and assayed for Sod activity. A higher level of Sod activity was indeed detected in cells grown at the lower pH (Fig. 1B).

Cloning and sequencing of the *sodA* gene. To clone the *L. lactis sod* gene, use was made of the *E. coli* strain from which

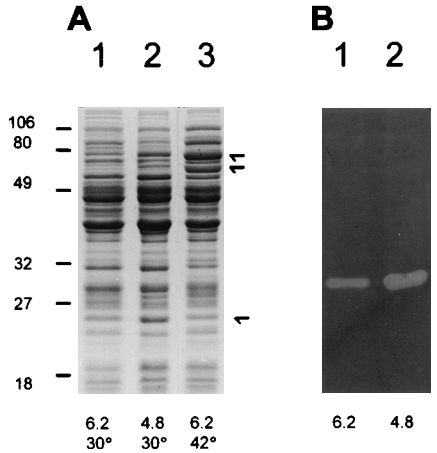

FIG. 1. (A) SDS–12.5% PAGE analysis of cell extracts of acid-stressed *L. lactis* cells. Lanes: 1, cells grown overnight in buffered MRS; 2, cells grown overnight in unbuffered MRS; 3, cells taken from exponential growth phase and incubated 1 h at 42°C. (B) Nondenaturing (12.5%) PA gel assayed for Sod 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.

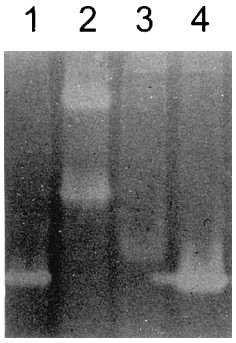

FIG. 2. Nondenaturing (12.5%) PAGE of cell extracts; the gel was stained for Sod activity. Lanes: 1, *L. lactis* MG1363; 2, *E. coli* NM522; 3, *E. coli* OX326A; 4, *E. coli* OX326A(pSOD2). Thirty micrograms of protein was applied per lane.

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 *Pvu*II digest of one of these clones (pSOD1) was ligated into the dephosphorylated *Sma*I 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 *Pvu*II 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 kcal/mol (−58.3 kJ/mol) (47). Upstream of the ribosome binding site a promoter-like structure was present, consisting of the −35 hexanucleotide TTGGCA, 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.

