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Synthesis of Species-Specific Stress Proteins by Virulent Strains of *Listeria monocytogenes*†

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Listeriolysin is a virulence factor that appears to be necessary for the intracellular survival of *Listeria monocytogenes*. As shown in this investigation, listeriolysin is produced in only small amounts by clinical isolates of *L. monocytogenes* belonging to the serogroup 1/2a, but its synthesis can be induced by heat shock and to a lesser extent by oxidative stress. In addition to about 15 heat shock proteins that appear to be common to *L. monocytogenes* and *Listeria* species that are nonpathogenic for humans, at least five heat shock proteins are specifically coinduced with listeriolysin in all *L. monocytogenes* strains under heat shock conditions but not in the other *Listeria* species. One type of *L. monocytogenes* mutant blocked in the expression of listeriolysin failed to synthesize several of these specific heat shock proteins.

Listeria monocytogenes belongs to the facultative intracellular bacteria, which are able to replicate in professional phagocytes (macrophages and monocytes) and in nonprofessional phagocytic mammalian cells (3, 10, 15, 18). The intracellular environment, especially that of phagocytes, imposes hostile conditions on invading bacteria. The diverse antibacterial factors include toxic oxidative products, lysosomal and granular proteins and peptides, low pH, and low levels of essential nutrients. It is therefore to be expected that intracellular bacteria such as *L. monocytogenes* require specific products to cope with intracellular stress conditions. The only known factor that is required for survival of *L. monocytogenes* within the mammalian host cell is listeriolysin (LisO). We have recently shown (20) that LisO is efficiently synthesized under heat shock conditions in two different strains of *L. monocytogenes*, both of which belong to the serotype 1/2a.

There is recent evidence that heat shock proteins (HSPs) may play an important role in infections by intracellular bacteria; HSPs are immunodominant antigens in infections by a number of invasive bacteria, including *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Coxiella burnetti*, and *Legionella pneumophila* (19, 21, 23). The analysis of proteins specifically expressed by *Salmonella* species within macrophages has revealed a large set of proteins that includes two major HSPs, DnaK and GroEL (N. Buchmeier and F. Heffron, personal communication).

To study the putative role of stress proteins in intracellular survival of *L. monocytogenes*, we further analyzed the specific stress proteins of *L. monocytogenes*. All tested virulent strains of *L. monocytogenes* that belong to serogroup 1/2a exhibited similar patterns of HSPs, including several proteins that are specific for *L. monocytogenes* and that are not synthesized by nonvirulent *Listeria* species. One of the *L. monocytogenes*-specific HSPs in all tested strains was LisO, which was also efficiently synthesized under oxidative stress conditions.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The wild-type *L. monocytogenes* EGD Sv1/2a strain SLCC 5764 (provided by R. J. North, Trudeau Institute, Saranac Lake, N.Y.; also referred to as strain Mackaness), *Listeria ivanovii* 5 ATCC 19119, *L. innocua* 6a NCTC 11288, and *L. seeligeri* SLCC 3954 were obtained from the *Listeria* strain collection of H. P. R. Seeliger, Hygiene and Microbiology Institute, University of Würzburg, Federal Republic of Germany. The *L. monocytogenes* Sv1/2a strains (referred to as strains 2 through 8) are clinical isolates obtained from different sources. *Listeria* strains were always grown in brain heart infusion (Difco Laboratories) at 37°C with aeration.

Labeling of HSPs. For heat shock experiments, 0.2 ml of an overnight culture was used to inoculate 10 ml of brain heart infusion. Bacteria were grown at 37°C to an optical density at 600 nm of 1.0. After centrifugation (8,000 rpm, 20 min, 4°C), the cell pellet was washed and then suspended in 5 ml of cold methionine assay medium (Difco). Cultures were incubated with shaking for 30 min at 48°C before the addition of 10 μCi of [^{35}S]methionine (specific activity, 800 Ci/mmol) per ml. Labeling was carried out at the same temperature for another 30 min. The cells were harvested by centrifugation (6,000 × *g*, 20 min, 4°C), and the proteins from the supernatant were precipitated with trichloroacetic acid (final concentration, 7%) at 4°C. The cell pellet was washed twice with cold methionine assay medium and incubated with 100 μl of lysozyme (3 mg/ml) for 30 min at 4°C. After centrifugation, the cells were prepared for gel electrophoresis.

