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Cloning, Characterization, and Expression in *Escherichia coli* of a Gene Encoding *Listeria seeligeri* Catalase, a Bacterial Enzyme Highly Homologous to Mammalian Catalases

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A gene coding for catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase; EC 1.11.1.6) of the gram-positive bacterium *Listeria seeligeri* was cloned from a plasmid library of *Eco*RI-digested chromosomal DNA, with *Escherichia coli* DH5α as a host. The recombinant catalase was expressed in *E. coli* to an enzymatic activity approximately 50 times that of the combined *E. coli* catalases. The nucleotide sequence was determined, and the deduced amino acid sequence revealed 43.2% amino acid sequence identity between bovine liver catalase and *L. seeligeri* catalase. Most of the amino acid residues which are involved in catalytic activity, the formation of the active center accession channel, and heme binding in bovine liver catalase were also present in *L. seeligeri* catalase at the corresponding positions. The recombinant protein contained 488 amino acid residues and had a calculated molecular weight of 55,869. The predicted isoelectric point was 5.0. Enzymatic and genetic analyses showed that there is most probably a single catalase of this type in *L. seeligeri*. A perfect 21-bp inverted repeat, which was highly homologous to previously reported binding sequences of the Fur (ferric uptake regulon) protein of *E. coli*, was detected next to the putative promoter region of the *L. seeligeri* catalase gene.

The gram-positive bacterium *Listeria seeligeri* (41) is a member of the genus *Listeria*, which comprises seven species, two of which are facultative intracellular bacteria: *Listeria monocytogenes* (pathogenic for animals and humans) and *Listeria ivanovii* (pathogenic for animals only). In particular, *L. monocytogenes* is responsible for mostly foodborne severe illness (abortion, septicemia, and meningitis) in immunocompromised hosts, pregnant women, and elderly persons (7, 40, 46). Listerial catalase has been proposed to be a potential virulence factor (4, 39, 52, 53) which may counteract the oxygen-dependent defense mechanisms of infected phagocytes. On the other hand, it has been reported that catalase-negative Tn*1545* mutants of *L. monocytogenes* are fully virulent (24). To clarify the situation, we have conducted an investigation into the biochemistry and genetics of a catalase from *L. seeligeri*.

Catalase (EC 1.11.1.6) cDNAs have been isolated from a number of eukaryotic organisms, including humans, rats, cattle, yeasts, maize, and sweet potatoes. All of these catalases share a high degree of amino acid sequence identity (see reference 3 and Fig. 3). However, knowledge of the structure of bacterial catalase genes is very poor. To date, isolation of the genes encoding both *Escherichia coli* catalases, HPI (*katG*; 27, 50) and HPII (*katE*; 35), has been reported, and the nucleotide sequence of the HPI gene has been published recently (49). Furthermore, *katF*, a genetic locus in *E. coli* affecting the synthesis of HPII in vivo, was isolated (26, 35). The genetic loci of the *E. coli* catalases and *katF* are not physically linked on the *E. coli* chromosome, being positioned at 38 min (*katE*), 59 min (*katF*), and 89 min (*katG*) (1a). Interestingly, *E. coli* HPI is unusual in its biochemical features compared with eukaryotic catalases; e.g., it shows catalase and broad-range peroxidase activities. The deduced amino acid sequence of HPI does not show homology with those of eukaryotic catalases (49). For *Listeria* catalases, some reports on the catalase activity of *L. monocytogenes* have been published (e.g., references 11, 12, 52, 53, and 55), and it has been reported that *L. monocytogenes* strains defective in catalase activity possess higher superoxide dismutase activity (24, 52, 53) and that log-phase *L. monocytogenes* cells show higher catalase activity than do cells from stationary-phase cultures (4).

Here, we report on the cloning and expression in *E. coli* of a gene encoding catalase from *L. seeligeri*, a nonpathogen which is closely related to pathogenic *Listeria* species (29, 40, 41).

MATERIALS AND METHODS

Bacterial strains and plasmids. *L. seeligeri*, *L. monocytogenes* Sv1/2a Mackaness (SLCC 5764), *Listeria innocua* Sv6a (NCTC 11288), *Listeria grayi*, *Listeria welshimeri*, *Listeria murrayi*, and *L. ivanovii* (ATCC 19119) were obtained from the *Listeria* strain collection of the Institute of Hygiene and Microbiology at the University of Würzburg, Würzburg, Germany. *Bacillus thuringiensis* was from our strain collection. *E. coli* DH5α [F^- *endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1* λ^- *recA1 gyrA96 relA1* Δ(*argF-lacZYA*)*U169* φ80d*lacZ*ΔM15] was used throughout the cloning and expression experiments. *E. coli* UM120 (*katE12*::Tn*10*), defective in the synthesis of *E. coli* catalase HPII, and *E. coli* UM202 (*katG*::Tn*10*), defective in the synthesis of HPI, have been described previously (45) and were kindly supplied by P. Loewen (University of Manitoba, Winnipeg, Manitoba, Canada). The multipurpose plasmid vector pTZ18R was described previously (32).

Media and reagents. *Listeria* strains and *B. thuringiensis* were grown overnight at 37°C and 160 rpm in brain heart infusion broth (Difco Laboratories, Detroit, Mich.); *E. coli* was grown in Luria-Bertani (LB) broth. When needed, ampicillin was added to a final concentration of 100 mg/liter.

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For solid media, 15 g of agar per liter was included. Restriction enzymes, T4 DNA ligase, and nucleotides were purchased from Boehringer Mannheim Biochemicals. All other chemicals were of reagent grade or better. [^{32}P]dATP (800 and 3,000 Ci/mmol) was purchased from Amersham International.

