Proteomic Analysis of a Hypochlorous Acid–Tolerant Listeria monocytogenes Cultural Variant Exhibiting Enhanced Biofilm Production

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ABSTRACT

Following exposure of Listeria monocytogenes Scott A (SA) to hypochlorous acid, rough colony variants were identified that were tolerant of hypochlorous acid and produced increased amounts of biofilm. A derivative of one of these variants was smooth, produced even more biofilm, and exhibited greater biofilm chlorine resistance. The objective of this research was to compare the protein expression of a cultural variant to SA and to identify proteins that might be associated with biofilm production and chlorine tolerance. Suspension chlorine tolerance for several cultural variants (SAR, SARS, and SBS) was determined by exposure to 60 to 120 ppm of hypochlorous acid for 5 min. Hypochlorous acid tolerance of biofilms was determined after growing biofilms on stainless steel and then exposing them to 200 ppm of hypochlorous acid for 5 min. All cultural variants were able to survive 120 ppm of hypochlorous acid in suspension. There was little difference in the hypochlorous acid tolerance of the cultural variant planktonic cells. The cultural variants produced greater amounts of biofilm than the common form of L. monocytogenes and were more tolerant of hypochlorous acid. The SBS variant was selected for proteomic comparison because it was the variant that produced the most biofilm and was the most tolerant of hypochlorous acid when grown as a biofilm. Protein expression of planktonic and biofilm cells of SBS was compared to SA by two-dimensional difference gel electrophoresis. The 50s ribosomal protein, L10, was down-regulated in biofilm SBS. Other proteins down-regulated in planktonic SBS were the peroxide resistance protein (Dpr) and a sugar-binding protein (LMO0181). This sugar-binding protein was also up-regulated in biofilm SBS. One protein spot down-regulated in planktonic SBS contained both 50s ribosomal protein L7/L12 and an unknown protein (LMO1888).

The common form of Listeria monocytogenes, known as the S-form, produces smooth, circular colonies with a glistening surface and dissociates into a variety of cultural variants. Rough cultural variants (R-forms) of L. monocytogenes produce rough colonies on agar and flaky growth in broth with a surface pellicle (32). R-forms of L. monocytogenes do not exhibit a blue sheen with Henry illumination and lack the translucence normally exhibited by the S-form. While the R-form does not revert to the S-form (32), both may develop into further cultural variants that produce colonies with smooth edges (18).

Dissociation of the S-form with the R-form occurs more frequently under a variety of circumstances. Colonies of L. monocytogenes incubated for several days on plates often dissociate to produce these R-forms (9, 14), and lowering the pH increases the conversion rate (9). Rowan and Anderson (29) isolated R-forms from milk heated after inoculation with L. monocytogenes. R-forms have also been isolated from infected mice (27) and result from growth as a biofilm (18). These observations may indicate that this variation in morphology is associated with a stress response; these R-forms exhibit increased adherence and greater heat resistance than the S-form (29).

There are several classes of R-form L. monocytogenes. Kuhn and Goebel (12) studied a small number of R-forms and found that three (n = 4) of them produced lower amounts of a protein they named p60 (iap or cwhA) in the supernatant. These R-forms (type I or MCR) grow as filaments with regular septation (30) without separation of cells and exhibit reduced virulence (10, 12, 27). However, it is not clear if p60 plays a role in the R-form, as Wuenschler et al. (39) found that complementation of an R-form with the gene for p60 resulted in smooth colony morphology. However, it was later found that p60 null mutants produce smooth colonies on agar (25) and that similar strains contain mutations in a gene (secA2) responsible for the secretion of p60 and other proteins (14). This recent observation is consistent with another observation of Wuenschler et al. (39) that complementation with the gene encoding p60 did not restore full expression of p60 in the supernatant. Another class of R-forms that produces normal amounts of p60 are virulent and produce filaments with variable septation with no separation of cells (type II or FR); these have also been found (14, 30, 31), and this class does not exhibit mutations in secA2 (14). Still other R-forms of L. monocytogenes exist. Monk et al. (18) described R-forms that produce low levels of p60 without exhibiting mutations in secA2 and also produced filaments of unseparated cells. No genes have been identified to account for these R-form cultural variants.
Two-dimensional differential gel electrophoresis (2D-DIGE) is an improved method for the comparison of protein expression that alleviates the gel-to-gel variation associated with conventional 2D gel electrophoresis. To accomplish 2D-DIGE, protein samples labeled with different fluorescent dyes are combined in equal amounts prior to 2D separation, and images of the individual proteins are produced. This technique allows meaningful comparisons to be made with one gel. Presently, there are three dyes available, allowing up to three different protein samples to be compared on a single gel, or two samples can be compared with replication with an internal standard (1, 7, 15, 36, 38).

