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Philippe Velge, Tatiana Vallaeys, Pierre Pardon, Eugénie Huillet. LadR, a new PadR-related transcriptional regulator fromListeria monocytogenes, negatively regulates the expression of the multidrug efflux pump MdrL. FEMS Microbiology Letters, 2005, 254, pp.87-94. 10.1111/j.1574-6968.2005.00014.x . hal-04731442

HAL Id: hal-04731442
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LadR, a new PadR-related transcriptional regulator from *Listeria monocytogenes*, negatively regulates the expression of the multidrug efflux pump MdrL

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Received 5 August 2005; revised 6 October 2005; accepted 10 October 2005.
First published online 14 November 2005.

doi:10.1111/j.1574-6968.2005.00014.x

Editor: Jan-Ingmar Flock

Keywords
PadR family; repressor; LadR; bacterial multidrug efflux pump; MdrL: *Listeria monocytogenes*.

Abstract

The *Listeria monocytogenes* genome encodes putative multidrug efflux transporters but only the MdrL transporter has been partially characterized in the wild-type LO28 strain. Here, we show in the LO28 strain, that the expression of MdrL is repressed at the transcriptional level, under standard growth conditions, by the product of a new gene *ladR* (lmo1408), and the expression of MdrL is induced in the presence of rhodamine. Phylogenetic analysis in related firmicutes shows that LadR, conserved in all sequenced *Listeria* genomes, forms an independent group from the large and diverse PadR transcriptional regulator family (PF03551). This is the first report of a bacterial multidrug transporter controlled by a member of the PadR family.

Introduction

Multidrug-resistance (MDR) transporters often specify resistance to a range of structurally and functionally different hydrophobic toxic substances (drugs) such as antibiotics, dyes and disinfectants. Drugs are actively exported from the cytoplasm to the outer membrane. These systems are widespread among bacterial species and are often found in several copies in bacterial genomes. However, it remains unknown whether the efflux of diverse drugs is the primary function of the MDR transporters or whether MDR pumps have more specific, but presently unknown, physiological functions (Grkovic *et al.*, 2001; Markham & Neyfakh, 2001). The concentrations of many of these MDR transporters are tightly controlled by transcription regulators, located in the immediate vicinity of the transporter (Grkovic *et al.*, 2001; Markham & Neyfakh, 2001).

Among the food-borne pathogens, *Listeria monocytogenes* is of main concern because it causes severe diseases such as septicaemia, meningitis, stillbirth and abortion, with immune-compromised patients and pregnant women being high-risk groups (Khelef *et al.*, 2005). In the *L. monocytogenes* EGDe genome, six MDR transporters based on predicted protein sequence similarity have been identified (Glaser *et al.*, 2001) but only one, the MdrL transporter, has been partially characterized using a genetic approach. An insertion mutant in the *mdrL* gene failed to pump out ethidium bromide and presented lower minimal inhibitory concentrations (MICs) of macrolides, cefotaxime and heavy metals (Mata *et al.*, 2000). MdrL has also been implicated in salt tolerance (Gardan *et al.*, 2003). The clinical significance of this MdrL pump (Mereghetti *et al.*, 2000) and its role in bacterial physiology remain to be elucidated (Mata *et al.*, 2000; Gardan *et al.*, 2003).

We previously identified a new locus in the *L. monocytogenes* strain LO28 involved in cellobiose-dependent repression of *hly* expression (Huillet *et al.*, 1999). The gene *hly*

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encodes listeriolysin, a major virulence factor of *L. monocytogenes*. A bank of Tn*917* insertion mutants was screened for deregulated expression of listeriolysin in the presence of cellobiose known to repress *hly* expression. In one mutant with a higher expression of *hly*, the transposon Tn*917* was inserted into the putative promoter region of *orfA*, a gene with an unknown function. Upstream of *orfA* and in the opposite direction *mdrL*, the gene encoding the MdrL pump, was found. Transcriptional analysis of the *mdrL* and *orfA* genes in this insertion mutant showed that the transcription of *orfA* was abolished whereas the expression of *mdrL* was activated under standard growth conditions (Huillet *et al.*, 1999), thus suggesting that OrfA could be a transcriptional repressor of the *mdrL* gene in *L. monocytogenes*. The OrfA protein belongs to the large and poorly characterized PadR transcriptional regulator family.

