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Stefanie Evans Gilbreth
University of Nebraska - Lincoln

Andrew K. Benson
University of Nebraska - Lincoln, abenson1@unl.edu

Robert W. Hutkins
University of Nebraska - Lincoln, rhutkins1@unl.edu

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Catabolite Repression and Virulence Gene Expression in *Listeria monocytogenes*

Stefanie Evans Gilbreth, Andrew K. Benson, and Robert W. Hutkins

Department of Food Science and Technology, University of Nebraska–Lincoln, Lincoln, NE 68583-0919, USA

Corresponding author: R. W. Hutkins; *email:* rhutkins1@unl.edu

Abstract. Previous studies have suggested that carbohydrates may affect expression of virulence genes in *Listeria monocytogenes*. Which carbohydrates influence virulence gene expression and how carbohydrates mediate expression, however, is not clear. The goal of this work was to examine how carbohydrates affect virulence gene expression in *L. monocytogenes* 10403S. Growth studies were conducted in medium containing glucose and various sugars. Metabolism of arbutin, arabitol, cellobiose, mannose, maltose, trehalose, and salicin were repressed in the presence of glucose. Only when glucose was consumed were these sugars fermented, indicating that catabolite repression by glucose had occurred. To determine whether virulence gene expression was also influenced by catabolite repression, we performed primer extension experiments, using primers for *hly* and *prfA,* which encode for a hemolysin and the regulator protein PrfA, respectively. In the presence of cellobiose and arbutin, transcription of hemolysin was reduced. However, none of the sugars affected transcription of *prfA*. The results demonstrate that catabolite repression occurs in *L. monocytogenes* and suggests that, at least in strain 10403S, cellobiose and arbutin repress expression of hemolysin.

Listeria monocytogenes is the causative agent of listeriosis, a disease whose symptoms are usually very similar to the flu. However, in immunocompromised individuals, the elderly, and children, the disease can cause meningitis, septicemia, and death. In pregnant women, listeriosis may cause spontaneous abortion [12]. The mortality rate associated with listeriosis is much higher than that associated with illness caused by other enteric pathogens. Approximately 28% of all food-borne, pathogen-related deaths in the U.S. are attributed to *L. monocytogenes* [7].

In its natural habitat (i.e., plants, soil, vegetation), *L. monocytogenes* is subject to and survives in a wide range of temperatures and environmental conditions. Compared with other food-borne pathogens, *L. monocytogenes* is particularly capable of surviving under inhospitable conditions. In foods and food processing environments, it survives low pH, high salt (10–20%), and low moisture conditions, and it is able to grow at temperatures ranging from 4°C–45°C [5]. Inside a host, *L. monocytogenes* encounters many other hurdles, including the low pH in the stomach and oxidative stress from the host immune system. Remarkably, *L. monocytogenes* is able to survive in both of these disparate environments.

The ability of this pathogen to sense in which environment it is and then to switch on and off the appropriate genes that are needed may be key to its survival. One of the ways in which it may discriminate differences in the environment may be by sensing the presence or availability of carbohydrates. Previously, the availability of carbohydrates was shown to affect the expression of virulence genes in *L. monocytogenes* [2– 4, 8–11]. There are conflicting reports however, on which carbohydrates contribute to this regulation and the precise means by which regulation occurs. Initially, it was shown that cellobiose, a plant-derived carbohydrate, repressed virulence gene expression and that repression could be seen at concentrations as low as 1 mM [11]. The repression of virulence genes was believed to be a signal-sensing response and not a general effect caused by substrate utilization. However, it was also claimed that any utilizable

sugar could repress virulence gene expression, indicating that this phenomenon might be due to a more general mechanism of catabolite repression [8]. Recently, Brehm et al. [4] characterized the *bvrABC* locus, which is responsible for sensing β-glucosides, and showed that both of these hypotheses could be correct. In this study we provide physiological evidence that catabolite repression occurs in *L. monocytogenes* 10403S and that virulence gene expression is repressed by specific sugars.