H_2O_2 treatment. Overnight cultures were used to inoculate 10 ml of fresh BHI medium, and cells were grown at 37°C to an optical density at 600 nm of 0.4. After centrifugation (8,000 rpm, 20 min, 4°C), the cell pellet was washed and suspended in 5 ml of cold methionine assay medium. Labeling was performed by preincubating the bacteria with 0.5 mM hydrogen peroxide with shaking. After 30 min, hydrogen peroxide was added to an final concentration of 30 mM; incubation was continued for another 10 min without shaking, and then [^{35}S]methionine (10 μCi/ml) was added. The mixture was incubated with shaking for 30 min. Samples were centrifuged (6,000 × *g*, 20 min, 4°C), and the supernatant proteins were precipitated with trichloroacetic acid. The cell pellet was washed in cold methionine assay medium and

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† Dedicated to F. Lingens on the occasion of his 65th birthday.

incubated with lysozyme (3 mg/ml) for 30 min at 4°C. After centrifugation, cells were prepared for gel electrophoresis.

Gel electrophoresis. The extracellular proteins and cell pellet were dissolved in Laemmli sample buffer (12) and heated at 95°C for 5 min. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed by the method of Laemmli (12) in a discontinuous system in 13% acrylamide gels. Gels were stained with Coomassie brilliant blue R250 and destained as described by Laemmli. The gels were then dried and exposed to X-ray film. For two-dimensional gel electrophoresis, the cells were disrupted by ultrasonic treatment, and the lysates were examined by the O'Farrell procedure (17).

Immunoblotting. After SDS-polyacrylamide gel electrophoresis the proteins were transferred from the gel to nitrocellulose filters (BA 85; Schleicher & Schüll) by the semidry electroblotting method of Kyhse-Andersen (11). The nitrocellulose filters were treated with serum from a horse that was hyperimmune to streptolysin O or anti-LisO antiserum as previously described (8) and peroxidase-labeled anti-immunoglobulin G antibodies. The peroxidase reaction was developed with 4-chloro-1-naphthol and H_2O_2.

Isolation of total RNA. Total RNA was isolated by the hot phenol method of Hattman (4). After heat shock and H_2O_2 treatment, the cell pellet was washed with 10 mM Tris hydrochloride (pH 7.6)–25 mM EDTA–150 mM NaCl and suspended in 0.5 ml of lysis buffer (20% saccharose, 20 mM Tris hydrochloride [pH 7.6], 10 mM EDTA, 50 mM NaCl, 10 mg of lysozyme per ml). Bacteria were incubated for 15 min on ice, following by the addition of 0.5 ml of 2% SDS. After shaking for 15 min at room temperature with 100 μl of proteinase K (5 mg/ml), the mixture was frozen on dry ice-methanol and thawed at 45°C three times. The phenol extraction was carried out at 65°C for 5 min, and the aqueous phase was extracted with chloroform. RNA was precipitated with 2 volumes of ethanol and treated with RNase-free DNase (Worthington Biochemicals) as described by von Gabain et al. (22). RNA was suspended in sterile distilled water and quantitated spectrophotometrically by A_{260} measurements. Samples were stored at −70°C.

Analysis of mRNA by dot blotting. RNA was denatured for 5 min at 80°C; 0.7 μg of RNA was diluted in 1:2 and 1:10 serial dilution steps. Each 10-μl sample was mixed with 20 μl of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and spotted onto 20× SSC-pretreated nitrocellulose filters. The nitrocellulose dot blots were baked for 2 h at 80°C, prehybridized for 2 h, and hybridized with labeled DNA probes. DNA probes were labeled with [^{32}P]dATP (specific activity, 3,000 Ci/mmol) by the random priming technique of Feinberg and Vogelstein (2). Nitrocellulose filters were washed in 2× SSC–1.0% SDS at room temperature and in 0.1× SSC–0.1% SDS twice for 30 min at 55°C, and the filters were exposed to X-ray film.