This research describes the isolation of the R-form and the smooth colony cultural variants of \textit{L. monocytogenes}, which exhibit increased biofilm production and hypochlorous acid tolerance compared to the S-form. We selected one of these cultural variants for proteomic study to identify differences in protein expression and to identify proteins that may be related to increased biofilm production and hypochlorous acid tolerance.

**MATERIALS AND METHODS**

**Preparation of hypochlorous acid solution.** Hypochlorous acid solutions of desired concentrations were prepared each day from a sodium hypochlorite stock solution (100 ml/liter) (Mal- linkrodt Baker, Inc., Paris, Ky.). The solutions were prepared in water free of organics (deionized 18 million \(\Omega/cm\) buffered with 0.015 M KH\(_2\)PO\(_4\). Glassware was thoroughly cleaned, soaked overnight in 1% sodium hypochlorite solution, and rinsed in de-ionized water. After preparation, the hypochlorous acid solution was adjusted to pH 7.00 \(\pm\) 0.05 with 10 N NaOH, and the concentration of hypochlorous acid was verified by UV spectroscopy with the molar absorption constants OCl\(^1\) = 9.96 M\(^{-1}\) cm\(^{-1}\) at 235 nm and 26.9 M\(^{-1}\) cm\(^{-1}\) at 290 nm, and HOCl, 7.8 M\(^{-1}\) cm\(^{-1}\) at 235 nm and 350.4 M\(^{-1}\) cm\(^{-1}\) at 290 nm (19).

**Isolation of cultural variants.** Cultural variants were derived from \textit{L. monocytogenes} strain Scott A (SA) by exposing cell suspensions to 20 through 80 ppm of hypochlorous acid in 20-ppm increments. SA was inoculated from beads (Microbank Bead Vials, PRO-LAB Diagnostics, Richmond Hill, Ontario, Canada) stored at \(-80^\circ\)C into 5 ml of tryptic soy broth (TSB; Becton Dickinson Microbiology Systems, Sparks, Md.) and grown for 20 h statically at 32°C. The inocula used for hypochlorous acid exposure were enumerated on tryptic soy agar with 6% yeast extract (TSAYE; Becton Dickinson) with a Spiral Biotech AP4000 (Advanced Instruments, Norwood, Mass.). Aliquots of inocula (100 \(\mu\)l) were placed into a test tube, and 5 ml of standardized sodium hypochlorite solution was added to each tube and vortexed. After 5 min, the hypochlorous acid was quenched with 1 ml of a 10% (wt/vol) solution of sodium thiosulfate (final concentration, 0.07 M). Four milliliters of double-strength TSB was then added to each tube, and the tubes were incubated at 32°C. These tubes were observed for 5 days, and any turbidity was noted. Exposures to hypochlorous acid were in triplicate per replication, and the experiment was replicated three times. The contents of any tubes exhibiting growth were streaked onto RAPID’L.MONO media (Bio-Rad Laboratories, Hercules, Calif.) to confirm \textit{L. monocytogenes} growth. The number of tubes with surviving \textit{L. monocytogenes} was recorded for each isolate at each level of hypochlorous acid.

