Production of a New Extracellular Cytotoxin from Listeria monocytogenes Serotype 4b and ATCC 15313 Serotype 1/2a in Relation to Growth Stage and Growth Temperature

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(Received for publication September 9, 1991)

ABSTRACT

Both virulent (4b) and avirulent (ATCC 15313;1/2a) strains of Listeria monocytogenes synthesize a previously unrecognized extracellular cytotoxin over a broad temperature range of 4-37°C. The highest level of cytotoxin production was observed during extracellular cytotoxin over a broad temperature range of 4-37°C. of Listeria monocytogenes milk, at the same rate as in the semisynthetic growth medium. production was detected in early to mid-log phase. The cytotoxin was stable in the growth medium at both 37 and 4°C. Both strains synthesized the cytotoxin at 4 and 10°C in either whole or skim milk, at the same rate as in the semisynthetic growth medium.

The presence of Listeria monocytogenes in a wide variety of foods has become of great concern during the last decade (2,4,11,13) and confirmed outbreaks of human listeriosis due to the consumption of contaminated foods have been reported (6,9,15). L. monocytogenes is one of the foodborne pathogens that has a very wide growth temperature range (1-44°C) (77); hence, contamination of refrigerated foods could represent a significant health hazard. Bacterial cultures that may play a role in infection have been reported (6,9,15). L. monocytogenes is one of the foodborne pathogens that has a very wide growth temperature range (1-44°C) (77); hence, contamination of refrigerated foods could represent a significant health hazard (3,5,6,14,20).

We recently isolated and characterized a new extracellular cytotoxin from both virulent and avirulent strains of L. monocytogenes that may play a role in infection (17). Our objective was to examine the influence of growth temperature and population density on the production of the cytotoxin. Such studies are important since the psychrotrophic properties of the organism are known to affect its pathogenicity and epidemiology (1,10,19), in particular in dairy based products. Further, many foods carry low numbers of the organism; thus, an evaluation of population density in relation to cytotoxin production is crucial.

MATERIALS AND METHODS

Bacterial cultures

Listeria monocytogenes serotype 4b was originally isolated from the spinal fluid of a listeric man (V.A. Hospital, Gainesville, FL). The avirulent, nonhemolytic L. monocytogenes strain ATCC 15313, serotype 1/2a (12) was obtained from the American Type Culture Collection (Rockville, MD). Both strains were stored at 4°C on tryptafavin nalidixic acid agar (16) with periodic subculturing. The virulence of each strain was determined as described previously (17).

Growth stage and temperature

Cytotoxin production in relation to the growth stage was determined at four temperatures (4, 10, 25, and 37°C). One-liter volumes of proteose peptone #3 broth (PP #3) (17) were equilibrated at each of the four different temperatures for 5 h. The medium was then inoculated from an overnight culture grown at 37°C without agitation, with either L. monocytogenes 4b or 15313, to a final concentration of 10^6 cells/ml and incubated at the respective temperature. Samples were taken in relationship to the incubation temperature as follows: every 2 h at 25 and 37°C, every 24 h at 10°C, and every 72 h at 4°C. At each sampling time the absorbance at 600 nm was measured using a Beckman DU7 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA), and the pH and cytotoxin levels were also determined. The absorbance at 600 nm was chosen because of the lowest background reading (wavelength scan from 400-700 nm). PP #3 broth is yellow and therefore strongly absorbs in the 400-500 nm range. The number of CFU at each sampling time was also determined to construct a growth curve. Tenfold serial dilutions were made in 0.1% peptone water and 100-μl aliquots of the appropriate dilutions were plated in duplicate on tryptose agar (Difco Laboratories, Detroit, MI) and incubated at 37°C for 24 h. The pH was determined using a Coming (Fisher Scientific, Springfield, NJ) 130 pH meter. Cytotoxin production at a particular population density was determined using 20-ml samples from each sampling time. Each sample was centrifuged at 27,000 x g for 15 min at 4°C to remove the cells. The supernatant was then taken to 50% ammonium sulfate saturation for 1 h at 4°C with constant stirring, followed by centrifugation at 27,000 x g for 15 min at 4°C. The pellet was resuspended in 1 ml of 50 mM potassium phosphate buffer, pH 7.0 (PB50) and dialyzed in Spectropore (Fisher) tubing (MW cutoff 3,500) against two changes of PB50 for 90 min at 4°C. The protein concentration of the dialyzed sample was estimated as described (8). All dialyzed samples were sterile filtered through 0.22-μm acrodisc filters (Gelman Sciences, Ann Arbor, MI) and assayed for their level of cytotoxicity using 1 x 10^6 Chinese hamster ovary cells as previously described (17). For the growth/temperature experiments the results are the averages of three independent experiments, with each experiment performed with duplicates (threefold replications).
Cold incubation (shock) and milk studies