Here, we present a new gene *orfA* renamed *ladR* in this study, involved in the negative control of the expression of the MdrL pump. We also analysed the distribution of the locus *ladR–mdrL* in *Listeria* spp. and present the first phylogenetic study of the PadR family in firmicutes (low GC% Gram-positive bacteria).

Materials and methods

Bacterial strains, plasmids and media

Listeria strains and plasmids are listed in Table 1. *Escherichia coli* TG1 was routinely grown in Luria–Bertani (LB) medium and used as a host for DNA manipulations (Sambrook & Russell, 2001). Antibiotics were used at the following concentrations: ampicillin, 100 μg mL^{-1} for *E. coli*; erythromycin, 8 μg mL^{-1} for *L. monocytogenes* and 300 μg mL^{-1} for *E. coli*; and kanamycin 20 μg mL^{-1} for *L. monocytogenes*.

Table 1. List of *Listeria* strains, plasmids and primers used

Strain, plasmids or primers	Characteristics	Reference or source
LO28	Serotype1/2c	Huillet *et al.* (1999)
prfA	LO28Δ*prfA* (BUG 802)	P. Cossart
m3.7	LO28::Tn*917*;Er^R	Huillet *et al.* (1999)
ladR	LO28Δ*ladR*;Km^R	This work
prfA–ladR	LO28Δ*prfA*Δ*ladR*;Km^R	This work
pAUL-A	Shuttle vector, Er^R	Schaferkordt & Chakraborty (1995)
pDG784	pKS with *apha-3* gene;Km^R	Trieu-Cuot & Courvalin (1983)
pKm1	*Bam*HI Km^R cassette of 850 bp	This work
pAH2	pAUL-A with a 2.65 kb *Xba*I *Kpn*I insert	This work
O1*	5′ gctctagattgtaaagcttcaggagtgca	This work
O2*	5′cgggatccacgattagatttgattca	This work
O3*	5′cgggatccagacatttggatcataca	This work
O4*	5′ggggtaccatcagagacgccaggcca	This work

*Restriction sites are underlined in the primer sequence.

Construction of a ladR null mutant and a double ladR–prfA null mutant

A *L. monocytogenes ladR* null mutant was constructed by double crossing over using the temperature-sensitive suicide plasmid pAUL-A (Schaferkordt & Chakraborty, 1995). Construction of the *ladR* allele was performed as follows: a region of 1 kb upstream of *ladR* (5′) and a region of 0.8 kb below *ladR* (3′) were amplified with primer pairs O1/O2

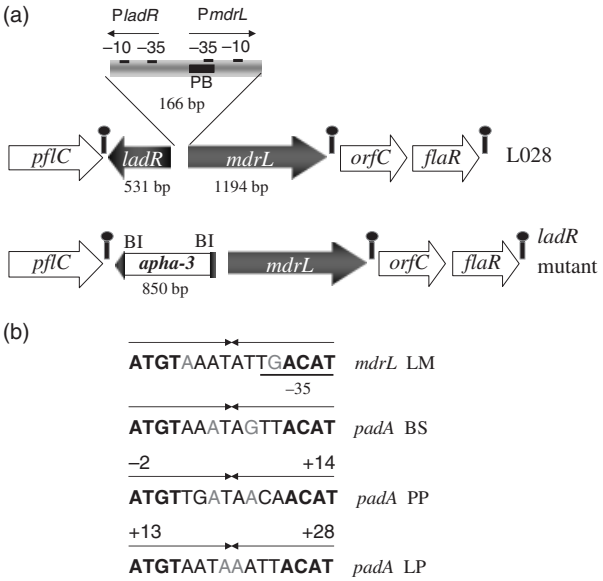

Fig. 1. (a) Genetic organization of the *ladR–mdrL* locus in LO28 strain and characterization of physical deletion/insertion in the *ladR* mutant. Large open arrows represent the different genes and their orientation. Stem–loop structures are used to illustrate putative terminator regions. A promoter/terminator-less kanamycin cassette (the *apha-3* gene) was cloned in place of the internal deleted fragment. The putative −10, −35 and PadR boxes (PB) are located in the intergenic region of the *ladR–mdrL* locus. The sequence of the locus in LO28 (EMBL accession no. AJ009627) has been published previously (Huillet *et al.*, 1999). The corresponding genes in EGDe with the nomenclature according to the ListiList server (Glaser *et al.*, 2001) are: lmo1407 (*pflC*), lmo1408 (*ladR*), lmo1409 (*mdrL*) and lmo1412 (*flaR*). BI, *Bam*HI restriction enzyme. (b) Comparison of the putative PadR-binding sites at the *mdrL* promoter in *Listeria monocytogenes* and at the *padA* promoter from relevant organisms. Convergent arrows indicate the palindromic sequence. The conserved motif is shown in bold and the nonpalindromic amino acids are shown in grey. The putative −35 box of *mdrL* is underlined. Initiation of transcription of the *padA* gene was identified both for *Pediococcus pentosaceus* (Barthelmebs *et al.*, 2000) and *Lactobacillus plantarum* (Gury *et al.*, 2004). LM, *Listeria monocytogenes*; BS, *Bacillus subtilis*; PP, *Pediococcus pentosaceus*; LP, *Lactobacillus plantarum*.

