Use of Two-Dimensional Electrophoresis To Study Differential Protein Expression in Divercin V41-Resistant and Wild-Type Strains of *Listeria monocytogenes*

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The use of bacteriocins from food-grade lactic acid bacteria to fight against the food-borne pathogen *Listeria monocytogenes* has been gaining interest. However, the emergence of resistant cells is frequently reported when *Listeria* is exposed to such antibacterials. A two-dimensional electrophoresis study of whole-cell protein expression of *Listeria monocytogenes* variants sensitive or resistant to the action of a bacteriocin produced by *Carnobacterium divergens* V41, divercin V41, is reported in this paper. The resistant variant obtained from the sensitive strain of *L. monocytogenes* P was also resistant to piscicin V1 and SF668, but remained sensitive to nisin. Its growth rate was 50% less than the sensitive strain, and the MIC for it was 10^4 times higher. No reversion of the resistance was observed after 20 successive cultures in the absence of divercin V41. Comparison of the protein patterns by two-dimensional gel electrophoresis analysis showed clear differences. In the resistant variant pattern, at least nine spots had disappeared and eight new ones were observed. One of the newly synthesized proteins was identified as a flagellin of *L. monocytogenes*. Direct interaction between flagellin and divercin V41 was not evidenced. Intracellular synthesis of flagellin is probably an indirect effect of a modification in transcriptional regulation with widespread effects through a sigma factor. An intense protein, only present in the sensitive strain, was identified as a non-heme iron-binding ferritin displaying strong similarities to Dps proteins. Common modifications in the transcriptional regulation for these two proteins are discussed.

During the past decade, *Listeria monocytogenes* has been incriminated in numerous food-borne outbreaks and several sporadic episodes of listeric illness (25). The emergence and persistence of *L. monocytogenes* on a large variety of dairy, ready-to-eat, and processed foods has led to enhanced interest in antimicrobials for its control. In addition to conventional antimicrobials (organic acids, radiation, packaging, etc.), interest in the use of bacteriocins from food-grade lactic acid bacteria (LAB) has increased. Bacteriocins were defined as ribosomally produced precursor polypeptides or proteins that, in their mature (active) form, exert an antibacterial effect against a narrow spectrum of closely related bacteria. Most of the reported bacteriocins are produced by LAB, which are naturally present in a lot of food products or are added for their technological and preserving characteristics (40).

However, in most studies, when *Listeria* is exposed to such antibacterial activity, emergence of resistant cells is frequently reported (35). The mechanisms underlying the bacteriocin resistance phenomenon are largely unknown. Because bacteriocin acts mainly in the cytoplasmic membrane, potential modifications of bilayer lipid content and quality have been investigated. Resistance to nisin has been correlated with both modified fatty acid and phospholipid composition (27).

Even if differences in protein expression between sensitive target cells and resistant cells are potentially numerous, the roles of proteins in bacteriocin resistance are unclear. In some target cell species, specific membrane-located bacteriocin receptors of a proteic nature have been identified (42). Modifications or the absence of such receptors could lead to resistance. Some killer toxin-resistant mutants of *Saccharomyces cerevisiae* expressed much smaller amounts of a protein which acts as a docking protein, facilitating toxin binding to the membrane, where it forms lethal ion channels, like bacteriocins do (38). Synthesis of new membrane proteins could interfere with bacteriocin anchorage on the receptor or in the membrane. In *Lactococcus lactis* subsp. *lactis* biocar diacyrlactis, the nisin resistance gene *nsr* encodes a putative protein with a molecular mass of 35 kDa. A strongly hydrophobic region supports the prediction that this protein is an integral membrane protein which could decrease bacteriocin activity. Decreased bacteriocin penetration could also appear to result from membrane protein oversynthesis, as observed by Koch et al. (22) in multidrug-resistant mouse and hamster cells. Synthesis of an enzyme able to degrade the bacteriocin is also a potential efficient resistance mechanism. Jarvis (20) described a nisinase which inactivated nisin. Moreover, cell wall proteins could play a crucial role in resistance, as clearly demonstrated by Dielband-hoising et al. (10) for two cell wall proteins in nisin resistance of yeast cells. Knowledge of the involvement of proteins in bacteriocin resistance, even if studied in gram-positive bacteria, could also highlight the role of outer membrane proteins in gram-negative resistance, which is probably essential, as demonstrated for Omp4 for the bacteriocin 28B resistance phenotype in *Escherichia coli* (18).