**Hypochlorous acid tolerance of planktonic cells.** The hypochlorous acid tolerance of prepared planktonic cells of the cultural variants was determined by exposing cell suspensions to 60 through 120 ppm of hypochlorous acid in 20-ppm increments. Each was inoculated from beads (Microbank Bead Vials) into 5 ml of TSB and grown for 20 h statically at 32°C in screw-cap test tubes prior to its use for hypochlorous acid challenge. Hypochlorous acid challenge inocula were enumerated on TSAYE with a Spiral Biotech AP4000 (Advanced Instruments). After growth, aliquots of culture (100 \(\mu\)l) were placed into a test tube, and 5 ml of standardized sodium hypochlorite solution was added to each tube and vortexed. After 5 min, the hypochlorous acid was quenched with 1 ml of 0.63 M sodium thiosulfate. Four milliliters of double-strength TSB was then added to each tube, and the tubes were incubated at 32°C. These tubes were observed for 5 days, and any turbidity was noted. Exposures to hypochlorous acid were in triplicate per replication, and the experiment was replicated three times. The contents of any tubes exhibiting growth were streaked onto RAPID’L.MONO media (Bio-Rad Laboratories, Hercules, Calif.) to confirm \textit{L. monocytogenes} growth. The number of tubes with surviving \textit{L. monocytogenes} was recorded for each isolate at each level of hypochlorous acid.

**Hypochlorous acid tolerance of biofilm cells.** Biofilms of the cultural variants were grown on stainless steel (type 304, finish 4b) coupons measuring 11 by 7.5 cm. For this experiment, each isolate was inoculated from beads into 5 ml of TSB and grown for 20 h statically at 32°C in screw-cap test tubes prior to transferring 2 to 200 ml of fresh TSB and incubating at 32°C for 20 h. Two coupons per strain were submerged in 200 ml of each culture of \textit{L. monocytogenes} and incubated at 32°C for 4 h in sterile plastic pans (Cambro H-Pan 62HP with lid 60HPC, Cambro, Huntington Beach, Calif.). Pans containing coupons were shaken at 70 rpm in a C24 incubator shaker (New Brunswick Scientific, Edison, N.J.). After 4 h, coupons were removed and placed in sterile sampling bags (Fisher brand 01-815-25, Fisher Scientific, Atlanta, Ga.) with 200 ml of 0.015 M KH\(_2\)PO\(_4\) adjusted to pH 7.0 (phosphate buffer) and hand shaken vigorously for 10 s. After rinsing, the coupons were transferred to sterile plastic pans containing 200 ml of fresh TSB and incubated for an additional 24 h at 32°C and 70 rpm. The coupons were rinsed with a wash bottle (no. 2405-0500, Nalgene, Rochester, N.Y.), with the nozzle widened by trimming (bore shape/diameter, oval/3.4 by 2.2 mm). Phosphate buffer (50 ml) was applied only to the top front of the coupon, and the back was rinsed by directing the stream of buffer evenly from top to bottom. After rinsing, coupons were placed biofilm side up into separate stainless steel pans (Super Pan II no. 3062-2/L33, Ollrath, Sheboygan, Wis.). The prepared biofilms of each strain were treated with 200 ml of 200 ppm of hypochlorous acid or phosphate buffer. A sterile stir bar was placed underneath the coupon during hypochlorous acid treatment to prevent the en-
trapment of cells, and the release of viable cells after the hypochlorous acid was neutralized.

Biofilms prepared and treated with phosphate buffer (untreated controls) were agitated for 10 min at 70 rpm (Orbit shaker, Lab Line Instruments, Inc., Melrose Park, Ill.), after which they were again rinsed with about 20 ml of phosphate buffer as previously described and transferred to a petri dish. Attached cells were enumerated by scraping with a Teflon policeman (VWR no. 13197-416, Bel-Art, Pequannock, N.J.) and rinsing with phosphate buffer three times, such that cells were collected with a total of 100 ml of phosphate buffer. The detached cells were shaken in milk dilution bottles for 10 min on a wrist action shaker (model 75, Burrel Scientific, Pittsburgh, Pa.) at maximum speed for 10 min to break up cell clumps prior to enumeration. Biofilm cells were enumerated on TSAYE (32°C, 18 h), and the number of attached biofilm cells was reported as log CFU/50 cm².

Biofilms prepared for treatment with hypochlorous acid were agitated for 5 min before the hypochlorous acid was neutralized with 175 ml of 0.2 M sodium thiosulfate (final concentration, 0.1 M). Neutralization of hypochlorous acid was verified with Aquacheck total-free hypochlorous acid test strips (Hach Company, Loveland, Colo.). The aliquots were then agitated for another 5 min, rinsed with about 20 ml of buffer, and transferred to a petri dish. It was necessary to determine surviving numbers of L. monocytogenes by overlaying the aliquots with TSAYE, as scraping and enumeration were not sufficiently sensitive to detect survivors. TSAYE was supplemented with sodium pyruvate at 1 g/liter to enhance the recovery of hypochlorous acid–injured cells and with potassium tellurite at 0.03 g/liter to improve the visualization of colonies. The overlays were incubated at 32°C for 48 h, and survivors were reported as log CFU/50 cm².