Materials and Methods

Catabolite repression studies. *L. monocytogenes* 10403S were grown at 37°C, with shaking, in a defined medium (DM) described by Becker et al. [1]. Each flask contained 25 mL of DM supplemented with 5 mM glucose and 5 mM of either arabitol, arbutin, fructose, maltose, mannose, trehalose, salicin, or cellobiose. At various times, growth was determined by optical density measurements. Samples (1 mL) were also removed, centrifuged, and the supernatants were stored at –20°C for sugar analysis.

Most of the sugars were analyzed by HPLC, as described previously [6]. When HPLC separation was not adequate, Boehringer Mannheim enzyme-linked assay kits (Roche, Indianapolis, IN) were used. Samples containing cellobiose or arbutin were incubated with α-glucosidase (Sigma, St. Louis, MO) or β-glucosidase (Worthington Biochemicals, Lakewood, NJ), respectively, to release free glucose, which was then measured.

Virulence gene expression. To determine the effect of growth conditions on transcription of virulence genes, primer extension reactions were performed. *L. monocytogenes* 10403S were grown in DM at 37°C, with shaking, containing 25 mM of one of the following: arabitol, arbutin, cellobiose, fructose, glucose, maltose, mannose, salicin, or trehalose. Total RNA was extracted from cells with TRI-REAGENT (Molecular Research Center Inc., Cincinnati, OH) according to the manufacturer's instructions. Samples were homogenized (4200 rpm for six 1-min cycles) with a bead beater (Biospec Products, Bartesville, OK). The concentration of RNA was determined by spectroscopy and agarose gel electrophoresis.

Primer extensions were performed with *hly* (cctaataatgccaaataccg) and *prfA* (ttgaattcttctgcttgagcg) primers (Sigma Genosys, St. Louis, MO) by using the method described by Becker et al. [1]. The samples were run next to a sequencing ladder (T7 Sequenase v 2.0, Amersham, Piscataway, NJ) prepared by using the primers above and the p163 and p154 template DNA specific for the *prfa* and *hly* genes. Autoradiographs were developed after 24 h of exposure. The intensity of the bands was also measured by cutting the bands out of the gel and counting radioactivity in a scintillation counter (Beckman LS 3801, Fullerton, CA).

Results

Catabolite repression studies. Although proteins that are involved in catabolite repression, such as HPr and CcpA, are present in *L. monocytogenes,* no physiological data havebeen published that demonstrate catabolite repression occuring in this organism. When two or more sugars are present, catabolite repression (CR) or the preferential use of one sugar before another sugar can occur. To assess CR, cells were grown in DM containing glucose and either cellobiose, salicin, fructose, arbutin, arabitol, mannose, maltose, or trehalose. The latter group of sugars were metabolized by the majority of *L. monocytogenes* strains. All sugars were added at 5 mM, which was shown in preliminary experiments to allow cells to reach a maximum optical density of 0.4 –0.5. In contrast, with 10 mM sugar present, *L. monocytogenes* grows to an optical density near 1.0. Most of the sugars tested were indeed under the control of CR. Results are shown only for salicin and arabitol (Fig. 1). Only when glucose was completely consumed was either the salicin (Fig. 1A) or arabitol (Fig. 1B) utilized. Diauxic growth was also observed for both sugars. The only exception was for fructose, which was used concomitantly with glucose. Most of the sugars tested were eventually metabolized completely, except for trehalose and maltose. Even after the glucose had been fully metabolized, these sugars were incompletely used. According to Bergey's Manual, it can take up to 4 days for *L. monocytogenes* to metabolize maltose; therefore, there may not have been