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and O3/O4, respectively (Table 1) (start and stop codons of *ladR* were, respectively, included). A promotorless and terminatorless *Bam*HI kanamycin cassette (the *apha-3* gene Trieu-Cuot & Courvalin, 1983) (Table 1) was cloned at the *Bam*HI site of a 5′–3′ fusion fragment of 1.8 kb. The obtained recombinant plasmid, pAH2 (Table 1), was introduced into LO28 and its *prfA* deletion mutant via electroporation (Schaferkordt & Chakraborty, 1995). Selection of recombinants, presenting an internal deletion of 400 bp replaced by a kanamycin cassette, was performed as described elsewhere (Schaferkordt & Chakraborty, 1995). Analysis of the genetic organization of the recombinant *ladR* allele was performed using PCR and Southern analysis. Chromosomal DNA extraction, DNA electrophoresis, Southern blotting and hybridizations were performed according to standard protocols (Sambrook & Russell, 2001).

RNA extraction and transcription analysis

Total RNA extraction from *L. monocytogenes* strains was performed with the Ambion Gram-positive RNA extraction kit (Huntingdon, UK). Northern blot analysis with an *mdrL* probe was conducted as described previously (Huillet *et al.*, 1999).

Drug resistance assay by metabolic activity monitoring

Bacterial drug resistance was assessed by monitoring the microbial metabolic activity of bacterial cultures via electrical capacitance measurements using the Bactometer automat (BioMérieux, Marcy L'Etoile, France) in sterile disposable 16 well modules (Firstenberg-Eden & Eden, 1984). The temperature was set at 37 °C. Bacterial growth of individual cultures was followed in well modules (1.5 mL) under nonagitated conditions by measuring the capacitance every 6 min for 48 h, resulting in a capacitance curve. MIC was estimated as the lowest concentration of drug that completely prevents growth (no capacitance variation). Drug resistance was assessed by measuring the capacitance detection time (CDT) defined as the lag phase of the capacitance curve. Two- and $1\frac{1}{2}$-fold serial dilutions were performed in LB medium from a 100 μg mL^{-1} stock solution for each drug in order to determine the MIC. The bacterial inoculum density was 1.4×10^5 logarithmic cells per well. Rhodamine 6G (R6G), acriflavine and norfloxacin were purchased from Sigma (Lyon, France), and ethidium bromide was obtained from Euromedex (Mundolsheim, France).

Growth conditions in order to detect mdrL expression with R6G

Rhodamine 6G at subMIC (6.58 μg mL^{-1}) was added for 1 h in *L. monocytogenes* culture in LB medium at $OD_{600\,nm}$ 0.4 (logarithmic phase). Culture without R6G was also collected at $OD_{600\,nm}$ 0.4 as a control.

Sequence analysis

ESPript, a bioinformatic tool (Gouet *et al.*, 1999), was used to display sequence and structure information of both LadR and AphA protein sequences (PDB accession no. 1YG2 for AphA) (Fig. 2). Megablast was used to search for LadR-like deduced amino-acid sequences at http://www.ncbi.nlm.nih.gov/sutils/genom_tree.cgi. When multiple hits were obtained in a same genome such as in *Listeria* and *Bacillus* species, blast bi-directional best hits (BDBH) were used to define the most probable orthologous gene among the candidate gene sequences. ClustalW sequence alignments (Higgins *et al.*, 1994) were obtained and a phylogenetic tree (Fig. 3) was constructed using the neighbour-joining method (Saitou & Nei, 1987) from the PHYLIP package (Felsenstein, 1989). The stability of the grouping was estimated by bootstrap analysis (100 replications) (Felsenstein, 1989). Relative gene positions in *Listeria* species and related genomes were defined from genome mining at http://genolist.pasteur.fr/

