Bioenergetic Mechanism for Nisin Resistance, Induced by the Acid Tolerance Response of Listeria monocytogenes

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This study examined the bioenergetics of Listeria monocytogenes, induced to an acid tolerance response (ATR). Changes in bioenergetic parameters were consistent with the increased resistance of ATR-induced (ATR+) cells to the antimicrobial peptide nisin. These changes may also explain the increased resistance of L. monocytogenes to other lethal factors. ATR+ cells had lower transmembrane pH (ΔpH) and electric potential (Δψ) than the control (ATR−) cells. The decreased proton motive force (PMF) of ATR+ cells increased their resistance to nisin, the action of which is enhanced by energized membranes. Paradoxically, the intracellular ATP levels of the PMF-depleted ATR+ cells were ~7-fold higher than those in ATR− cells. This suggested a role for the FOF1 ATPase enzyme complex, which converts the energy of ATP hydrolysis to PMF. Inhibition of the FOF1 ATPase enzyme complex by N-1,3-dicyclohexylcarbodiimide increased ATP levels in ATR+ cells, where ATPase activity was already low. Spectrometric analyses (surface-enhanced laser desorption ionization–time of flight mass spectrometry) suggested that in ATR+ listeriae, the downregulation of the proton-translocating c subunit of the FOF1 ATPase was responsible for the decreased ATPase activity, thereby sparing vital ATP. These data suggest that regulation of FOF1 ATPase plays an important role in the acid tolerance response of L. monocytogenes and in its induced resistance to nisin.

Listeria monocytogenes causes ~2,500 cases/year of listeriosis out of an estimated 76 million cases/year of food-borne disease in the United States (23). The pathogen targets mainly newborn, pregnant, elderly, and immunocompromised individuals, and it is associated with mortality rates of up to 37%. Control of L. monocytogenes is difficult, due to its widespread presence in nature, intrinsic physiologic resistance, adaptation capacity, and ability to grow at low temperatures (37).

The responses of L. monocytogenes to acid, osmotic, and thermal stresses increase its resistance and virulence (16, 28). The acid tolerance response (ATR) is the abnormal resistance to lethal acid after an exposure to mild acidic conditions (21). The regulation of stress response proteins changes during induction of the ATR (29, 31). These proteins include chaperones, transcriptional regulators (13), the glutamic acid decarboxylase system, and the FOF1 ATPase enzyme complex (10, 31). The ATR also increases virulence and cross-protects listeriae from other stressors, such as elevated temperatures (16) and antimicrobials (28).

The specific acids affect the pH range at which the ATR is induced and the range within which the pH becomes lethal; lactic acid is a stronger inducer than HCl (2). The ATR also confers resistance to the bacteriocin nisin, an antimicrobial peptide that is approved for food use in >40 countries (6). ATR-induced L. monocytogenes cells (ATR+) but not the control cells (ATR−) survive for at least 30 days at 4°C in a model fermented system where Lactococcus lactis produced lactic acid (pH 5.7) and nisin (50 μg/ml) (2). The mechanism by which the ATR protects L. monocytogenes against nisin is uncertain. Analysis of membrane lipids of constitutively nisin-resistant listeriae shows that their membrane is more rigid, due to changes in the proportion of fatty acids (11, 24, 25). Similar temperature-induced changes in membrane composition cause measurable changes in membrane fluidity as demonstrated by fluorescence anisotropy (22). However, these changes in membrane lipid composition do not fully explain the increased nisin resistance of ATR+ listeriae (38).

Cell membranes have low permeability to protons, which are subjected to specific transport mechanisms such as FOF1 ATPase, Na+/H+ antiporters, and electron transport systems (31). This enables living cells to build a potential across their membranes, which is essential for energy transduction (41). The peptide nisin targets energized cell membranes, and its insertion is activated by the difference in free available energy across the membrane (12). Nisin molecules insert cooperatively into the cell membrane, which is disrupted by transient pore formation (4). Destruction of the membrane integrity collapses the proton motive force (PMF), causing cell death.

The PMF-dependent action of nisin suggested a bioenergetic contribution to nisin resistance in ATR+ listeriae. We hypothesized that decreased PMF contributes to the increased nisin resistance of ATR+ L. monocytogenes. In L. monocytogenes, the PMF is generated primarily by the membrane-associated FOF1 ATPase, which builds a PMF using energy derived from ATP hydrolysis (8). We present surprising evidence that ATR+ listeriae have significantly higher intracellular ATP (ATP) concentrations than ATR− cells. This vital ATP sparing in ATR+ L. monocytogenes is correlated to the downregulation of the FOF1 ATPase c subunit.