Preparation of cells for protein extraction. Planktonic cells of SA and SBS were inoculated from beads into 5 ml of TSB and grown for 20 h statically at 32°C in screw-cap test tubes prior to transferring 2.5 ml into 250 ml of fresh TSB and incubating for 20 h at 32°C. Cells were harvested by centrifugation (5,000 × g, 5 min, 20°C), washed three times in 40 mM Tris buffer (pH 8.5), and suspended in 1 ml of lysis buffer (40 mM Tris, 8 M urea, and 4% 3-[3-cholamidopropyl]-dimethyl-ammonio]-l-propanesulfonate [pH 8]) before protein extraction. Biofilm cells of SA and SBS were prepared in a fashion similar to the method of Trémoulet et al. (37). Planktonic cells of SA and SBS were inoculated from beads into 5 ml of TSB and grown for 20 h statically at 32°C in screw-cap test tubes prior to transferring 100 μl to 10 ml of fresh TSB and incubating for 20 h at 32°C. Cells were harvested by centrifugation (5,000 × g, 5 min, 20°C) and resuspended in 13 ml of phosphate buffer. Ten sterile glass fiber filters (extra-thick glass fiber filter, Pall no. 66084, Pall Life Sciences, Ann Arbor, Mich.) were placed in 150-mm petri dishes (one filter per dish), and the inoculum was dispensed onto the filter. Filters were incubated at room temperature for 5 min to allow attachment. The unattached cells were washed from the filter by swirling in 200 ml of phosphate buffer on an orbital shaker at 60 rpm for 1 min. Individual filters were washed in a sterile Pyrex dish (150 by 75 mm). After washing, the filters were transferred to a 150-mm petri dish containing TSAYE. A sterile glass fiber filter was placed between the agar surface and the inoculated filter to provide for the diffusion of nutrients from the agar surface to the inoculated filter and to avoid a collection of cells grown on the agar surface. Filters were incubated at 32°C for 72 h, washed in ice-cold phosphate buffer in the manner described previously, and placed in filtered stomacher bags (Nasco Whirl-Pak B01318, Fisher). The filters were kept refrigerated (≈5°C, <1 h) until 100 ml of ice-cold 40 mM Tris (pH 8.5) was added, after which the filters were stomached for 5 min. The bacterial suspension was harvested and kept on ice. The contents of the stomacher bag were allowed to drain through another filtered stomacher bag in order to remove large pieces of the glass wool (Nasco Whirl-Pak B01318). The filtrate was then centrifuged at 10,000 × g for 30 min, and the pellet was resuspended in 40 ml of cold Tris (pH 8.5) and filtered with 100-μm Sterifold filters (Millipore, Billerica, Mass.). The biofilm cells were washed two more times with cold Tris (pH 8.5) and suspended in 1 ml of lysis buffer. The cells were stored at −80°C until protein extraction.

Protein extraction and purification. Protein extraction was accomplished by sonication of planktonic cells and biofilm cells on ice (50% power/50% pulse/2-min increments for 10 min; Omni Ruptor 400, Omni International Inc., Marietta, Ga.). The lysate was centrifuged at 7,700 × g for 10 min at 4°C, and the pellets were extracted with a ReadyPrep sequential extraction kit according to the manufacturer’s instructions for the second and third extractions (Bio-Rad). The resulting protein was further purified with an Ettan 2-D cleanup kit (GE Healthcare, Piscataway, N.J.), resuspended in reagent 3 (ReadyPrep sequential extraction kit, Bio-Rad), and frozen at −80°C. The three fractions were combined, and the protein concentration was determined by Bradford’s method with the Bio-Rad protein assay (no. 500-0001) according to the manufacturer’s instructions.