Two-dimensional electrophoresis (2DE) of proteins is currently the highest-resolution analytical technique available for the study of protein expression patterns. This technique has already been used for studying minocycline-susceptible and -resistant *Mycobacterium smegmatis* (44). Comparative proteome analysis of *Mycobacterium tuberculosis* virulent and non-virulent vaccine strains was carried out with the help of 2DE.
(21). D2E can be an important resource in identifying proteins involved in bacteriocin resistance. Thus, D2E is a powerful tool to highlight the biochemical mechanisms governing development of cell resistance and then will help in the design of new efficient molecules or mixing of molecules with different cell targets.

In this paper, we report physiological and metabolic differences between *Listeria monocytogenes* variants sensitive (wild type) and resistant to the action of divercin V41, a bacteriocin produced by the LAB *Carnobacterium divergens* V41. Moreover, D2E was carried out to study differential protein expression in these two characterized variants.

**MATERIALS AND METHODS**

**Bacterial strains, culture conditions, and bacteriocin production.** Experiments were carried out with *Listeria monocytogenes* P (serotype 4b), a wild-type sensitive strain isolated from vacuum-packed cold-smoked salmon (Escola Superior de Bioteconomia, Porto, Portugal) and its bacteriocin-resistant variant (RV41) obtained as described below. The bacteriocin used for resistance studies was divercin V41, the bacteriocin produced by *Carnobacterium divergens* V41 (34).

Bacteria were subcultured and cultured overnight aerobically at 37°C in Elliker broth (Biokar, France). Growth was determined by optical density at 550 nm (OD_{550}) measurements and by enumeration on Elliker agar after incubation for 24 h at 37°C.

Divercin V41 was purified as described by Métivier et al. (29).

**MIC determination.** The MIC of divercin V41 was determined after growth on Elliker broth or the simulated cold smoked fish system (SCSFs) (12) at 37°C in microtiter plates containing 100 μl of 1% glucose-supplemented Muller-Hinton broth (Biokar) in each well. The total protein content of the purified bacteriocin stock solution was found to be 700 μg/ml as determined by the bicinchoninic acid assay (Pierce, Rockford, Ill.). The divercin V41 was serially diluted in Mueller-Hinton broth (Biokar) in each well. The microtiter plate was incubated overnight at 37°C.

**Isolation of a divercin V41-resistant variant of *L. monocytogenes*.** After MIC experiments, *L. monocytogenes* P cells were picked from the well containing 87.5 μg of divercin per ml and grown in divercin-free Elliker medium. One of the resistant variants, *Listeria monocytogenes* RV41, was isolated on a Palcam plate (selective agar and supplement; Merck, Nagar sur Marne, France), and divercin sensitivity and resistance were verified according to the spotting method described by Pilet et al. (34). The frequency of isolation of resistant variants was determined according to the method of Dykes and Hastings (13), in the presence of divercin V41 at five times the MIC.

**Antibiogram of the two *L. monocytogenes* variants.** Serotyping and lysotyping of the two *L. monocytogenes* variants. Serotyping of *Listeria monocytogenes* strains was performed as described previously (39). Phage typing was carried out according to reference 37 with 29 well-characterized phages isolated from lysogenic strains.

**Two-dimensional PAGE.** 2D polycrylamide gel electrophoresis (PAGE) experiments were performed essentially according to the method of Gormond and Phan-Thanh (16). When not indicated, chemicals and materials were from Pharmacia-Biotech (Orsay, France).