Construction and screening of the DNA library. All DNA manipulations were done in accordance with manufacturer suggestions and as described by Maniatis et al. (30). Chromosomal DNA from *Listeria* strains was isolated as described by Flamm et al. (15). For the construction of the DNA library, chromosomal DNA from *L. seeligeri* was digested to completion with *Eco*RI and ligated into the unique *Eco*RI site of pTZ18R. Calcium chloride-competent *E. coli* DH5α cells (30) were transformed with the ligation mixture and plated onto LB agar containing 50 mg of isopropyl-β-D-thiogalactoside (IPTG) per liter, 40 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per liter, and ampicillin. Incubation was done at 30°C for 30 h. β-Galactosidase-negative *E. coli* colonies containing *L. seeligeri* DNA fragments were isolated. For screening of the recombinant clones for increased catalase activity, 3,000 recombinant *E. coli* cells were replica plated onto LB agar (100 mg of ampicillin per liter) and grown overnight at 30°C. The plates were overlaid with a solution of 3% hydrogen peroxide in water, and colonies with high catalase activities were identified by their enhanced oxygen formation.

Construction of deletion mutations, DNA sequencing, and DNA sequence analysis. To construct deletion derivatives of pAHA1 (harboring the *L. seeligeri* catalase gene) for plasmid DNA sequencing, we performed exonuclease III-triggered digestion (19) with restriction enzymes *Pst*I and *Bam*HI to generate a suitable DNA substrate. Some of the *E. coli* DH5α clones, transformed with the mixture of deletion plasmids, were analyzed for their plasmid content by an alkaline lysis method (30). Plasmids of different molecular weights were used for plasmid DNA sequence determinations by the dideoxy chain termination method (44) with a T7 sequencing kit from Pharmacia. Reactions were performed as described in the manufacturer's manual with universal or reverse oligonucleotide primers. Oligonucleotides were constructed on the basis of the determined first DNA strand sequences (Applied Biosystems DNA synthesizer) and used for sequence analysis of the second DNA strand sequences by the same method. Electrophoresis was done in 6% polyacrylamide–42% (wt/vol) urea gels at 40 W, and the dried gels were autoradiographed for 14 h at −20°C. DNA sequences and derived amino acid sequences were analyzed with a microcomputer system and PC/Gene (IntelliGenetics Inc.) software; protein sequence comparisons were done on a VAX computer system with the Genetics Computer Group sequence analysis software package, version 6.1 (13).

Southern blot analysis. DNA was digested, fractionated on Tris-borate (90 mM Tris base, 90 mM H_3BO_4, 2 mM Na-EDTA [pH 8.0])–1% agarose gels and transferred to nitrocellulose filters by capillary transfer as described previously (30). For generation of DNA probes, DNA fragments were electroeluted from agarose gels (Biotrap device; Schleicher & Schuell). Radioactive labeling of DNA probes was performed with a Boehringer Mannheim random primer DNA labeling kit and [α-^{32}P]dATP (3,000 Ci/mmol). Prehybridization reactions were carried out at 56°C for 2 h in a solution containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's reagent (30), 20 mM sodium phosphate buffer (SPB) (pH 6.8), and 100 mg of denatured salmon sperm DNA per liter. The labeled and denatured probes were added, and hybridization was performed for 14 h at 56°C. The nitrocellulose filters were washed twice at 60°C (see Fig. 4B and C) in 1× SSC–0.1% sodium dodecyl sulfate (SDS) or at 45°C in 5× SSC–0.1% SDS (see Fig. 4A) for 30 min each time and exposed to X-ray films (Fuji RX-NIF) at −20°C for 6 to 48 h.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis (12.5% acrylamide) was done as described previously (23). Nondenaturing polyacrylamide gels (6.25% acrylamide) were essentially the same as SDS-polyacrylamide gels but contained no SDS or mercaptoethanol, and the samples were not heated prior to electrophoresis. Gels were run at 100 V until the bromophenol blue dye track left the gel. Denaturing gels were stained for protein content with Coomassie blue; nondenaturing gels were stained for enzyme activity as described below. Scanning of Coomassie blue-stained acrylamide gels was done with a Hirschmann Elscript 400 device at 258 nm.

Preparation of cell lysates. *E. coli* cells were harvested from LB broth-ampicillin cultures and washed in 50 mM SPB (pH 7.0). The pellet was resuspended in 1/100 the original culture volume of SPB. The solution was sonicated three times for 30 s each time at 60 W with a Branson Sonifier. *Listeria* cells were incubated with 20 μg of mutanolysin (Sigma) per ml for 1 h at 37°C prior to sonication (three times for 1 min each time). Debris was removed by centrifugation in an Eppendorf microcentrifuge at 4°C for 15 min. Protein concentration was determined with a Bio-Rad protein assay kit with bovine serum albumin as a standard. The samples were used immediately after preparation or were stored at −20°C until needed (no significant loss of activity was detected when these lysates were stored frozen for several weeks).

Assays for catalase and peroxidase activities. In situ activity staining for catalase in native polyacrylamide gels was done as described previously (54). For detection of both catalase and 3,3′-diaminobenzidine-peroxidase activities within the same polyacrylamide gel in situ, the method of Wayne and Diaz (51) was used, but during staining for catalase activity, 120 μl of a 30% hydrogen peroxide solution per 100 ml of water was used for better contrast. All samples were centrifuged for 5 min at 4°C in a microcentrifuge prior to electrophoresis to minimize smearing of bands. Quantitative determination of catalase activity was performed by monitoring the decomposition of hydrogen peroxide by catalase spectrophotometrically at 240 nm as described previously (2). Catalase activity in *L. seeligeri* culture supernatants was determined with 0.2-ml aliquots prepared by centrifugation of overnight cultures. For determination of the effect of IPTG on *kat* expression, *E. coli* was grown overnight at 37°C and 180 rpm in LB broth containing 100 μg of ampicillin per ml. A 350-μl sample of this culture was used to inoculate 50 ml of fresh LB broth containing 100 μg of ampicillin per ml. Before inoculation, 0.5 ml of 100 mM IPTG or distilled water was added. Cells were shaken at 37°C and 180 rpm for 4 h and harvested.

