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# Influence of Environmental Parameters on Phosphatidylcholine Phospholipase C Production in *Listeria monocytogenes*: a Convenient Method To Differentiate *L. monocytogenes* from Other *Listeria* Species

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**The ability to produce phosphatidylcholine phospholipase C (lecithinase) is associated with virulence in pathogenic species of *Listeria*. Levels of production vary greatly among members of the genus, and this virulence factor is not readily detectable in many members of the pathogenic species on conventional agar media containing egg yolk, a common substrate for the enzyme. In this study, the influence of a variety of environmental parameters, including temperature, pH, and salt concentration, on the production of lecithinase by a number of strains was evaluated. Lecithinase production by *Listeria monocytogenes* LO28 in brain heart infusion medium was optimal at 1.75 to 2.0% NaCl, pH 7.0 to 7.3, and 37 to 40°C, and the presence of oxygen had no effect. In a chemically defined medium, the optimal NaCl concentration and temperature were lower at 0.75 to 1.0% NaCl and 33.5°C. As detection of virulence factors is useful to assist in the identification and differentiation of *Listeria* species, this report shows that lecithinase activity can conveniently be detected within 36 h on a relatively inexpensive medium. Under the conditions described, *L. monocytogenes* could be distinguished from other members of the genus as a result of distinct lecithin degradation which was not evident in *L. innocua*, *L. seeligeri*, *L. ivanovii*, *L. welshimeri*, or *L. murrayi/grayi*.**

The genus *Listeria* is divided into six species among which *Listeria monocytogenes* is recognized as a food-borne pathogen of major significance (10, 13). The remaining species, *L. ivanovii* (an animal pathogen), *L. seeligeri*, *L. welshimeri*, *L. innocua*, and *L. grayi*, are considered non-pathogenic for humans (16). *L. monocytogenes* is widely distributed in the environment and has commonly been isolated from foodstuffs, in which it can survive and multiply. Its ability to grow at refrigeration temperatures, at pH values from 5 to 9, and at salt concentrations up to 10% renders it a considerable threat to public health, as consumption of contaminated foods can result in serious disease (listeriosis). Outbreaks of listeriosis have been associated with a variety of foods including meat products, raw vegetables, coleslaw, and milk and dairy products (13).

In recent years, much effort has been focused on the genetic analysis of the virulence locus in *Listeria* spp., and this, in combination with the exploitation of tissue culture models of infection, has resulted in the characterization of several virulence factors in the genus (4, 11, 15, 24). However, there is a lack of information pertaining to the influence of environmental parameters on the activity of many enzymes associated with virulence. In *L. monocytogenes*, lecithinase is necessary for the breakdown of the two plasma membranes that surround the bacterium after cell-to-cell spread in the human host during infection (24). Like all zinc metallophospholipases C, it is synthesized as a single polypeptide and posttranslationally activated, after export from the cell, by the removal of 27 N-terminal amino acids by the action of another virulence factor, the zinc metalloprotease (22, 26, 28). Levels of lecithinase production vary greatly among strains, and the enzyme is not readily detectable in many members of the pathogenic species on conventional agar media containing egg yolk (12). The properties of lecithinase have been described by Geoffroy et al. (14). This work examines the impact of temperature, pH, oxygen, and salt concentration on production and detection of this enzyme. On the basis of enhancement of lecithinase production by manipulation of environmental parameters, *L. monocytogenes* alone can be conveniently distinguished from other *Listeria* species. This observation may be exploited to complement existing methods for distinguishing *L. monocytogenes* from the other five species.