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,

```
                PvuII
1             CAGCTGTTTCGGGAATAAATGTTGGTGGAGCTGGCGGAACTAATTTTGC
1               A  V  S  G  I  N  V  G  G  A  G  G  T  N  F  A

50    TTGGATTGAGCGTAAACGGAGCAAAAATGGTTTTGATTTGGACGATTTTGGTTTTCAAC
17     W  I  E  R  K  R  S  K  N  G  F  D  L  D  D  F  G  F  S  T

110   TCTCGAAAGCCTGCTGGAGGCTAAAACGGCAGAGAATACCAAAAGCTTAGTTGCTACTGG
37     L  E  S  L  L  E  A  K  T  A  E  N  T  K  S  L  V  A  T  G

170   GGGAATTTCCTCTGCTCAAGATATTTTTAAATCTTTGATTTTAGGTGCCGATTTGGCAAG
57     G  I  S  S  A  Q  D  I  F  K  S  L  I  L  G  A  D  L  A  S

230   TTCAGCGGGTTTTATTTTGAAAAATCTCATGCAAACAGGTCCTGAAAAGGTAGAAGAAAT
77     S  A  G  F  I  L  K  N  L  M  Q  T  G  P  E  K  V  E  E  I

290   TCTTGAGCAATGGAAGCAAGATTTGAATAAATTATTTGTTCTGACAGGTTCAAAAAACAT
97     L  E  Q  W  K  Q  D  L  N  K  L  F  V  L  T  G  S  K  N  I
                    BglII
350   AGCAGAATCCCACAATGTAGATCTACTTTATTCAGCGAAAATGCTCGATTTTATCCAACA
117    A  E  S  H  N  V  D  L  L  Y  S  A  K  M  L  D  F  I  Q  Q
                                    -35                  -10
410   AAGAAAAAAATAAATTATAAATCAAATCATTTGGCAATGATTTCAAAAACGACTATAATG
137    R  K  K  ***          ------->    <--------
                                  EcoRI
470   AGAATAGAATTAAAAAATAATCTAACTGAATTCCATTCTCAATCTGGTCAAAATACCCAA
        ↑
                                          rbs          start sodA
530   GTATTAAGACTTCAAAATGGATTCACATCTTAaaaggagAATTACTATGGCATTTACATT
1                                                    M  A  F  T  L
                 NdeI
590   ACCTGAACTTCCATATGCGCCAAATGCGCTTGAACCTTTCTTTGATGAAGCAACAATGCG
6      P  E  L  P  Y  A  P  N  A  L  E  P  F  F  D  E  A  T  M  R

650   TTTGCATCATGGAAAACATCATCAAACTTATGTGAATAATCTTAATGCAGCAATTGAAAA
26     L  H  H  G  K  H  H  Q  T  Y  V  N  N  L  N  A  A  I  E  K

710   ACACAATGAACTTGATGACCTTAGCCTCGAAGAATTATTGACTGATTTGTCAGCAATCCC
46     H  N  E  L  D  D  L  S  L  E  E  L  L  T  D  L  S  A  I  P
                PvuII
770   AGAAGACATTCGTACAGCTGTTCGTAACAACGGTGGTGGTCATTTGAATCATAGTCAATT
66     E  D  I  R  T  A  V  R  N  N  G  G  G  H  L  N  H  S  Q  F

830   CTGGCTTTGGCTTCGTCCAAATACTGATGGTTCTGAAAACCATGCTGACGGTGAAATTGG
86     W  L  W  L  R  P  N  T  D  G  S  E  N  H  A  D  G  E  I  G
                                                 EcoRI
890   CGATGCAATTGCAAAAGAATTTGGTAGTTTTGAAACTTTCAAAACAGAATTCAAAGCTGC
106    D  A  I  A  K  E  F  G  S  F  E  T  F  K  T  E  F  K  A  A

950   TGCCACAGGCCGTTTTGGTTCAGGCTGGGCTTGGTTAGTTGTTGATGAAGCTGGCAAATT
126    A  T  G  R  F  G  S  G  W  A  W  L  V  V  D  E  A  G  K  L

1010  GAAAGTTGTATCAACTGCAAATCAAGATAATCCAATTTCTGAAGGATTGACACCAGTTTT
146    K  V  V  S  T  A  N  Q  D  N  P  I  S  E  G  L  T  P  V  L

1070  GGGACTTGATGTTTGGGAACATGCTTACTACCTTAAATATCACAATGTACGTCCTGACTA
166    G  L  D  V  W  E  H  A  Y  Y  L  K  Y  H  N  V  R  P  D  Y

1130  CATTGAAGCATTCTTTAACCTTGTAAACTGGGATAAAGTCAATGAACTTTACGCTAAGGC
186    I  E  A  F  F  N  L  V  N  W  D  K  V  N  E  L  Y  A  K  A