Assays for biochemical characteristics of *L. seeligeri* catalase. Cellular lysates from overnight cultures of *L. seeligeri* prepared as described above were dialyzed overnight (10-kDa exclusion) at 4°C against several thousand volumes of 50 mM SPB (pH 7.0). For determination of the effect of 20 mM 3-amino-1,2,4-triazole (Sigma) plus 4 mM ascorbate (Sigma) on *L. seeligeri* catalase activity, dialyzed cell lysates (at a dilution of 1 mg/ml) were incubated with gentle shaking at 37°C for 120 min with the latter reagents in 50 mM SPB (pH 7.0). Controls were either not treated or treated with

sodium ascorbate, and bidistilled water was included instead of 3-amino-1,2,4-triaziole. Treated lysates were immediately dialyzed overnight at 4°C against several thousand volumes of 50 mM SPB (pH 7.0). Subsequently, protein concentrations and catalase activities were determined spectrophotometrically. To study the effect of chloroform-ethanol treatment on *L. seeligeri* catalase activity, we treated dialyzed cell lysates as previously described (dialyzed lysate-ethanol-chloroform, 10:5:3 [vol/vol/vol]; 17). To determine the pH dependency of catalase activity, we adjusted 50 mM SPB to pHs 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0. For pH values beyond the buffering capacity, the pH was adjusted by the addition of HCl or NaOH directly before the assay as previously described (38). For the spectrophotometric determination of catalase activity, hydrogen peroxide concentrations were adjusted immediately before activity assays.

RESULTS

Screening of the plasmid library. Three thousand recombinant *E. coli* clones, each harboring an *Eco*RI fragment of *L. seeligeri* chromosomal DNA ligated into the unique *Eco*RI recognition site of plasmid pTZ18R, were replica plated and screened for an increased production of molecular oxygen upon addition of 3% hydrogen peroxide. Of these 3,000 recombinant *E. coli* clones, 15 showed increased liberation of oxygen. Upon repetition of the experiment, only three of these clones showed extraordinarily strong oxygen production on LB agar plates compared with the other recombinant clones and negative controls. These three *E. coli* clones were chosen for further analysis. A 3.57-kb DNA insert was present in the plasmids of each of these transformants. Both possible orientations of the *L. seeligeri* DNA insert were present (pAHA1 and pAHA2). (As determined later by nucleotide sequencing, transcription from the *lacZ* promoter in pTZ18R occurs in the same orientation as does transcription of the catalase gene in pAHA2 and occurs in the opposite orientation in pAHA1; see Fig. 3A for a restriction map of pAHA1.)

Analysis of positive *E. coli* clones with electrophoretograms. To determine whether the *L. seeligeri* DNA fragment in pAHA1 contained the genetic information for *L. seeligeri* catalase or whether the increased catalase activity originated from a recombinant *L. seeligeri* factor which enhances the expression of *E. coli* catalase activity by acting in *trans*, we analyzed cell lysates from *E. coli* recombinants and from *L. seeligeri* by nondenaturing polyacrylamide gel electrophoresis and staining for catalase activity. *E. coli* DH5α (HPI$^+$ HPII$^+$), HPI$^-$ strain UM202 (not shown), and HPII$^-$ strain UM120 harboring pAHA1 or pTZ18R were used. Figure 1A shows a nondenaturing polylacrylamide gel which was stained first for 3,3'-diaminobenzidine-peroxidase activity and subsequently for catalase activity. Staining for peroxidase activity resulted in dark bands on an unstained background (positive stain). Subsequent staining for catalase activity resulted in darkening of the entire gel, except at the positions of catalase activity (negative staining). Enzymes having both peroxidase and catalase activities appeared as dark bands on a locally light background. *E. coli* HPI showed this pattern (Fig. 1A, lanes A to D). Most of its catalase activity was masked by strongly positive peroxidase activity (this feature is inherent to the type of assay). *E. coli* HPI has indeed previously been reported to possess both activities (8). In contrast to *E. coli* HPI, we could not detect 3,3'-diaminobenzidine-peroxidase activity associated with *E. coli* HPII (in agreement with the results of Claiborne et al.

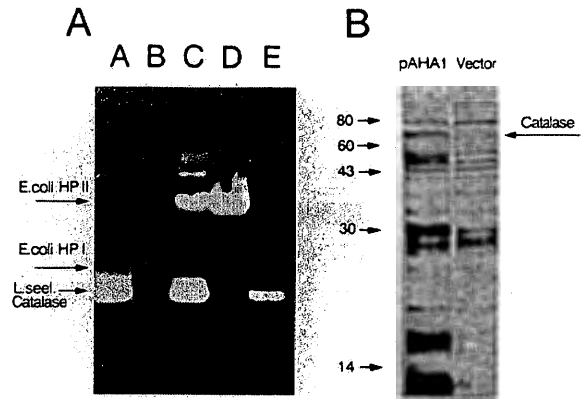
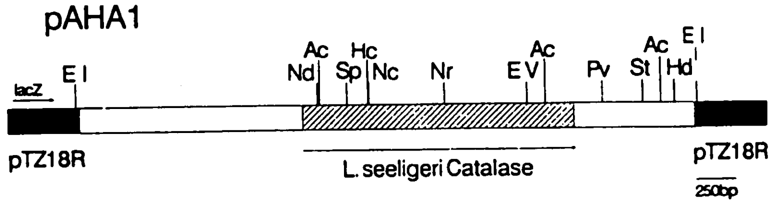


FIG. 1. Expression of the recombinant *L. seeligeri* catalase gene in *E. coli*. (A) Nondenaturing polyacrylamide gel stained for 3,3'-diaminobenzidine-peroxidase activity and, subsequently, for catalase activity. Lanes: A, UM120 (HPI$^+$ HPII$^-$) harboring pAHA1; B, UM120(pTZ18R); C, DH5α (HPI$^+$ HPII$^+$) harboring pAHA1; D, DH5α(pTZ18R); E, *L. seeligeri*. In lanes A, C, and E, cleared cell lysates containing 50 μg of protein each were applied to the gel; in lanes B and D, 200 μg of protein was applied. The positions of *E. coli* HPI and HPII and of *L. seeligeri* (L. seel.) catalase are indicated on the left. (B) Denaturing SDS-polyacrylamide gel stained for proteins with Coomassie blue. Cell lysates containing 50 μg of protein from *E. coli* DH5α(pAHA1) and DH5α(pTZ18R) (Vector) were applied to the gel. The recombinant catalase can be seen as an additional protein band corresponding to a molecular weight of 67,000 (left lane). Numbers at the left of panel B are molecular weights in thousands.