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**MATERIALS AND METHODS**

Bacterial strains, cultivation conditions, and chemicals. *L. monocytogenes* Scott A® (serotype 4b, containing plasmid pGK12) was originally obtained from P. Foegeding (North Carolina State University, Raleigh, NC) (14) and maintained as described in our previous studies (2). Broth cultures were prepared in tryptic soy broth augmented with 0.5% yeast extract (TSBYE) and incubated statically for 18 h at 37°C. Unless otherwise noted, TSBYE acidification was done using 30% (vol/vol) l(+)-lactic acid (80% [wt/vol] commercial solution; Purac America, Lincolnshire, IL). All pH measurements were conducted using a recording potentiometer (Merkatron, Honolulu, HI) at 37°C. Acidified media, solutions, and supernatants were sterilized using 0.2-μm membrane filters (Millipore Corporation, Bedford, MA). Dehydrated media and their major components were acquired from Difco/Becton Dickinson and Company. Inorganic substances, enzymes, nisin preparation, antibiotics, and ionophors were purchased from Sigma Chemicals (St. Louis, MO). Fluorescent probes were purchased from Molecular Probes (Eugene, OR).

**Induction of ATR in *L. monocytogenes***. The ATR was induced as previously described. Briefly, 10 μl of a stationary-phase TSBYE *Listeria monocytogenes* Scott A® culture (which had been inoculated with a loopful of the agar slant stock culture and incubated for 18 h at 37°C) was used to inoculate TSBYE (40 ml) and incubated at 37°C until early-exponential-growth phase (Δ(log10) = 0.1; 4.1 × 10⁷ CFU/ml) Cells were centrifuged (5,095 × g at 4°C for 10 min) and resuspended in an equivalent volume of TSBYE (10 M; ATR™ control) or in TSBYE acidified with lactic acid to pH 5.5 (ATR® induction) at 37°C for 60 min.

**Determination of intracellular pH (pH<i></i>i)**. (i) Cell preparation and probe uptake. Determinations of pH<i></i>i were conducted using the probe BCECF-AM (2′,7′-bis(2-carboxyethyl)-5′- and 6′-carboxyfluorescin, acetoxymethyl ester). ATR® or ATR™ *L. monocytogenes* (10 ml) was centrifuged at 5,095 × g at 4°C for 10 min, and the pellets were suspended in 500 μl potassium phosphate buffer (50 mM) at pH 7.5 or pH 7.0, respectively. Suspensions were washed twice (10,000 × g for 30 s) using the respective buffer. Aliquots (each, 200 μl) were collected by centrifugation (10,000 × g for 30 s) and resuspended in 20 μl buffer containing 1 μl of BCECF-AM solution (5 μg BCECF dissolved in 8 μl dimethyl sulfoxide [DMSO]). After 15 min at room temperature to allow for probe uptake and hydrolysis by cell esterases, preparations were washed twice, each time in 500 μl of the appropriate buffer and resuspended.

(ii) Calibration curves for pH<i></i>i. Individual calibration curves (to account for different cell numbers in the ATR® and ATR™ preparations) correlating pH<i></i>i and fluorescence were obtained by measuring the fluorescence (Fluorescent Spectrometer model LS10B, Perkin-Elmer Instruments, Shelton, CT) of each cell preparation at various external pH values (pH<i></i>e). Potassium phosphate buffer (50 mM) at pH 7.0 or pH 7.5 was used to establish pH<i></i>e values. Cuvettes containing 2,000 μl of 50 mM potassium phosphate buffer at the appropriate pH<i></i>e were aliquoted with 50 μl of a 20% (wt/vol) wt/vol) solution glucose (ft) curve to determine their pH<i></i>i values.

(iii) Calibration curves for internal pH (pH<i></i>i). To assess pH<i></i>i during exposure to nisin, 12.5 μl of a nisin solution (10 mM) to give a final concentration of 50 μg/ml was added at time zero to a cuvette containing the cell preparations in acidified buffer and glucose, as described above.