Fluorescence 2D-DIGE. A total of 50 or 100 μg of protein was labeled with cyanine dye (Cy) DIGE dyes (Cy3 or Cy5, GE Healthcare). Briefly, the protein suspension was mixed with 1 or 2 μl of Cy dye (200 pmol in dimethylformamide) and incubated on ice for 30 min. The labeling reaction was stopped by the addition 1 μl of lysine solution (10 mM), and the samples were incubated on ice for 10 min. After labeling, the volume was brought up to 150 μl with reagent 3. The Cy3- and Cy5-labeled proteins were multiplexed onto one 17-cm ReadyStrip IPG strip (IPG strip, Bio-Rad) for isoelectric focusing (IEF).

All 17-cm IPG strips were rehydrated (50 V for 12 h) with the multiplexed samples. IEF (protein IEF cell, Bio-Rad) was performed according to the programming recommended by the manufacturer for each pH range. Proteins were focused on IPG strips at three different pH ranges (3 to 10NL, 4 to 7, and 4.7 to 5.9; Bio-Rad). After IEF, the IPG strips were equilibrated for sodium dodecyl sulfate–polyacrylamide gel electrophoresis with equilibration buffers I and II (Bio-Rad) according to the manufacturer’s instructions. IPG strips were loaded onto 1 mM Tris-glycine gradient (8 to 15%) gels (Jule, Inc., Milford, Conn.). Electrophoresis of gels was performed with an Ettan Dalt II (GE Healthcare) at 5 W per gel for 1 h and 2 W per gel overnight. Electrophoresis was completed at 45 mA per gel. For each pH range, proteins from planktonic cells of SBS and SA were compared on a single gel, and proteins from biofilms of those strains were compared. For the 3 to 10NL and 4 to 7 strips, 100 μg of protein was loaded, and for the 4.7 to 5.9 strips, 50 μg was loaded.

Gel imaging. Labeled proteins were imaged with a Typhoon 9400 imager (GE Healthcare), resulting in two images. Gel images were analyzed with DeCyder DIA software (GE Healthcare), and protein spots were chosen if expression changed more than twofold. Gels were then stained with SYPRO Ruby (Invitrogen, Carlsbad, Calif.), and DeCyder BVA was used to align the picked spots from the Cy dye images with the SYPRO Ruby images.

Peptide sequencing. Gel plugs of 2.0 mm were picked and automatically prepared for mass spectrometry with a Spot Handling Workstation (GE Healthcare). The plugs were washed twice
with 50 mM ammonium bicarbonate in 50% methanol for 20 min at room temperature and then washed with 75% acetonitrile for 20 min at room temperature. The plugs were dried (at 40°C for 10 min) and then incubated in 10 mM dithiothreitol in 20 mM ammonium bicarbonate at 37°C for 1 h, followed by 100 mM iodoacetamide in 20 mM ammonium bicarbonate (at room temperature for 30 min). Protein in the plug was digested with trypsin (200 ng of trypsin, 37°C for 2 h), peptides were extracted twice with 50% acetonitrile in 0.1% trifluoroacetic acid (at room temperature for 20 min), and solvents were removed by evaporation at 40°C for 30 min (SpeedVac, Jouan, Winchester, Va.). Approximately 25% of the resulting peptides were applied to the mass spectrometry target plate with partially saturated ammonium bicarbonate in 50% methanol for 20 min at room temperature. L. monocytogenes isolates studied in this article were cultural variants of the same strain; thus, the small spectrum obtained with mass accuracy, molecular weight, and isoelectric point.

RESULTS

Description of L. monocytogenes cultural variants.