Results

Sequence analysis and phylogenetical inference of *LadR*

The genetic organization of the *ladR–mdrL* locus (EMBL accession no. AJ009627) in *Listeria monocytogenes* LO28 published previously (Huillet *et al.*, 1999) was found to be conserved in all sequenced *Listeria* genomes: the *ladR* gene (ListiList accession no. lmo1408) lies upstream of the *mdrL* gene (lmo1409) in the opposite direction (Fig. 1a). The intergenic region of the *ladR–mdrL* genes is 166 bp long and contains two nonoverlapping putative promoter regions with putative −35 and −10 consensus sequences and ribosome-binding site sequences (Fig. 1a). We found an inverted-repeat corresponding to the putative PadR-binding site of the PadR regulators group (ATGT-8N-ACAT) (Barthelmebs *et al.*, 2000; Gury *et al.*, 2004) in the −35 position of the *mdrL* gene (Fig. 1b).

The *ladR* gene belongs to the large (more than 300 members), diverse and poorly characterized PadR family (Pfam accession no. PF03551). As defined from the Pfam database of protein domains, this new family appears to be characterized by an N-terminal motif including amino acids 1–89. Multiple alignment with selected PadR-related protein sequences in firmicutes and in *Vibrionaceae* family allowed us to find an N-terminal motif ($Lx_9GY(D/E)x_{16-20}Yx_2(L/I)x_{18-21}$(K/R)(K/N)x(Y/F)xxTxxG) presented in Fig. 2. In the LadR protein sequence, this PadR motif is made up of amino acids 11–74 (Fig. 2).

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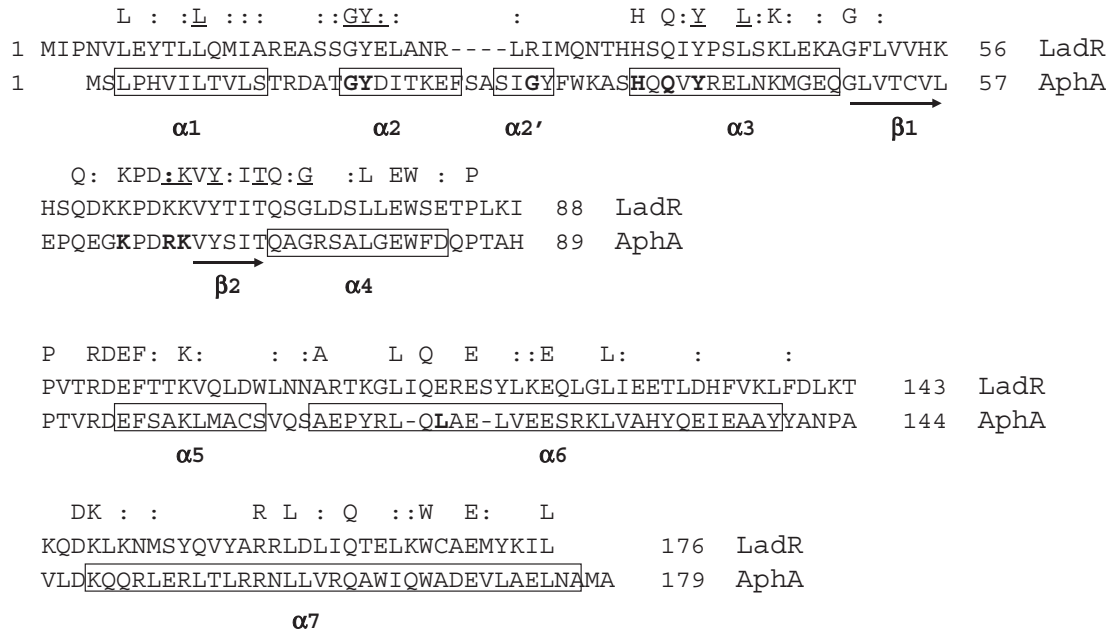

Fig. 2. Sequence alignment of LadR from *Listeria monocytogenes* and AphA from *Vibrio cholerae*. The consensus sequence is presented above the LadR sequence and 11 residues belonging to our proposed N-terminal PadR motif (see Results) are underlined. Secondary structure elements for AphA (ESPript, Protein Data Bank code 1YG2) are shown below its sequence. α, α-helix; β, β-strand. The positions of mutations in the AphA sequence, which influence DNA-binding and dimerization activities, are shown in bold (De Silva *et al.*, 2005).