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## MATERIALS AND METHODS

**Bacterial strains and growth media and conditions.** *Listeria* species were routinely grown at 37°C in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) which included 1.5% agar for solid medium. Chemically defined minimal medium of Premaratne et al. (23) was supplemented with 0.01% glycine to optimize growth and with 1% bacteriological agar (Oxoid, Basingstoke, United Kingdom) for solid medium. All amino acids were obtained in the L configuration from Sigma Chemical Co., St. Louis, Mo. When necessary, glucose was replaced with cellobiose (Sigma). When required, the osmoprotectants betaine and carnitine (Sigma) were incorporated at a concentration of 1 mM each (1). Bacteria used were selected on the basis of their ability or inability to exhibit lecithinase activity on BHI broth and are described in Table 1.

**Analysis of lecithinase activity.** Overnight cultures (2 μl) of *Listeria* spp. were inoculated onto BHI agar containing 5% egg yolk previously diluted in 0.15 M NaCl (1:2, vol/vol) (BioTrading Benelux B. V., Mijdrecht, The Netherlands). Following incubation, plates were examined for precipitation of degraded egg yolk surrounding the colonies. Prior to use, media were adjusted to various pH values between 6.0 and 8.2 and to various NaCl concentrations from 0.5 to 3.0%. Plates were incubated at temperatures ranging from 4 to 43°C. For anaerobic incubation, plates were placed in an anaerobic jar, using Anaerocult A (Merck, Darmstadt, Germany) to generate an anaerobic environment. The degree of lecithinase activity was estimated from the extent of the zone of degraded egg yolk precipitate surrounding the colonies. When relevant, the presence of lecithinase was evaluated by incubating 1.0 ml of liquid culture supernatant with 2 ml

TABLE 1. Species and strains of *Listeria* used in the study and their ability to form a precipitate on BHI agar containing egg yolk and various amounts of supplementary NaCl

| *Listeria* species and strain | Lecithinase activity (avg diam of zone of precipitate [mm]) | | |
|---|---|---|---|
| | 0.5% NaCl | 1.0% NaCl | 1.75% NaCl |
| *L. monocytogenes* | | | |
| ATCC 23074 | 0 | 1.0 | 2.0 |
| 106 | 0 | 1.0 | 2.0 |
| 4B | 0 | 1.0 | 2.0 |
| 13 | 0 | 1.0 | 2.0 |
| L4492 | 0 | 1.0 | 2.0 |
| 1 | 0 | 1.0 | 2.0 |
| 311 | 0 | 1.0 | 2.0 |
| ScottA | 0 | 1.0 | 2.0 |
| LO28 | 1.75 | 2.75 | 3.75 |
| NCTC 7973 | 3.5 | 4.5 | 6.0 |
| EGD | 0 | 1.0 | 2.0 |
| *L. innocua* | | | |
| 6A46 | 0 | 0 | 0[a] |
| NCTC 1128867 | 0 | 0 | 0[a] |
| 6B47 | 0 | 0 | 0[a] |
| NTCT 1128966 | 0 | 0 | 0[a] |
| *L. ivanovii* | | | |
| 5A | 0 | 0 | 0[a] |
| 5B | 0 | 0 | 0[a] |
| *L. seeligeri* | | | |
| 6B | 0 | 0 | 0[a] |
| *L. welshimeri* | | | |
| 117t | 0 | 0 | 0[a] |
| 148t | 0 | 0 | 0[a] |
| *L. murrayi/grayi* | | | |
| 149v | 0 | 0 | 0[a] |
| 150d | 0 | 0 | 0[a] |
| 151v | 0 | 0 | 0[a] |

[a] Denotes a faint precipitate which fades as it extends outward from the colony.

of purified lecithin solution (Merck) and 7.0 ml of 0.15 M NaCl at 37°C for 4 h, as described previously (14). In such cases, the extent of enzyme activity was correlated with the amount of titratable $H^+$ produced during lecithin degradation by lecithinase (19).

## RESULTS

**Effect of environmental parameters on lecithinase production.** A range of strains which included the six species of *Listeria* was selected for study (Table 1). Strains were streaked on BHI agar supplemented with egg yolk, allowing detection of lecithinase activity by the appearance of a zone of precipitated degraded egg yolk around the lecithinase-producing colony following incubation at 37°C. Under these conditions, the majority of strains failed to exhibit zones of the type shown in Fig. 1. Two of the 11 strains of *L. monocytogenes* were capable of degrading egg yolk in this medium (Table 1).