[9]) or with *L. seeligeri* catalase (Fig. 1A, lanes A, C, and E) in this assay; their positions were marked by very light bands in the stain of gels for 3,3-diaminobenzidine- or *o*-dianisidine-peroxidase activity (Fig. 1A and data not shown, respectively). The catalase activity from recombinant *E. coli* carrying pAHA1 comigrated with the *L. seeligeri* catalase activity in the gel system used, whereas *E. coli* HPI and HPII showed different migration behaviors. These data supported the suggestion that a full-length *L. seeligeri* catalase structural gene (*kat*) had been cloned and was functionally expressed in *E. coli* hosts.

Analysis of proteins and of catalase activities in recombinant *E. coli* strains. SDS-polyacrylamide gel electrophoresis of cell lysates from *E. coli* DH5α(pAHA1) and DH5α(pTZ18R) showed that the pAHA1-harboring *E. coli* strain contained two additional protein bands corresponding to molecular weights of approximately 67,000 and 50,000. The latter protein is most probably a degraded *kat* gene product, as (i) there is no other open reading frame on the *Eco*RI insert DNA which could encode such a protein and (ii) analysis of radiolabeled proteins from *E. coli* minicells harboring pAHA1 revealed that of these two proteins, only the 67-kDa protein was present, concomitant with a high recombinant catalase activity (16a). Scanning of polyacrylamide gels revealed that the presumptive *L. seeligeri* catalase protein (molecular weight, 67,000) accounted for approximately 2.0% of the total soluble protein present on the gels. Cell lysates from *E. coli* DH5α cells containing pAHA1, pAHA2, or pTZ18R and grown with or without 1 mM IPTG were assayed spectrophotometrically (2) for catalase activity. *E. coli* clones containing either pAHA1 or pAHA2 produced equal activities (1,080 to 1,260 U/mg of soluble protein)

A



B

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1     AAAAAGCATTTCTATTTAAACGATGTTGTAGAAATGAAAAAACCACATCCTTGTGGGACTAATCGTTTCAAGATTATCCGCATGGGAATG     90

91    GACATTCGAATTAAATGTGAAGGTTGCGGACATAGTGTAATGATTCCGCGCCGTGAGTTTGAGCGAAAAGTGAAAAAGATTTTAGTTAAG     180

181   GCTGAGGAAGAATAAAATAATCTCTTCATTATCTGGGGTTTAAGATAATGAAGAGATTATTTTTATATAAGCCTTTTAGTTGAAGTTTGG     270
                                                                                         -35
271   GACAACCTTCTTTAAAATATAAATAGTATCATTTAATAATTATTATAAATAAAAATGATAATACTATTTTAATGGAGGTATACATATGAC     360
                 -10      +1                                                    S D          M T
361   CGATAGAAGAAACTTAACGACGAATCAAGGAGTGCCGATTGGGGACAACCAAAATTCGATGACAGCGGGATTAAAAGGACCAACTTTGTT     450
       D  R  R  N  L  T  T  N  Q  G  V  P  I  G  D  N  Q  N  S  M  T  A  G  L  K  G  P  T  L  L
451   AGAAGATTATGTGTTAATTGAGAAATTGGCGCATTTTGATAGAGAACGTGTTCCAGAACGTGTGGTGCATGCTCGTGGTGCTGGTGCGCA     540
       E  D  Y  V  L  I  E  K  L  A  H  F  D  R  E  R  V  P  E  R  V  V  H  A  R  G  A  G  A  H
541   CGGCAAATTTGTAACGAAAAAAAGCATGAAAAAATATACGAAAGCACAATTTTTACAAGAAGAAGGAACAGAGACAGAGGTTTTTGCGCG     630
       G  K  F  V  T  K  K  S  M  K  K  Y  T  K  A  Q  F  L  Q  E  E  G  T  E  T  E  V  F  A  R
631   TTTTTCTACAGTAATCCATGGTCAACATTCACCAGAAACGCTTCGTGATCCACGTGGTTTTTCGGTTAAATTTTATACGGAAGAAGGTAA     720
       F  S  T  V  I  H  G· Q  H  S  P  E  T  L  R  D  P  R  G  F  S  V  K  F  Y  T  E  E  G  N
721   CTATGACTTTGTCGGTAACAACTTACCTGTATTCTTTATCCGTGATGCTATCAAGTTTCCTGACGTAATTCACTCCTTGAAGCCAGATCC     810
       Y  D  F  V  G  N  N  L  P  V  F  F  I  R  D  A  I  K  F  P  D  V  I  H  S  L  K  P  D  P
811   ACGTACTAACATTCAAGATGGCAATCGTTACTGGGACTTCTTTAGTTTAACTCCGGAAGAGACGACGATGATTACTTATTTATTTAGCGA     900
       R  T  N  I  Q  D  G  N  R  Y  W  D  F  F  S  L  T  P  E  A  T  T  M  I  T  Y  L  F  S  D
901   TGAGGGGACTCCGGCATCTTACCGCGAAATTCGTGGTTCAAGCGTACATGCCTTTAAATGGATAAACGAAGAAGGTAAGACTGTTTATGT     990
       E  G  T  P  A  S  Y  R  E  I  R  G  S  S  V  H  A  F  K  W  I  N  E  E  G  K  T  V  Y  V
991   AAAACTACGCTGGGTTCCAAAAGCAGGAATCGTCAATCTTTCAACTGATCAAGCAGCACAAATTCAAGCAAAAGAATTTAACCATGCTAG     1080
       K  L  R  W  V  P  K  A  G  I  V  N  L  S  T  D  Q  A  A  Q  I  Q  A  K  E  F  N  H  A  S
1081  TCGCGATTTGTATGAAGCAATTGAGAATGGTGATTATCCAGAGTGGGATTTATATGTGCAAGTGCTAGATCCAAAAGACTTGGATAATTA     1170
       R  D  L  Y  E  A  I  E  N  G  D  Y  P  E  W  D  L  Y  V  Q  V  L  D  P  K  D  L  D  N  Y
1171  CGACTTCAATCCGCTTGATGCAACCAAAGACTGGTTTGAAGATGTATTTCCATACGAGCATGTTGGAACAATGACATTAAATCGTAATCC     1260
       D  F  N  P  L  D  A  T  K  D  W  F  E  D  V  F  P  Y  E  H  V  G  T  M  T  L  N  R  N  P
1261  GGATAATATTTTTGCTGAAACAGAATCAGTTGGCTTTAATCCAGGTGTGCTTGTGCCGGGGATGTTACCTTCTGAGGACCGTTTACTACA     1350
       D  N  I  F  A  E  T  E  S  V  G  F  N  P  G  V  L  V  P  G  M  L  P  S  E  D  R  L  L  Q
1351  GGGGCGATTGTTCTCTTACTCTGATACGCAAAGACACCGCGTTGGACCTAACTACTTACAATTACCAATCAACAGCCCAAAAACTCCTGT     1440
       G  R  L  F  S  Y  S  D  T  Q  R  H  R  V  G  P  N  Y  L  Q  L  P  I  N  S  P  K  T  P  V
1441  TGATAACAACCAACGTGATGGACAGATGCCGTTTAAACAGCAAACAAGTTCGATTAATTATGAACCAAATAGTTATGATACAGAACCAAA     1530
       D  N  N  Q  R  D  G  Q  M  P  F  K  Q  Q  T  S  S  I  N  Y  E  P  N  S  Y  D  T  E  P  K
1531  AGAAAACCCTGCATATATCGAGCCTGAGCAAGAAATTCGTGGGGATATCTCTGGCCGACTAGTGGCAGAAAAGCCAAATAACTTTGGTCA     1620
       E  N  P  A  Y  I  E  P  E  Q  E  I  R  G  D  I  S  G  R  L  V  A  E  K  P  N  N  F  G  H
1621  TGCTAAAGAAGTTTGGAAGCGTTACTCAGATGCAGAACGTGCGGCTCTTGTGAAAAATATTGTAGACGATTGGGAAGGTGTGCGCGAAGA     1710
       A  K  E  V  W  K  R  Y  S  D  A  E  R  A  A  L  V  K  N  I  V  D  D  W  E  G  V  R  E  D
1711  TATTAAGATTCGCAACTTGCGCAATTTCTATCAAGTAGAGCCGGAATTTGCAGAACGTGTGGCTGCTGGAACTGGAATTAACCTTGCTGA     1800
       I  K  I  R  N  L  R  N  F  Y  Q  V  E  P  E  F  A  E  R  V  A  A  G  T  G  I  N  L  A  E
1801  ACATGTGATAGATTTAAAATAAGCAAAAGAAAAACGGAAATCCCATGATGTTTAAGGGGTTTTCGTTTTTTTAGTGGGTGGCTTTTTGTT     1890
       H  V  I  D  L  K  *
1891  TGAAAAACCTTTATAATATACGTTAGTGAAAAAATGTCTATT                                                     1932
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regardless of whether IPTG was added. This catalase activity was about 50 times the combined activity of *E. coli* HPI and HPII (23 U/mg of soluble protein) under the same cultivation conditions. This result indicated that in both DH5α(pAHA1) and DH5α(pAHA2), expression was not dependent on the *lacZ* promoter located on the vector.