The LadR protein (176 amino acids) presented a low overall sequence identity (24–29%) with the known PadR regulators from *Pediococcus pentosaceus* (176 amino acids) (Barthelmebs *et al.*, 2000) and from *Lactobacillus plantarum* (181 amino acids) (Gury *et al.*, 2004). PadR repressors from *P. pentosaceus* and from *L. plantarum* control the expression of a phenolic acid decarboxylase (encoded by the *padA* gene) in the phenolic acid stress response (Barthelmebs *et al.*, 2000; Gury *et al.*, 2004).

LadR also presented a low overall sequence identity of 25.5% with the repressor/coactivator AphA (179 amino acids) of *Vibrio cholerae* (Kovacikova *et al.*, 2003, 2004; De Silva *et al.*, 2005). AphA is a quorum sensing-regulated activator that initiates the virulence cascade in *V. cholerae* by cooperating with the regulator AphB and is also a repressor of the penicillin amidase activity (*pva* gene) (Kovacikova *et al.*, 2003, 2004). However, the comparison of the amino-acid sequences of LadR and AphA suggests that the secondary structures of AphA, determined recently from the analysis of the crystal structure of the AphA dimer (De Silva *et al.*, 2005), are remarkably conserved in the sequence of LadR (Fig. 2). The alignment (Fig. 2) shows that LadR contains seven putative α-helices and two putative β-strands, as determined for AphA. The protein AphA folds into two domains: a globular N-terminal DNA-binding domain made up of amino acids 1–86, and a distinctive C-terminal coiled-coil dimerization domain including amino acids 98–179. The crystal structure shows that the N-terminal DNA-binding domain adopts a winged helix fold similar to that of the multiple antibiotic-resistance repressor MarR (De Silva *et al.*, 2005). We can conclude that albeit being different in function, AphA and LadR are putative structural homologues. This result is also supported by the following phylogenetical analysis.

Data mining in available *Listeria* genome sequences (including *L. monocytogenes, L. innocua*, *L. ivanovii* and *L. grayi*) allowed us to identify two to three copies of genes whose deduced amino-acid sequences present an N-terminal PadR motif. Among the *padR*-related genes, a BDBH approach in *L. monocytogenes*-sequenced genomes (1/2a EGDe, 1/2a F6854, 4b H7858, 4b F2365) allowed us to define that *ladR*, present in a single copy, is the closest relative of the canonical *padR* gene from *P. pentosaceus*. The same result was obtained with the *ladR* orthologous sequences in other *Listeria* species (*L. innocua*, *L. ivanovii* and *L. grayi*) genomes.

This result was also supported by the construction of a phylogenetic tree (Fig. 3) of *padR*-like genes in related firmicutes (*Listeria*, *Bacillus, Staphylococcus, Enterococcus, Lactobacillus, Streptococcus, Clostridium*) plus relevant organisms. The *padR*-related genes fall into two distinct families: subfamily I with longer sequences (approximately 176 amino acids) and a more distant family, subfamily II presenting shorter sequences (approximately 110 amino