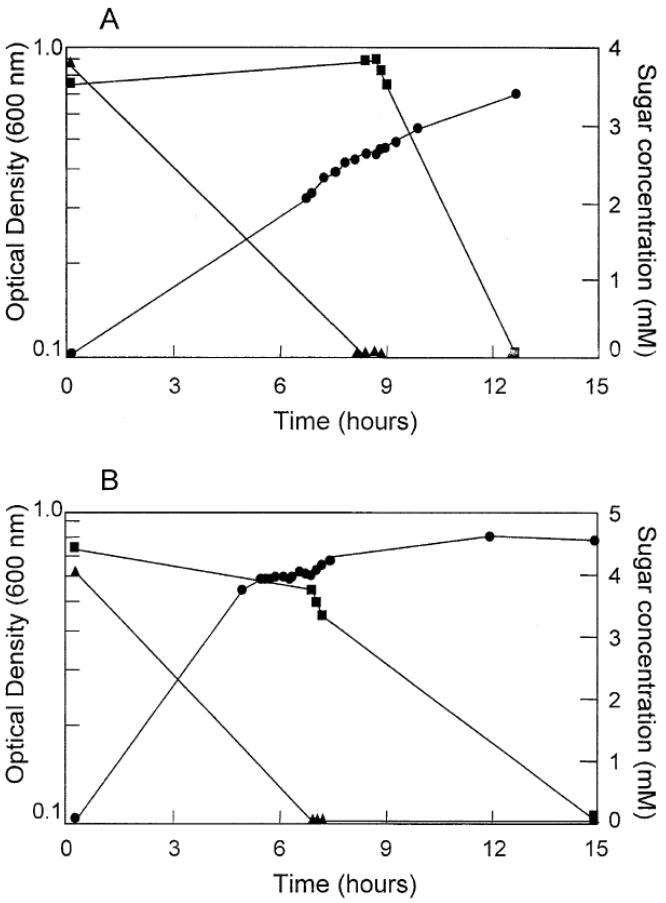

Figure 1. Catabolite repression in *Listeria monocytogenes* 10403S during growth on salicin (A) and arabitol (B). Cells were grown in DM containing 5 mM glucose (▲) and either salicin or arabitol (■). Cell growth (●) was determined by optical density measurement at 600 nm.

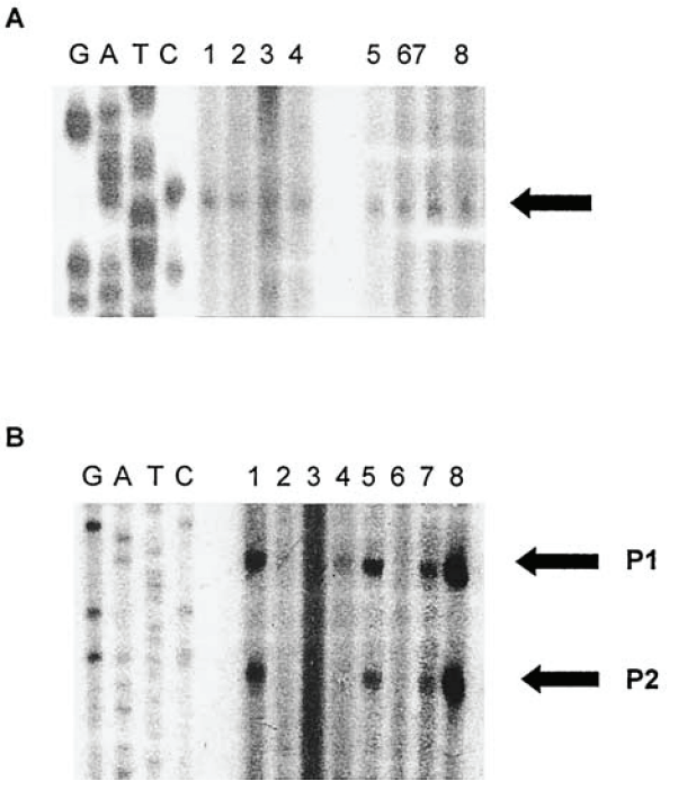

Figure 2. Primer extension analysis of *prfA* (A) and *hyl* (B) transcripts. Extension reactions were performed on cells grown with arabitol (lane 1), arbutin (lane 2), cellobiose (lane 3), fructose (lane 4), glucose (lane 5), mannose (lane 6), salicin (lane 7), or trehalose (lane 8). Sequence ladders were derived from sequencing reactions by using *prfA* and *hly* primers.

adequate time to utilize it to completion [13]. However, unlike the trehalose sample, the maltose concentration did not decrease significantly until the glucose was absent, indicating that catabolite repression occurred.

Virulence gene transcription. We next examined the effect each of these sugars had on expression of the virulence genes, *prfA* and *hly.* Previously, it was shown that pronounced repression of virulence genes, including *hly,* occurred when cells were grown in medium containing cellobiose and other plant-derived carbohydrates [2, 9,10]. In contrast, the transcriptional activator, PrfA, was shown to be unaffected by cellobiose by Western blot analysis [8]. However, very few sugars have been examined for their ability to affect PrfA transcription.