Analysis of the catalase nucleotide sequence and the deduced amino acid sequence. The nucleotide sequence of the open reading frame of *kat* was determined. The sequence comprised 1,464 bp and encoded a protein of 488 amino acids with a calculated molecular weight of 55,869. The codon usage was similar to that detected in previously described *L. monocytogenes* genes (22, 34). The low G+C content (41.1%) was typical for *L. seeligeri* DNA (36% [41]). No typical amino-terminal signal sequence was found. The nucleotide sequences upstream of the coding region, which may function as promoter sequences in *E. coli* and which may be fortuitously present, are indicated in Fig. 2B. A −10 box similar to the *E. coli* consensus sequence was detected upstream of the reading frame of the catalase gene (TAAAAT in the *L. seeligeri* DNA at position 283; TATAAT in *E. coli* [21]). A −35 box which shares high identity with the proposed *E. coli* consensus sequence (TTGAAG at position 260 in the *L. seeligeri* sequence; TTGACA in *E. coli* [21]) was also found 17 bp upstream of the proposed −10 box. Furthermore, a putative ribosome binding site present on the isolated *Listeria* DNA is homologous to the 16S rRNA of *L. monocytogenes* (TGGAGG [28]; *E. coli* consensus sequence, AGGAGG) and probably also functions in *E. coli* (22). A potential rho-independent transcription terminator sequence was also detected downstream of the catalase gene (position 1830; free energy, −20.7 kcal/mol [ca. −86.6 kJ/mol]). The assumption that *kat* is monocistronically transcribed was supported by DNA-RNA hybridization experiments with total RNA from *E. coli*(pAHA1) and from *L. seeligeri* (data not shown). The deduced amino acid sequence of *L. seeligeri* catalase was compared with previously published catalase sequences; a high degree of homology and identity was detected with mammalian catalases (Fig. 3, indicated by boxed amino acid residues).