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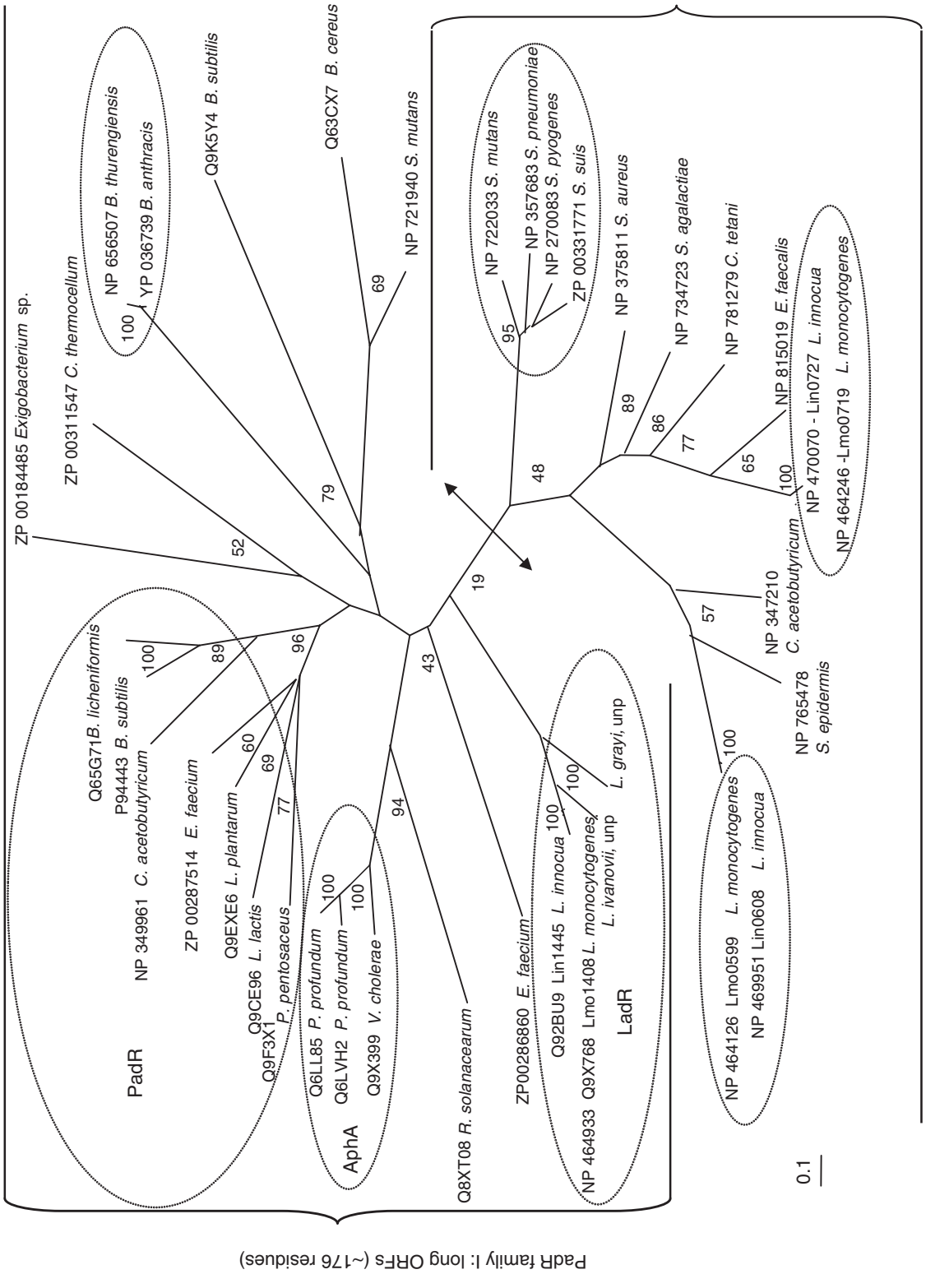

Fig. 3. Phylogenetic tree for PadR-related proteins in firmicutes plus a few additional relevant organisms. The *aphA* gene from *Vibrio cholerae* and related *padR* genes from *Photobacterium profundum* (*Vibrionaceae* family) and the phytopathogenic betaproteobacterium *Ralstonia solanacearum* were initially introduced as outgroup sequences but fall into subfamily I. The arrow indicates the position of the split between the two PadR subfamilies (I and II). Accession numbers are shown for each member of the two subfamilies. Bootstrap values are indicated for each node.

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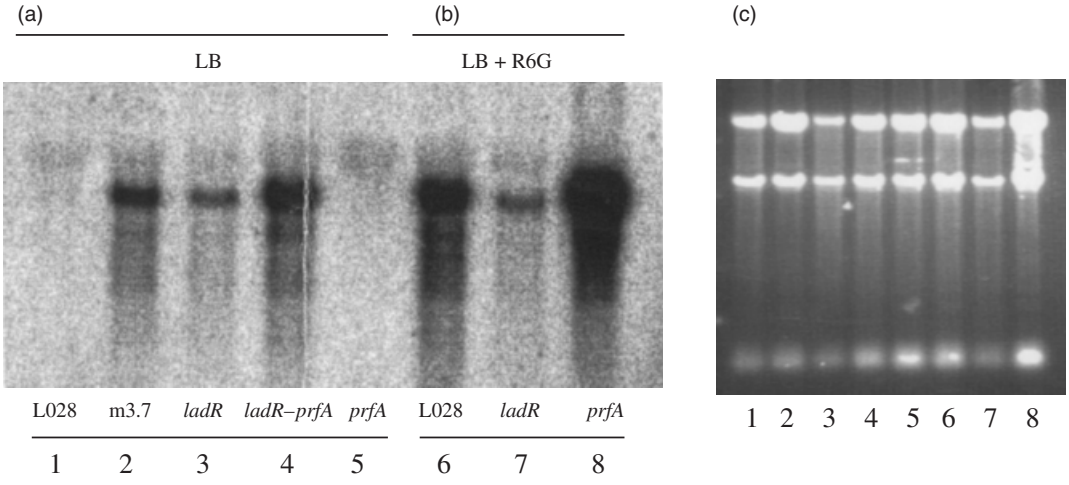