**Sample preparation.** Sensitive *Listeria monocytogenes* and RV41 cells were grown at 37°C until the middle exponential phase (OD_{550} ≈ 0.5, which corresponds to 10^8 CFU/ml) in 10 ml of Elliker broth. Cells were harvested by centrifugation (4,000 × g, at 25°C for 30 min). Pellets were washed three times in 10 ml of physiological water (8.5 g of NaCl per liter) and recovered in 0.2 ml of Tris (2-amino-2-hydroxyethyl)-1,3-propanediol) buffer (10 mM, pH 7.2) containing 5 mM MgCl2, 5 μl of a cocktail of protease inhibitors containing leupeptin, pepstatin, and PEFABLOC [4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; Boehringer, Mannheim, Germany] at 2 mg/ml, 5 μl of deoxyribonuclease and ribonuclease (100 mg/ml; Boehringer), and 20 μl of a solubilization solution containing 20% CHAPS (1-[3-cholamidopropyl]dimethylammonio)propane sulfonate), 10% DTT (dithiothreitol) and 20% IPG (immobilized pH gradient) 4.7 buffer. Cells were sonicated (Vibra cell; Bioblock, Illkirch, France) three times for 1 min at a power setting of 5 and 50% pulse at 4°C. The mixture was incubated at room temperature for 30 min. Urea (7 M) was added, samples were vigorously agitated (Vortex level 5) at room temperature for 15 min, and samples were finally centrifuged (20,000 × g, at 4°C for 20 min). Supernatants could be stored at −20°C. Proteins were quantified by the Bradford method (5).

**IEF.** The 1D separation was carried out on immobilized pH gradients (4/7 Immobiline dry strips of 18 cm) as described by Gorg et al. (15) with Multiphor II apparatus. The following voltage gradient was applied: from 0 to 50 V in 0.02 h; 50 V for 1 h; from 50 to 150 V in 0.02 h; 150 V for 1 h; from 150 to 300 V in 0.02 h; 300 V for 2 h; from 300 to 3,500 V in 5 h; and 3,500 V for 1 h. Protein samples (100 μg) were loaded into cups at the anode end. After isoelectric focusing (IEF), strips were equilibrated in a solution containing 50 mM Tris (pH 6.2), 6 M urea, 30% glycerol (vol/vol), 2% (wt/vol) sodium dodecyl sulfate (SDS), and 0.3% (wt/vol) DTT followed by a second bath with the same buffer, but with 4.5% (wt/vol) iodacetamide in place of DTT.

**SDS-PAGE.** The 2D separation was performed, essentially according to the method of Laemmli (24), in an IsoDalt apparatus ( Hoeffer, San Francisco, Calif.) with a 14% acrylamide separating gel, but without a stacking gel and at constant voltage (below 180 V). Large plate gels (200 by 250 by 1 mm) were used to improve resolution. Ten gels were run simultaneously in one tank to improve reproducibility. A low-molecular-weight electrophoresis calibration kit (Amer- sham-Pharmacia-Biotech, Buckinghamshire, England) was used for protein molecular weight determination (daihatsu) reference standards (phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100; and α-lactalbumin, 14,400).

**2D SDS-PAGE.** Also was performed (Protein II or Mini-Protein; Bio-Rad, Paris, France). Samples were diluted twice in a mixture of 62.5 M Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, and 5% β-mercaptoethanol and heat treated at 100°C for 2 min before loading (20 μl) on wells of SDS-PAGE gels at a 14% polyacrylamide concentration.

In the same way, delay gels were performed with flagellin extracted from cells grown at 20 and 37°C (see below) and purified divercin V41. Interaction studies were performed with flagellin extract of RV41 (200 μg) and purified divercin V41 (3.5 μg) in phosphate-buffered saline (PBS) (0.01 M sodium phosphate with 0.15 M sodium chloride [pH 7.6]) for 10 min at room temperature. Separations were carried out in either the presence or absence of SDS to visualize interactions between the two proteins.

