JOURNAL OF BACTERIOLOGY, Dec. 1997, p. 7591–7594 Vol. 179, No. 23
0021-9193/97/$04.00+0

The *rpoN* (σ^{54}) Gene from *Listeria monocytogenes* Is Involved in Resistance to Mesentericin Y105, an Antibacterial Peptide from *Leuconostoc mesenteroides*

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Received 21 July 1997/Accepted 29 September 1997

To gain insight into the mode of action of mesentericin Y105, a bacteriocin bactericidal agent against *Listeria monocytogenes*, we undertook to identify the listerial factors mediating this susceptibility by using a genetic approach. Transposon mutants resistant to the bacteriocin were obtained. One of them corresponded to a transposon insertion in a gene (*rpoN*) encoding a putative protein (447 amino acids) with strong homologies to alternative transcriptional σ^{54} factors, including that of *Bacillus subtilis* (38% identity). Complementation experiments with the wild-type *rpoN* gene demonstrated that the insertion in *rpoN* was responsible for the resistance phenotype in *L. monocytogenes*. Moreover, expression of the *L. monocytogenes rpoN* gene in an *rpoN* mutant strain of *B. subtilis* promoted transcription of a σ^{54}-dependent operon in the presence of the associated regulator. These results demonstrate that the *L. monocytogenes rpoN* gene encodes a new σ^{54} factor.

In addition to conventional antibiotics, the use of ribosomally synthesized peptides is increasingly envisioned (8, 25). These include bacteriocins, which are antibacterial peptides, produced by most of the bacterial genera in which they have been sought. In gram-positive bacteria, most of the reported bacteriocins are produced by lactic acid bacteria (12), which have long been used as food preservatives since they produce several compounds, such as lactic acid, which limit the development of pathogenic or spoilage bacteria. Several classes of bacteriocins from lactic acid bacteria have been described (14). A subclass (IIa) of nonlantibiotic peptides contains specific antilisterial bacteriocins. We have previously described mesentericin Y105, a bacteriocin synthesized by *Leuconostoc mesenteroides*, showing a narrow spectrum of bactericidal activity towards *Listeria monocytogenes* (7, 11). This peptide (37 amino acids [aa]) has been demonstrated to kill target bacteria via membrane permeabilization (17). Pore formation was observed for several bacteriocins. It occurs in the cytoplasmic membrane of the target cells, and it is followed by an efflux of ions and small molecules (1, 31). However, bacterial resistance to bacteriocins was described. For example, *Listeria* resistance to nisin has been reported (5, 24), but the molecular basis of this resistance remains mostly unexplained. One noticeable exception concerns nisinase, a specific peptidase present in some bacterial species (2, 13). To our knowledge, there is no information concerning acquired resistance to bacteriocins, at least at the molecular level.

We describe here the molecular characterization of a transposon-induced mutant that displays resistance to mesentericin Y105. The interrupted gene, *rpoN*, encodes a protein similar to σ^{54}, which is an alternate subunit of bacterial RNA polymerase (20) only described once previously in a gram-positive organism, i.e., *Bacillus subtilis* (6). It directs the RNA polymerase holoenzyme to a specific class of promoter sequences with a −12/−24 consensus sequence. Complementation experiments with *B. subtilis* indicate that the interrupted gene in this mutant encodes a bona fide σ^{54}.

Characterization of a mesentericin-resistant mutant of *L. monocytogenes* LO28. A library of 3,000 Tn*917-lac* insertion mutants of *L. monocytogenes* LO28 (18) was screened for susceptibility to mesentericin Y105 extracts. Wells of microtiter plates containing 150 μl of brain heart infusion (BHI) medium (supplemented with erythromycin at 5 μg/ml for mutant culture) were inoculated (1%) with each mutant. After 3 h at 37°C with shaking, 50 μl of mesentericin concentrated extract prepared by ammonium sulfate precipitation as previously described (11) was added to each well and growth was monitored by measuring optical density at 570 nm. This approach led us to identify 18 bacteriocin-resistant mutants which had variable resistance to the bacteriocin. One of them, LUT758, was chosen for further studies on the basis of its resistance to mesentericin Y105 (Fig. 1). Both the 50% lethal dose, determined as previously reported (22), and motility were unaffected by the transposon insertion (data not shown).