Fig. 4. Northern blot analysis of *mdrL* expression in *Listeria monocytogenes* wild-type strain LO28 and isogenic mutant strains: insertional mutant m3.7 (Huillet *et al*., 1999), null mutants *ladR*, *prfA-ladR* and *prfA*. (a) *MdrL* expression in cells grown in Luria–Bertani (LB) during log phase. Lane1: LO28; lane2: m3.7; lane 3: *ladR*; lane 4: *ladR–prfA*; lane 5: *prfA*. (b) Effect of 1 h incubation with rhodamine 6G (R6G) in LB medium during log phase on the *mdrL* expression. Lane 6: LO28; lane 7: *ladR* and lane 8: *prfA*. (c) Agarose gel picture of total RNA loaded per lane: 10 μg of total RNA was loaded per well, except for lanes 3 and 7 (< 10 μg) and lane 8 (> 10 μg).

acids). This result was supported by a very low bootstrap value at the node between the two corresponding branches from the phylogenetic tree.

According to our classification (BDBH and phylogeny), *padR* homologues *sensus stricto* corresponded to subfamily I that contains the PadR group and the AphA group (Fig. 3). We found that the *ladR* gene and the *ladR* orthologues from both nonpathogenic and pathogenic *Listeria* species constitute one independent group (LadR group, Fig. 3) probably with a similar function.

The product of the *ladR* gene represses the mdrL gene transcription under standard growth conditions in *Listeria monocytogenes*

Allelic exchange technique was successfully used to construct an *ladR* null mutant (Fig. 1a). Under standard conditions (brain heart infusion (BHI), LB broth supplemented or not with sugars), no defect in growth was observed for this mutant (data not shown). Under these conditions, in a wild-type strain, *ladR* is expressed (Huillet *et al*., 1999). Northern blot analysis of *mdrL* expression in *L. monocytogenes* strains LO28 and two isogenic mutant strains (m3.7 transposon mutant (Huillet *et al*., 1999) and the *ladR* null mutant) were performed in LB medium, during the exponential growth phase (Fig. 4a). A transcript of 1400 bp approximately corresponding to the expected size of the *mdrL* gene was detected in the *ladR* null mutant and, as observed previously, in the m3.7 mutant, while no signal could be detected for the parental strain. The same results were obtained in BHI medium (data not shown). This result shows unambiguously that the product of the *ladR* gene represses the *mdrL* gene expression under standard growth conditions and suggests that LadR is a transcriptional repressor of the *mdrL* gene in *L. monocytogenes*.

Comparison of multidrug resistance phenotype between *Listeria monocytogenes* LO28 and its *ladR* null mutant

In order to define induction conditions for the expression of MdrL, resistance to diverse drugs was assessed by monitoring the culture's metabolic activity (as electrical capacitance). Ethidium bromide (dye), R6G (dye), acriflavine (disinfectant) and norfloxacin (fluoroquinolone) were selected as known artificial substrates of the Bmr multidrug transporter of *Bacillus subtilis* (Grkovic *et al*., 2001; Markham & Neyfakh, 2001). It was shown in *B. subtilis* that these substrates are transported with substantially different effectiveness and the degree of resistance was different (Grkovic *et al*., 2001; Markham & Neyfakh, 2001). the MIC of LO28 and its null *ladR* mutant did not differ for the drugs used: 3.12 μg mL^{-1} for norfloxacin (low resistance), 13.17 μg mL^{-1} for R6G (resistance) and 66.6 μg mL^{-1} for acriflavine and for ethidium bromide (strong resistance).

Dose/response, for the four drugs tested, was observed for both LO28 and *ladR* following serial dilutions (data not shown). Interestingly, at these dilutions and notably at the first dilution below the MIC (subMIC), the *ladR* null mutant, which expresses the MdrL transporter constitutively, exhibits a significantly shorter lag phase as evidenced by a shorter CDT (data not shown).