Gels were stained either with silver (41) or with Coomassie brilliant blue (PhastGel Blue R; Amer sham-Pharmacia-Biotech) and stored in a 20% ethanol solution at 4°C for several weeks.

**Analysis of protein spots on 2D gels.** Images were scanned (CanoScan FB620 P, Canon, France). Protein gel analysis was performed with Melanie II 2D PAGE software (release 2.2; Bio-Rad, Ivry sur Seine, France). Reference points (landmarks) were marked on images to align and match gels. After gel alignment and matching, pairs (spots present in several gels) could be highlighted. The reverse function evidenced the differences between each gel. Ten gels per sample were analyzed and compared. Spots present in at least nine gels were considered to be consistent spots and were taken into account.

**Protein identification.** Coomassie blue-stained proteins which had been separated by 2D gel electrophoresis were excised and washed five times with 30 μl each 80% n-propanol, followed by five washes with 30 μl each of 2% 2-ammonium carbonate containing 50% acetonitrile. The gel pieces were completely dried under reduced pressure (Speed-Vac; Savant). To the dried gel...
piece, 0.5 μg of trypsin in 10 μl of 100 mM ammonium carbonate (digestion buffer) was added to allow reswelling of the gel pieces. About 15 μl of digestion buffer was added to completely immerse the gel pieces. Digestion was carried out at 37°C for 2 h. The supernatant was collected, and the gel pieces were extracted with 15 μl of 0.1% formic acid, followed by 15 μl of acetonitrile. Extraction was repeated twice, and all supernatants were pooled and dried in a Speed Vac. To be able to recover supernatants from the gel pieces, two 500-μl Eppendorf tubes arranged concentrically were used. The tube containing gel pieces was pierced with a hypodermic needle to generate a hole large enough to allow the liquid to be centrifuged into the lower tube, but to retain the gel pieces. Also, in order to avoid possible cross-contamination, all buffers and wash solutions were pipetted with different pipettes, which were rinsed thoroughly for the handling of the same solutions, making sure that the needle never touched the gel pieces.

For mass spectral analysis, the peptides were dissolved in 10 μl of 0.1% trifluoroacetic acid, and 5 μl was used for identification. Separation of the digests was carried out on 100-μm capillary columns which were packed with POROS R2 material (PerSeptive Biosystems, Framingham, Mass.). In short, fused silica capillaries (100 by 280-μm LC Packings; Polymer Laboratories, Marseille, France) were drawn to an aperture of 1 to 2 μm on a laser puller (Sutter Instruments, Science Products AG, Geneva, Switzerland). A column frit was constructed by introducing a few grains of 5-μm-diameter silica beads. The POROS material was then packed into the capillary with the aid of a stainless steel capillary syringe to a final length of 15 cm to perform the liquid chromatography. A MicroTAS system was capable of delivering pressures up to 6,000 lb/in². After the packing process, the columns were cut to approximately 2.5 cm and inserted into a microsource. The microcapillaries were connected in parallel to a glass microapore (150-μm diameter) which was rinsed solubly for the handling of the same samples, making sure that the needle never touched the gel pieces.

Preparation of crude flagella. One liter of culture was harvested by centrifugation at 4,000 × g for 10 min to remove any particulate matter. The cells were washed twice in 10 mM sodium phosphate–0.15 M sodium chloride (pH 7.6) (PBS) buffer, and resuspended at a ratio of 5 ml of PBS/liter of broth culture. Samples, in bottles containing glass beads, were shaken vigorously for 30 min at 20°C. The suspension was centrifuged at 5,000 × g for 15 min, and the supernatant was retained. The pellet was washed twice with PBS by vigorous pipetting to remove sheared flagella trapped within the cell mass. The supernatant and cell washing were pooled and centrifuged at 14,000 × g for 40 min to clear the remaining bacteria, and the resulting supernatant was centrifuged at 200,000 × g for 90 min to harvest crude flagella (33). One milligram of crude flagellar protein was recovered in each experiment and solubilized in 50 μl of PBS.