Several cultural variants were isolated by chlorination of L. monocytogenes SA. The first cultural variant (SAR) was more tolerant of hypochlorous acid than SA, and after testing the suspension chlorine resistance of SAR and streaking out growth for isolation, a colony was picked and designated SAR5. SAR5 exhibited morphological characteristics similar to SAR. A smooth spontaneous mutant (SBS) was isolated from a sample of SAR that was plated for enumeration. SBS produces smooth colonies on TSA that differed from the colonies of SA in color and had slight surface texturing not present on SA colonies (Fig. 1). Hypochlorous acid–tolerant variants SAR and SAR5 produced large, rough, light beige colonies that were opaque on TSA, while SBS produced smooth, beige, opaque colonies on TSA that were slightly smaller than the S-form (Fig. 1). These morphologies are unlike the white or bluish translucent colonies of typical L. monocytogenes strains (Fig. 1). When grown on Listeria selective agar, all cultural variants were smooth and difficult to discern from the S-form (data not shown). Growth of all three cultural variants in TSB was flaky, with a surface pellicle as described by Seeliger (32); however, our cultural variants did not produce filaments like other reported R-forms (Fig. 1). Cells of these cultural variants tended to clump (Fig. 1), and when challenged with hypochlorous acid, the survivors grew only as colonies attached to the glass surface of the test tubes for many hours before resuming planktonic growth. A similar occurrence was described by Monk et al. (18). All variants exhibited biochemical and morphological characteristics consistent with L. monocytogenes. R-form cultural variants of L. monocytogenes with colony morphology and growth characteristics similar to the strains used in this study have been previously described (9, 10, 12–14, 29, 32). To et al. (35) also observed survivors growing only as colonies attached to the glass surface when challenging L. monocytogenes strains with benzalkonium chloride.

Suspension exposure to 120 ppm of hypochlorous acid was not sufficient to eliminate the cultural variants (Table 1), whereas the parent strain SA survived only 60 ppm of hypochlorous acid (6). There was no difference among the variants with regard to suspension hypochlorous acid tolerance (Table 1). Two hundred parts per million of hypochlorous acid was insufficient to kill all the cells in biofilms produced by the cultural variants (Table 2). In contrast to the results obtained on cell suspensions, the biofilms of cultural variant SBS were significantly more tolerant of hypochlorous acid than the other cultural variants. SBS also produced a biofilm that contained 10-fold more cells than the others. While all cultural variants survived a much higher level of hypochlorous acid than the S-form, they also produced over 1,000-fold more biofilm cells than SA when grown as a biofilm (Table 2). SBS was chosen for proteomic comparison because of its superior biofilm production and biofilm chlorine tolerance.

Fifteen proteins were down-regulated in SBS compared to planktonic SA cells, and four were identified. Five of these proteins were found on the 3 to 10 range gel, eight on the 4 to 7 range gel, and two on the 4.7 to 5.9 range gel. Four proteins were up-regulated in planktonic SBS, but none could be identified; two were found on the 4 to 7 range gel, and two were found on the 4.7 to 5.9 range gel. Fifteen proteins were down-regulated in biofilm-grown SBS cells, of which one was identified. Two were found on the 3 to 10 range gel, nine were found on the 4 to 7 range gel, and four were found on the 4.7 to 5.9 range gel. Seven proteins were up-regulated in biofilm-grown SBS cells, of which one was identified, with four of those found on the 4 to 7 range gel and the other three on the 4.7 to 5.9 range gel. Detailed descriptions of the identified proteins are listed in Table 3.

DISCUSSION

The procedures used to assess the hypochlorous acid tolerance in our previous study (6) were modified to accommodate the increased biofilm produced by the cultural variants. Despite agitation of the medium during growth, the biofilms of SBS were easily removed by the previously described rinsing procedures (6). This necessitated the use of a more gentle procedure. Monk et al. (18) also experienced difficulty washing the biofilms of cultural variants grown in a bioreactor, noting that biofilm structure would be lost to washing, despite growth in turbulent flow.

While exposure to hypochlorous acid improved biofilm production and increased hypochlorous acid tolerance, subculturing these cultural variants in the absence of hypochlorous acid exposure resulted in diminished surface pellicle, flakiness, and diminished biofilm production, with no changes in colony morphology and no reversion to the S-form. Because of this phenomenon, subculturing was minimized when possible.

The L. monocytogenes isolates studied in this article were cultural variants of the same strain; thus, the small
FIGURE 1. Images of colonies illustrating the colony and cell morphology of L. monocytogenes strains SA (a), SBS (b), SAR (c), and SAR5 (d). Images on left, phase-contrast images (original magnification ×1,000, bar = 10 μm). Images on right, stereoscope image (original magnification ×30, bar = 1 mm).

TABLE 1. Survival of planktonic cultural variant strains of Listeria monocytogenes exposed to hypochlorous acid for 5 min

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of tubes of 9 with growth (ppm):</th>
<th>Inoculum, log (CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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</tbody>
</table>

<sup>a</sup> Cultural variant exhibiting rough colony morphology.
<sup>b</sup> Cultural variant exhibiting smooth colony morphology.