1190  AAAATAAAATAATCAATGAAAGACTCTCCTTTTTGGGGGAGTCTTTTTATTTTACAGAAG
206    K  ***          ---------->      <----------

1250  AATTAGAAAATGAAAAGAATTTTCTGTGTAAAATTCATCTTGTATTTTACAAAAAGATGT

1310  TTTAAAGAGATTTTTTTAAACATAAATGCTTATCTTTTTGATTAAATAGTGCTAAAATAT
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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); −10, −35, putative promoter sequence (with the bases underlined and in boldface type); vertical arrow, transcription start site. Stop codons are given in boldface type and indicated with asterisks. The doubly underlined amino acid sequence is identical to that determined from the isolated 24-kDa protein. A number of relevant restriction enzyme sites are indicated.

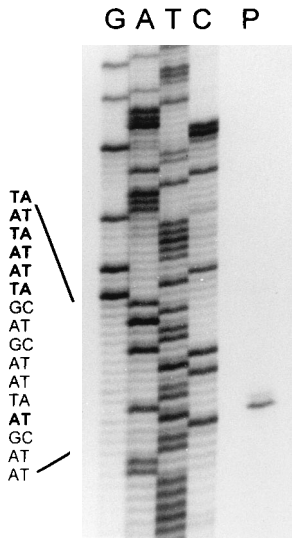

FIG. 4. Determination of the transcriptional start point of *sodA*. Lane P, primer extension product. The sequence ladder was obtained with the same primer on *sodA* template DNA. At the left is indicated the sequence of both the transcribed strand and the sequenced strand. The −10 region of the promoter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

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

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                                                                  ■
L.lactis    M-----------------------AFTLPELPYAPNALEPFFDEATMRLH  27
B.stearo    M-----------------------PFELPALPYPYDALEPHIDKETMNIH  27
S.mutans    M-----------------------AILLPDLPYAYDALEPYIDAETMTLH  27
E.coli Mn   -------------------------SYTLPSLPYAYDALEPHFDKQTMEIH  26
E.coli Fe   M-----------------------SFELPALPYAKDALAPHISAETIEYH  27
Human       MLSRAVCGTSRQLAPALGYLGSRQKHSLPDLPYDYGALEPHINAQIMQLH  50
                                      **.***  .**.* .. ...  *

               □□  □                                        ×
L.lactis    HGKHHQTYVNNLNAAIEKHNELDDLSLEELLTDLSAIPEDIRTAVRNNGG  77
B.stearo    HTKHHNTYVTNLNAALEGHPDLQNKSLEELLSNLEALPESIRTAVRNNGG  77
S.mutans    HDKHHATYVANANAALEKHPEIGE-NLEVLLADVEQIPADIRQSLINNGG  76
E.coli Mn   HTKHHQTYVNNANAALESLPEFANLPVEELITKLDQLPADKKTVLRNNAG  76
E.coli Fe   YGKHHQTYVTNLNN-LIKGTAFEGKSLEEIIRSSEG-------GVFNNAA  69
HUMAN       HSKHHAAYVNNLNVTEEKYQ-------EALAKGDVTAQIALQPALKFNGG  93
            ..***..**.* *              * .              .  *..

            ×   ■  ×
L.lactis    GHLNHSQFWLWLRPNTDGSENHADGEIGDAIAKEFGSFETFKTEFKAAAT 127
B.stearo    GHANHSLFWTILSPNGGG---EPTGELADAINKKFGSFTAFKDEFSKAAA 124
S.mutans    GHLNHALFWELLSPE----KTKVTAEVAAAINEAFGSFDDFKAAFTAAAT 122
E.coli Mn   GHANHSLFWKGLKKG-----TTLQGDLKAAIERDFGSVDNFKAEFEKAAA 121
E.coli Fe   QVWNHTFYWNCLAPNAGG---EPTGKVAEAIAASFGSFADFKAQFTDAAI 116
HUMAN       GHINHSIFWTNLSPNGGG---EPKGELLEAIKRDFGSFDKFKEKLTAASV 140
               **. .*  *  .          ... .**   *** . **. .. *.

               □              ××                ■
L.lactis    GRFGSGWAWLVVDEA-GKLKVVSTANQDNPISEGLT------PVLGLDVW 170
B.stearo    GRFGSGWAWLVVNN--GELEITSTPNQDSPIMEGKT------PILGLDVW 166
S.mutans    TRFGSGWAWLVVDKE-GKLEVTSTANQDTPISQGLK------PILALDVW 165