Sample preparation for scanning electron microscopy. Five milliliters of the cultures was filtered over membranes (0.2-μm-pore-diameter GTTP 01300; Millipore Polycarbonate, Molsheim, France). Membranes were loaded on the surface of a 0.1 M sodium cacodylate (pH 7.2) buffer containing 2.5% glutaraldehyde. Cells or flagella were fixed for at least 48 h at room temperature. Cells or flagella were washed in sucrose solution (10, 25, 50, and 100%); for 10 to 20 min. Samples were dissected by introducing them into a pressurized enclosure where ethanol was replaced by liquid CO2 (10°C). Samples were desiccated by heating until the critical point was reached (31°C and 73.8 bars) without deterioration and then were metalized with gold and observed.

RESULTS AND DISCUSSION

Physiological comparison of Listeria monocytogenes sensitive and RV41 variants. Sensitive and resistant variants present the same phenotypic characters, the same serotype (4b), and the same lysotype (1444, 1317, 3274, 2671, and 340) (data not shown). Thus, no drastic surface modifications could be postulated on these bases. The MICs for the sensitive and resistant RV41 strains were determined after growth on Elliker and SCFS broths. In Elliker broth, the MIC for the resistant variant was at least 104 times higher than that for the sensitive strain (higher than 104 and 0.01 μg/ml, respectively). This result was in accordance with that obtained by Mêtivier (28) with L. monocytogenes Scott A. In the SCFS medium, the MIC for RV41 was 105 times higher than that for the sensitive strain (higher than 104 and 5.7 10−5 μg/ml, respectively). For the latter strain, the MIC was 175 times higher on Elliker broth than on SCFS. These results could be explained by two facts. (i) SCFS is a less nutritious medium in which L. monocytogenes had difficulty in growing, and thus it was more sensitive to bacteriocin activity. (ii) Elliker broth contains many more molecules which are able to interfere with the bacteriocin and artificially decrease the number of molecules free to interact with the target cells. These observations underlined the impact of environmental conditions on bacterial growth and bacteriocin sensitivity to antibacterial agents.

The frequency of appearance of resistance was 3.5 × 10−5 at five times the MIC. The stability of the resistant variant was tested against diverscin V41, and no reversion of the resistance was observed after 20 successive cultures in the absence of diverscin V41 as observed by Rekhif et al. (35) for mesentericin 52, curvatinic 13, and plantaricin C19. On the contrary, Dykes and Hastings (13) found a reversion frequency within the range of 10−4 to 10−5 with leucocins A, B, and E and sakacin A. The stability or instability of the resistant phenotype remained unexplained, but it is reasonable to argue for several resistance mechanisms among the different species, and possibly within the same species, leading to different mechanisms and thus different frequencies of reversibility.

The resistant variant obtained from the wild-type strain of L. monocytogenes P was resistant to diverscin V41, piscicolin V1, and piscicolin SF668, but kept its sensitivity to nisin (data not shown). The cross-resistance between nonlantibiotic bacteriocins has been already observed by Rekhif et al. (35) and Mêtivier (28). The difference in sensitivity of RV41 to diverscin V41 and nisin has been correlated with a potential difference in the mode of action of lantibiotics and nonlantibiotic bacteriocins.

The resistance or sensitivity of both strains to several antibiotics, acting on different cell targets was investigated to observe potential differences or similarities between bacteriocin and antibiotic resistance. The results are presented in Table 1 and show, first, that there are no differences between the two spectra and, second, that the resistances or sensitivities measured correspond to the common phenotype of L. monocytogenes 4b. These results show that resistance to diverscin does not confer any resistance to the antibiotics tested. This had already been observed for the nisin resistance phenotype (8).