**Determination of transmembrane electric potentials (ΔΦ).** (i) Assay optimization and cell preparation. Transmembrane electric potentials (ΔΦ) were determined at 22°C using the fluorescent probe 3,3′-dipropylthiadicarbocyanine iodide (di-S-C3) (5). Prior to determinations, the ΔΦ values of various di-S-C3 (5) and cell concentrations were evaluated to optimize assay conditions and to avoid inner filter effects (the scattering and absorption of the excitation and emission light by turbid material). Briefly, solutions of the probe ranging from 490 to 4,900 nM were prepared in 50 mM sodium–HEPES–morpholinoneethanesulfonic acid (MES) buffer and their ΔΦ values were measured in the absence and presence of cell suspensions (ΔΦ<sub>50</sub> = 0.19; 9 × 10⁷ CFU/ml). Once the extinction coefficient (ε) of the probe and optimal assay conditions were established, 10 ml of ATR® or ATR™ cells was washed twice (5,095 × g for 4°C for 10 min), suspended to an A<sub>600</sub> of 0.19 using 50 mM potassium–HEPES–MES buffer at pH 7.0 or 5.5, respectively, and kept ice cold.

(ii) Estimation of [K<sup>i</sup>]. Intracellular potassium concentrations [K<sup>i</sup>] must be determined to calculate ΔΦ (37). The fluorescence of cells suspended in buffers ranging from 0 to 250 mM potassium phosphate [K<sup>i</sup>], was evaluated after the addition of the potassium uniporter valinomycin. To impose various [K<sup>i</sup>], values, appropriate amounts of 3.0 F KCl were added to 50 mM sodium–HEPES–MES buffers at pH 5.5 (for ATR®) or pH 7.0 (for ATR™). The probe di-S-C3, (100 μM solution) (5) was added (10 μl; final concentration, 330 mM) to cuvettes containing 2,000 μl buffer at pH 7.0 (ATR®) or 5.5 (ATR™). Continuous fluorescence readings were made at 120 s, 1,000 μl of ATR® or ATR™ cells (each previously resuspended in the test potassium concentration) was mixed into the cuvette. At ~150 s, 12 μl of a 2 mM ethanolic valinomycin solution was added to a final concentration of 8 μM. Excitation and emission wavelengths were 643 and 666 nm, and band-pass widths were 5.0 and 10.0 nm, respectively. The assay was set at 22°C for 300 s, with readings every 0.1 s. The ΔΦ was determined from potassium diffusion potentials (39) as follows:

\[ \Delta \Phi = -59 \log_{10} \left[ \frac{[K^+]_i}{[K^+]_o} \right] \tag{2} \]

(iii) Effect of nisin on transmembrane electric potential (ΔΦ). Assays were conducted as described above, with modifications. Prior to fluorescence measurements, 75 μl of 20% (wt/vol) glucose solution (final concentration, 0.5% [wt/vol]) was added to cuvettes containing 2,000 μl of 50 mM potassium–HEPES–MES buffer at pH 5.5 or pH 7.0. The cell preparation (1,000 μl) was then added, followed by nigericin (1 μl of 5 mM ethanolic solution; final concentration, 1.6 μM) to eliminate the ΔΦ component of the PMF. The cuvette was subjected to baseline fluorescence readings; at ~40 s, 10 μl of 100 μM di-S-C3 (final concentration, 320 nM) (5) was added and quickly mixed in by pipetting. After probe equilibration, 15 μl of 10,000 IU nisin ml⁻¹ was mixed in to a final concentration of 50 μg ml⁻¹. In some assays, valinomycin (8 μM) was added at 400 s to deplete residual ΔΦ. To establish a background fluorescence level, assays were also performed with dead cells (autoclaved at 121°C for 15 min) of the corresponding cell preparations.

(iv) De novo protein synthesis and response to nisin. The influence of de novo protein synthesis on ΔΦ and cellular response to nisin was examined at pH 5.5 using elevated, inhibitory levels of chloramphenicol (70 μg/ml). Assays were conducted as described above, but the assay time was extended to 1,000 s. Nisin (final concentration, 50 μg ml⁻¹) was added at 650 s, followed by valinomycin (8 μM) at 500 s.