DNA-DNA hybridization experiments. To determine the number of related catalase genes within *L. seeligeri* chromosomal DNA and the degree of homology of *L. seeligeri kat* to chromosomal DNA from other *Listeria* species, we performed DNA-DNA hybridization studies. The 493-bp *Nru*I-*Eco*RV fragment from pAHA1, which contains an internal nucleotide sequence of *kat*, was used as a probe. With chromosomal DNA from *L. seeligeri* digested with six different restriction enzymes, there was only a single hybridizing DNA fragment in each of the digests (Fig. 4B). This result strongly suggests that a single catalase gene of this type is present within the *L. seeligeri* chromosome. These data are in good agreement with the data obtained during analysis of catalase electropherograms of cell lysates from *L. seeligeri*. Regardless of the amount of protein applied to the acrylamide gel, only a single band of catalase activity was detected (see, e.g., Fig. 1A, lane E). Chromosomal DNA from *Listeria* species other than *L. seeligeri* also contained closely related nucleotide sequences. With chromosomal DNA from any *Listeria* species tested, only a single DNA fragment strongly hybridized with the heterologous *Nru*I-*Eco*RV probe (Fig. 4C). This result suggested that closely related catalase genes are present in the other *Listeria* species. To determine whether nucleotide sequences related to the pAHA1 insert DNA are also present in bacteria of other genera, we hybridized *Eco*RI-digested chromosomal DNAs of *Legionella pneumophila*, *Bacillus thuringiensis*, *Acholeplasma laidlawii*, *Mycobacterium tuberculosis*, and *Halobacterium halobium*, in this case using the *Eco*RI insert fragment of pAHA1 as a probe. Fragments of chromosomal DNAs of the first three species hybridized specifically to the DNA probe (Fig. 4A), suggesting that related catalase genes may be present in these species.

Enzymatic studies of *L. seeligeri* catalase. To characterize the enzymatic activity of *L. seeligeri* catalase, we performed activity inhibition assays on dialyzed cell lysates of *L. seeligeri*.

(i) pH dependency of activity. We assayed catalase activity from pH 5.0 to pH 10.0. Catalase activity was 100% from pH 7.0 to pH 10.0, 80% at pH 6.0, and 46% at pH 5.0, indicating a broad pH spectrum for activity, particularly in the neutral and alkaline pH ranges.

(ii) Treatment with 3-amino-1,2,4-triazole. Treatment of *L. seeligeri* cell lysates with 3-amino-1,2,4-triazole, a potential irreversible inhibitor of catalase activity, together with ascorbate, a source of hydrogen peroxide, resulted in a 99% loss of catalase activity (13% inhibition for treatment with ascorbate alone, compared with that for a sample treated equally but without aminotriazole or ascorbate). A sample of twice-crystallized bovine liver catalase (Sigma) was used as a positive control; there was a 98% loss in specific catalase activity after treatment with aminotriazole plus ascorbate and a 29% loss after treatment with ascorbate alone.

(iii) Treatment with a chloroform-ethanol solution. Treatment of typical catalases (17) with a chloroform-ethanol solution does not result in the inhibition of catalytic activity, whereas many other proteins are inactivated (17). In the present study, the specific catalase activity of the cell lysates was 180% after treatment, compared with 100% in the control (treated equally but without the addition of chloroform-ethanol). This increase in specific activity was due to a loss of hydrophilic proteins from the lysates after treatment with the organic solvents and subsequent centrifugation. Cell lysates from *E. coli* UM120 ($HPII^-$ HPI^+) were used as a control. As expected (38), catalase activity in this case was strongly affected by the treatment (8% residual specific activity). These data clearly indicated that *L. seeligeri* catalase, like other typical catalases, is not inactivated by chloroform-ethanol treatment.

FIG. 2. Restriction endonuclease mapping, nucleotide sequence, and predicted amino acid sequence of the catalase gene from *L. seeligeri* (*kat*). (A) Restriction endonuclease map of pAHA1. The positions of the cleavage sites are indicated for *Acc*I (Ac), *Eco*RI (E I), *Eco*RV (E V), *Hin*cII (Hc), *Hin*dIII (Hd), *Nco*I (Nc), *Nde*I (Nd), *Nru*I (Nr), *Pvu*II (Pv), *Sph*I (Sp), and *Stu*I (St). The orientation of the *lacZ* promoter of pTZ18R is shown. (B) Nucleotide sequence analysis of the cloned *L. seeligeri* catalase gene. The sequences which are probably responsible for expression of the recombinant catalase in *E. coli* are underlined (−35 box at position 260; −10 box at position 283; ribosome binding site SD at position 343; and transcriptional start site at position 295, marked +1; the stop codon is marked +; a putative rho-independent transcription terminator sequence is also underlined). The perfect 21-nucleotide inverted repeat which shows high homology to *Fur* protein binding sequences (see Discussion) is indicated by arrows.