RESULTS

***L. monocytogenes* 1/2a strains synthesize similar patterns of HSPs.** Heat shock provides a simple experimental tool with which to study the induction of stress proteins. *L. monocytogenes* induces the specific heat shock response at a relatively high temperature (Sokolovic, unpublished results). We have chosen 48°C as the optimal temperature. Treatment of the bacteria under these conditions for 60 min results in survival rates of 40 and 60%. Previous data have shown (20) that two *L. monocytogenes* strains of serotype 1/2a, which differ significantly in the amount of LisO produced under normal growth conditions (37°C), synthesize similar amounts of LisO under heat shock conditions. In addition, the other HSPs produced under these conditions are also similar in quality and quantity. We have extended this study to a series of other *L. monocytogenes* strains belonging to serotype 1/2a. All 1/2a strains (Fig. 1A, lanes 1 through 8) synthesized at 48°C a set of HSPs similar to those observed previously (20). This HSP pattern always included the 58-kilodalton (kDa) protein, which was LisO as shown by immunoblotting with anti-LisO antiserum (Fig. 1B). The immunoblot also indicated that all of these 1/2 a strains, with the exception of the reference strain Mackaness 1/2a (lane 1), produced very low levels of LisO at 37°C, which is in agreement with our previous observation (20). Note in the immunoblot at 37°C the reaction of the LisO antiserum with a protein above the LisO band. This protein, termed p60 (9), is highly immunogenic, and the LisO protein fraction used for the preparation of the LisO antiserum was apparently slightly contaminated with p60. The p60 protein is not induced under heat shock

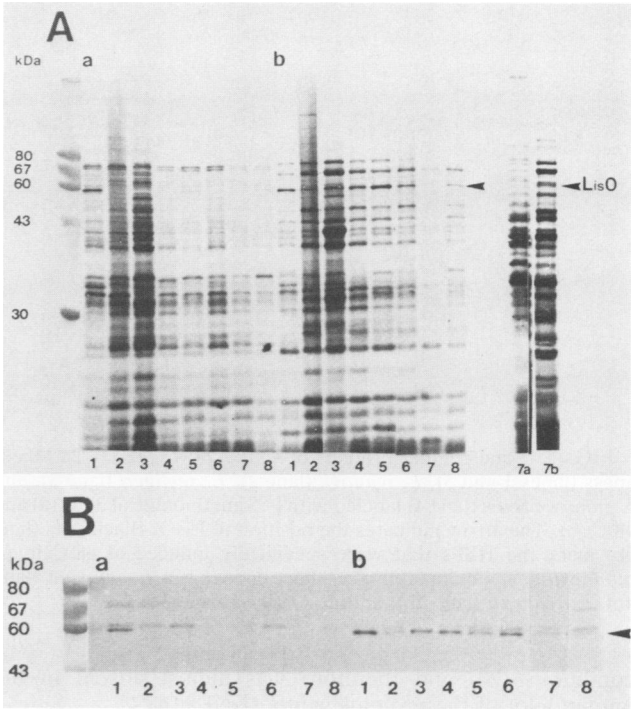

FIG. 1. Proteins synthesized by *L. monocytogenes* isolates at 37°C (a) and 48°C (b). Total cellular proteins were labeled with [^{35}S]methionine and separated by SDS-polyacrylamide gel electrophoresis. (A) ^{35}S-labeled proteins of *L. monocytogenes* 1/2a Mackaness (lane 1) and the protein patterns of different *L. monocytogenes* clinical isolates 2 through 8 of serogroups 1/2a (lanes 2 through 8, respectively). The additional lanes 7a and 7b show a repetition of the heat shock experiment with *L. monocytogenes* 1/2a strain 7, which was not properly labeled in the experiment indicated in lane 7. The position of listeriolysin (58 kDa) is marked by an arrow. (B) Immunoblots of the proteins in the same order as in panel A. Total cellular proteins were labeled with anti-SLO antiserum. Note in lanes 1 through 8 at 37°C (a) the cross-reaction of the anti-listeriolysin antibody preparation used with the p60 protein that migrated slightly above the listeriolysin. The concentration of listeriolysin synthesized at 37°C in the *L. monocytogenes* 1/2a isolates (lanes 2 through 8) was too low to be detected with the antibody used. Only *L. monocytogenes* 1/2a Mackaness (lane 1) produced listeriolysin already at 37°C in amounts that were detectable with the antibody preparation used.