**Determination of ATP levels**. ATP levels were determined by the luciferin–luciferase method previously described (24), with modifications. Calibration curves for luminescence versus ATP concentrations were generated by the addition of the enzyme-substrate preparation (diluted 1:10 with the dilution buffer provided) to serially diluted ATP standards in the same buffer, followed immediately on a luminometer (Luminoskan TL Plus; Labsystems Oy, Helsinki, Finland). ATP was determined as the difference between total ATP and extracellular ATP. ATR® or ATR™ cells were washed twice (each, 5,095 × g at 4°C for 10 min) and resuspended at equal cell densities (A<sub>600</sub> = 0.19) in 50 mM sodium–HEPES–MES buffer at pH 7.0 or 5.5, respectively, and kept ice cold. For determination of extracellular ATP, cell suspensions (each, 100 μl) were diluted 1:10 in 50 mM sodium–HEPES–MES buffer at pH 7.8, followed by the mixing of 100 μl with an equal volume of enzyme-substrate preparation in a round cuvette immediately before readings were carried out. To determine total (extracellular plus intracellular) ATP levels, 100-μl cell suspensions were diluted 1:10 in their respective suspension buffers and centrifuged at 10,000 × g for 30 s. The resulting pellets were mixed with 10 μl DMSO for complete cell permeabilization (32). After 5 min of permeabilization at room temperature, 990 μl of 50 mM sodium–HEPES–MES buffer, pH 7.8, was added to the pellets to give a final volume of 1,000 μl. Samples (each, 100 μl) were added with equal amounts of enzyme-substrate preparation to determine their ATP concentrations.

Time-dependent examination of ATP levels in the presence of glucose and nisin. ATP determinations in the presence of glucose (0.5% [wt/vol]) and nisin (50 μg ml⁻¹) were conducted as described above with the following modifications. Portions (each, 1,000 μl) of ice-cold cell preparations were centrifuged (10,000 × g at 4°C for 30 s) and resuspended (A<sub>600</sub> = 0.19) in 50 mM sodium–HEPES–MES buffer (pH 5.5) containing glucose and nisin. At predetermined intervals, 100-μl portions were diluted 1:10 in 50 mM sodium–HEPES–MES buffer, pH 7.8, to decrease the nisin concentration and minimize its action and then centrifuged. Pellets were treated with 10 μl DMSO and resuspended in 1,000 μl.
buffer, and 100 µl was used for each luminescence assay. Controls were performed with glucose in the absence of nisin.

**Role of the F$_{0}$F$_{1}$ ATPase complex on ATP levels.** ATP concentrations of cells treated with 1 mM N-$'$-$'$N-$'$-$'$N-$'$-$'$N-$'$-L-1,3-dicyclohexylcarbodiimide (DCCD), an F$_{0}$F$_{1}$ ATPase inhibitor (22), during ATR induction were determined as described above, except that 50 mM potassium-HEPES-MES buffers (containing 1 mM DCCD) were used to avoid the sodium-induced decrease of DCCD inhibitory activity (3).

**Regulation of F$_{0}$F$_{1}$ ATPase.** (i) **Sample preparation for surface-enhanced laser desorption ionization–time of flight mass spectrometry (SELDI-TOF MS).** ATR$^+$ or ATR$^-$ L. monocytogenes cells (100 ml) were centrifuged at 5,995 × g at 4°C for 10 min, washed in potassium phosphate buffer (50 mM) at pH 5.5 or pH 7.0, respectively, and centrifuged again. The pellets were resuspended in 1 ml aquous chloramphenicol solution (3.5 µg · ml$^{-1}$) to inhibit de novo protein synthesis during sample preparation and frozen at −20°C overnight. The A$_{600}$ was fixed at 0.50 (2.5 × 10$^{7}$ CFU/ml) by the addition of lysis buffer (30 mM Tris-HCl [pH 7.4], 100 mM NaCl, 5 mM EDTA, and 1% sodium dodecyl sulfate) to the resuspended pellets. Twenty milliliters of each preparation was subjected to 20,000 ln/m$^2$ for 1 min with a French pressure cell. Protein was quantified by the extinction coefficient determined by a standard curve generated using bovine serum albumin (200 mg/ml; Sigma) in lysis buffer.

(ii) **Chip preparation.** Samples (each, ca. 4 µl) having equal amounts of protein (6.6 µg) were spotted in quadruplicate on a hydrophobic H4 protein chip (Ciphergen Biosystems, Fremont, CA) and dried at room temperature for 15 min. The co-crystalization matrix was a saturated ninhydrine acid (Sigma-Fluka) solution prepared in equal volumes of 1% trifluoroacetic acid and 50% acetonitrile. Two microliters of the matrix was added to each spot prior to assay and allowed to dry for an additional 15 min.

