TABLE 1. PCR primers used in this study.

Name	Sequence (5′–3′)
nisRF	AATGA**TCGA**TAAACAATCGGAGGT[a]
nisKB	ACTAGTGGATCCCCCGGG
NZlisRF	GGGTTAGGCC**CC**ATG**G**ATAGAATACTA[b]
NZlisRB	AATGG**T**CTAG**A**CGTCATGTACGCAT[c]
lisRRTPCRF	CAGCGGTTGCTAATGATG
lisRRTPCRB	GATAACAGATCACGTGCG
HSOEA	CAGGTAGAGCGGA**A**TTCATTG[d]
HSOEB	TTCATCGATTTCACTCTCCTTC**CGTGTGT GTTAAGCGGATCCAT**[a,e]
HSOEC	AT**GGA**TCCGCTTAACACACACG[f]
HSOED	CC**TCTAG**AGAATCTGCTTTTACCGTC[c]
PBP 1	GCGACAAGGCCGGGGAAC
PBP 2	CGGCGATTAGTCGCTTTG
CP 1	AGACGCCCAGAACCGACTCCA
CP 2	AATCGTACTCACGACGAGG
HKP 1	CGGTACGGTCGGTTACTAT
HKP 2	TGTCGCGCTTTTTTCACTCT

[a] Base changes made to incorporate a *Cla*I site are in boldface.
[b] Base changes made to incorporate an *Nco*I site are in boldface.
[c] Base changes made to incorporate an *Xba*I site are in boldface.
[d] Base changes made to incorporate an *Eco*RI site are in boldface.
[e] Overhang complementary to HSOEB primer is underlined.
[f] Base changes made to incorporate a *Bam*HI site are in boldface.

Basingstoke, Hampshire, England) supplemented with 0.6% yeast extract (Difco) (TSA-YE or TSB-YE) or brain heart infusion (BHI) agar or broth (Oxoid). Plasmid pKSV7, used for SOEing PCR, was a kind gift from Kathryn Boor, Cornell University, Ithaca, N.Y. Plasmids pNZ8048 (8) and pNZ9530 (21) were gifts from Michiel Kleerebezem, NIZO, Ede, The Netherlands. Plasmid pNZ44 (29) was a gift from Stephen McGrath, University College Cork, Cork, Ireland.

Growth in the presence of nisin. The rates of growth of *L. monocytogenes* LO28 and LO28Δ*lisK* in the presence of different levels of nisin (2% inoculum in TSB-YE containing 50, 100, 150, 200, or 300 μg of nisin powder [Sigma, St. Louis, Mo.]/ml) were compared by monitoring optical density at 600 nm (OD_{600}) with a Spectra Max 340 spectrophotometer (Molecular Devices, Sunnyvale, Calif.) over a 20-h period.

Antibiotic assays. Assays to determine the sensitivities of LO28 and LO28Δ*lisK* to a wide range of antibiotics were carried out by agar diffusion. Overnight cultures were diluted to 10^6 CFU/ml and swabbed onto TSA-YE. Commercially purchased disks (6 mm in diameter; Oxoid) containing 30 μg (unless otherwise stated) of the antibiotics to be studied were then placed on the surfaces of agar plates. Following overnight incubation of the plates at 37°C, the diameters of the zones of bacterial growth inhibition surrounding the filter disks were measured. The relative susceptibilities of different strains to the various antibiotics tested were correlated with the sizes of the zones of inhibition, with increased zone size reflecting increased susceptibility. Initially the antibiotics assayed were cefotaxime, cefuroxime, vancomycin, erythromycin, kanamycin, fosfomycin (50 μg), minocycline, polymyxin B (300 μg), streptomycin (25 μg), fusidic acid (10 μg), oxytetracycline, penicillin (10 μg), clindamycin (10 μg), spectinomycin (25 μg), ampicillin (25 μg), novobiocin, rifampin, nalidixic acid, gentamicin, colistin sulfate (25 μg), chloramphenicol (CAM), and tetracycline.

In addition to this assay, designed to compare antibiotic susceptibilities in general, further studies involved disks containing antibiotics of the cephalosporin family. These were cefuroxime, ceftazidime, cefaclor, cephalothin, cefoxitin, ceftriaxone, cefotetan, cefoperazone, cephradine, cephalexin, and cefotaxime (all 30 μg).