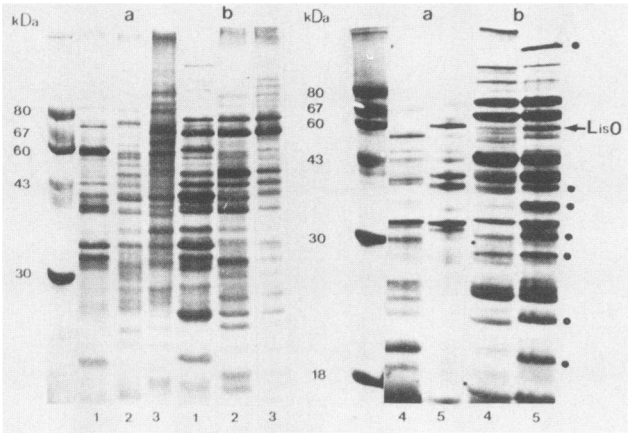

FIG. 2. Total cellular proteins of *L. monocytogenes* 1/2a Mackaness (lanes 1 and 5), *L. ivanovii* (lane 2), *L. seeligeri* (lane 3), and *L. innocua* Sv6a (lane 4) labeled with [^{35}S]methionine at 37°C (a) and 48°C (b). The arrow indicates the position of LisO. Black dots (lane 5b) mark the HSPs that were specifically induced at 48°C in *L. monocytogenes* compared with those induced in *L. innocua* (lane 4b), *L. ivanovii* (lane 2b), and *L. seeligeri* (lane 3b).

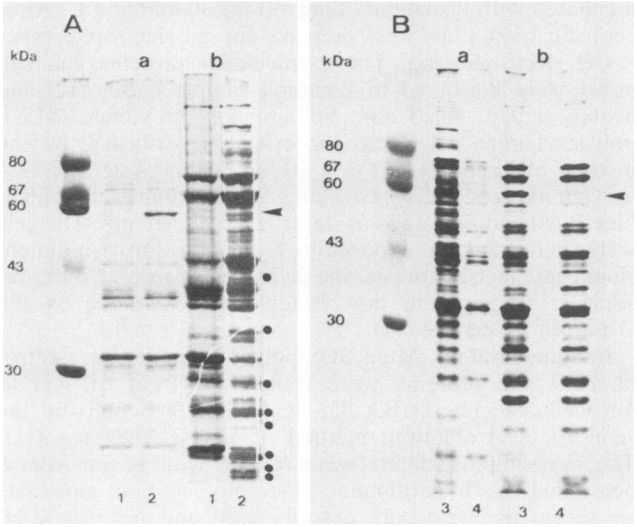

FIG. 3. HSPs synthesized by two types of regulatory mutants of *L. monocytogenes* deficient in the expression of the listeriolysin gene. (A) Pattern of total cellular proteins (labeled with [^{35}S] methionine) of mutant SLCC 53 (lane 1) compared with that of the parental strain 1/2a Mackaness (lane 2) at 37°C (a) and 48°C (b). Black dots mark the proteins that are absent in SLCC 53. The arrow indicates the position of LisO. (B) Pattern of total cellular proteins of Tn*916* mutant M3 (lane 4) compared with that of the parental strain (lane 3) at 37°C (a) and 48°C (b).

conditions (20) and therefore was almost absent in the immunoblot of the proteins synthesized at 48°C.

Species-specific HSPs of *L. monocytogenes*. We next compared the HSP patterns of *L. monocytogenes* 1/2a with those of the two other hemolytic *Listeria* species, *L. ivanovii* and *L. seeligeri* (13), and with a nonhemolytic *L. innocua* 6a strain.