The influence of pH on lecithinase production was evaluated initially by adjusting the pH of BHI (containing egg yolk) agar media to a range of pH values between 6.0 and 8.2 and measuring the size of the zone of degraded egg yolk surrounding the colonies after incubation at 37°C for 36 h (Fig. 2). Optimal degradation was observed between pHs 7.0 and 7.3. This was confirmed by growing *L. monocytogenes* LO28 in BHI broth at each pH, removing supernatant at the beginning of the stationary phase, and monitoring reduction in the pH of the lecithin solution due to release of $H^+$ during degradation over a 4-h incubation in the presence of supernatant (data not shown).

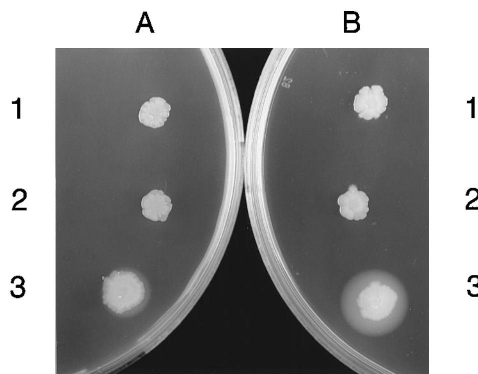


FIG. 1. Enhancement of lecithinase production, visualized by precipitate on BHI-egg yolk agar containing 0.5% NaCl (A) and 1.0% NaCl (B). 1, *L. innocua* NCTC 1128867; 2, *L. ivanovii* 5A; 3, *L. monocytogenes* LO28.

Variations in culture incubation temperature also influenced the production of lecithinase. Plates were incubated at 4, 7, 10, 15, 20, 25, 30, 37, 40, and 43°C until colonies were fully grown. Only those incubated at 20°C or higher exhibited egg yolk degradation, with maximum lecithinase activity evident between 37 and 40°C (Fig. 3). An identical trend was observed for lecithinase production when liquid culture supernatants from each incubation temperature were added to purified lecithin as already described.

Cultures incubated anaerobically exhibited the same ability to degrade lecithin as those incubated aerobically, indicating that lecithinase production was independent of the presence of oxygen.

The influence of NaCl was assessed by plating strains on the agar medium at pH 7.3 supplemented with a range of NaCl concentrations from 0.5 to 3.0%, in increments of 0.25%, and incubating at 37°C. Zone sizes for strain LO28 were at a maximum when the medium contained 1.75 to 2.0% NaCl (Fig. 4). Replacing NaCl with KCl also had a stimulatory influence on lecithinase activity, suggesting a general osmotic effect. On plates containing 1.75% NaCl, all *L. monocytogenes* strains

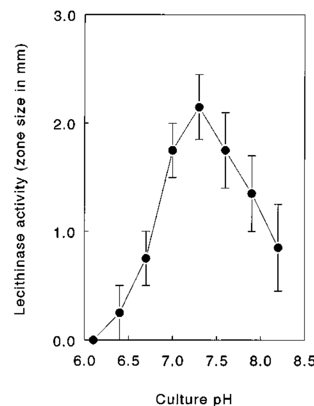


FIG. 2. Effect of pH on lecithinase activity in *L. monocytogenes* LO28. Activity is correlated with the size of the zone of egg yolk precipitation surrounding bacterial colonies on BHI agar.

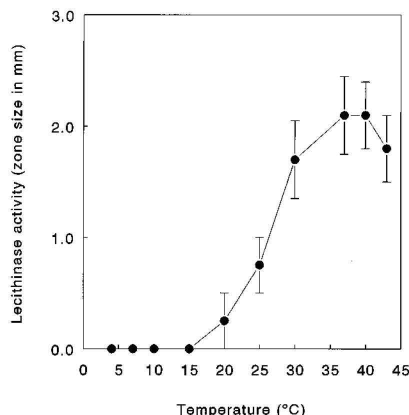


FIG. 3. Effect of temperature on lecithinase activity in *L. monocytogenes* LO28.