Implementation of the nisin-controlled expression (NICE) system in *L. monocytogenes* LO28Δ*lisK*. A strategy to replace the hemolysin gene, *hly*, on the *L. monocytogenes* chromosome with the *nisRK* genes was devised. Primers were designed to amplify the chromosomal regions flanking the *hly* gene (primers HSOEA, -B, -C, and -D; Table 1). The resultant A-B and C-D fragments were spliced by overlap extension PCR (19) using primers HSOEA and -D to create a single A-D fragment, representing the region surrounding *hly* but with the gene precisely removed. This fragment was subsequently cloned in temperature-sensitive plasmid pKSV7 (41). Since primers B and C have built-in restriction sites, we were able to clone the *nisRK* operon (amplified, by using primers nisRF and nisKB, from pNZ9530 [21]) into the location formerly occupied by *hly*. This plasmid construct, pCPL-53, was electroporated into *L. monocytogenes* LO28Δ*lisK*, and transformants were selected on BHI agar with 10 μg of CAM/ml (BHI/CAM). Chromosomal integration of the plasmid at 41°C was selected by serial passage of a transformant in prewarmed BHI/CAM broth and streaking onto prewarmed BHI/CAM agar. Plasmid excision was accomplished by continuous passage in BHI at 30°C, and clones in which *nisRK* had replaced *hly* on the chromosome were identified by plating them onto blood agar plates at 30°C. Replica plating of nonhemolytic colonies onto BHI and BHI/CAM at 30°C identified cured derivatives. PCR and sequencing analysis of one such strain confirmed that the *nisRK* operon had replaced the *hly* gene. This strain was designated LO28Δ*lisK*-NICE.

Overexpression of *lisR*. PCR primers NZlisRF (containing the *lisR* start codon) and NZlisRB (containing the *lisR* stop codon) with incorporated *Nco*I and *Xba*I sites, respectively, were used to amplify *lisR* from LO28. The resultant PCR product was digested with *Nco*I and *Xba*I restriction enzymes (Roche) and cloned into similarly digested pNZ8048, resulting in the generation of a translational fusion between the nisin-inducible *nisA* promoter on pNZ8048 and the *lisR* gene. This event was confirmed by sequence analysis. This strain was designated LO28Δ*lisK*-NICE(pNZ8048-*lisR*). pNZ8048 was also introduced into the LO28Δ*lisK*-NICE background to create LO28Δ*lisK*-NICE(pNZ8048). As an alternative to inducible overexpression, *lisR* (amplified by primer pair NZlisRF and NZlisRB) was also cloned into plasmid pNZ44, a derivative of pNZ8048 in which the P_{nis} promoter was replaced by a constitutive P_{44} promoter (29), resulting in the creation of LO28Δ*lisK*(pNZ44-*lisR*). LO28Δ*lisK*(pNZ44) was also created to serve as a control for subsequent experiments.

RT-PCR. RNA isolation and reverse transcription-PCR (RT-PCR) were carried out as described previously (6). RNA was isolated from overnight cultures (constitutive overexpression system) or following nisin induction (inducible overexpression system). For induction with nisin cultures were grown to an OD_{600} of 0.2 and preinduced with 4.5 μg of nisin powder/ml for 1 h, followed by induction with 45 μg of nisin powder/ml (a concentration at which there is no difference in the nisin sensitivities of the two strains) for 30 min and then isolation of RNA. In all cases cDNA was amplified by PCR with specific primers and samples were taken at regular intervals and run on agarose gels. Primers for the 16S rRNA of *L. monocytogenes* LO28 were used as controls (36).

RESULTS AND DISCUSSION

Nisin resistance of the Δ*lisK* mutant. Because deletion of the *L. monocytogenes* histidine kinase-encoding gene, *lisK*, results in an altered response to environmental parameters (5), we examined the susceptibility of *L. monocytogenes* to the lantibiotic nisin. It was found that the levels of nisin required to inhibit growth of wild-type LO28 are high relative to those required to inhibit the growth of a number of other gram-positive bacteria (9). This is a feature of this strain which we have previously observed in relation to other bacteriocins such as lacticin 3147 and enterocin A (our unpublished data). Growth curves were carried out to determine whether deletion of *lisK* affected the response to nisin. In the absence of nisin the growth rates of the parent and mutant strains were identical, but in the presence of increasing levels of nisin differences became apparent, primarily manifested as a significantly longer lag phase in wild-type LO28 (Fig. 1). This is most obvious when one observes that the lag period for LO28 in the presence of 300 μg of nisin/ml is 6 h longer than that for LO28Δ*lisK* (Fig. 1E). There is no difference in the growth rates once the lag phase has been exited (e.g., both are 0.046 U/h during logarithmic growth in the presence of 200 μg of nisin/ml). Therefore, the LisK mutation results in a culture that is able to rapidly initiate growth in the presence of nisin, whereas the parent apparently needs a significant period in which to adapt to the presence of the inhibitor. We speculate that, during prior growth in the absence of nisin, the LisRK system plays a role in creating a particular cell envelope composition that renders the cell more susceptible to nisin (and also alters its