No clear morphological difference was observed between the sensitive and RV41 variants either by optical or by scanning electron microscopy (data not shown). The absence of morphological differences between sensitive and diverscin-resistant cells was in accordance with the results of Crandall and Montville (8) on L. monocytogenes ATCC 700302 resistant to nisin.

Comparative growth of the L. monocytogenes sensitive and resistant variants in Elliker broth at 37°C is represented in Fig. 1. The sensitive strain had a 4-h lag phase, followed by a rapid exponential phase (μ = 0.13 h−1) up to the 10 hours of growth. For the resistant variant, the lag phase was shorter (2 h), but the growth rate was twofold lower (μ = 0.07 h−1). The decrease in the growth rate could be explained by the energy cost of the potential resistance metabolic pathway(s) which reduces the fitness for growth (13). However, this rather important difference in growth efficiency could be the result of a more wide alteration of the cell metabolism than only a difference in bacteriocin resistance, which is the unique phenotypic characteristic observed here.

Comparison of the protein patterns of L. monocytogenes sensitive and RV41 variants. No significant difference was observed between the two 1DE patterns of the total proteins from the two variants of L. monocytogenes P (data not shown). The protein extractability was similar for both, as demon-
FIG. 1. Growth curves of the *L. monocytogenes* wild type (solid symbols) and the resistant variant (open symbols) on Elliker broth at 37°C.

Stratified by the protein content of preparations (2.10 ± 0.12 and 2.14 ± 0.11 mg/ml for RV41 and the sensitive strain, respectively). When minor differences between sample preparations occurred, standardization of the amount of protein of the samples was performed before 2DE experiments in order to allow direct comparison of the patterns.

Ten 2D gels were run for each *L. monocytogenes* variant. Figure 2 shows two typical patterns of the *L. monocytogenes* strain sensitive to divercin V41 (Fig. 2a) and the resistant variant (Fig. 2b). 211 and 278 spots could be detected on each gel, respectively. Silver staining is not quantitative; only proteins present or absent on nine of the two patterns were considered. Arrows indicate the main differences between the two patterns and were determined after computer-assisted analysis with Melanie II software.

Nine intense spots, lacking in the resistant pattern, were chosen in the sensitive strain pattern (Fig. 2a). Three of them are small (<20 kDa) and acidic (pI of <5.20) proteins; four have average size (about 30 kDa) proteins with a pI ranging from 4.6 to 5.8; two are bigger proteins (<35 kDa) with pIs higher than 5.4 and 5.85. In parallel, in the protein pattern derived from the resistant strain, we selected eight new and intense protein spots absent in the sensitive one. All of these proteins have a molecular mass ranging from 25 to 65 kDa and a pI between 4.5 and 5.8 (Fig. 2b).

Among the spots analyzed by mass spectroscopy, only spots R1 and S86 were identified as *L. monocytogenes* or *L. innocua* proteins, respectively (Table 2). The masses and the segment sequences of the other spots did not share any homology with identified proteins of *Listeria* spp. (available in the SWISS PROT database). The protein R1, present only in the RV41 variant, has been identified as a flagellin of *L. monocytogenes* characterized by Dons et al. (11). Flagellin is the main component of the flagellar filament. This is the engine of mobile bacteria which allows movement to high-nutrient-concentrated zones or away from toxic substances. How could flagellin be involved in the bacteriocin resistance phenomenon? Two hypotheses are presented: the flagella could act directly in the resistance phenomenon. It could play the role of a biological magnet, attracting, by electrostatic forces (14), molecules of divercin which then become unavailable to interact with *L. monocytogenes* membrane. A second hypothesis is that in RV41 cells, flagellin synthesis is indirectly affected by modification of gene control expression with widespread effects.

