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Identification and Disruption of *lisRK*, a Genetic Locus Encoding a Two-Component Signal Transduction System Involved in Stress Tolerance and Virulence in *Listeria monocytogenes*

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***lisRK* encodes a two-component regulatory system in the food pathogen *Listeria monocytogenes* LO28. Following identification of the operon in an acid-tolerant Tn*917* mutant, a deletion in the histidine kinase component was shown to result in a growth phase variation in acid tolerance, an ability to grow in high ethanol concentrations, and a significant reduction in virulence.**

The success of *Listeria monocytogenes* as a pathogen owes much to its ability to sense and respond to its environment. A number of studies have demonstrated that the ability of *L. monocytogenes* to respond to stress has consequences in terms of the virulence of the organism (18, 20, 22). In many other bacteria, the link between variations in environmental factors and enhanced or reduced virulence has been shown to result from the sensing and regulatory activities of two-component signal transduction systems (8, 11). A typical two-component system consists of a membrane-associated histidine kinase, which monitors a specific environmental parameter, and a cytoplasmic response regulator, which enables the cell to respond, often via regulation of gene expression, when this parameter varies (21, 27). In this study our aim was to identify mutants of *L. monocytogenes* with altered abilities to respond to stress and to investigate whether such alterations influence virulence. We describe the identification of a two-component signal transduction system and demonstrate that it functions in stress response and plays a role in in vivo survival. This represents the only description, other than that of the *cheA-cheY* system (7), of genes encoding a two-component regulatory system in *L. monocytogenes*.

Isolation and characterization of a mutant of *L. monocytogenes* with enhanced stationary-phase acid tolerance. An *L. monocytogenes* LO28 (serotype 1/2c) Tn*917* bank was created by using the temperature-sensitive plasmid pTV1-OK (12). Isolated transformants were grown overnight in tryptic soy broth-yeast extract (TSB-YE) containing kanamycin (50 μg/ml) at 30°C, subcultured (1%) into TSB-YE containing erythromycin (0.04 μg/ml) at 42°C, and selected for kanamycin-sensitive Tn*917* integrants on tryptic soy agar-yeast extract containing erythromycin (10 μg/ml). Overnight cultures of the transposon bank were exposed to TSB-YE (adjusted to pH 4) for 36 h. While no survivors were recovered from the LO28 control, some 140 pinpoint and 3 regularly sized colonies were recovered from the bank. Southern hybridization showed that Tn*917* had integrated only once and in different locations in each of the three isolates which had given rise to the regular colonies (data not shown). Inverse PCR was used to isolate chromosomal DNA from one isolate, LO28-M9; in this method *Hpa*I was used to generate upstream flanking DNA, and *Xba*I and *Hin*dIII were used to generate downstream DNA. The inverse-PCR products were cloned into pBluescript.

In total, 3,619 bp flanking the point of insertion was sequenced from both strands. Analysis showed that this region contained two partial and two complete open reading frames (ORFs) (Fig. 1A). The ORF into which the transposon had inserted demonstrates a high degree of similarity to regulator components of many bacterial two-component systems. The predicted amino-terminal receiver domain of this ORF includes the invariant aspartate and lysine residues conserved in this family of regulatory proteins (Fig. 1B). Alignments indicated greatest similarity in both the receiver and output domains to the group A *Streptococcus* regulator of hyaluronic acid capsule synthesis CsrR (17), the uncharacterized *Bacillus subtilis* putative regulator YkoG (GenBank accession no. AJ002571), and a *Synechocystis* sp. regulator (accession no. D64002), all of which are members of the OmpR-PhoB subfamily of response regulators. The carboxy termini of members of this subfamily are thought to have DNA-binding roles, and several have been shown to target specific sites upstream from the promoters that they regulate. It is thus probable that the putative protein is also a transcriptional activator. The listerial regulator gene was subsequently assigned the designation *lisR*. The start codon for this gene can be predicted from the high degree of similarity exhibited at the N termini of response regulators. A putative ribosome-binding site (5′-AGAGG-3′) was identified 9 bp upstream from this location.

Immediately downstream from *lisR*, an ORF encoding a histidine kinase was identified. The carboxy terminus of the predicted histidine kinase, designated LisK, most resembles LlkinA from *Lactococcus lactis* (19), CsrS (the partner of CsrR) (17), and YkoH (the partner of YkoG)—all of which have transmitters typical of the EnvZ-NarX family of histidine kinases. An alignment (Fig. 1C) also demonstrates the presence of the H, N, G1, and G2 boxes conserved among transmitter domains. In contrast to the carboxy termini, which are usually cytoplasmic in location, the amino termini of histidine kinases are quite often membrane bound, containing at least two membrane-spanning regions with a periplasmic loop. The membrane-spanning regions, which can be identified as stretches of hydrophobic amino acids, are also present in LisK (data not shown). It is not surprising that a procedure designed