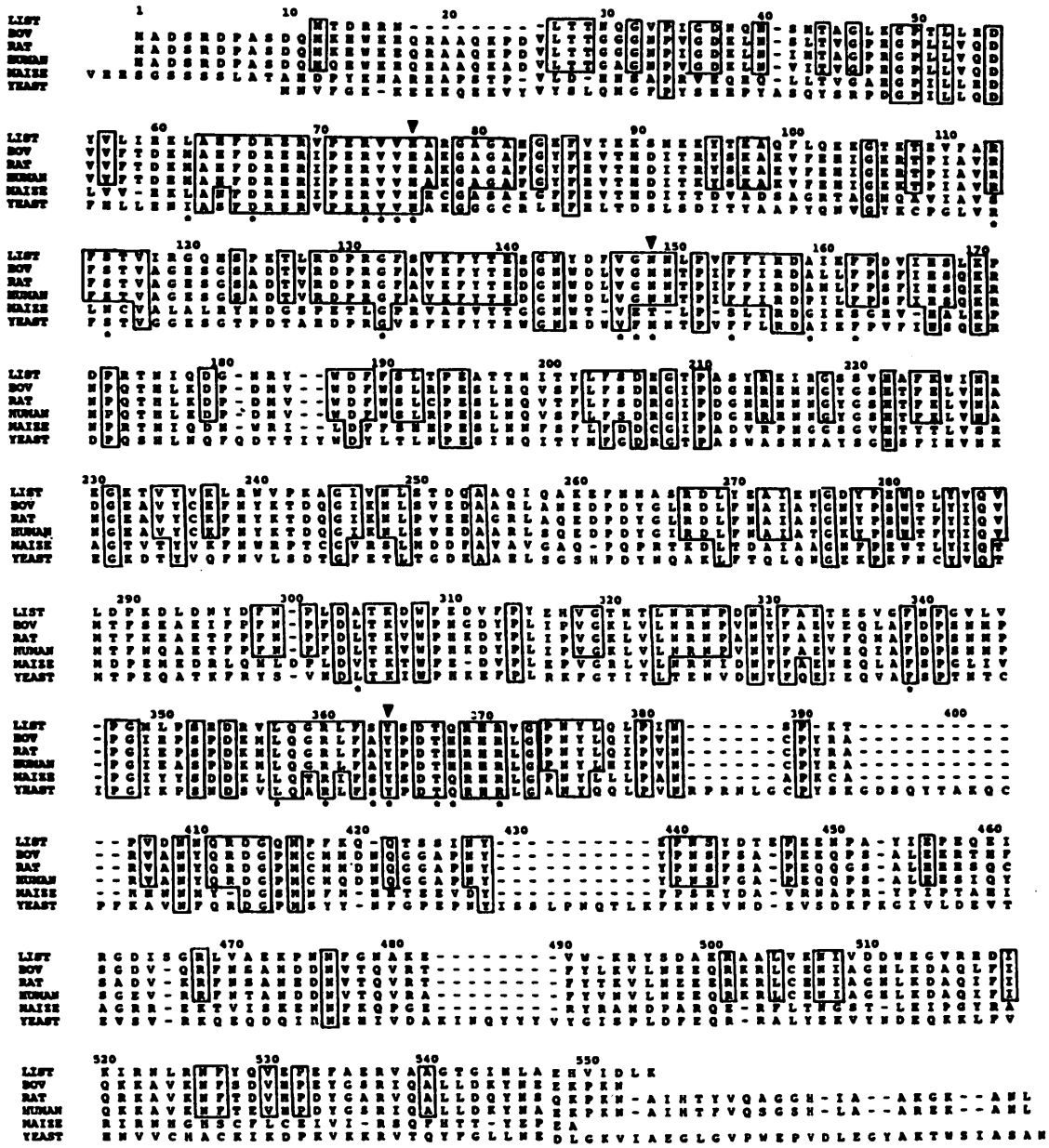

FIG. 3. Comparison of the deduced amino acid sequences of catalases of mammalian, plant, yeast, and bacterial origin. The amino acid sequences of catalases from *L. seeligeri* (LIST), bovine liver (BOV), rats, humans, maize, and yeasts are shown (for all eukaryotic sequences, see reference 3). Amino acid residues which are identical between *L. seeligeri* and mammalian catalase sequences or among all the shown sequences are indicated by boxes. Amino acid residues which are involved in the catalytic activity of the bovine enzyme are marked by a triangle, and those which have been identified to be in close contact with the heme cofactor in bovine catalase are marked by an asterisk.

DISCUSSION

In this study, we present evidence for the cloning of a gene encoding catalase (*kat*) from *L. seeligeri*. For this purpose, we have used an approach similar to that described by Loewen et al. (27) for the cloning of *E. coli katG*. The *L. seeligeri kat* gene was expressed to high enzymatic activities within the gram-negative host, the activity being about 50 times the combined activity of wild-type *E. coli* catalases. The recombinant protein accounted for approximately 2.0% of the total soluble protein. The high expression was most probably directed by regulatory nucleotide sequences present on the cloned *L. seeligeri* DNA, as the enzymatic activity of the gene product in *E. coli* was independent of the orientation of the cloned insert DNA and the induction of the plasmid *lac* promoter by IPTG. Also, nucleotide sequence analysis revealed transcription- and translation-directing se-

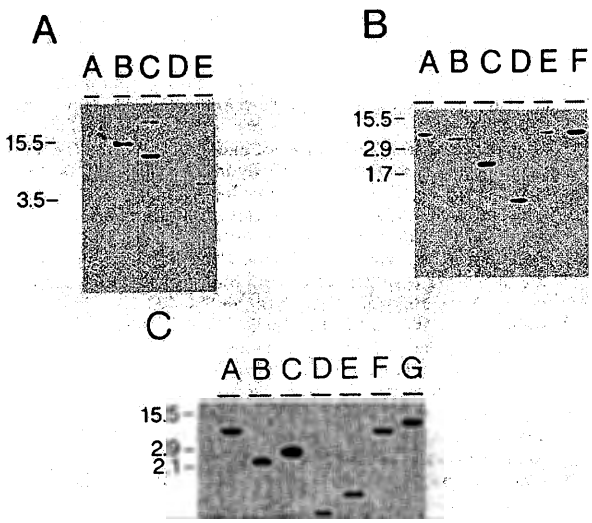

FIG. 4. DNA-DNA hybridization experiments with DNA probes derived from pAHA1. (A) The full-length *Eco*RI insert DNA of pAHA1 was used to probe *Eco*RI-digested chromosomal DNAs from *M. tuberculosis* (lane A), *L. pneumophila* (lane B), *B. thuringiensis* (lane C), *H. halobium* (lane D), and *A. laidlawii* (lane E). (B) Chromosomal DNA from *L. seeligeri* was digested with *Nco*I (lane A), *Nde*I (lane B), *Nru*I (lane C), *Acc*I (lane D), *Pst*I (lane E), and *Bam*HI (lane F). The 493-bp *Nru*I-*Eco*RV fragment of pAHA1 internal to the recombinant catalase gene (Fig. 2A) was used as a probe. (C) Chromosomal DNAs from various *Listeria* species were digested and tested with the same probe as that used in panel B. Lanes: A, *L. innocua* (*Eco*RI); B, *L. grayi* (*Eco*RI); C, *L. welshimeri* (*Eco*RI); D, *L. monocytogenes* (*Eco*RI-*Hin*dIII); E, *L. monocytogenes* (*Hin*dIII); F, *L. monocytogenes* (*Eco*RI); G, *L. ivanovii* (*Eco*RI). The gel wells are indicated by broken lines beneath the lane labels. Numbers at left are kilobase pairs.

quences similar to *E. coli* consensus sequences. Although these nucleotide sequences are not necessarily used in *Listeria* species, they probably direct the strong expression in *E. coli* of *kat*, located on a high-copy-number plasmid (as a pUC18 derivative in approximately 500 copies per cell [43]).