Cloning and sequence analysis of the *rpoN* gene. The plasmids used in this study are described in Table 1. A 1.6-kb *Hin*dIII fragment corresponding to the downstream Tn*917-lac* chromosome junction from the LUT758 mutant was identified by Southern blotting experiments by using a 0.5-kb probe spanning the 3′ end of the transposon (27) and cloned at the *Hin*dIII site into pBluescript II SK+ (Stratagene). Listerial chromosomal DNA was prepared as previously reported (19). The nucleotide sequence of the chromosomal region located next to the transposon in the resulting pRT758 recombinant plasmid was then determined by dideoxy-chain termination. A search of data banks showed that insertion had taken place within an open reading frame (ORF) very similar to bacterial σ^{54} factors. By using the known partial *rpoN* sequence to generate a PCR probe, we cloned the wild-type gene from *L. monocytogenes* LO28 as a 4.4-kb *Spe*I fragment in pUC18 previously cleaved by *Xba*I, leading to plasmid pEL80. This re-

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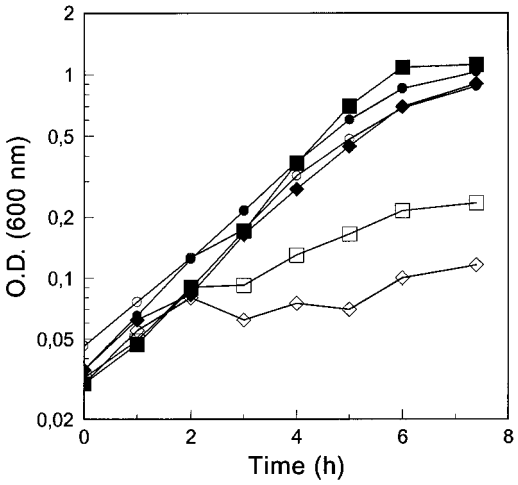

FIG. 1. Growth curves of *L. monocytogenes* LO28, LUT758, and LUT758/pRPO10 with or without mesentericin Y105. Strains were inoculated in duplicate at 5% in 1 ml of BHI medium (supplemented with appropriate antibiotics), and optical density (O.D.) at 600 nm was monitored. A concentrated extract of mesentericin Y105 (50 μl) was added after 2 h. Symbols for untreated cultures: ■, LO28; ●, LUT758; and ◆, LUT758/pRPO10. Similar open symbols are used for mesentericin-treated cultures.

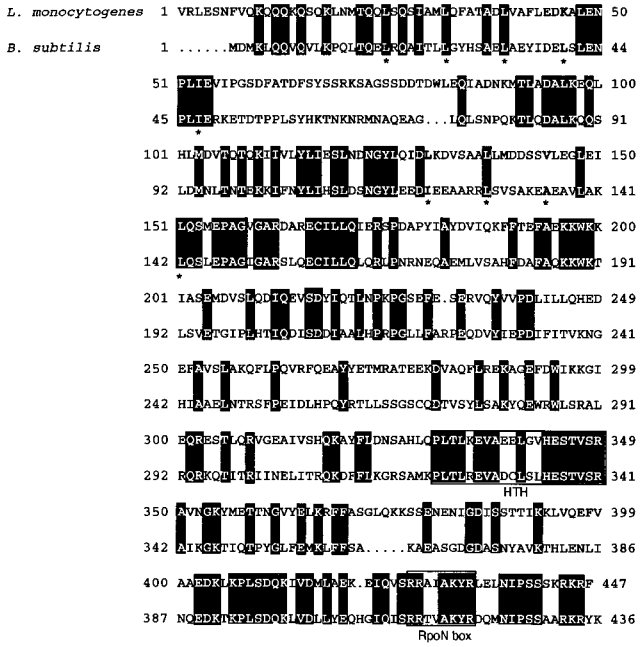

FIG. 2. Alignment of the amino acid sequences deduced from the *rpoN* gene of *L. monocytogenes* and the *sigL* gene of *B. subtilis*. The consensus patterns, helix-turn-helix (HTH) motif, and RpoN box of the σ^{54} factor family are boxed. The asterisks indicate amino acids involved in a heptad hydrophobic repeat that could form a leucine zipper structure.

