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Synthesis of Listeriolysin in *Listeria monocytogenes* under Heat Shock Conditions

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Listeriolysin, produced by all virulent *Listeria monocytogenes* isolates, is an essential virulence factor which appears to be necessary for the intracellular survival of these bacteria. It has been postulated that the intracellular environment imposes stress conditions similar to heat shock on invading bacteria. We show here that listeriolysin was still very efficiently synthesized in one *Listeria monocytogenes* strain even intracellularly and induced under heat shock conditions in another *L. monocytogenes* strain. Listeriolysin appears to be the only major extracellular protein synthesized under heat shock conditions; all other heat shock proteins remain cell associated.

Listeria monocytogenes is a facultative intracellular bacterial pathogen which can cause severe infections (septicemia and meningitis) in immunocompromised adults and neonates. Although the natural mammalian host cells for *L. monocytogenes* appear to be the professional phagocytes (macrophages and monocytes) (12), virulent strains of this microorganism can also penetrate nonprofessional phagocytic cells, such as mouse fibroblasts (3T6 cells) or enterocytelike cells (Caco-2 cells) (4, 9, 13). Recently, evidence has been provided for the involvement of an extracellular protein (p60) of *L. monocytogenes* in the penetration step (10). Intracellular survival of *L. monocytogenes* appears to require synthesis of listeriolysin, since transposon-induced listeriolysin-negative mutants are unable to survive within macrophages and nonprofessional phagocytic cells (4, 9). These data suggest that listeriolysin is produced in the intracellular environment. In contrast, the extracellular protein p60 does not seem to be required for intracellular survival, as mutants unable to synthesize p60 penetrate mouse fibroblasts when treated with p60 outside of the mammalian cells and survive within these cells after penetration. It has been postulated that the intracellular environment which the penetrating bacteria encounter (low pH, presence of H_2O_2 and other oxidating agents) may impose a stress on the bacterial cells to which the bacteria may react by inducing stress proteins, e.g., heat shock proteins. Indeed, recent experimental data provide circumstantial evidence for this assumption, as it has been shown that some heat shock proteins represent important antigens for B- and T-cell responses during infection (15, 16).

We have analyzed the heat shock proteins of *Listeria monocytogenes* by labeling proteins synthesized at 48°C with [^{35}S]methionine. Labeling was performed by first incubating the bacteria in methionine assay medium (Difco) for 30 min at 48°C prior to the addition of [^{35}S]methionine (11). Labeling was carried out at the same temperature for another 30 min. Proteins from the supernatant were precipitated with trichloroacetic acid. This extracellular protein fraction and the pool of cellular proteins (prepared by lysozyme treatment) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In addition to the two *L. monocytogenes* SV1/2a wild-type strains (8), EGD strain SLCC5764 (provided by R. J. North, Trudeau Institute, Saranac Lake, N.Y.; also referred to as strain Mackaness) and the EGD strain provided by S. H. E. Kaufmann, Ulm, Federal Republic of Germany (hereafter referred to as the EGD Strain), two nonhemolytic transposon mutants (M3 and M20) derived from strain Mackaness (7) were included in this study. As shown previously (8), the two *L. monocytogenes* wild-type strains differ considerably in the amount of listeriolysin which they synthesize at the normal growth temperature. Nevertheless, the virulence of both strains is comparable in the mouse model.

Proteins of both *L. monocytogenes* wild-type strains were labeled at 37°C (Fig. 1A, lanes a and b) and at 48°C (Fig. 1A, lanes c and d), and total cellular proteins were separated by SDS-PAGE. The proteins synthesized at 48°C were remarkably similar in both strains. These 12 to 14 heat shock proteins ranged in size from 20 to 120 kilodaltons (kDa). The patterns of proteins labeled at 37°C differed in several bands between the two strains. One major difference was observed in a protein migrating at 58 kDa which was synthesized in a relatively large amount in strain Mackaness but not in strain EGD (Fig. 1A, lanes a and b). On the other hand, at 48°C a protein of the same molecular mass was synthesized in both strains in comparable concentrations. As shown previously (3, 7), listeriolysin has a molecular mass of 58 kDa. To identify the 58-kDa protein as listeriolysin, the proteins synthesized at 37 and 48°C were immunoblotted after electrotransfer on nitrocellulose filters, with antibodies against streptolysin O (SLO), which, as shown previously (3, 7), cross-react with listeriolysin. In addition, the extracellular proteins of both strains labeled at 48°C were also immunoblotted with anti-SLO antibodies. The results shown in Fig. 1B clearly demonstrate that the 58-kDa protein was listeriolysin and indicate that listeriolysin production was heat-induced in strain EGD, whereas similar amounts of listeriolysin were synthesized at 37 and 48°C in strain Mackaness.