One-dimensional SDS-polyacrylamide gel electrophoresis showed that these three nonpathogenic (for humans) *Listeria* species synthesized fewer HSPs (Fig. 2, lanes 2 through 4) than did *L. monocytogenes* (lanes 1 and 5). Most of these proteins seemed to correspond to HSPs that were also present in the HSP pattern of *L. monocytogenes*, but *L. monocytogenes* synthesized at least six additional HSPs, with sizes ranging from 108 to 17 kDa, that were absent in the other three *Listeria* species (marked with dots in Fig. 2). The HSP patterns of *L. seeligeri*, *L. ivanovii*, and *L. innocua* were remarkably similar. Interestingly, in *L. seeligeri* and *L. ivanovii* strains, which are hemolytic and synthesize LisO-related listeriolysins at 37°C (13), no induction of listeriolysin synthesis occurred under heat shock conditions.

HSPs synthesized in mutants that are blocked in the expression of the listeriolysin gene. Two types of nonhemolytic *L. monocytogenes* mutants were recently characterized (6, 14); neither mutant expresses listeriolysin, although the *hlyA* gene encoding listeriolysin is unaltered. The first mutant type (exemplified by M3) carries a Tn*916* transposon insertion in a noncoding region about 200 base pairs upstream of the start codon of *hlyA*, whereas the other mutant (exemplified by SLCC 53) has suffered a 300-base-pair deletion in an open reading frame located about 1.3 kilobases upstream of the *hlyA* gene (14). Both types of mutants are blocked in the transcription of the *hlyA* gene (14). Since listeriolysin is induced under heat shock conditions, we expected that the second mutant type (represented by mutant SLCC 53, which is a derivative of *L. monocytogenes* 1/2a Mackaness) may be impaired in the synthesis of other HSPs as well. This was indeed the case (Fig. 3A). In addition to listeriolysin there were at least five proteins that were synthesized at a significantly reduced rate under heat shock conditions compared with the rate of the wild-type strain. In contrast, the patterns of proteins synthesized at 37°C in the wild-type and the mutant strains were indistinguishable with the exception of listeriolysin, which in *L. monocytogenes* 1/2a Mackaness is already derepressed at 37°C (20). Two-dimensional gel electrophoresis of the HSPs of wild-type strain *L. monocytogenes* 1/2a, the mutant SLCC 53, and *L. innocua* Sv6a (Fig. 4) further demonstrated the absence of several HSPs in the mutant. All of these proteins were also absent in *L. innocua*, but *L. innocua* lacked even more HSPs than mutant SLCC 53 (Fig. 4). This is obvious when the HSP patterns of the latter two strains are compared with that of the wild-type strain. These data suggest that a putative positive regulator that is defective in SLCC 53 controls a subset of HSPs in *L. monocytogenes* that includes listeriolysin. No difference in the HSP patterns (with the exception of listeriolysin) could be recognized between the wild type and mutant M3, which belong to the other class of regulatory Hly$^-$ mutants (Fig. 3B).

Listeriolysin is synthesized under oxidative stress conditions. Multiple stimuli may regulate the expression of proteins required for the survival of *L. monocytogenes* within macrophages. The presence of oxidants may cause the induced expression of specific gene products that help to overcome this stress. Hydrogen peroxide has been shown to induce a variety of proteins in *Salmonella typhimurium* (16). DnaK protein was reported to be induced by heat and H_2O_2 (16). *L. monocytogenes* strains can tolerate relatively high levels of H_2O_2. However, the resistance to H_2O_2 is strain dependent; the two related strains *L. monocytogenes* 1/2a Mackaness and EGD, both of which belong to serotype 1/2a, differ significantly with respect to survival in the presence of H_2O_2, with 50% lethal doses of 2 and 60 mM H_2O_2, respectively. As expected, a catalase-negative transposon Tn*916* mutant (S. Kathariou, unpublished results) exhibits a significantly reduced resistance against H_2O_2 (50% lethal dose, 0.3 mM H_2O_2). The optimal oxidative stress response for strain