The *kat* gene product has a molecular weight of 55,869, as calculated from the deduced amino acid sequence. This value is in good agreement with the molecular weights previously determined for mammalian catalases (55,000 to 67,500 per subunit [17]).

Our DNA-DNA hybridization experiments revealed that most probably a single catalase gene is present in *L. seeligeri* and presumably also in other *Listeria* species, in agreement with the single catalase activity detected in *L. seeligeri* cell lysates in catalase activity gels. In contrast, there are multiple catalases in the related gram-positive bacterium *Bacillus subtilis* (25). Interestingly, we detected nucleotide sequences homologous to the *Eco*RI insert DNA of pAHA1 (containing *kat*) in chromosomal DNA from bacteria of other genera (*Legionella*, *Bacillus*, and *Acholeplasma*) (Fig. 4C), suggesting that there may be related catalase genes in these bacteria.

For *E. coli*, it has been demonstrated that HPI is present in both the periplasm and the cytoplasm. *E. coli* HPII, however, is a cytoplasmic enzyme (18). Our nucleotide sequence analysis revealed no indication for the presence of a typical export-directing amino acid sequence in *L. seeligeri* catalase. Furthermore, we did not detect catalase activity in culture supernatants of *L. seeligeri*. Thus, *L. seeligeri* catalase is most probably a cytoplasmic protein.

Comparison of the deduced amino acid sequence of *L. seeligeri* catalase with previously reported catalase sequences revealed a high homology of the bacterial enzyme to mammalian catalases (Fig. 3). Identical or highly homologous regions between bovine liver catalase and *L. seeligeri* catalase include (i) the catalytically important residues His-76 and Asn-149; (ii) the ligand for heme iron, Tyr-364 (37); (iii) the heme cofactor binding domain (20 of 25 amino acid residues are identical [14]); and (iv) the amino acid residues which form the entrance of the active center accession channel (three of five residues are identical) and those which form the channel itself (six of eight residues are identical). Also taking into consideration the facts that the number of amino acid residues and the molecular weights are about the same in the bacterial catalase and the mammalian catalases, these data reveal a very high structural conservation of typical catalases during evolution. Homology between *L. seeligeri* catalase and typical eukaryotic catalases is minimal in the amino- and carboxy-terminal regions, as has also been observed for other typical, nonmammalian enzymes (such as *Candida tropicalis* and sweet potato catalases [36, 42]). Comparison of the deduced *L. seeligeri* catalase amino acid sequence with the amino acid sequence for a previously reported "flavodoxinlike domain" of *Penicillium vitale* catalase (33) did not reveal any homology.

The only deduced amino acid sequence published so far for a bacterial catalase (*E. coli* HPI [49]) does not share any amino acid identity or homology or a similar hydrophobicity pattern with those of typical (mammalian) catalases. This fact is not really surprising, as *E. coli* HPI possesses both catalase activity and broad-range peroxidase activity (8). In contrast to *E. coli* HPI (49), *L. seeligeri* catalase was inhibited by 3-amino-1,2,4-triazole which, together with hydrogen peroxide, inhibits typical catalases by interaction with the catalytically important His-76 residue (1, 31). Furthermore, *L. seeligeri* catalase functioned over a broad pH range, was not inhibited by chloroform-ethanol treatment, and did not posses detectable peroxidase activity, all of which are characteristics of mammalian catalases (17). Thus, we concluded that *L. seeligeri* catalase is, both enzymatically and structurally, very similar to mammalian hydroxyperoxidases. Bacterial catalases with structural and enzymatic features of mammalian catalases have been previously described for, e.g., *Micrococcus luteus* (20), *Rhodopseudomonas sphaeroides* (10), and *Rhodospirillum rubrum* (38). These catalases, together with *L. seeligeri* catalase, probably constitute one form of bacterial catalase.

By computer analysis of the determined nucleotide sequence, a 21-nucleotide perfect inverted repeat which showed considerable homology to previously reported ferric uptake regulon (Fur) protein binding nucleotide sequences of *E. coli* was identified upstream of *kat* (6). Such nucleotide sequences have been identified in several gram-negative species (6), and one promoter for a gene from a gram-positive bacterium, the *tox* promoter from *Corynebacterium diphtheriae* (47), has initially been described as being Fur regulated in *E. coli*. A very recent molecular study of *tox* regulation, however, could not confirm this latter datum (5). The possible Fur binding sequence presented here shares more homology with the deduced *E. coli* Fur binding consensus sequence than do some of the *E. coli* genes for which Fur regulation has been suggested or proven. The nucleotide sequence reported here is GATAATGAAGAGATTATT

TTT; the previously reported consensus sequence is GAT(A/T)ATGAT(A/T)AT(C/T)ATTTTC (6). The *L. seeligeri* sequence shows 2 additional nucleotides, in comparison with the *E. coli* consensus sequence (in the corresponding region of the *tox* promoter in *C. diphtheriae*, 10 inserted nucleotides can be detected [47]). This nucleotide sequence homology alone is not sufficient to define the catalase gene as being regulated by a Fur-like protein, but catalase is an enzyme which in most cases includes an iron-centered cofactor, and catalases participate in the prevention of hydroxyl radical formation by the iron-catalyzed Haber-Weiss reaction (16). Thus, catalytic activity may be regulated on a transcriptional basis by the concentration of available iron. By computer analysis, we did not detect nucleotide binding sites for other possible regulatory factors, such as OxyR, which in *E. coli* influences *katG* expression (48).

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ADDENDUM

After submission of this paper, the nucleotide sequence and the derived amino acid sequence of catalase HPII of *E. coli* were published (I. von Ossowski, M. R. Mulvey, P. A. Leco, A. Borys, and P. C. Loewen, J. Bacteriol. **173**:514–520, 1991). The core part of the enzyme (i.e., amino acid residues 57 to 528 of a total of 753 amino acid residues) also showed considerable amino acid sequence similarity to mammalian catalases (e.g., 53.6% with bovine liver catalase; for the *L. seeligeri*-bovine liver catalase pair, we have calculated a similarity of 64.0%).

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