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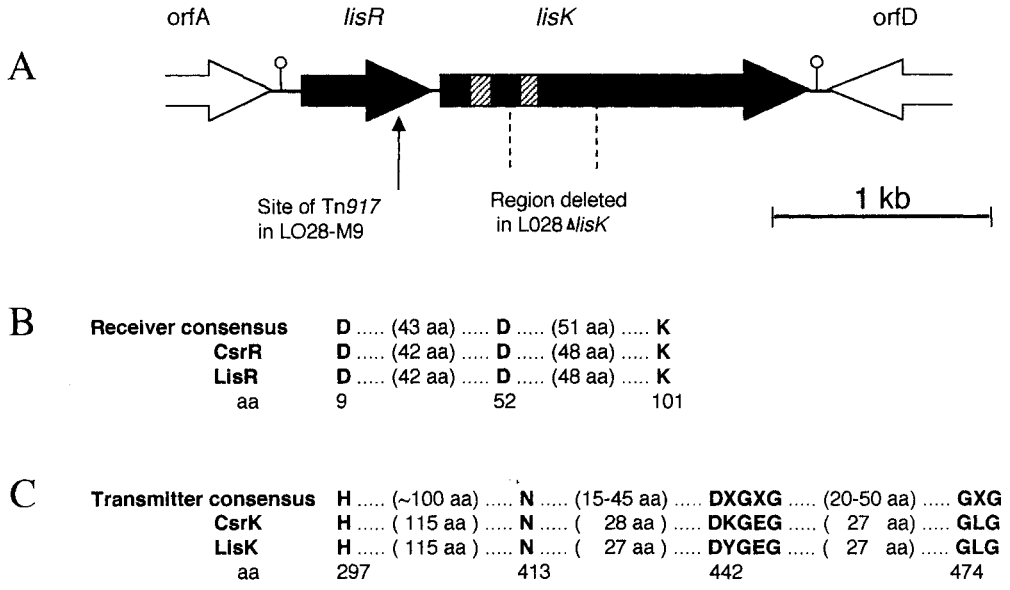

FIG. 1. Graphic illustration of the *lisRK* locus. (A) Line drawing of the orientation of ORFs flanking the Tn*917* insertion site. The striped regions within *lisK* indicate the potential membrane-spanning regions, while the lollipops indicate putative rho-independent terminators. The 498-bp region deleted in the mutant LO28Δ*lisK* is also indicated. (B) Alignment of the predicted receiver domains of both *lisR* and *csrR* and the consensus sequence motif for the receiver element of response regulators (27). (C) Alignment of the predicted transmitter domain of both *lisR* and *csrR* and the consensus sequence motif for the transmitter element of histidine kinase sensor proteins (27). aa, amino acids.

to identify genes involved in sensing and responding to acid stress should have identified a two-component regulatory system, the most-characterized mechanism by which environmental signals can be sensed in bacteria. There are numerous examples of two-component systems which regulate virulence factor production in response to specific environmental signals (8, 11).

The amino acid sequence deduced from the incomplete upstream *orfA* showed high identity with the *Escherichia coli* enzyme 6-phosphogluconate dehydrogenase, while the downstream *orfD* most closely resembled a variety of inner membrane proteins found in *E. coli*. However, due to the presence of putative rho-independent terminators (hairpin loops) both upstream of *lisR* and immediately downstream of *lisK*, it appears that *lisRK* constitutes a discrete transcriptional unit.

Creation and analysis of a *lisK* deletion mutant. A mutant, with a nonpolar 498-bp deletion in *lisK* from nucleotide 1872 to 2369, was created to confirm that the Tn*917* insertion event was responsible for the enhanced survival at low pHH displayed by LO28-M9. The SOE (splicing by overlap extension) PCR procedure (13) was used to splice two 348-bp PCR products from either side of the sequence to be deleted. This hybrid was subsequently cloned into the temperature-sensitive shuttle vector pKSV7 (25) and transformed into LO28. An allelic exchange between the SOE product on pKSV7 and the intact gene resulted in the removal of a 498-bp sequence encoding one of the hydrophobic regions and the conserved histidine of the histidine kinase (Fig. 1A). The mutation was confirmed by PCR analysis, and the mutant was subsequently designated LO28Δ*lisK*.