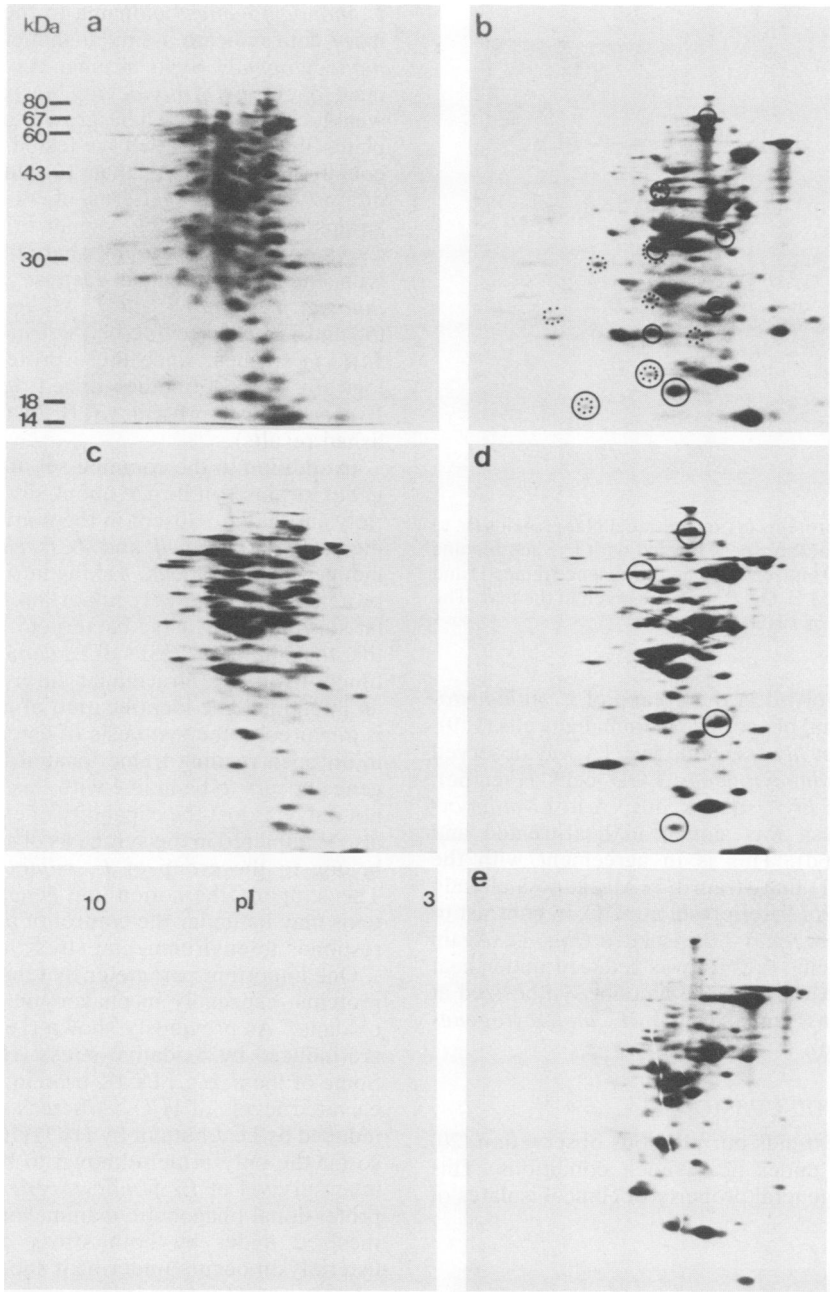

FIG. 4. Two-dimensional gel electrophoresis of total cellular proteins labeled with [^{35}S]methionine at 37°C (a, c) and 48°C (b, d, e) of *L. monocytogenes* 1/2a Mackaness (a, b), mutant SLCC 53 (c, d), and *L. innocua* Sv6a (e). Symbols: ○, HSPs specific for the *L. monocytogenes* wild-type strain and the mutant SLCC 53 when compared with the HSPs of *L. innocua*; ⁘, HSPs absent in SLCC 53 compared with the wild-type strain.