(iii) **Spectrometric analysis.** Samples were analyzed by mass spectrometry using a series PBS II ProteinChip reader (Ciphergen) with optimization between 2 and 15 kDa. Spectra from continuous laser excitation of three independent spots for each sample were averaged and analyzed.

(iv) **Protein databases.** Computational studies were performed primarily using information on the Listeria monocytogenes complete genome available from the Pasteur Institute at http://genolist.pasteur.fr/ListiList/index.html and applicable links. Protein hydropathy plots were performed using Kite-Doolittle computations available at http://www.vivo.colostate.edu/molkit/hydropathy/.

**Statistical analysis.** Experiments were reproducible and performed in at least duplicate unless stated otherwise. Where applicable, means were compared using Student’s t test, and inferences were made at a 5% probability level.

**RESULTS**

pH$_{i}$ in ATR$^+$ and ATR$^-$ cells; effect of nisin on pH$_{i}$. Intracellular pH values were calculated from separate calibration curves ($r^2 > 0.98$) which accounted for the different cell densities of ATR$^+$ and ATR$^-$ cells. When cell preparations were subjected to nisin and glucose at pH 5.5, the pH$_{i}$ of ATR$^+$ listeriae at time zero was 5.8, resulting in a transmembrane pH (ΔpH) of −0.3 U. In contrast, ATR$^-$ cells had a higher initial pH$_{i}$ of 6.2, resulting in a ΔpH of −0.7 U (Fig. 1). The reduced ΔpH of the ATR$^+$ cells decreased the action of nisin during at least the first 20 s. The pH$_{i}$ of the ATR$^-$ cells rapidly approached a pH$_{i}$ of 5.5. These results did not change in the absence of nisin (data not shown), indicating that pH$_{i}$ responses were due to the penetration of lactic acid.

**Transmembrane electric potential (∆ψ) in ATR$^+$ and ATR$^-$ cells.** (i) **Assay optimization and cell preparation.** The ∆ψ component of the PMF was also examined. Assay conditions were optimized to avoid detrimental inner-filter effects, and the extinction coefficient (ε) of di-S-C$_3$ (5) was determined. The initial optimal probe concentration was 490 nM. The 490 nM probe solution had an $A_{443}$ value of 0.015, producing an extinction coefficient (ε) of 30,612 M$^{-1}$ cm$^{-1}$. The $A_{443}$ increased to 0.07 as cell suspensions were added to a ratio of one part cell suspension to two parts buffer with probe, while the probe concentration fell to about 320 nM. These conditions (final $A_{443}$ <0.10) ensured that inner-filter effects (15) were minimized.

(ii) **Estimation of [K$^+$]$_{i}$.** Intracellular potassium concentrations of ATR$^+$ or ATR$^-$ cells prepared in buffer containing 0 or 50 mM K$^+$ were determined. When assayed in buffer without potassium, cells were hyperpolarized, due to valinomycin-mediated potassium efflux. Cell hyperpolarization (negative inside) caused the uptake of positively charged di-S-C$_3$ (5), resulting in quenched fluorescence. Conversely, cell depolarization was indicated by the increase in fluorescence after valinomycin addition. When [K$^+$]$_{o}$ was equal to [K$^+$]$_{i}$, fluorescence was unchanged after valinomycin addition. The [K$^+$]$_{i}$ values for cells prepared at a [K$^+$]$_{o}$ of 50 mM were 50 and 25 mM for ATR$^+$ and ATR$^-$ cells, respectively (data not shown). When the cells were prepared in buffer without potassium, the approximate [K$^+$]$_{i}$ was accordingly smaller ([K$^+$]$_{i}$ = 8 versus 4 mM for ATR$^+$ and ATR$^-$, respectively), but the twofold difference between them remained constant. Using equation 2 to convert differences in potassium concentrations into millivolts, a [K$^+$]$_{i}$ value of 50 mM resulted in a ∆ψ of ~0 mV for ATR$^+$ cells and −18 mV for ATR$^-$ cells. Using equation 1 and the previously determined ∆ψ values, the PMF was −18 mV for the ATR$^+$ versus −59 mV for ATR$^-$ listeriae in medium containing 50 mM K$^+$ (Table 1). For cells prepared in the absence of potassium, the ∆ψ in buffer with [K$^+$]$_{o}$ at 50 mM was −47 and −65 mV for ATR$^+$ and ATR$^-$ cells, respectively.