One-liter volumes of sterile PP #3 broth were allowed to equilibrate for 12 h at either 4°C or 37°C. Overnight cultures of either 4b or 15313, grown in PP #3 at 37°C, were used to inoculate the preincubated media to a final concentration of 10⁶ cells/ml. Thus, the cells transferred to the 4°C preincubated flask received a cold shock, while those transferred to 37°C did not. The cultures were then incubated at 37°C without agitation and allowed to grow until the OD₆₀₀ reached 0.45. The 4°C preincubated culture took approximately 1 h to reach 37°C. All experiments were threefold replicated.

The synthesis of cytotoxin was examined in both sterile skim and whole milk, using the same procedures described previously. Growth rates and cytotoxin synthesis were examined at 4 and 10°C for both 4b and 15313. All experiments were threefold replicated.

RESULTS AND DISCUSSION

Effect of temperature on growth and cytotoxin production

Upon incubation at 37°C in PP #3 semisynthetic broth, the 4b and 15313 strains reached stationary phase after 16 and 20 h, respectively, with doubling times of 100 and 90 min. From an initial pH of 7.5, small incremental decreases in pH occurred during growth until the cell concentration was 10⁶/ml, after which the pH dropped to a final value in the range 5.0-5.2 (Fig. 1). The change in pH versus cell density was similar to Fig. 1, for all temperatures examined. The cytotoxicity of the culture medium in relation to the population density of the two strains are distinctly different (Fig. 2a). Both strains show slight cytotoxin production in early-log phase growth which was unexpected. Increased cytotoxin synthesis began at 10⁵ CFU/ml for 4b and 10⁶ CFU/ml for 15313, and 10⁶ CFU/ml for 4b. The greatest level of cytotoxicity was observed in stationary phase and was higher in 15313 (72% cell death) when compared to 4b (42% cell death).

At 25°C, 4b and 15313 strains reached stationary phase after 22 and 38 h, respectively, with doubling times of 180 and 215 min. The changes in pH in relation to population density and absorbance at 600 nm were the same as for 37°C. Cytotoxin synthesis showed a similar general pattern to that for 37°C (Fig. 2b). However, cytotoxin production by 4b was observed at 10⁵ CFU/ml, that is, one log of growth earlier to that at 37°C. Cytotoxin synthesis by 15313 was similar to that at 37°C. Both strains showed maximum cytotoxicity at stationary phase, and again, there was a small production of cytotoxin in early-log phase which disappeared.

At 10°C, both the 4b and 15313 strains reached stationary phase after 7 d, with a doubling time of approximately 15 h. The changes in pH in relation to population density and absorbance at 600 nm showed similar patterns as for 25 and 37°C, however, cytotoxin synthesis was very different. For 15313, the highest cytotoxicity was detected in very early-log phase (10⁶ CFU/ml), with cytotoxicity gradually decreasing until stationary phase, at which time cytotoxicity increased (Fig. 2c). The 4b strain showed a low level of cytotoxin production starting in early-log phase growth (10⁵ CFU/ml) which remained relatively constant throughout the rest of the growth cycle. For both 4b and 15313, the highest cytotoxin levels detected were low (15% cell death), compared to that obtained at 25°C.