Carbohydrate utilization (as assayed by API-CH50), listeriolysin O production on blood agar plates, and phospholipase production on egg yolk emulsion plates (all at 37°C) were found to be unaffected in LO28Δ*lisK* (data not shown). As LO28-M9 was isolated as an acid-tolerant mutant, the acid resistance of the deletion mutant was tested. The growth phase acid sensitivities of LO28Δ*lisK* and the parent strain were tested by inoculating (2%) overnight cultures into TSB-YE, removing aliquots at regular intervals, and inoculating them into TSB-YE adjusted to pH 3.5 with 3 M lactic acid. Survivors were counted after 45- and 140-min periods. With the longer acid exposure time, a significant difference ($0.01 < P < 0.02$) was observed, in that the mutant was more resistant to acid than the parent during stationary phase (Fig. 2A), thus confirming the M9 phenotype. This mutant was used for further analysis. No difference could be observed at lower cell densities because this treatment regimen reduced bacterial numbers to below detectable levels. However, when the time of exposure to low pH was reduced to 45 min, the mutant was shown to be significantly more sensitive ($0.01 < P < 0.02$) than the parent strain during early logarithmic growth (Fig. 2A). No difference between stationary-phase cells could be observed due to the reduced exposure to acidified media. These results indicate that the relative resistance of the mutant is growth phase dependent. These results also matched those obtained with the original LO28-M9 mutant, confirming that transposon insertion in *lisR* was responsible for the mutant's selection in acidified broth (data not shown). It is tempting to speculate that a signal which would normally stimulate LisRK to induce acid tolerance is missed during the early stages of growth, leading to an extremely sensitive cell. However, this supposes that a separate induction event which overcompensates for the lack of LisRK by inducing even greater tolerance subsequently occurs. In response to the altered acid resistance profile of LO28Δ*lisK*, the effects of several other stresses were examined. Overnight cultures were inoculated (2%) into TSB adjusted to pH 5.75 with lactic acid, with 7% NaCl, or with 5% ethanol. Growth was determined with a Spectra max 340 spectrophotometer (Molecular Devices) over a 24-h period. The mutant proved able to grow in the presence of 5% ethanol, a concentration which is bacteriostatic for the parent strain (Fig. 2B). This result was representative of a trend that was also apparent for a number of other ethanol concentrations (data not shown). Ethanol stress, and alcohol stress in general, is thought to be

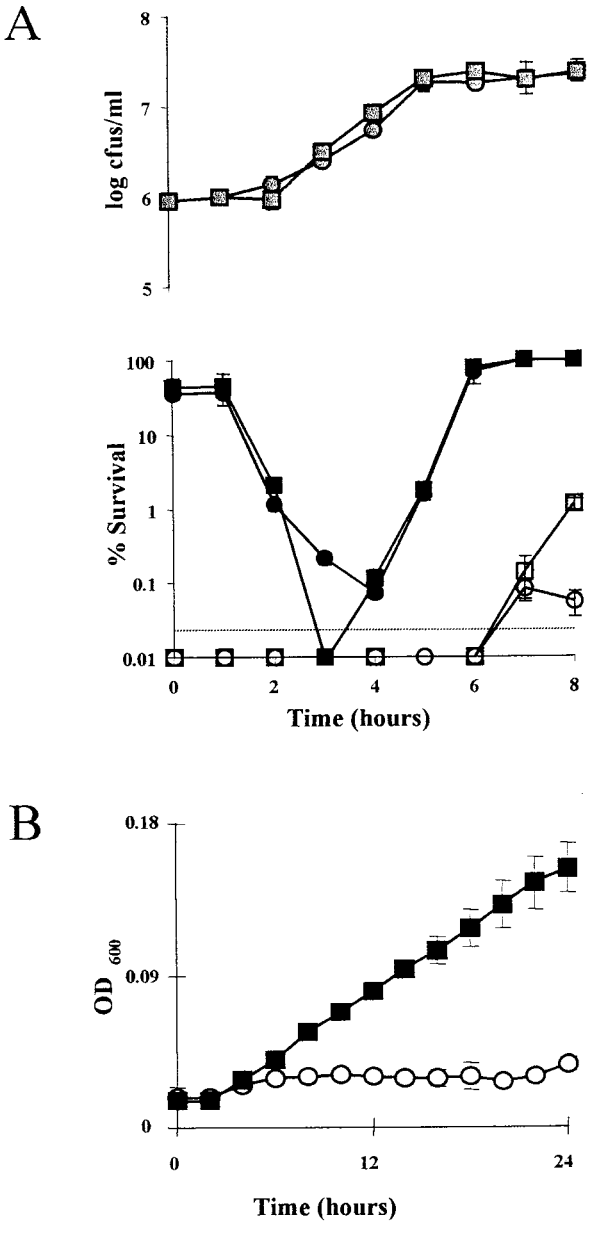

FIG. 2. (A) Percent survival of cultures of LO28 and LO28Δ*lisK* in TSB-YE (pH 3.5) after 45 min (● and ■, respectively) and 120 min (○ and □, respectively), at different points during growth (◍ and ▤, respectively). Points below the dotted line indicate that there were no survivors. (B) Growth of LO28 (○) and LO28Δ*lisK* (■) in TSB-YE supplemented with 5% ethanol at 37°C. Error bars represent the standard deviations of triplicate experiments. OD_{600}, optical density at 600 nm.

exerted by a reduction of cytoplasmic membrane integrity (15, 23). An increase in unsaturated-fatty-acid content has been shown to aid growth and survival when the organisms are exposed to ethanol (14). It may be at the membrane that the two-component system ultimately exerts an influence, as it has been shown that the composition of the cell membrane reflects the relative acid tolerance of a bacterial strain (2, 4, 5).