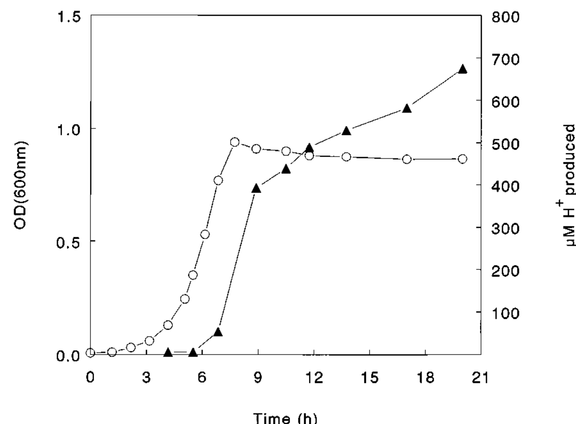


FIG. 5. Effect of growth phase on production of lecithinase in *L. monocytogenes* LO28. ○, bacterial optical density (OD) at 600 nm; ▲, lecithinase activity during growth correlated with $H^+$ produced during lecithin degradation following incubation of culture supernatant with purified lecithin.

which exhibited no activity on plates with 0.5% NaCl had a distinct zone of precipitate. At this NaCl concentration, a degree of precipitation was also observed with the species *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, and *L. grayi*. However, in these species, the zone of precipitate was quite faint and faded as it extended outwards. In all *L. monocytogenes* strains, the zone was of uniform intensity as it extended outwards and had a distinct limit (Fig. 1 and Table 1).

Lecithinase activity also varied with the stage of growth at which the enzyme assay was performed. Enzyme activity was evident at the beginning of the stationary phase and increased significantly during the stationary phase (Fig. 5).

**Lecithinase production on chemically defined minimal medium.** All strains described in Table 1 were incubated for up to 5 days on chemically defined minimal agar medium containing 5% egg yolk. *L. monocytogenes* LO28 was the only strain to exhibit lecithinase production on this medium, which has a pH between 7.1 and 7.2. Manipulation of salt concentration and temperature did not lead to enzyme production in any of the other *L. monocytogenes* strains employed. For strain LO28, optimal enzyme production on this medium was observed after 36 to 48 h of incubation at 33.5°C and when the salt concentration was adjusted to between 0.75 and 1.0%. The addition of the osmoprotectants betaine and carnitine at 1 mM each to this medium, however, did not allow higher levels of enzyme to be produced at higher NaCl concentrations.

Defined medium was also employed to assess the influence of carbon source on lecithinase production in *L. monocytogenes* LO28. When the glucose usually employed in this medium was replaced by the same concentration of cellobiose, lecithinase production was unaffected. This contrasts with the expression of phosphatidylinositol-specific phospholipase C (Fig. 6), a virulence factor active at an early stage in the infectious process, which was repressed when *L. monocytogenes* was grown on cellobiose (21).

**Choice of differentiation medium.** The use of the BHI medium adjusted to 1% NaCl at its natural pH of 7.3, containing 5% egg yolk and incubated at 37°C for 36 h, provided minimum egg yolk degradation by strains of *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, and *L. grayi* while still allowing a visible zone of precipitation with all *L. monocytogenes* strains. This medium was thus effectively employed to complement existing methods to differentiate *L. monocytogenes* strains from other *Listeria* strains in this laboratory (Table 1).