At 4°C, both 4b and 15313 showed an extended lag phase for approximately 20 d, with a cell doubling time of approximately 4 d. The changes in pH showed a similar pattern to that for growth at 25 and 37°C. Cytotoxin production for both 4b and 15313 was again different to that previously observed. Cytotoxicity for 4b was high in early-lag phase but was significantly reduced until early-log phase growth (10⁶ CFU/ml). At this stage, cytotoxicity increased very briefly and returned again in late-log phase (10⁶ CFU/ml) (Fig. 2d). Strain 15313 did not show early-lag phase cytotoxicity as was observed at 10°C. However, this discrepancy may be due to sampling time differences, 72 h at 4°C compared with 24 h at 10°C. Cytotoxin production by 15313 was not detected until mid-log phase growth.

Cold/warm shock: Cytotoxin production in milk

Under the conditions used in this study, cold shock did not stimulate cytotoxin synthesis in the 4b strain. This strain appears to prefer a continuous warm incubation for cytotoxin expression. However, in strain 15313, cytotoxin synthesis was stimulated by the cold shock (data not shown). Results from the inoculated milk study indicated that cytotoxin was synthesized by both strains at 4 and 10°C. Cytotoxin synthesis in both milk types was comparable to cytotoxin synthesis in PP #3 media.

The results from this study suggest that the conditions for cytotoxin production for the two different L. monocytogenes strains, virulent 4b and avirulent 15313, have some similarities. Both strains produce cytotoxin over a broad temperature range 4-37°C. The highest level of cytotoxin production was observed during stationary phase. However, for all growth temperatures, cytotoxin production was detected in early- to mid-log growth. The spike in cytotoxin production during early-lag phase was consistent in all experiments and cannot at present be explained. Strain 15313 could be stimulated by cold incubation to produce cytotoxin, while 4b was stimulated by steady warm temperatures. The different origins of the two strains may
account for this unusual observation. The 4b strain was recently isolated from a clinical patient and might be adapted to mammalian body temperatures. Strain 15313 could be considered a laboratory strain due to its multiple transformations on cultivation media and prolonged cold storage at 4°C.

Previously, we noted that the isolated and partially purified cytotoxin produced at 37°C was cold labile (17). This observation does not, however, hold true for cytotoxin production at refrigeration temperatures, where the cytotoxin appears to be very stable for extended periods. This suggests several alternatives; possibly the cytotoxin produced at 37°C is slightly different to that produced at 4°C. That is, the active site remains the same; however, the temperature stability is altered. There is some evidence that temperature can affect translation of the mRNA so that miscoding occurs (7,18). Alternately, if the cytotoxin is composed of a number of different subunits, as is suggested by our previous observations (17), then different combinations of subunits may affect temperature stability. A further possibility is that during purification, the cytotoxin loses a cofactor which stabilizes the molecule at low temperature. However, this would appear incongruous, since the cytotoxin is still highly thermostable after purification.

That the cytotoxin can be produced by low levels of both virulent and avirulent strains over a broad range of temperatures, may be of potential concern, since many foods contain low levels of both virulent and avirulent \textit{L. monocytogenes} (2). However, the problem is that the role or significance of the cytotoxin in infection is, at present, unresolved. We have no evidence that a disease can be caused by ingestion of preformed toxin, or that the cytotoxin is produced in vivo during infection. There are several areas we are examining in further work. First, is the cytotoxin produced by most, if not all \textit{L. monocytogenes} strains, and is this cytotoxin a means to discriminate between pathogenic and nonpathogenic \textit{Listeria}. We feel this is a worthwhile pursuit, since there have been a number of questions relating to what are the true indicators of virulence in \textit{Listeria}. Second is an examination of cytotoxin expression at the molecular level, which may help in elucidating its method of induction.
REFERENCES