E.coli Mn   SRFGSGWAWLVL-KG-DKLAVVSTANQDSPLMGEAISGASGFPILGLDVW 169
E.coli Fe   KNFGSGWTWLVKNSD-GKLAIVSTSNAGTPLTTDAT------PLLTVDVW 159
HUMAN       GVQGSGWGWLGFNKERGHLQIAACPNQDP-----LQGTTGLIPLLGIDVW 185
               ****.**   .  ..* ... .*..              *.*..***

            ■  □
L.lactis    EHAYYLKYHNVRPDYIEAFFNLVNWDKVNELY--AKAK   206
B.stearo    EHAYYLKYQNRRPEYIAAFWNVVNWDEVAKRYSEAKAK   204
S.mutans    EHAYYLNYRNVRPNYIKAFFEVINWNTVARLYAEALTK   203
E.coli Mn   EHAYYLKFQNRRPDYIKEFWNVVNWDEAAARFAA--KK   205
E.coli Fe   EHAYYIDYRNARPGYLEHFWALVNWEFVAKNLAA----   193
HUMAN       EHAYYLQYKNVRPDYLKAIWNVINWENVTERYMACK-K   222
            *****....* **.*.  .....**. ..
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FIG. 5. Alignment of the deduced amino acid sequences of *L. lactis* SodA with amino acid sequences of MnSods and the *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 *Bacillus stearothermophilus* (*B. stearo*) (5), *S. mutans* (33), and *E. coli* (*E. coli* Mn) (44); the FeSod of *E. coli* (*E. coli* Fe) (9); and human MnSod (Human) (12).

TABLE 2. β-Galactosidase activity of *sodA*::*lacZ* fusions

Strain	β-Galactosidase activity (U/mg)[a] ± SD in:	
	Standing culture	Shaken culture
LL108(pSOD3)	3,740 ± 120	7,310 ± 170
LL108(pSOD4)	29.0 ± 6.3	47.6 ± 3.0
MGSOD4	16.8 ± 1.8	37.9 ± 2.5
LL108(pSOD5)	5.7 ± 1.3	11.9 ± 1.6
LL108(pSOD6)	16.6 ± 1.7	15.1 ± 3.2

[a] Values are means of at least three independent experiments.

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 *L. lactis* SodA belongs to the class of MnSods.

Analysis of *sodA*::*lacZ* fusions. Since in *L. lactis* higher levels of Sod activities have been reported at the higher O_2 concentrations (24), we examined the expression of *sodA* under aerobic conditions. For this purpose transcriptional fusions of *sodA* to the *E. coli lacZ* gene were constructed. A 0.8-kb *Pvu*II 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 *L. lactis* cells carrying this construct, pSOD3, an unusually high level of β-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 *Bam*HI site (Fig. 6). In this way a frameshift was introduced in the truncated *sodA*, resulting in a stop of translation 21 bp upstream of *lacZ*. As a consequence, the overlap by 1 bp of the *sodA* stop codon and the *lacZ* start codon, as in pSOD3, was eliminated in pSOD4. Both plasmids were introduced in the Rep^+ lactococcal helper strain LL108, which provides the plasmid replication protein in *trans* (28a). The 100-fold-higher level of β-galactosidase activity observed in strain LL108(pSOD3) as compared with that of LL108(pSOD4) (Table 2) strongly suggests that the high β-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 *L. lactis* MG1363 by Campbell-type integration. The level of β-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 β-galactosidase activity was measured (data not shown). An overall higher level of β-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.