combinant plasmid was used to transform *Escherichia coli* DH5α as described by Hanahan (10). Part of this 4.4-kb *L. monocytogenes Spe*I fragment from pEL80 was sequenced by gene walking on both strands by Genome Express (Grenoble, France) using an Applied Biosystems sequencer. The sequence of 2,482 bp at the 3′ end of the cloned fragment appears in the EMBL nucleotide sequence database under accession no. X93169. Sequence analyses, performed with the programs of the GCG sequence analysis software package, showed two ORFs. The first one, ORFD, encodes an incomplete protein of 290 aa obviously lacking an NH_2 terminus. It shows about 33% identity with two putative proteins with unknown functions located in the region of the pyrrolo-quinoline-quinone biosynthesis gene cluster of *Klebsiella pneumonia* (21) and *Acetinobacter calcoaceticus* (9). The second ORF, *rpoN*, is located 160 bp downstream from ORFD. The complete gene encodes a putative protein of 447 aa. This protein displays strong similarities to the 17 RNA polymerase σ^{54} subunits already described in several bacterial genera, e.g., 38% identity and 59% similarity with that of *Bacillus subtilis* (6) (Fig. 2) and 32% identity and 57% similarity with that of *E. coli* (26). Moreover, it shares the same domains already described, (i) a relatively well-conserved N-terminal glutamine-rich region of about 50 residues that contains a potential leucine zipper motif, (ii) a less conserved central region, and (iii) a conserved C-terminal region that contains a potential DNA binding helix-turn-helix motif (PLTLKEVAEELGVHESTVSR) and a conserved octapeptide, named the RpoN box (RRTVA/TKYR), whose function is not known. However, the deduced sequence of the *L. monocytogenes* σ^{54} factor displays two differences within the latter motif in that the first threonine is replaced with an alanine (position 428 in the *L. monocytogenes* sequence) and the valine residue is replaced with an isoleucine at position 429. Based on sequence alignments with the other σ^{54} factors described, the GTG codon located at position 1034 in the nucleotide sequence is more likely to be the start codon than is the ATG located 20 codons downstream or 27 codons upstream. It is preceded 8 bp upstream by a consensus ribosome-binding site sequence (GGAGG), and an upstream putative promoter region was also found by sequence analysis. Finally, one putative transcription terminator (with a calculated free energy of formation of −33.1 kcal) was detected between positions 878 and 913 upstream from *rpoN* and preceding the putative promoter.

TABLE 1. Plasmids used in this study

Plasmid	Relevant properties	Source or reference
pUC18	Amp^r	Life Technologies
pEL80	pUC18::4.4-kb *Spe*I	This study
pMK4	Shuttle vector, Amp^r Cm^r	28
pRPO10	pMK4::*rpoN*	This study
pG+host6	Thermosensitive shuttle vector, Amp^r Ery^r	E. Maguin
pRPO1	pG+host6::*rpoN*	This study
pHT315	Shuttle vector, Amp^r Ery^r	3
pSIG7	pHT315::*sigL*	6

Transcription analysis of the *rpoN* gene. The expression of *rpoN* was studied by Northern blotting of RNA from *L. monocytogenes* LO28 as described by Mengaud et al. (19), except that, due to the high instability of the RNA, the gel was run immediately after extraction. RNAs were separated in a 1.2% (wt/vol) agarose-formaldehyde gel and transferred to a Hybond-N (Amersham) nylon membrane. Only one transcript was detected by hybridization with a 0.5-kb fragment internal to *rpoN* and showed a size of approximately 1,650 nucleotides (Fig. 3). This result indicates that *rpoN* is transcribed as a monocistronic unit, unlike most of the *rpoN* genes but like *sigL* of *B. subtilis* (two stem-loop structures are found upstream and downstream from *sigL* [unpublished result]). Assuming that the above-mentioned putative promoter is efficient, this implies that the transcript should extend to a transcription ter-

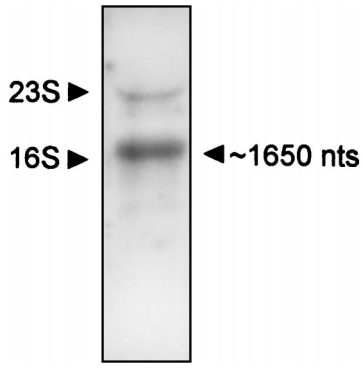

FIG. 3. Northern hybridization analysis of the total RNA from *L. monocytogenes* LO28. The probe is the 0.5-kb fragment internal to *rpoN* (see the text for details). The rRNAs indicated by arrowheads on the left were used as size standards (2,904 nucleotides [nts] for 23S and 1,540 nucleotides for 16S). The approximate size of the *rpoN* transcript is indicated by the arrowhead on the right.

minator located approximately 100 nucleotides downstream from the 3′ end of our cloned region.