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R6G induces transcription of the *mdrL* gene

The induction of *mdrL* expression was demonstrated for R6G, at a subMIC value (6.58 μg mL^{-1}) using Northern analysis: a transcript of 1400 bp approximately, corresponding to the size of the *mdrL* gene, was detected both in the wild type and its *ladR* null mutant strain (Fig. 4b). At subMIC level, R6G induces transcription of the *mdrL* gene in the wild-type strain and has no apparent effect on *mdrL* expression in the *ladR* mutant (Fig. 4, lanes 3, 7). Expression of the *mdrL* gene is constitutive in the *ladR* mutant and inducible in *L. monocytogenes* LO28 in the presence of R6G (Fig. 4, lanes 1, 6). This result suggests that in the presence of R6G, the product of *ladR* does not repress the transcription of the *mdrL* gene.

PrfA, the global virulence transcriptional regulator is not involved in the regulation of the MdrL transporter

We previously showed that the locus *ladR–mdrL* is involved in the expression of listeriolysin, a PrfA-dependent virulence factor (Huillet *et al*., 1999). PrfA is the pleiotropic transcriptional activator of virulence genes in *L. monocytogenes* (Khelef *et al*., 2005). Recently, the PrfA regulon has been characterized: it comprises three groups of genes differentially regulated by PrfA and not all involved in virulence (Khelef *et al*., 2005). LadR–MdrL could belong to the PrfA regulon. Of note, the PrfA box is not present in the promoter region of the *ladR* gene or the *mdrL* gene as observed for recent *prfA*-controlled genes (Khelef *et al*., 2005).

To test this hypothesis, we constructed a double null mutant *ladR–prfA* and analysed the *mdrL* expression in *prfA* and *ladR–prfA* mutants in LB medium (Fig. 4a). As observed for the LO28 strain under standard growth conditions, no transcript of *mdrL* could be detected in the *prfA* mutant, while one transcript was detected in the *ladR–prfA* mutant as observed for the *ladR* mutant. Induction of *mdrL* gene expression in the *prfA* mutant was observed in the presence of R6G, as observed for the parental strain LO28 (Fig. 4b). Therefore, in our conditions, PrfA is not involved in the global regulation of the MdrL transporter.

Discussion

The LadR protein is not similar to known MDR regulators such as BmrR or QacR. To date, the known transcription activators of the MDR pump genes belong to the MerR, AraC or LysR families, whereas the known repressors of the MDR pump genes fall into the TetR, MarR or LacI families (Grkovic *et al*., 2001). This is the first report of the transcriptional control of an MDR transporter with a member of the PadR family.

We also first report that the MdrL pump expression is inducible and identify rhodamine as a synthetic inducer. Rhodamine is both a substrate and an inducer of the two best-characterized bacterial MDR transporter systems: Bmr/BmrR of *B. subtilis* and QacA,QacB/QacR of *Staphylococcus aureus* (Grkovic *et al*., 2001; Markham & Neyfakh, 2001). The interaction of rhodamine with the QacR repressor or BmrR activator results in a conformational change, leading to the inactivation of the regulator (Grkovic *et al*., 2001; Markham & Neyfakh, 2001). With the hypothesis that rhodamine is also a substrate of MdrL and from our results, we propose a reasonable model for the regulation of the MdrL pump in *L. monocytogenes*: under standard growth conditions, the LadR repressor is active and binds within the *mdrL* promoter at the PadR box, preventing the transcription of *mdrL*; in the presence of rhodamine, LadR is inactive and transcription of MdrL can proceed; and rhodamine present in the bacterial cell is consequently expulsed via the MdrL transporter.

A structure–function analysis of LadR should allow determination of the regions involved in ligand-binding, DNA-binding and dimerization activities. Natural inducers of the MdrL transporter remain to be identified and the physiological roles of the MdrL transporter in pathogenic and nonpathogenic *Listeria* species need to be clarified.

Acknowledgements

We thank P. Glaser and C. Burchrieser for providing access to the unpublished *L. ivanovii* and *L. grayi ladR–mdrL* sequences. We thank P. Cossart for giving us the BUG 802 strain, T. Chakraborty for giving us the pAUL-A plasmid and I. Martin-Verstraete for providing plasmid pDG784. We are very grateful to P. Cossart, D. Lereclus, S. Dramsi and H. Bierne for critically reviewing the manuscript and to A. Richard and J. Pizarro-Cerda for English correction. We thank G. André, M. Grana and P. Dehoux at Pasteur Institute and I. Virlogeux-Payant at INRA Institute for their constructive discussions. We also thank E. Bottreau, S. Aligé and F. Mompart for their technical support at INRA Institute.

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