## DISCUSSION

In this communication we examine the role of environmental parameters on the level of production of lecithinase in *L. monocytogenes*. Initial observations with agar media containing egg yolk indicated that pH, temperature, salt concentration, and nutrient availability all had a significant impact on the production of this enzyme. It is notable, however, that the optimum pH for detection of the enzyme (7.0 to 7.3) is distinct

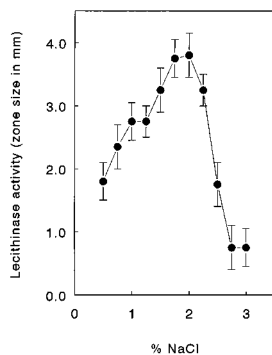


FIG. 4. Effect of NaCl concentration in BHI agar on lecithinase activity in *L. monocytogenes* LO28.

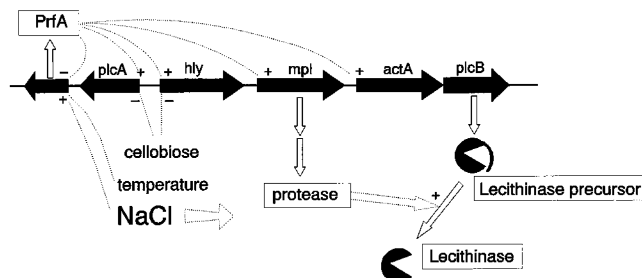


FIG. 6. Representation of virulence locus of *L. monocytogenes*, adapted from Dramsi et al. (9), summarizing the role of environmental parameters on activity of lecithinase and including the possible effects of NaCl. PrfA, positive regulatory factor; *plcA*, phosphatidylinositol-specific phospholipase C determinant; *hly*, hemolysin determinant; *mpl*, metalloprotease determinant; *actA*, actin polymerizing factor determinant; *plcB*, lecithinase determinant.

from the optimum pH for enzyme activity, which has been shown by Geoffroy et al. (14) to be between 6.0 and 7.0. Nevertheless, it is difficult to assess accurately the direct effect of pH on enzyme production, as pH values lower than 6.7 were observed to reduce bacterial growth slightly. In this study, production of lecithinase in BHI cultures was significantly reduced below pH 6.5. It is interesting that when the bacterium was incubated at a range of temperatures which permitted growth, lecithinase was not detectable at 15°C or lower. It reached its optimum in BHI at 37 to 40°C and in defined medium at 33.5°C. This observation suggests that reduced temperature is a major inhibitory factor for lecithinase production in the natural environment of *L. monocytogenes*. Indeed, high temperatures have been shown to have a positive effect on expression of other virulence factors in this bacterium (17, 25).

Major differences in enzyme production were detected at different NaCl concentrations at which growth rate did not appear to be significantly affected. The high level of lecithinase at 1.75 to 2.0% NaCl could in fact be correlated with increased amounts of metalloprotease, which is responsible for maturation of lecithinase (8, 22), in culture supernatants at these NaCl concentrations (5). This may be due to a direct effect of NaCl on the metalloprotease or to an indirect effect resulting in enhanced expression of PrfA, the positive regulator of virulence. The fact that NaCl has previously been shown to enhance Hly activity in *L. monocytogenes* would support the latter view (7). Such an indirect effect of salt on lecithinase may be explained by osmotic adaption in the cytoplasm, inducing increased expression of PrfA (1, 24). Significantly, at the higher salt concentrations, some egg yolk degradation on BHI agar was evident in all six species of *Listeria*. However, the activity associated with *L. monocytogenes* was manifested by a defined zone of precipitate surrounding the colony. Here, the precipitation was of uniform intensity as it extended from the colony and then ended abruptly. It is important to note that zones of precipitation in *L. monocytogenes* strains that did not exhibit activity on BHI lacking supplementary NaCl were quite faint by comparison with strong lecithinase producers like strains LO28 and NCTC 7973. Colonies of the other five *Listeria* species exhibited faint halos of precipitate which gradually faded as they extended from the colonies. The degradation of egg yolk by these strains is intriguing since *L. seeligeri* and *L. ivanovii* are the only members of these five species which are known to possess genetic determinants with homology to the lecithinase operon associated with the virulence locus (16). The other three strains apparently lack this genetic information. This suggests that the lecithinase activity seen around *L. monocytogenes* colonies is indeed directed by the *plcB* determinant of the lecithinase operon in the virulence locus. This genetic determinant in *L. ivanovii* and *L. seeligeri* may be regulated in a different manner than in *L. monocytogenes* and thus is not expressed in BHI supplemented with NaCl. The origin and nature of the egg yolk degradation in the five non-human pathogenic *Listeria* species may be due to sphingomyelinase (27), which may be active under these conditions. It is noteworthy that the hybridization data of Gouin et al. (16) suggest that in the cases of the species *L. ivanovii* and *L. seeligeri* the virulence locus in the region of *plcB* may exhibit differences from that in *L. monocytogenes*. It is also interesting that in another study (14) it was not possible to detect lecithinase in *L. ivanovii* supernatants with antibodies against *L. monocytogenes* lecithinase.