L. monocytogenes 1/2a was obtained by pretreating the bacterial cells with 0.5 mM H_2O_2 during the logarithmic growth phase and challenging these cells with a final concentration of 30 mM H_2O_2 for 40 min. In strain *L. monocytogenes* EGD we could not observe a clear oxidative stress response. Some proteins were induced under the oxidative stress conditions in *L. monocytogenes* 1/2a (Fig. 5a); others continued to be synthesized at slightly elevated rates in the presence of H_2O_2. Listeriolysin belongs to the latter group of proteins, which were still efficiently synthesized and transported under the oxidative stress conditions (Fig. 5). None of the H_2O_2-induced proteins seemed to represent catalase, since the catalase-negative mutant produced the same proteins as the wild-type strain 1/2a Mackaness (data not shown). In contrast to the *L. monocytogenes* 1/2a Mackaness strain, no clear response to oxidative stress could be obtained with *L. monocytogenes* 1/2 EGD.

Dot blot analysis of the *hlyA*-specific mRNA of H_2O_2-treated and untreated cells indicated a slight increase of LisO-specific mRNA under oxidative stress (Fig. 6a). This is in contrast to the synthesis of the p60-specific mRNA, which was significantly reduced under oxidative stress (Fig. 6b). The latter protein is another major extracellular protein of *L. monocytogenes* that is not required for intracellular bacterial

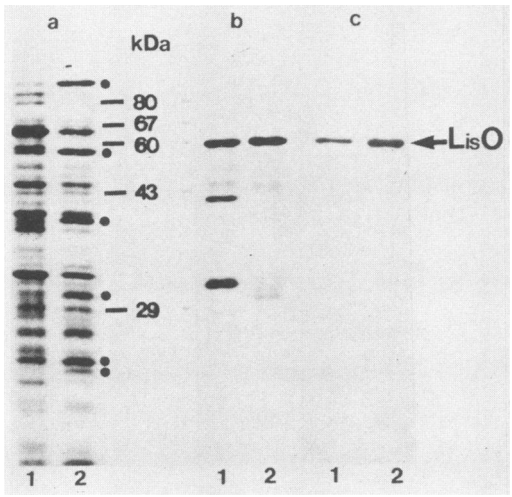

FIG. 5. Total cellular proteins (a) and extracellular proteins (b, c) of *L. monocytogenes* 1/2a Mackaness labeled with [^{35}S]methionine (a, b) or anti-streptolysin 0 antiserum (c) in the absence (lanes 1) and presence (lanes 2) of 30 mM H_2O_2. Details are given in the text. The arrow marks the position of LisO.

growth but may be involved in the uptake of *L. monocytogenes* by nonprofessional phagocytic mammalian cells (1, 9). A significant increase of *hlyA*-specific mRNA was observed in heat-shocked *L. monocytogenes* EGD cells (Fig. 6c), whereas the amount of *hlyA*-specific mRNA in *L. monocytogenes* 1/2a Mackaness was similar in heat-treated and untreated cells (Fig. 6d). This is in agreement with the previous observation (1) that strain 1/2a Mackaness already synthesizes high levels of listeriolysin at 37°C, in contrast to most other *L. monocytogenes* 1/2a isolates (Fig. 1). Again the amount of p60-specific mRNA was reduced under heat shock conditions compared with the amount synthesized at 37°C (Fig. 6e). This was similar in both *L. monocytogenes* strains (data not shown).