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<th>Cell preparation</th>
<th>Calculated (mV)</th>
<th>PMF (mV)</th>
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<td>$\Delta p$H</td>
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</tr>
<tr>
<td>ATR$^+$</td>
<td>50</td>
<td>−18</td>
</tr>
<tr>
<td>ATR$^-$</td>
<td>25</td>
<td>−41</td>
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<td>−18$^a$</td>
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<td>ATR$^-$</td>
<td>4</td>
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$^a$ Intracellular potassium concentrations ([K$^+$]$_{i}$) were estimated from changes in fluorescence using different extracellular potassium concentrations ([K$^+$]$_{o}$) in the presence of the ionophor valinomycin. Transmembrane pH (∆pH) values were determined using a calibrated pH-sensitive fluorescent probe. Transmembrane electrical potentials (∆ψ) were calculated using equation 2 for [K$^+$]$_{i}$ = 50 mM. See the text for more details.

$^a$ Assumes that [K$^+$]$_{i}$ has a negligible affect on ΔpH.

**FIG. 1.** Determination of pH$_{i}$ of ATR$^+$ (thick line) or ATR$^-$ (thin line) L. monocytogenes suspended in potassium phosphate buffer at pH 5.5 (acidified with lactic acid) containing 0.5% glucose. Cells were exposed to nisin (50 µg ml$^{-1}$) from time zero. Data are averages of two independent and reproducible spectra.
(iii) Response of the transmembrane electric potential ($\Delta \psi$) to nisin. The data in Table 1 demonstrate that the ATR$^+$ listeriae have both lower $\Delta$ pH and lower $\Delta \psi$ values than the ATR$^-$ listeriae. Since nisin action is enhanced by energized membranes, these results suggest that the lowered PMF of ATR$^+$ L. monocytogenes made them less sensitive to nisin. To test this hypothesis, the $\Delta \psi$ of cells during exposure to nisin was initially studied with TSBYE acidified with lactic acid to pH 5.5 (ATR$^+$) or with unacidified TSBYE at pH 7.0 (ATR$^-$). Once the probe equilibrated in the system (200 s), the ATR$^+$ cells had lower $\Delta \psi$ values than the ATR$^-$ cells. Nisin addition at 250 s depleted the $\Delta \psi$ of ATR$^+$ cells but not of ATR$^-$ cells. Interestingly, when ATR$^-$ cells were assayed at pH 5.5, they behaved similarly to the ATR$^+$ cells (Fig. 2a). To further support these observations, the $\Delta \psi$ values of ATR$^+$ and ATR$^-$ cells were studied during longer periods (500 s) at pH 5.5 or 7.0 as they were treated with nisin, followed by valinomycin to deplete residual $\Delta \psi$. After the probe equilibrated in the system (250 s) at pH 5.5, the $\Delta \psi$ values of live and dead ATR$^-$ cells were similar and equal to zero (Fig. 2b). This confirmed our previous estimate using various extracellular potassium concentrations ([K$^+$]o) and valinomycin. Neither nisin nor valinomycin addition increased fluorescence, indicating that neither depleted the $\Delta \psi$. In contrast, ATR$^-$ cells assayed at their original pH of 7.0 had measurable $\Delta \psi$ values relative to the dead cell control (Fig. 2c). Nisin addition depleted the $\Delta \psi$ of ATR$^-$ cells to levels close to the dead cell control in ~50 s. Subsequent addition of valinomycin further reduced $\Delta \psi$ to zero (Fig. 2c). This suggests that lactic acid, besides reducing the pH, also reduced the $\Delta \psi$ of ATR$^-$ cells after 250 s, rendering them energetically resistant to nisin. Conversely, when ATR$^+$ cells were assayed in buffer at pH 7.0, they had increased $\Delta \psi$ and therefore were energetically sensitized to nisin (Fig. 2d).

De novo protein synthesis and response to nisin. Additional assays were conducted to investigate if the reduced $\Delta \psi$ of ATR$^-$ cells following exposure to pH 5.5 required de novo protein synthesis, a characteristic of the ATR. Such protein synthesis and therefore full ATR can be blocked if chloramphenicol is added no later than 10 min after exposure to mild acid (28). The $\Delta \psi$ decreased similarly in the presence or absence of chloramphenicol added at time zero (data not shown). This suggests that the changes in bioenergetics imposed by the
acidified buffer and the resulting energetic protection to nisin
were independent of de novo protein synthesis.