TABLE 2. Biofilm growth of cultural variants of Listeria monocytogenes and survival of variant biofilms exposed to 200 ppm of hypochlorous acid for 5 min<sup>a</sup>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Adherent cells, log (CFU/50 cm&lt;sup&gt;2&lt;/sup&gt;)</th>
<th>Hypochlorous acid survivors (CFU/50 cm&lt;sup&gt;2&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rep 1</td>
<td>Rep 2</td>
</tr>
<tr>
<td>SA</td>
<td>6.71</td>
<td>1</td>
</tr>
<tr>
<td>SAR</td>
<td>10.11</td>
<td>16</td>
</tr>
<tr>
<td>SBS</td>
<td>10.98</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>SAR5</td>
<td>10.03</td>
<td>5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Rep, replication.
TABLE 3. Changes in the expression of proteins identified from the peptides sequenced

<table>
<thead>
<tr>
<th>Protein name</th>
<th>IEF range</th>
<th>Gene name</th>
<th>Size (kDa)</th>
<th>pI</th>
<th>% protein covered</th>
<th>X-fold expression change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosomal protein L10</td>
<td>4–7</td>
<td>RplJ</td>
<td>17.8</td>
<td>5.4</td>
<td>44</td>
<td>-2.2</td>
</tr>
<tr>
<td>ABC transport: sugar binding</td>
<td>4–7</td>
<td>LMO0181</td>
<td>46.8</td>
<td>4.6</td>
<td>45</td>
<td>+2.4</td>
</tr>
<tr>
<td>ABC transport: sugar binding</td>
<td>4.7–5.9</td>
<td>LMO0181</td>
<td>46.8</td>
<td>4.6</td>
<td>26</td>
<td>+2.0</td>
</tr>
</tbody>
</table>

Protein expression changes in biofilm-grown SBS

ABC transport: sugar binding

Peroxide resistance protein (Dpr)         | 4–7       | fri       | 18.2       | 4.9| 49                | -2.2                     |
Hypothetical protein                      | 4–7       | LMO1888   | 12.9       | 4.6| 48                | -2.1d                    |
Ribosomal protein L7/L12                  | 4–7       | RplL      | 12.5       | 4.6| 20                |                          |

Protein expression changes in planktonic SBS

ABC transport: sugar binding

Peroxide resistance protein (Dpr)         | 4–7       | fri       | 18.2       | 4.9| 49                | -2.2                     |
Hypothetical protein                      | 4–7       | LMO1888   | 12.9       | 4.6| 48                | -2.1d                    |
Ribosomal protein L7/L12                  | 4–7       | RplL      | 12.5       | 4.6| 20                |                          |

Proteins are listed with size, isoelectric point (pI), protein coverage by the peptides (%), and the isoelectric focusing (IEF) range of the strip used.

a ListilList gene name (8).
b pI and sizes presented are the calculated results.
d Protein spot yielded two proteins.

c

The number of proteins (19 to 22) found to be altered in expression was expected. In addition, the two-way comparison employed in this study would likely yield fewer differences in expression than a three-way comparison that included the SAR. Because there are no protein expression changes due to strain difference, we expect that proteins found in this research are involved with the cultural variation and may include proteins associated with increased biofilm production or hypochlorous acid tolerance. However, it is possible that many more proteins exhibited altered expression, as much of the protein extracted was not well resolved because of the preponderance of proteins with an isoelectric point of between pH 4 and 6 (5). The pH 3 to 10NL and 4 to 7 IPG strips became overloaded (Fig. 2), resulting in a large area on the 2D gels where protein spots overlapped. This area was better resolved by narrowing the pH range and lowering the protein load, but some proteins outside the pH range of 4 to 6 may not have been detected, since overloading may impede proper IEF.