However, relative growth rates were not affected under other stress conditions, such as media adjusted to various pHs or containing various concentrations of salt (data not shown). These results were similar to those seen in LO28-M9. As it is likely that the Tn*917* insertion in LO28-M9 resulted in a polar mutation in *lisK*, it is possible to attribute the phenotypic changes common in both mutants to *lisK* mutations.

LO28Δ*lisK* is affected in virulence compared with LO28. A mouse model was used to measure any possible impact of the Δ*lisK* mutation on virulence. The number of bacteria in the spleen was recorded, as this reflects the progress of the infection (16). The data show that, regardless of whether a low (1×10^5-CFU) or high (2×10^6-CFU) dose is used, the number of LO28Δ*lisK* cells in the spleen 1 day postinfection is much lower than that for wild-type-infected mice (Fig. 3). This represents a significant attenuation of the virulence of the bacteria. It is necessary to understand the consequence of intraperitoneal inoculation to appreciate how alterations in stress-induced responses can alter the virulence of *Listeria*. Initially, the host immune system responds via the arrival of infiltrating polymorphonuclear neutrophils and subsequently of macrophages (9). Following phagocytosis, *Listeria* produces hemolysin and phosphatidyl inositol phospholipase C to enable its escape from the phagosome (1, 3, 10). It seems to be at this stage of escape that the sensing of and response to stresses in vivo are most important: firstly, to adapt to the presence of toxic radicals, acidification, and degradative enzymes (24) and, secondly, to activate the production of virulence factors, many of which have been shown to be regulated by environmental conditions (6, 26). Thus, there is increasing evidence that stress responses, possi-

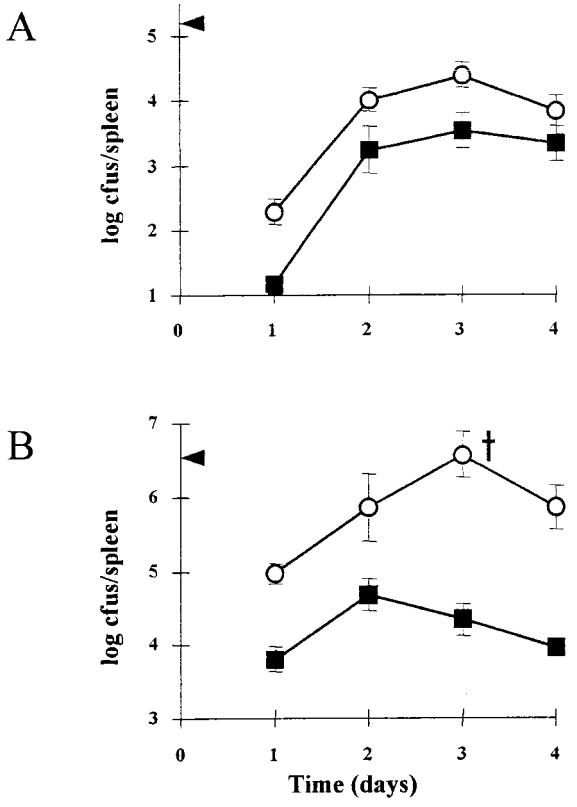

FIG. 3. Growth of LO28 (○) and LO28Δ*lisK* (■) in the spleens of BALB/c mice. Mice were inoculated with 1×10^5 (A) and 2×10^6 (B) *Listeria* cells by the intraperitoneal route. The arrowheads indicate the numbers inoculated into the peritoneal cavity on day 0. The cross indicates that one mouse died at that time point. Each datum point represents the mean $\log_{10}$ number of *Listeria* cells per spleen for four mice. Error bars represent the standard deviations of triplicate experiments. (A) Differences were significant on days 1 and 3 ($0.1 < P < 0.5$). (B) Differences were significant on days 1, 3, and 4 ($0.01 < P < 0.02$). In all cases, significance was calculated by using the Student *t* test.

bly through a number of pathways, can regulate the virulence of *L. monocytogenes*.

It is thus significant that genes encoding LisRK, initially identified as having a role in stress response, are also important in the virulence of this pathogen. These results contribute to the increasing evidence of the importance of functional stress-responsive mechanisms for a successful infection.

Nucleotide sequence accession number. The sequence of the 3,619-bp DNA determined in this study has been deposited in GenBank under accession no. AF139908.

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