Determination of ATP levels. Since the PMF can be inter-
converted with energy stored as ATP via the F$_{0}$F$_{1}$ ATPase
complex, we examined the [ATP]$_{i}$ of cell preparations. Extra-
cellular ATP levels were negligible compared to [ATP]$_{i}$ (data
not shown), allowing total ATP determinations to be reported
as intracellular ATP. For ATR$^{+}$ cells, the [ATP]$_{i}$ of 7.64 ±
1.78 mM was significantly ($P < 0.05$) higher than that of ATR$^{-}$
cells (1.12 ± 1.38 mM) when tested immediately after induc-
tion. After treatment with 50 µg ml$^{-1}$ nisin, the [ATP]$_{i}$ of the
ATR$^{+}$ cells decreased significantly to 0.29 ± 0.03 mM, a level
similar to those of ATR$^{-}$ cells. These results were confirmed
by kinetic experiments.

(i) Kinetics of ATP concentrations in the presence of glucose
and nisin. ATR$^{+}$ and ATR$^{-}$ cells suspended in buffer at pH
5.5 were exposed to glucose or glucose and nisin. In the pres-
ence of glucose but absence of nisin, the [ATP]$_{i}$ of the
ATR$^{+}$ cells maintained higher [ATP]$_{i}$ relative to ATR$^{-}$ cells (Fig. 3). Nisin
addition caused ATP depletion in both ATR$^{+}$ and ATR$^{-}$ cells.

FIG. 3. Time-dependent intracellular ATP levels of ATR$^{+}$ and ATR$^{-}$ listeriae during treatment with nisin and 0.5% glucose in 50 mM
Na$^{+}$-HEPES–MES buffer at pH 5.5. Data are averages of duplicate experiments, and error bars indicate 1 standard error.

FIG. 4. The intracellular ATP levels of listeriae after ATR induction in the absence or presence of 1 mM DCCD, prior to ATP determinations. ATR$^{+}$
cells (closed symbols) and ATR$^{-}$ cells (open symbols) were suspended in 50 mM K$^{+}$–HEPES–MES buffer at pH 5.5 and 7.0, respectively, in the presence
of glucose (triangles) or glucose and nisin (circles). Data are averages of triplicate experiments, and error bars indicate 1 standard deviation.
became energetically sensitized to nisin and valinomycin addition. Conversely, resuspension of ATR\(^+\) in buffer at pH 5.5 immediately decreased their \(\Delta \psi\) to levels similar to those observed with ATR\(^-\) cells, and as expected, rendering them energetically unresponsive to nisin and valinomycin.

To confirm that the bioenergetic changes were independent of de novo protein, experiments with ATR\(^+\) cells using inhibitory levels of chloramphenicol from time zero were extended to 1,000 s. By excluding a role for de novo protein synthesis, we were able to focus on other early physiological events, including extended bioenergetic changes. When treated with nisin at 650 s and subsequently valinomycin, the ATR\(^+\) cells had reduced \(\Delta \psi\) values and therefore resistance to nisin and valinomycin, similar to that for the ATR\(^-\) cells. This also suggests that de novo protein synthesis was not involved in the bioenergetic changes.

The lisRK gene, a two-component signal transduction system in *L. monocytogenes*, is important in stress responses and in virulence regulation (7, 10) and has also been implicated in resistance to nisin and antibiotics using *L. monocytogenes* mutants (9). However, it has not been established whether *lisRK* is specifically upregulated during ATR induction. Even if it is, our results suggest that an earlier sequence of bioenergetic events prevents nisin action.

**Increased ATP levels in ATR\(^+\) cells.** Immediately after ATR induction, the [ATP], of the ATR-induced cells was significantly higher than for ATR\(^-\) cells. This was a conceptual disconnect with the low PMF of the ATR\(^+\) cells. Since the *L. monocytogenes* genome lacks all the components required for a functioning respiratory electron transport chain (36), the F\(_0\)F\(_1\) ATPase enzyme complex must function unidirectionally, building a PMF at the expense of energy from ATP hydrolysis. For control purposes, treatment with nisin strongly depleted ATP levels in both preparations; however, absolute comparisons were not possible because the different pH values of each preparation affected nisin’s action (12). The ATP kinetic assays were performed with ATR\(^-\) and ATR\(^+\) cells suspended in buffer at pH 5.5. The increased [ATP], of ATR\(^+\) relative to ATR\(^-\) cells persisted for the duration of the assay, suggesting that the 5-min exposure of ATR\(^-\) cells to pH 5.5 did not induce an ATP-sparing mechanism. However, exposure to nisin depleted intracellular ATP from time zero, and the depletion was maintained for the duration of the experiment despite the presence of glucose. This indicates that ATR\(^+\) and ATR\(^-\) cells instantly use their ATP reserves almost fully to survive exposure to nisin, underscoring the importance of ATP during nisin’s action.