Listeriolysin and p60, apparently involved in the virulence of *L. monocytogenes* (9, 10), are produced by all virulent *L. monocytogenes* strains. The two proteins are difficult to separate by SDS-PAGE. The distinction of these proteins is achieved unambiguously, however, by the two types of listeriolysin-negative mutants M3 and M20, both of which produce normal amounts of p60 but no 58-kDa listeriolysin protein. As expected, p60 was labeled at 37°C in the wild-type strain Mackaness and the two mutants, and p60 was

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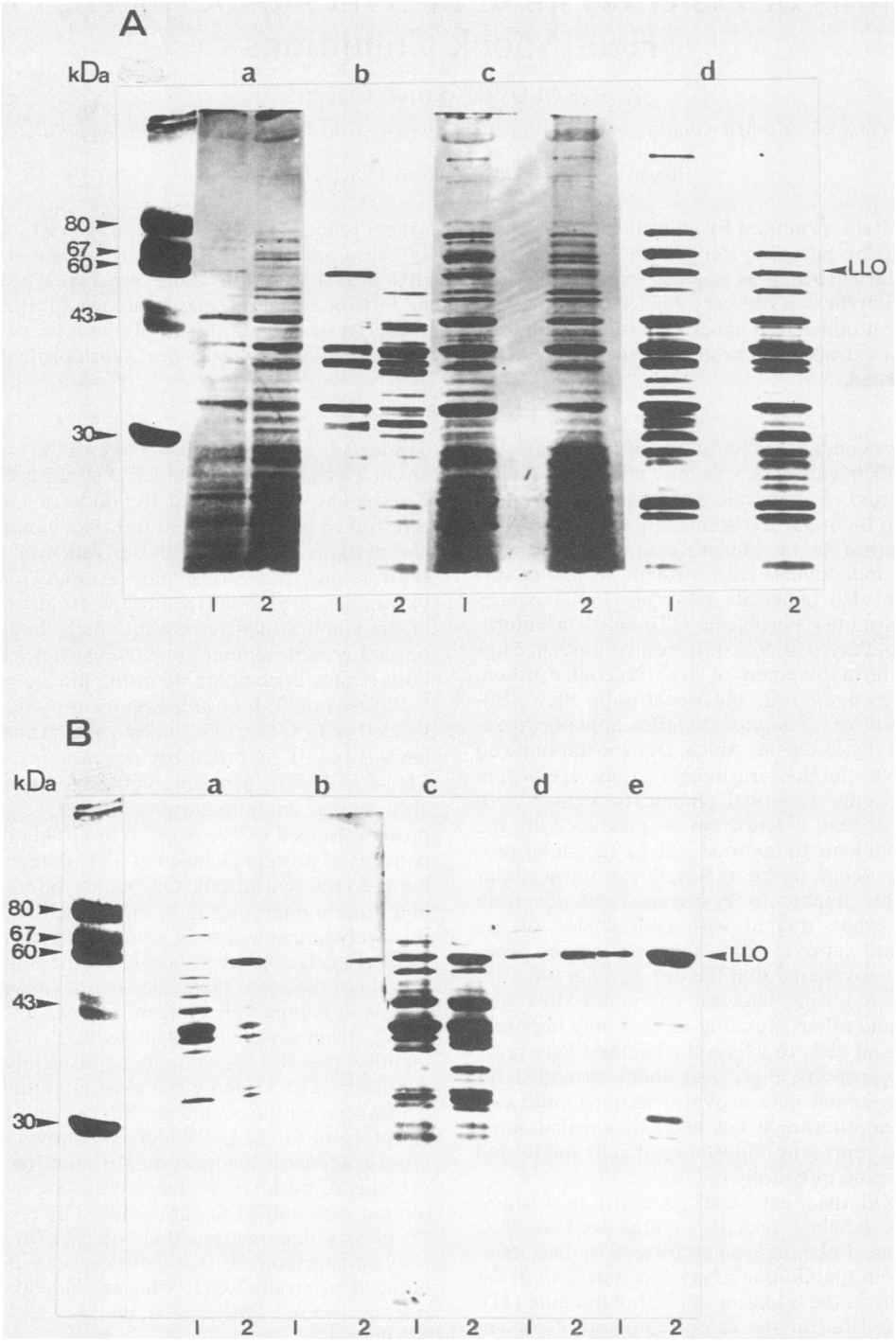

FIG. 1. Proteins of *L. monocytogenes* Sv1/2a Mackaness (lanes 1) and *L. monocytogenes* Sv1/2a EGD (lanes 2) synthesized at 37°C (a,b) or 48°C (c,d). Proteins were labeled with [^{35}S]methionine under these conditions (b,d). (A) Lane a shows total cellular proteins from cells grown at 37°C separated by SDS-PAGE and stained with Coomassie; lane b shows the same after autoradiography of the ^{35}S-labeled proteins; lanes c and d show the same protein profiles from cells grown and labeled at 48°C. Arrow marks the position of listeriolysin (LLO). Size markers and their molecular masses are shown on the left. (B) Lane a contains ^{32}P-labeled cellular proteins from bacterial cells grown at 37°C after electrotransfer to nitrocellulose filters, and lane b shows the same proteins after immunoblotting with anti-SLO antibodies; lanes c and d show the same for proteins labeled at 48°C, and lane e shows an immunoblot of the extracellular proteins labeled at 48°C and developed with anti-SLO. LLO marks the position of listeriolysin. Proteins from *L. monocytogenes* Sv1/2a EGD (lanes 1) and from *L. monocytogenes* Sv1/2a Mackaness (lanes 2) were run.

