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Effects of Glucose, Growth Temperature, and pH on Listeriolysin O Production in *Listeria monocytogenes*

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Expression of listeriolysin O of *Listeria monocytogenes* as a function of different growth conditions was studied by performing a direct hemolysin assay, immunoblotting experiments, and an enzyme-linked immunosorbent assay. Expression of listeriolysin O was reduced at a lower growth temperatures (26°C) and at higher glucose concentrations (≥0.3%) in the growth media. The effect of glucose appeared to be due to a change in the pH of the growth media.

Listeria monocytogenes, a gram-positive, intracellular, microaerophilic bacterium, is the causative agent of human listeriosis. The organism enters the human body primarily through ingestion of contaminated food (5). In the last decade, at least four major outbreaks and numerous sporadic cases of listeriosis have been reported throughout the world (5). All virulent strains of *L. monocytogenes* elaborate an extracellular hemolysin, listeriolysin O (LLO), which has been shown to be an essential virulence factor. In addition to LLO, several other virulence factors have been described (11).

Establishment of a successful pathogenic infection requires the presence of virulence genes and expression of these genes at various stages of infection. Expression of virulent genes is often controlled by various environmental factors of the host (10), including temperature, osmolarity, pH, and the concentrations of certain metal ions. It has been shown previously that the expression of *L. monocytogenes* LLO is controlled by growth temperature (3, 7) at the level of transcription (7). In this paper we describe the effect of glucose concentration and external pH on the expression of LLO in *L. monocytogenes* and provide additional data which support the previous finding of temperature regulation of LLO expression in this organism.

For the hemolysin assay, *L. monocytogenes* strains were routinely grown in Trypticase soy broth without glucose (BBL, Cockeysville, Md.) containing 0.6% yeast extract (TSBYE) (pH 7.5). This basal medium was supplemented with glucose and other sugars as needed. Cultures were grown overnight at different temperatures, and the supernatant fluids were assayed for hemolysin activity with sheep erythrocytes as described elsewhere (4). The results of these assays (Table 1) showed that hemolysin production in culture supernatants was reduced when the cells were grown at 26°C in the presence of 0.2% glucose or at 37°C in the presence of 1% glucose. The extents of these effects were not serotype dependent, as two different strains belonging to the same serotype exhibited different levels of response. Similar effects of temperature and glucose were also observed (data not shown) in *Listeria ivanovii* ATCC 19119 (serotype 5). This experiment and all of the experiments described below were conducted at least three times. Because of the nature of the assays, the values sometimes fluctuated considerably, although the basic trends remained the same. Therefore, the values in all of the tables were obtained from individual experiments.

To investigate the effect of glucose in greater detail, we measured the amount of hemolysin produced in *L. monocytogenes* Scott A (LS2). After overnight growth of this strain at 37°C in the presence of different concentrations of glucose, the culture supernatant fluids and sonic extracts of the cultures were assayed for hemolysin activity. Sonic extracts were prepared as previously described (4). Protein concentrations were determined by the method of Bradford (2). Maximum production of hemolysin occurred at a glucose concentration of 0.2%, and hemolysin activity progressively decreased as the glucose concentration increased (Table 2). Similar patterns obtained with both supernatant fluids and sonic extracts indicated that the reduced hemolysin activities in the culture supernatant fluids were not due to reduced secretion of hemolysin as a result of higher glucose concentrations in the media. To ensure that the higher glucose concentrations did not interfere with the hemolysin assay per se, we added different amounts of glucose directly to the assay buffer and showed that these additions did not interfere with the assay (data not shown).

To determine whether the reduction in hemolysin activity was actually due to decreased production of LLO at 26°C and at higher glucose concentrations and not due to inactivation of LLO (9), we used an immunoblotting procedure and a plate enzyme-linked immunosorbent assay (ELISA) with anti-LLO antibody. LS2 cultures were grown under the conditions described above, and the culture supernatant

TABLE 1. Effects of temperature and glucose concentration on production of hemolysin in *L. monocytogenes* strains

Strain	Serotype	Hemolysin titer under the following growth conditions[a]:		
		0.2% glucose and 26°C	0.2% glucose and 37°C	1% glucose and 37°C
LS1	1/2a	18	102	0
LS2	4b	0	115	0
LS55	4b	0	30	0
LS112	4b	0	112	0
LS115	1/2c	0	86	0
LS117	1/2b	0	18	0
LS120	3a	17	132	14
LS185	1/2a	0	58	0

[a] Expressed in arbitrary units per milliliter of supernatant per A_{600} of 1.0.