We first assessed *prfA* expression in cells grown in the presence of eight different carbon sources, all of which were shown to support growth in the previous catabolite repression studies. In these experiments, cells were grown in defined media with 25 mM of either arbutin, arabitol, cellobiose, fructose, maltose, mannose, salicin, or trehalose. Primer extension results consistently demonstrated that the abundance of the *prfA* transcript was unaffected in all of the carbon sources tested (Fig. 2A). However, expression of *hly,* encoding the listeriolysin O, was dependent on the sugar present in the growth medium. Transcription of *hly* originates from three promoters that are differentially regulated by PrfA. Two of the promoters, *hlyp1* and *hlyp2,* are PrfA-dependent, while a third promoter, *hlyp3,* is PrfA-independent. We studied the PrfA-dependent promoters, since PrfA and hemolysin are both essential for full virulence. A primer (HLYP2) that is complementary to positions +73 to +92 relative to the transcriptional start site at *hlyp1* and +84 to +103 relative to the transcriptional start site at *hlyp2* was end-labeled and used in primer extension analyses on *L. monocytogenes* 10403S mRNA isolated from log phase cells. Again, the cells were grown in eight different carbon sources previously shown to support growth. When cells were grown in arbutin and cellobiose, the amount of *hly* transcript was greatly reduced (Fig. 2B). In contrast, salicin, glucose, maltose, mannose, and arabitol had no effect on the amount of hemolysin transcript accumulated. Trehalose did not repress *hly* expression and actually appeared to increase the amount of transcript accumulated (Fig. 2). These results indicate that hemolysin expression is affected by sugars that are present in the media, especially arbutin, cellobiose, and trehalose. Transcript amounts, as determined by measuring the radioactivity in the bands (by scintillation counting), matched the band intensities and further confirmed the results.

Discussion

The ability of *L. monocytogenes* to metabolize cellobiose, salicin, and other plant-derived sugars supports the suggestion that plant material is the natural habitat for this saprophytic organism. Indeed, a sugar utilization analysis revealed that of 95 sugars tested, all 168 strains fermented glucose, fructose, mannose, cellobiose, salicin, and N-acetyl-D-glucosamine; 99% used arbutin; and 96% used trehalose (data not shown). In contrast, amygdalin, maltose, and dextrin were not consistently utilized. However, according to Bergey's Manual, trehalose was listed as variable among *Listeria* isolates, N-acetyl-Dglucosamine and arbutin were not tested, and amygdalin, maltose, and dextrin should have been used by all strains [13]. Most of the sugars tested, including arabitol, arbutin, maltose, mannose, trehalose, salicin, and cellobiose, were catabolite repressed by glucose. Growth of *L. monocytogenes* 10403S in DM containing limiting glucose and each of these sugars also gave diauxic growth during the transition period. Only fructose was co-metabolized with glucose.

In an earlier report, Park et al. [9, 10] reported that plant-derived carbohydrates were capable of repressing expression of virulence genes and that this signal-sensing mechanism was related to specific β-glucoside sugars. However, Milenbachs et al. [8] showed that several different carbohydrates can repress virulence gene expression. Thus, the hypothesis that the expression of virulence genes is under more global control, such as catabolite repression, was proposed [8]. Subsequently, Behari et al. [2] suggested that Catabolite Control Protein A (CcpA) and the complex it forms with HPr might regulate transcription of virulence genes. However, that a CcpA mutant retained the ability to express virulence genes indicated that CcpA was not involved.

Transcription analyses revealed that *prfA* transcription was unaffected by all of the carbon sources tested. However, when we examined transcription of the *hly,* which encodes for listeriolysin, a pore-forming toxin that is required for full virulence, the amount of *hly* transcript was greatly reduced in arbutin-and cellobiose-grown cells (Fig. 2B). Salicin, glucose, maltose, mannose, and arabitol had no effect on the amount of hemolysin transcript accumulated. Trehalose did not repress *hly* expression and actually appeared to increase the amount of transcript accumulated (Fig. 2). These results indicate that hemolysin expression is affected by sugars that are present in the media, especially arbutin, cellobiose, and trehalose.

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