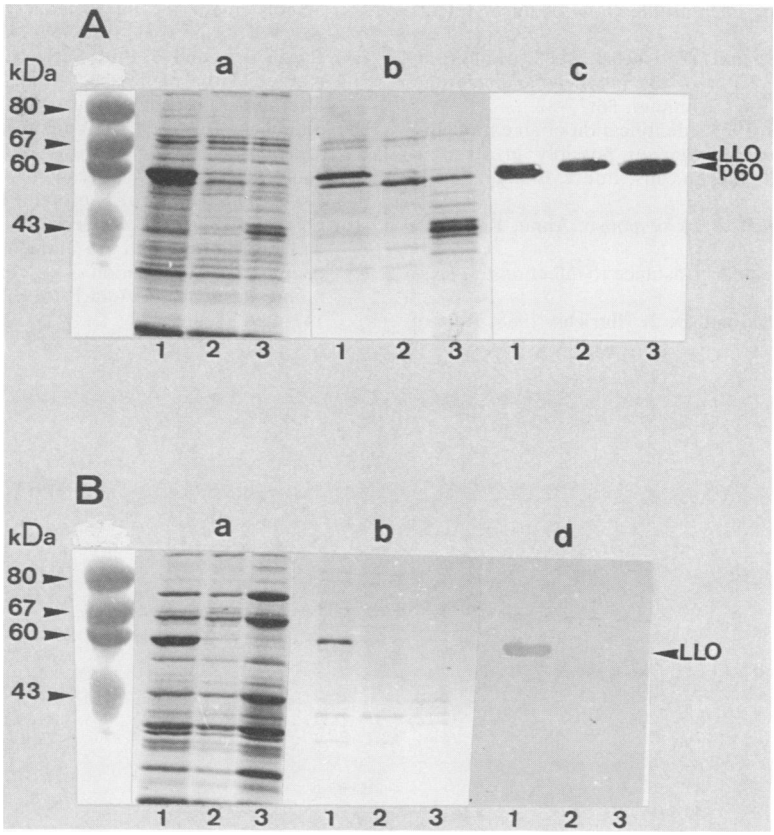

FIG. 2. Listeriolysin (LLO) and p60 from *L. monocytogenes* Sv1/2a Mackaness (lanes 1) and its Hly$^-$ mutants M3 (lanes 2) and M20 (lanes 3) synthesized at 37°C (A) and 48°C (B). Lanes a show total cellular proteins from the three listerial strains after autoradiography, lanes b show the extracellular proteins after autoradiography, and lanes c show immunoblots of the extracellular proteins developed with antibodies against p60. Note that the migration of the two proteins in this gel was altered compared with other reported patterns, in which p60 migrated ahead of listeriolysin (10). Lanes d show an immunoblot of extracellular proteins from the three strains labeled at 48°C and developed with anti-SLO. Size markers and their molecular masses are indicated on the left.

clearly identified in the supernatant of the three strains by immunoblotting with p60-specific antibodies (Fig. 2, lane c). At 48°C, p60 was no longer synthesized, but listeriolysin (58 kDa) was still produced in the wild-type strain but not in the two mutants (Fig. 2B). Mutant M3 synthesizes no listeriolysin (7), whereas M20 produces a truncated protein (7), which was visible at 37°C (Fig. 2A, lanes a and b) but absent at 48°C (Fig. 2B, lanes a, b, and d), suggesting that the truncated mutant listeriolysin is quickly degraded at the elevated temperature. It is also interesting that p60, which is a potent antigen of all virulent *L. monocytogenes* strains (Sokolovic and Goebel, unpublished results), had already stopped synthesis at 43°C (data not shown). This is in line with the assumption that p60 is only required for the penetration step, e.g., in the extracellular environment, where stress conditions are probably not imposed on the bacteria. Heat shock proteins, on the other hand, may support survival of bacteria in a hostile environment in several ways, e.g., by preventing unfolding of essential protein structures (1, 2) or inhibiting hydrolytic activities.

Apparently, listeriolysin is also still synthesized or even induced under stress conditions and may help the bacteria to escape the hostile phagosomal compartment by lysing the phagosomal membrane. It is known that *L. monocytogenes* is unable to multiply at low pH (5), which it encounters in the phagosome, whereas the cytoplasmic environment may provide more suitable growth conditions (higher pH, nutrients, iron, etc.) for multiplication of these bacteria.

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