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TABLE 2. Effects of various concentrations of glucose on production of hemolysin in *L. monocytogenes* LS2

Glucose concn (%)	Hemolysin titer in:	
	Supernatant (U/ml)[a]	Cell extract (U/mg of protein)
0	2.7	100
0.1	17	914
0.2	137	1,280
0.3	6.5	246
0.4	1.5	36
0.5	0	0
1.0	0	0

[a] Expressed in arbitrary units per milliliter of supernatant per A_{600} of 1.0.

fluids were concentrated by using Centriprep-30 ultrafiltration devices (Amicon, Beverly, Mass.). The concentrated supernatant fluids were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (6), using 8 to 25% gradient gels in a Phastsystem apparatus (Pharmacia-LKB Biotechnology, Piscataway, N.J.). The separated proteins were electroblotted onto nitrocellulose membranes (8) and were detected with anti-LLO antibody and anti-rabbit alkaline phosphatase conjugate and a Bio-Rad substrate kit. The results of these experiments showed that the amounts of LLO expressed were much smaller when the cultures were grown at 26°C or when the cultures were grown in the presence of glucose concentrations of 0.6 and 1% at 37°C (Fig. 1). These results correlated well with our hemolysin assay results (Tables 1 and 2) and also confirmed previous observations (3, 7) that expression of *L. monocytogenes hlyA* is controlled by the growth temperature. The overall banding patterns (Fig. 1A) in all of the lanes were similar, indicating that the effects of glucose concentration and low temperature on LLO production are specific. We further quantified the effects of temperature and glucose concentration on LLO expression by using a plate ELISA. Concentrated culture supernatant fluids were diluted in 0.1 M bicarbonate buffer (pH 9.6) and added to wells (50 μl per well) in a microtiter plate (Immulon; Dynatech Laboratories, Inc., Alexandria, Va.). After storage for 18 h at 4°C, the ELISA was performed as described previously (14), except that rabbit anti-LLO antibody (dilution, 1:1,000) was used as the antibody. Wells with A_{410} values of ≥0.2 as determined with a Microelisa Minireader apparatus (Dynatech) were considered positive. A minimum of 6 ng of purified LLO gave a positive result. Table 3 shows the correspondence among the results of all three assays, and these results indicated that the expression of LLO was about 64-fold lower in cultures grown at 26°C and about 128-fold lower in cultures grown at 37°C in the presence of 0.6 and 1% glucose compared with cultures grown at 37°C in the presence of 0.2% glucose.

TABLE 3. Influence of growth conditions on production of LLO by *L. monocytogenes* LS2

Glucose concn (%)	Growth temp (°C)	LLO concn (μg/mg of protein)
0.2	26	6
0.2	37	384
0.6	37	3
1.0	37	3

The effect of high glucose concentrations in the medium may be due to an increase in osmolarity (10). The exact mechanism by which high osmolarity controls gene expression is far from clear, although it has been suggested that DNA supercoiling and/or bending plays an important role in this effect. We investigated whether the observed effect of glucose on LLO expression was due to a change in the osmolarity of the medium. We grew LS2 cells in TSBYE containing 0.2% glucose and either 0.8% galactose, 0.8% lactose, 0.8% maltose, or 0.8% glycerol and assayed the supernatant fluids from these cultures for hemolysin activity. Maltose and glycerol are poorly utilized by *L. monocytogenes*, whereas lactose and galactose cannot be utilized by *L. monocytogenes* (12). Because none of these sugars affected the hemolysin production of LS2 (data not shown), we concluded that the glucose effect was not due to higher osmolarity.