```
pSOD3:
                    BamHI  XbaI     R.B.S.
gaagacattcgtacaggggatcctctagattcGGAGGaattttgaaATGaaaggggcc
E  D  I  R  T  G  G  S  S  R  F  G  G  I  L  K  ***
sodA                                        lacZ:  M  K  W  A

pSOD4:
            filled in BamHI  XbaI     R.B.S.
gaagacattcgtacaggggatcgatcctctagattcGGAGGaattttgaaATGaaaggggcc
E  D  I  R  T  G  G  S  I  L  ***
sodA                                        lacZ:  M  K  W  A
```

FIG. 6. DNA sequence of the fusion points of *sodA* and *lacZ* in pSOD3 and pSOD4. The translation of the two genes is given below the DNA sequence. The translational stops are indicated with three asterisks. In pSOD3 the *sodA* translation stop codon and *lacZ* translation start codon overlap.

FIG. 7. Nondenaturing PAGE of cell extracts of MG1363 (lane 1) and MG-SOD7 (lane 2). The gel was stained for Sod activity; 30 μg of protein was applied per lane.

Also in this situation, a twofold induction by aeration was observed.

To identify the DNA region responsible for oxygen-induced *sodA* expression, pSOD5 was constructed by cloning in pORI13 a 502-bp *Pvu*II-*Eco*RI fragment (coordinates 1 to 502 in Fig. 3) immediately upstream of the *E. coli lacZ* gene devoid of its own promoter. This plasmid contains the *sodA* promoter and part of the upstream ORF but no *sodA* coding sequences. The level of *lacZ* expression from this plasmid replicating in strain LL108 was lower than that from pSOD4 in LL108 but was still inducible. However, with another subclone, pSOD6, carrying a 130-bp *Bgl*II-*Eco*RI fragment in front of *lacZ* (coordinates 370 to 502, Fig. 3), β-galactosidase expression was similar in standing and in aerated cultures and comparable to that from LL108(pSOD5) under aerated conditions. In pSOD5 and pSOD6 the same *lacZ* fusion point was used. This indicates that the 370-bp *Pvu*II-*Bgl*II fragment (coordinates 1 to 370, Fig. 3) is involved in the regulation of *sodA* expression.

Growth of a Sod-negative *L. lactis* mutant is impeded in aerobic cultures. To examine possible phenotypic effects of SodA deficiency, the *sodA* gene was disrupted by the insertion of the integration vector pSOD7. Figure 7 shows that no SodA activity could be detected in the disruption mutant. The growth rate of MGSOD7 in aerated medium was lower than that of a $SodA^+$ Em^r control strain, whereas the growth rates of both strains were comparable in standing cultures (Fig. 8). The lag phase of MGSOD7 was consistently somewhat longer than that of the Sod^+ strain. Also the SodA-negative mutant grew only slowly on solid medium in the presence of O_2. Thus, although not essential, *L. lactis* SodA is important for optimal growth of the organism under aerobic conditions.

DISCUSSION

In the framework of a general interest in stress-induced protein expression in *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 *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 *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 *dnaK* and *groEL* in *L. lactis.*

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 evolutionary 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 *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 *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 *E. coli* (10) and *Streptococcus mutans* (33). A Sod-free *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 *L. pneumophila*, a bacterium that needs oxygen for growth (40). The inactivation of Sod of the obligate anaerobe *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 *E. coli* and *S. mutans* do have such systems. The ability of SodA-deficient *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 *E. coli* and in *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 *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 *L. lactis*. The results of the *sodA*::*lacZ* transcrip-

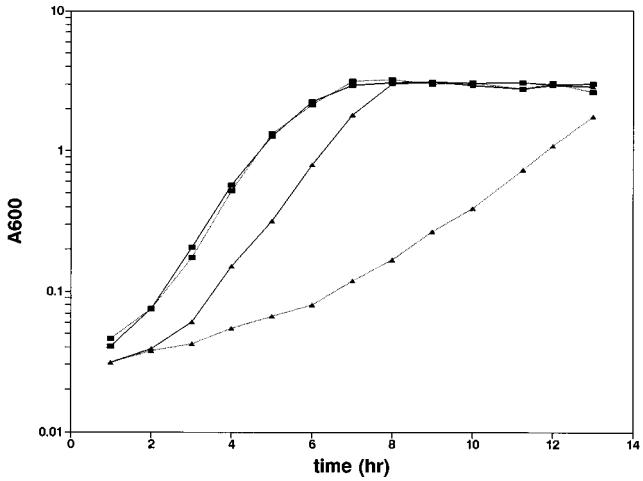

FIG. 8. Growth of *L. lactis* MG1363 derivatives in liquid GM17 medium, either as aerated (dotted lines) or standing cultures (solid lines). Strains: filled box, MGSOD8 ($SodA^+$ Em^r); filled triangle, MGSOD7 ($SodA^-$ Em^r). A600, Absorbance at 600 nm.

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 aeration, the inducibility of *sodA* expression is probably specified by the 370-bp *Pvu*II-*Bgl*II 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, making the expression of *sodA* dependent on growth conditions and on the presence of O_2, Fe, NO_3^-, 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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