In order to assess if flagella were directly implicated in bacteriocin resistance, the MICs for sensitive, RV41, and RV41 flagellum-free (obtained by vigorous shaking and centrifugation) strains were determined at 20 and 37°C, temperatures at which cells are mobile and immobile, respectively (33). No change in MICs was evidenced between the two temperatures and between RV41 and flagellum-free RV41 (data not shown).

The physical interaction between flagellin and divercin V41 was explored. Figure 3 shows that no migration delay was observed when flagellin and divercin V41 were comigrated in electrophoresis experiments under native and SDS conditions. This suggests that, under our experimental conditions, there is no interaction between these two molecules. The presence of flagella on the RV41 cell surface was investigated. Optical microscopy showed that, for whichever variant considered, *L. monocytogenes* cells were mobile at 20°C and not at 37°C. Scanning electron microscopy confirmed that both *L. monocytogenes* sensitive and RV41 cells possessed flagella on their surface at 20°C and not at 37°C. Thus it could be postulated that flagellin is present in a large amount in the intracellular fraction of the RV41 cells, cultivated at 37°C, but flagellin export at the cell surface does not occur. Thus direct involvement of flagellin in the resistance phenomenon is doubtful, except, maybe, it plays a potential unknown role at the internal face of the cytoplasmic membrane.

Variation in the level of flagellin synthesis in RV41 is probably the result of modification(s) in the transcriptional regulation of this protein. This hypothesis is based first on the fact that flagellin synthesis is thermoregulated at the transcriptional level (35). In our experiments, flagellin synthesis is repressed at 37°C in the sensitive variant and seems derepressed in the resistant one. A second element of response is based on the results obtained by Robichon et al. (36). They found a mesentericin Y105-resistant phenotype of *L. monocytogenes* obtained by transposition insertion. The insertion of the transposon was in the *posN* gene encoding an alternative transcriptional σ^34 factor. σ^34 is known to be involved in the control of many genes, including some genes of flagellar synthesis (36).

The flagellin role is of particular interest, because this protein has been hypothesized to increase the virulence of *L. monocytogenes* (9). PrfA, the transcriptional activator of virulence genes, which is maximally expressed at 37°C, down regulates motility genes in *Listeria* (31). If alteration(s) occurred in the transcriptional regulation of these genes, leading to flagellin synthesis as observed in our variant, we suppose that other genes encoding proteins directly involved in sensitivity and resistance phenomena are also deregulated. Moreover, such genes encoding entry and belonging to a multigene family have been observed near the virulence genes, on the same notA fragment of the physical map of *L. monocytogenes* (30). The expression of the gene encoding flagellin is frequently reported as being not only temperature regulated, but also influenced by stresses such as osmotic stress (19). In *E. coli*, the RNA polymerase σ^2 subunit, involved in the transcription of the flagellar and chemotaxis genes, possesses a strict promoter recognition property as found for minor sigma subunits involved in stress response. The transcription efficiency is salt dependent (23). Could we postulate that bacteriocin is considered by the target cell as a stress, like heat or osmotic stress, and that the cell response then uses the same kinds of mechanisms? This is an open question. This comparison was also suggested by O’Connor et al. (32), studying the response of *Salmonella enterica* serovar Typhimurium to deleterious conditions, including, besides oxidative and osmotic stresses, exposure to toxic cationic peptides. The regulation of the proteins involved in these resistance mechanisms is complex and overlapping. Moreover, a recent report (43) demonstrated that acid-adapted *L. monocytogenes* cells exhibit increased toler-
ance toward nisin and lactacin 3147. These results suggest common cell responses toward both types of attack.