It has previously been reported that the plant-derived disaccharide cellobiose has the effect of repressing PlcA and Hly activities (Fig. 6) in *L. monocytogenes*, reflecting the fact that in the primary natural saprophytic environment of *Listeria* species these membrane-damaging enzymes are not required for survival (21). In this work we have observed that cellobiose does not repress lecithinase activity. Since this virulence factor functions late in the infection within a double-membraned vacuole with limited growth substrates (11), plant-derived carbon sources would not be expected to play a role in regulation. Indeed, the continued production of lecithinase throughout the stationary phase of growth when nutrients are limiting supports this proposal, and it may well be that limiting growth substrates stimulate the expression of this virulence factor. It also agrees with the previous observation by Bohne et al. (4) that bicistronic mRNA species transcribed from *plcB* (and *actA* [Fig. 6]) have a significantly longer half-life and are present in higher concentrations than other mRNA species associated with the virulence locus when the cell is exposed to a nutrient-limiting environment. This high stationary-phase activity of lecithinase contrasts with observations made in the case of internalin, a virulence factor required for the initial entry of *L. monocytogenes* into a human cell, where stationary-phase activity is much reduced in comparison with that of the exponential phase (9). The reason for the inability of strain NCTC 7973, the strongest lecithinase producer on BHI, to degrade egg yolk in the defined medium remains to be seen. Nevertheless, it is apparent that within the species *L. monocytogenes* lecithinase activity exhibits significant variation among strains, which include hyperproducers (NCTC 7973), medium producers (LO28), and very weak producers (ScottA) (Table 1).

In light of the in vivo conditions under which *L. monocytogenes* is required to produce lecithinase, i.e., in the absence of excess growth factors, in the absence of oxygen, and at 37°C, it is not surprising that production of this enzyme responds to these environmental stimuli in the fashion we have observed. While the major influence of NaCl, and possibly other salts, on lecithinase activity may be explained by an effect on maturation of the metalloprotease or an effect on expression of Prf, it is not known exactly what salt concentrations are encountered within host cells during infection and intracellular growth.

The observations described here for *Listeria* spp. have been applied to the rapid differentiation of *L. monocytogenes* from other species. Existing methods used include the CAMP test, which depends on the synergistic lysis of erythrocytes by *L. monocytogenes* in the presence of extracellular enzymes produced by *Staphylococcus aureus* (18), and the detection of phosphatidylinositol-specific phospholipase C activity to distinguish *L. monocytogenes* and *L. ivanovii* from the nonpathogenic *Listeria* species (20). Other differentiation methods include a *Listeria*-specific API system (2), a hemolysis agar test (6), and a capillary air thermal cycler system (3). The results presented in this paper represent a simple and inexpensive method of differentiation based on the enhancement of lecithinase production by manipulating environmental parameters. It may be useful in complementing the existing methods for distinguishing *L. monocytogenes* from the other five species.

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