Disruption of the putative *rpoN* gene and complementation with the wild-type gene. To verify that the phenotype of the mutant was due to the transposon insertion and not to a secondary mutation, we generated a new chromosomal mutant by plasmid insertion in *rpoN* with a pHV1248ΔTn*10* integrative vector as previously described (15). This mutant acquired resistance to mesentericin Y105, indicating that the putative *rpoN* gene is indeed involved in mesentericin Y105 sensitivity. The LUT758 mutant was then complemented with the wild-type gene cloned into a pMK4 shuttle vector. A 1,650-bp *Bam*HI-*Sal*I fragment was digested from pRPO1 and cloned at the same sites in pMK4, leading to plasmid pRPO10. LUT758 was transformed by electroporation with pRPO10 as previously described (22) and selected on BHI medium supplemented with erythromycin (5 μg/ml) and chloramphenicol (7 μg/ml). It showed restored sensitivity to mesentericin Y105 at a level even higher than that of wild-type strain LO28 (Fig. 1). This finding supports the conclusion that disruption of *rpoN* is responsible for the resistance phenotype.

Complementation of an *rpoN* (*sigL*) mutant of *B. subtilis* with the *L. monocytogenes rpoN* gene. To test the functionality of the *L. monocytogenes* σ^{54} factor, we complemented *B. subtilis* QB5504 [*trpC2 levR8 amyE*::(*levR8 levD-lacZ*) *aphA3 recA*::*cat sigL*] lacking the endogenous *B. subtilis* σ^{54} factor (6). A recombinant plasmid carrying the *L. monocytogenes rpoN* gene was constructed by cloning a 1,650-bp *Bsa*BI-*Bam*HI fragment from pRPO10 into pG+host6 opened by *Eco*RV-*Bam*HI digestion (plasmid pRPO1) and introduced by transformation as previously described (16) into strain QB5504 and selected on SP plates (6) containing erythromycin (1 μg/ml) and lincomycin (25 μg/ml). In addition to the *sigL* mutation, this strain contains a constitutive allele of *levR* (*levR8*) and a *levD-lacZ* translational fusion. Transcription of the levanase operon strongly depends on SigL. Therefore, QB5504 displays a white phenotype on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates. The corresponding *sigL*$^+$ strain has a dark blue phenotype in Luria broth or minimal medium containing X-Gal (6). As controls, strain QB5504 was also transformed with either pG+host6 or pSIG7, which contains the *sigL* gene expressed in pHT315, or pHT315. For each of these transformants, β-galactosidase synthesis was assayed in cultures incubated at 30°C in Luria-Bertani medium containing erythromycin. Results, expressed as Miller units per milligram of protein (23), were 5 U/mg with the pHT315 or pG+host6 control plasmid, 2,165 U/mg with pSIG7, and 3,545 U/mg with pRPO1. This indicates that plasmid pRPO1 complements the *sigL* mutation, allowing full expression of the levanase operon in strain QB5504. These results thus establish that the *L. monocytogenes rpoN* gene encodes a protein functionally equivalent to the *B. subtilis* σ^{54} factor.

Conclusions. By searching for factors responsible for the sensitivity of *L. monocytogenes* to mesentericin Y105, we have identified the second σ^{54} factor found in gram-positive bacteria. When this transcriptional factor is not expressed in *L. monocytogenes*, this bacterium becomes resistant to mesentericin Y105, suggesting that genes responsible for sensitivity are controlled by σ^{54}. In other systems, these genes share a consensus promoter sequence at −24/−12 positions with respect to the transcription initiation site, and activation requires binding of an appropriate activator protein upstream at enhancer sequences (20). σ^{54} is required for expression of a wide variety of genes but is not essential for growth. Most of the genes under σ^{54} control are involved in nitrogen metabolism, as well as in pilus production, dicarboxylic acid transport, and toluene and xylene catabolism (20, 29). Hence, it is likely that the bacteriocin could enter the cell by using a receptor normally used for other purposes. It should be emphasized that some class II bacteriocins (e.g., mesentericin Y105), as well as colicins, have been demonstrated to interact with a specific membrane protein (4, 30), in contrast to lantibiotics (e.g., nisin), which seem to act directly on the membrane. Characterization of the other mesentericin Y105-resistant mutants, currently in progress, should help to identify the pathway(s) leading to sensitivity or establish the missing link between σ^{54} expression and sensitivity. Preliminary analysis of another resistant mutant revealed an insertion in a gene displaying a putative σ^{54}-dependent promoter and encoding a protein with a conserved hexapeptide motif found in surface proteins of gram-positive cocci. Other transposon insertions leading to mesentericin resistance have interrupted genes encoding putative proteins involved in different metabolic pathways. These results underline the possibility of multiple mechanisms leading to resistance, as previously described for antibiotic resistance.

We thank Xavier Varlet-Grancher for technical help and Christophe Fremaux and Randy Worobo for technical tips and stimulating discussions.

This work was supported by the French Ministère de l'Enseignement Supérieur et de la Recherche (scholarship to Denis Robichon) and in part by a partnership with TEXEL (groupe Rhône-Poulenc).

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