Spot S86, only present in the sensitive strain, has been identified as a DNA-binding protein already described by Bozzi et al. (4) in Listeria innocua. This non-heme iron-binding ferritin is able to sequester many iron atoms inside the protein cage. Bacteriocin activity leads to intracellular ion leakage through the altered membrane. The absence of such iron-chelating intracellular systems, as observed in the resistant variant, could be a major problem for the cell attacked by bacteriocin molecules, but the mechanisms are unknown. Divalent cations (Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, and Ba$^{2+}$) increased the resistance of a nisin-resistant strain of L. monocytogenes Scott A in a concentration-dependent manner (8). Iron was not tested. In their discussion, the authors described a model in which cations may interfere with the lipids of the membrane and the cell wall.
TABLE 2. Characterization of R1 and S86 proteic spots

<table>
<thead>
<tr>
<th>Spot</th>
<th>Mol mass (Da)</th>
<th>Amino acid sequencea</th>
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<tbody>
<tr>
<td>R1</td>
<td>30,445</td>
<td>MKVNTISLKTQELRKNNEGTOAQERLASG KRINSSLDIAAGLAVTRMNVKSTGDLDAASKNS SMDILDQIADSALSSMSILORMQLAVVSS NGFSFSDERRKQTAEGSLKIELKDHVATNTY NNKLLDDQATGAATQVSIOASDAKLINIDF NAKGLSIMTITLGGSTVAGYSALSVAADDSS QFATEIDELINNSGRALLAGMSRLSVNS NNVNSQIAETKASASIEEDAMAEMSEMETYK ILTQTSISMSLSQANQTPOPMLTLINS</td>
</tr>
<tr>
<td>S86</td>
<td>18,048</td>
<td>MKTINSDVKEFLNHOVANLNTVQKHQHWY MRGHNFHTHEKMDLDSYEGFEOQMDFEAER LLGIGSFPSLTKFELENASVVEAPYTPKTPMDQ LMDVGLTLELREDYEYKGIELTDKEGDTY NDMLIAFKASIDKHIMFKAFGLKAPLE</td>
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a Boldface letters represent sequence obtained by mass sequence analysis of tryptic peptides.

However the decreased bactericidal activity of lactostreptacin 5 on Streptococcus cremoris in the presence of Mg2+ and Ca2+ was attributed to their stimulative role on membrane-bound ATPase (45).

Moreover, this ferritin shows strong similarities to Dps proteins. These stress-induced widespread conserved polypeptides, present in diverse groups of bacteria, are involved in DNA protection during oxidative stress (26). Proteins induced by stress are considered to be members of global regulatory networks which comprise multiple unlinked genes and operons coordinately controlled by a common regulatory signal. In Escherichia coli, mutant cells lacking Dps show dramatic changes in the pattern of proteins synthesized during starvation. This result prompted Almiron et al. (1) to postulate that Dps plays a role in gene expression.

Altuvia et al. (2) found that Dps mRNA levels were controlled by RpoS and σ70 factors. These data had to be linked with the conclusions of Robichon et al. (36) on σ54 involvement in mesentericin resistance and confirm that genes responsible for divercin resistance are controlled by sigma factors. The role of Dps in bacteriocin sensitivity remains unexplored, but is of special concern because of the importance of iron in the infection process caused in human cells by L. monocytogenes (17). In Bacillus subtilis, mrgA, encoding a Dps protein, is a gene repressed by metal ions (6). Expression of virulence genes (intI) is positively iron regulated at the transcriptional level in L. monocytogenes (7). It could be interesting to test the influence of iron on L. monocytogenes sensitivity or resistance to bacteriocins and strain virulence.

We are currently trying to establish a relationship between variants exhibiting different levels of resistance and a quantitative evolution of the copy number differences between sensitive and resistant variants. Experiments to determine the role of transcriptional regulation with respect to resistance acquisition are under way, and the results will be reported later.

We will also investigate the resistance phenotype through, on one hand, identification of all the proteins detected as highly repressed or overexpressed in the resistant clone, and, on the other hand, 2DE of specially extracted membrane proteins from purified membrane fractions of wild and resistant variants.

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REFERENCES

15. Görg, A., W. Postel, J. Weser, S. Gunther, S. M. Strahler, S. M. Hanash, and...