The peroxide resistance–associated protein (Dpr/fri/flp) down-regulated in the SBS cultural variant is an iron-binding ferritin similar to the DNA-binding protein Dps of Escherichia coli; however, Dpr does not bind DNA (2). The mRNA of Dpr is transcribed by either $\sigma^A$ or $\sigma^B$ (26), and the gene is also under the regulatory control of the peroxide resistance regulon (23). The regulatory element of this regulon is PerR, a metal-binding protein whose negative regulation is relieved during oxidative stress, allowing transcription of a wide variety of peroxide resistance genes, including Dpr. Mutants lacking functional PerR are extremely resistant to hydrogen peroxide (28). Dpr is also linked to oxygen tolerance in other organisms (23, 40). This is a result of the iron-sequestering function of Dpr, which inhibits the formation of hydroxyl radicals formed via the Fenton reaction (40). While it might be expected that a decreased expression of Dpr in planktonic SBS would result in a decreased tolerance to hypochlorous acid, the ob-

FIGURE 2. Images of representative 2D gels with identified proteins indicated by ListilList gene name (8). (A) pH range, 3 to 10; (B) pH range, 4 to 7; and (C) pH range, 4.7 to 5.9. Regions of the gel where proteins were poorly resolved are indicated by arrows.
ervation that SBS is more tolerant of hypochlorous acid indicates that the peroxide resistance regulon does not have an important role in hypochlorous acid tolerance, as is the case with the E. coli homolog, the oxyR regulon (4). However, the flaky growth, diminished surface pellicle, and petite colony characteristics may be associated with reduced oxygen tolerance due to the decreased expression of Dpr.

Sugar-binding protein components of gram-positive ABC (ATP-binding cassette) transporters are anchored in the cell membrane by attachment of a lipid to an N-terminal cysteine. The first 20 amino acids of LMO0181 precede a cysteine and are consistent with other lipopidation signals (34). The LMO0181 gene coding for this protein is a member of an operon that includes seven proteins in total (LMO0178 through LMO0184). These genes encode a repressor of xylose metabolism and other genes related to the breakdown of carbohydrates and transport of sugars (8, 17). The regulation of this operon is uncertain, but evidence indicates that it is negatively regulated by the virulence regulator prfA (17). The reduced expression of this operon in planktonic SBS and the increased expression in biofilm-grown SBS may indicate that the protein expression changes found in SBS result in additional carbon sources being available for biofilm formation. Thus, these changes in regulation could be responsible for the increased biofilm production of SBS.

The organization of the genes encoding L7/L12 and L10 of L. monocytogenes is the same as found in E. coli (8). Ribosomal protein L7/L12 is one of many proteins that are associated with the 50s ribosomal subunit. Four L7/L12 proteins form a protein complex with ribosomal protein L10 and are required for protein elongation. Ribosomal protein L7 is formed from L12 by acylation of the N terminus and is functionally identical to L12 (22, 24). Although the ratio of L7 to L12 varies by growth conditions, it is not known why (3). Since the growth of SBS is not impaired when compared to SA, it is unlikely that protein synthesis is decreased by the decreased expression of L10 in biofilm-grown SBS or L7/L12 in planktonic-grown SBS. There must be an alternative function for these proteins. Research indicates the possibility that L7/L12 can be altered to perform other cellular roles (20, 21), and a protein structurally similar to L7/L12, whose gene is located in the genome in a position similar to L7/L12, functions as a surface-located virulence factor in Neisseria gonorrhoeae (33). These observations are consistent with the results of other researchers, who found that ribosomal protein L7/L12 expression is unaffected by biofilm growth (11) and transition into stationary phase (5). Since amino acid starvation reduces ribosome production (22), ribosomal proteins would be expected to be down-regulated under those conditions. Ribosomal protein L10, however, is down-regulated on entry into stationary phase (5), casting doubt on an alternative function. There is no evidence for other cellular roles for this protein.

DivIVA is involved with the initiation of cell division, and LMO1888 shares 51% homology with the Bacillus cereus DivIVA (NCBI accession no. AAP08541). While another protein has been ascribed the function of DivIVA in Listeria spp. (NCBI accession no. CAD00098), it seems likely that this protein plays some role in cell division because of this homology.

We have observed a change in expression of at least five proteins by cultural variants of L. monocytogenes isolated after exposure to hypochlorous acid. These patterns of expression were concomitant with hypochlorous acid tolerance and increased biofilm production. The proteins affected are diverse in function and likely regulated by several systems. The cultural variants described in this study arose after the wild type was exposed to a common food processing plant sanitizer. There is a need to understand the frequency with which such cultural variants arise in the food-processing environment. If they are common, then it will be important to determine the virulence of these strains, in addition to their survival characteristics.

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