The reduction of gene expression by glucose (catabolite repression) has been studied in various gram-negative and

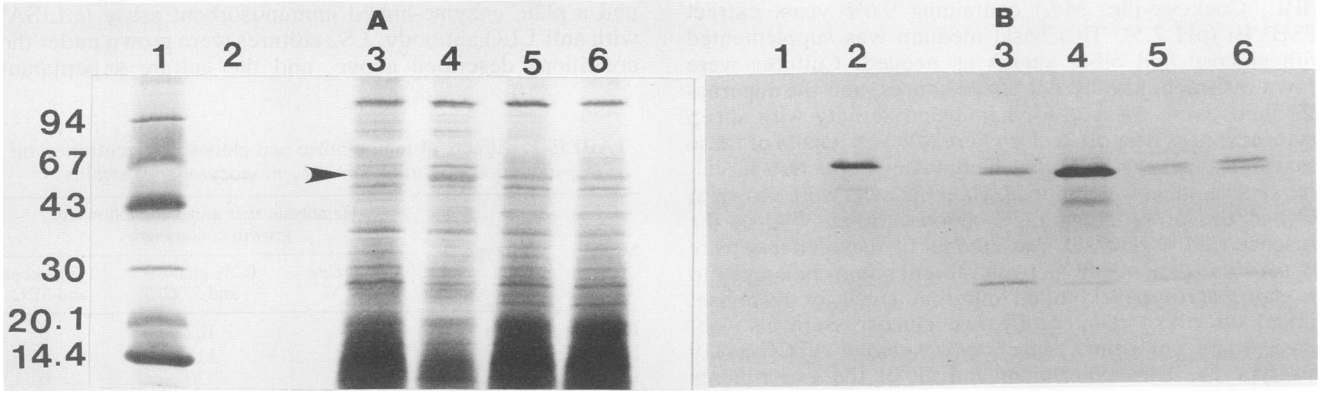

FIG. 1. SDS-PAGE and Western blot (immunoblot) analysis of LLO. (A) SDS-PAGE analysis. The gel was stained with silver nitrate. The arrowhead indicates the approximate position of LLO. (B) Western blot analysis. Bands were visualized by sequential probing with rabbit anti-LLO antibody, goat anti-rabbit alkaline phosphatase conjugate, and the phosphatase substrate. Lanes 1 contained molecular weight markers (molecular weights [10^3] are indicated on the left), and lanes 2 contained purified LLO (8.5 ng). Lanes 3 through 6 contained 100-ng portions of concentrated culture supernatant fluids from LS2 cultures grown in the presence of 0.2% glucose at 26°C (lanes 3), in the presence of 0.2% glucose at 37°C (lanes 4), in the presence of 0.6% glucose at 37°C (lanes 5), and in the presence of 1.0% glucose at 37°C (lanes 6).

TABLE 4. Effects of glucose and αMG on the production of LLO in *L. monocytogenes*

Growth conditions	pH in the presence of[a]:		LLO concn (ng/μg of protein) in the presence of:	
	No TES	200 mM TES	No TES	200 mM TES
0.1% Glucose	6.6	7.3	192	192
0.2% Glucose	6.0	7.3	96	192
0.6% Glucose	4.9	6.8	<3	384
0.1% Glucose + 0.8% αMG	5.5	7.0	<3	384
0.2% Glucose + 0.8% αMG	5.4	7.0	<3	96

[a] The pH values of the supernatants were determined after overnight growth of LS2; the initial pH was adjusted to 7.5.

TABLE 5. Effect of pH on LLO production in *L. monocytogenes* LS2

Growth conditions	pH before growth (after growth)	Hemolysin titer (U/ml)[a]	LLO concn (ng/μg of protein)
0.1% Glucose	5.0 (5.5)[b]	6.6	<6
	5.5 (5.6)[b]	6.6	<6
	6.0 (5.5)	2.6	<6
	6.5 (6.5)	67.3	96
	7.0 (6.9)	222	384
	7.5 (7.4)	222	384
0.1% Glucose + 0.8% αMG	7.5 (6.9)	111	384

[a] Expressed in artibtary units per milliliter of supernatant per A_{600} of 1.0.
[b] After 40 h of incubation at 37°C.

gram-positive microorganisms (1). Of particular interest is the report of glucose and/or pH effects on decreased production of *Staphylococcus aureus* alpha-hemolysin, enterotoxins A, B, and C, and toxic shock syndrome toxin (13). In LS2, we found that in addition to glucose, α-methylglucoside (αMG) (a nonmetabolizable glucose analog) also reduced hemolysin production and that the effects of glucose or αMG were not reversed by adding 5 mM cyclic AMP (cAMP) with and without 1 mM 3-isobutyl-1-methyl xanthene (an inhibitor of cAMP phosphodiesterase) in LS2 (data not shown).