DISCUSSION

The present report extends our previous observation (20) that LisO is induced under heat shock conditions. This appears to be a rather general property of clinical isolates of *L. monocytogenes* belonging to the serogroup 1/2a. Preliminary data indicate a similar induction of listeriolysin in *L. monocytogenes* Sv4b strains. As pointed out before (1), most of these *L. monocytogenes* clinical isolates are only weakly hemolytic when grown on standard blood agar plates. In all strains that we tested the amount of LisO was equally induced (more than 10-fold) under heat shock conditions. The HSP patterns of all *L. monocytogenes* 1/2a strains were remarkably similar in the tested strains. These *L. monocytogenes* strains shared a common set of HSPs with the nonpathogenic *Listeria* species tested, e.g., *L. innocua*, *L. ivanovii*, and *L. seeligeri*. The two major HSPs (68 and 75 kDa) seem to be the DnaK and GroEL analogs of *Listeria* species, since these proteins cross-react immunologically with antibodies raised against the corresponding *Escherichia coli* proteins (S. Brühl and Z. Sokolovic, unpublished results).

In addition to the common set of HSPs, the *L. monocytogenes* strains synthesize about seven HSPs, including listeriolysin, that are absent in the nonvirulent species. Interestingly, in *L. ivanovii* and *L. seeligeri* listeriolysin is not induced by heat shock. Taking into consideration that stress proteins are most likely important factors for the intracellular survival of invasive bacteria (5, 19, 21, 23), it is possible that the additional HSPs of *L. monocytogenes* serve specific functions in the intracellular survival of the bacteria during an infection. The identification of a nonvirulent mutant that is impaired in the synthesis of listeriolysin due to a deletion in an open reading frame located far upstream of the *hlyA* gene appears to be in line with this assumption. This mutant not only has lost the capability of expressing listeriolysin but also is impaired in the synthesis of several HSPs, all of which belong to the group of *L. monocytogenes*-specific HSPs. This supports the notion that genes determining stress proteins may be under the control of a common element(s) that responds to environmental stress factors.

One important parameter that may induce bacterial stress proteins, especially in phagocytic cells, is the presence of oxidants. As previously shown (16), several stress proteins are induced by oxidative stress (H_2O_2) in *S. typhimurium*. Some of them, e.g., DnaK protein, are induced by heat and elevated levels of H_2O_2, whereas others, e.g., GroEL, are induced by heat but not by H_2O_2 (16). Listeriolysin, which is so far the only protein known to be necessary for intracellular survival of *L. monocytogenes* in phagocytic and nonprofessional phagocytic mammalian cells, is efficiently synthesized under by both stress conditions. Induction of listeriolysin occurs under heat shock conditions and also to a lesser extent under oxidative stress conditions.

Increased levels of *hlyA*-specific mRNA suggest that induction occurs at the transcriptional level. This contrasts with the significant decrease in the level of mRNA and the amount of protein p60. This protein appears to be necessary for the uptake of *L. monocytogenes* by nonprofessional phagocytic host cells but not for intracellular survival (9). The fact that listeriolysin is efficiently synthesized under both stress conditions, heat and H_2O_2, underlines the central role of this toxin in intracellular survival.

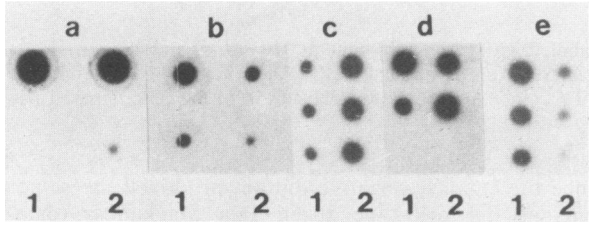

FIG. 6. Concentration of LisO-specific mRNA (a, c, d) and p60-specific mRNA (b, e) determined by dot blot analysis under H_2O_2 (a, b) and heat shock (c, d, e) conditions. Lanes: 1, control (without H_2O_2 or incubation at 37°C); 2, stress condition (with 30 mM H_2O_2 or incubation at 48°C). Equal concentrations of RNA were dotted on nitrocellulose filters and hybridized with ^{32}P-labeled *hlyA*-specific (a, c, d) and p60 gene-specific (b, e) DNA probes: LisO-specific RNA of *L. monocytogenes* 1/2a Mackaness (a, d), p60-specific RNA of *L. monocytogenes* 1/2a Mackaness (b, e), LisO-specific RNA of *L. monocytogenes* 1/2a EGD (c). Dilution steps: 1:10 (a, b) and 1:2 (c, d, e).

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