**Decreased activity of F\(_0\)F\(_1\) ATPase in ATR\(^+\) cells.** When DCCD was used to inhibit the F\(_0\)F\(_1\) ATPase during ATR induction, the [ATP], in ATR\(^+\) cells did not change. In contrast, the [ATP], in ATR\(^-\) listeriae increased significantly (\(P < 0.05\)) in the presence of DCCD, suggesting that their F\(_0\)F\(_1\) ATPase but not the ATPase in ATR\(^+\) cells was consuming ATP to maintain their higher PMF values (Table 1). [ATP], in the absence of DCCD did not differ significantly between ATR\(^+\) or ATR\(^-\) cells assayed in K\(^+\)– and Na\(^+\)–HEPES–MES.

The apparent contradiction that nisin resistance can be accompanied by acid sensitivity in genetically altered mutants (24) or acid tolerance in physiologically adapted cells (2) may be reconciled through the role of the F\(_0\)F\(_1\) ATPase, which is...
implicated in both phenotypes. The F$_1$F$_0$ ATPase complex has two main domains connected by a central stalk. The globular F$_1$ ATPase domain is coupled to the membrane-spanning F$_0$ proton-translocating domain (3). Certain chemicals and physiological conditions can uncouple ATP hydrolysis from proton pumping. This coupled or uncoupled state might explain how nisin-resistant cells can be acid resistant or generate an acid tolerance response.

**Implication of the F$_1$F$_0$ ATPase c subunit.** SELDI-TOF MS using a hydrophobic chip allowed the use of total cell lysates and investigation of the highly hydrophobic c subunit of the F$_1$F$_0$ ATPase. In *L. monocytogenes*, the c subunit contains 72 amino acid residues and has a molecular mass of 7.2 kDa (17). Spectral analysis suggests that the 7.4-kDa signal (a 2.9% mass variation) was due to the c subunit of the enzyme. The signal was decreased in the ATR$^+$ preparation ($P < 0.05$), indicating a reduction in the number of putative c subunits. The only other proteins identified in the *L. monocytogenes* genome which had a similar molecular weight were the cold shock proteins (17), but all of them are hydrophilic and would not be detected with a hydrophobic chip surface. Moreover, the downregulation of cold shock proteins during ATR induction has been excluded by others (1, 30, 40).

The c subunit of the F$_1$F$_0$ ATPase complex contains 9 to 14 subunits (41). The subunits are highly hydrophobic proteolipids and contain an Asp or Glu residue that supplies a proton-translocating carboxylate (18). The carboxylate and a conserved Arg residue of the a subunit apparently form a channel for proton transport. The F$_1$F$_0$ ATPase inhibitor DCCD specifically binds the proton-translocating carboxylate of the c subunit, inhibiting proton transport (18, 19). In *L. monocytogenes*, the c subunit lacks Asp, but side-chain carboxylates are located at Glu 56 and 39 (17).

In the mitochondrion and chloroplast, where the F$_1$F$_0$ ATPase operates in both directions, PMF positively regulates F$_1$F$_0$ ATPase across the internal membrane (35, 41). However, information on the regulation of *L. monocytogenes* F$_1$F$_0$ ATPase is scarce (8, 24). We propose that downregulation of the enzyme in ATR$^+$ *L. monocytogenes* could be triggered by decreased PMF, avoiding the futile waste of ATP against overwhelming conditions established by the acid. This would save vital ATP during ATR induction, preparing the cell for survival under potentially lethal conditions.

Several investigators suggest that changes in the number of c subunits per enzyme complex alter the proton/ATP ratio (27, 33, 35, 41). In *Escherichia coli*, where the enzyme works in both the hydrolytic and synthetic directions, cells have more c subunits in their ATPase when growing in the presence of glucose than in the presence of succinate (27). In this way, metabolic information on the regulation of *L. monocytogenes* F$_1$F$_0$ ATPase operon in the acid tolerance response. 

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