Next, we explored the possibility that the glucose-mediated reduction in LLO production is due to a decrease in pH as a result of growth of *Listeria* cells in media containing high glucose concentrations. Two series of experiments were performed to evaluate the role of low pH in LLO production. First, we grew strain LS2 in TSBYE containing various concentrations of glucose and αMG and measured the pH values of the supernatant fluids after overnight incubation at 37°C. One set of cultures was buffered with 200 mM tris-methyl-aminoethansulfonic acid (TES), and the other set was left unbuffered. After overnight incubation, the pH values of the culture supernatant fluids were determined, and the LLO concentrations were measured by the ELISA (Table 4). The effect of glucose was eliminated in those cultures in which the changes in pH were minimal because of the presence of TES buffer. These experiments also showed that the effect of αMG can also be explained by changing pH.

In the second experiment, we grew LS2 at 37°C overnight in TSBYE containing 0.1% glucose with or without 0.8% αMG. The media were adjusted to various pH values by adding 100 mM citrate (for pH 5.0 and 5.5), 2-(*N*-morpholino)ethansulfonic acid (for pH 6.0 and 6.5), 3-(*N*-morpholino)propanesulfonic acid (for pH 7.0), and TES (for pH 7.5). The supernatant fluids were then used for the hemolysin assay and measurements of LLO concentrations by the ELISA. Table 5 shows that hemolysin activity and LLO production were reduced considerably between pH 5.0 and 6.0 and that optimum LLO production occurred at around pH 7.0. Again, high concentration of αMG had very little effect at a high pH (pH 6.9). On the basis of the results of these two series of experiments, we concluded that LLO production in *L. monocytogenes* is controlled by external pH and that the effect of high glucose concentrations (≥0.3%) may be due simply to a lowering of the pH of the growth medium, resulting from its metabolism.

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REFERENCES

1. **Botsford, J. L., and J. G. Harman.** 1992. Cyclic AMP in prokaryotes. Microbiol. Rev. **56**:100–122.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
3. **Datta, A. R.** 1989. Expression of listeriolysin O gene of *Listeria monocytogenes*, abstr. B-254, p. 73. Abstr. 89th Annu. Meet. Am. Soc. Microbiol. 1989.
4. **Datta, A. R., B. A. Wentz, and J. Russel.** 1990. Cloning of listeriolysin O gene and development of specific gene probes for *Listeria monocytogenes*. Appl. Environ. Microbiol. **56**:3874–3877.
5. **Farber, J. M., and P. I. Peterkin.** 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. **55**:476–511.
6. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
7. **Leimeister-Wachter, M., E. Domann, and T. Chakraborty.** 1992. The expression of virulence genes in *Listeria monocytogenes* is thermoregulated. J. Bacteriol. **174**:947–952.
8. **Matsudaira, P.** 1987. Sequence from picomole quantities of protein electroblotted onto polyvinylidine difluoride membranes. J. Biol. Chem. **262**:10035–10038.
9. **McKellar, R. C.** 1992. Effect of reduced pH on secretion, stability and activity of *Listeria monocytogenes* listeriolysin O. J. Food Safety **12**:283–293.
10. **Mekalonas, J. J.** 1992. Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. **174**:1–7.
11. **Portnoy, D. A., T. Chakraborty, W. Goebel, and P. Cossart.** 1992. Molecular determinants of *Listeria monocytogenes* pathogenesis. Infect. Immun. **58**:3770–3778.
12. **Premaratne, R. J., W. Lin, and E. A. Johnson.** 1991. Development of an improved chemically defined minimal medium for *Listeria monocytogenes*. Appl. Environ. Microbiol. **57**:3046–3048.
13. **Regassa, L. B., R. P. Novick, and M. J. Betley.** 1992. Glucose and nonmaintained pH decrease expression of the accessory gene regulator (*agr*) in *Staphylococcus aureus*. Infect. Immun. **60**:3381–3388.
14. **Schultz, A. J., and B. A. McCardell.** 1988. DNA homology and immunological cross-reactivity between *Aeromonas hydrophila* cytotonic toxin and cholera toxin. J. Clin. Microbiol. **26**:57–61.