Microbiology (2013), **159**, 1328–1339 DOI 10.1099/mic.0.067744-0

Occurrence of mutations impairing sigma factor B (SigB) function upon inactivation of *Listeria monocytogenes* genes encoding surface proteins

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Received 12 March 2013
Accepted 2 May 2013

Bacteria of the genus *Listeria* contain the largest family of LPXTG surface proteins covalently anchored to the peptidoglycan. The extent to which these proteins may function or be regulated cooperatively is at present unknown. Because of their unique cellular location, we reasoned that distinct LPXTG proteins could act as elements contributing to cell wall homeostasis or influencing the stability of other surface proteins bound to peptidoglycan. To test this hypothesis, we used proteomics to analyse mutants of the intracellular pathogen *Listeria monocytogenes* lacking distinct LPXTG proteins implicated in pathogen–host interactions, such as InlA, InlF, InlG, InlH, InlJ, LapB and Vip. Changes in the cell wall proteome were found in *inlG* and *vip* mutants, which exhibited reduced levels of the LPXTG proteins InlH, Lmo0610, Lmo0880 and Lmo2085, all regulated by the stress-related sigma factor SigB. The ultimate basis of this alteration was uncovered by genome sequencing, which revealed that these *inlG* and *vip* mutants carried loss-of-function mutations in the *rsbS, rsbU* and *rsbV* genes encoding regulatory proteins that control SigB activity. Attempts to recapitulate this negative selection of SigB in a large series of new *inlG* or *vip* mutants constructed for this purpose were, however, unsuccessful. These results indicate that inadvertent secondary mutations affecting SigB functionality can randomly arise in *L. monocytogenes* when using common genetic procedures or during subculturing. Testing of SigB activity could be therefore valuable when manipulating genetically *L. monocytogenes* prior to any subsequent phenotypic analysis. This test may be even more justified when generating deletions affecting cell envelope components.

INTRODUCTION

Listeria monocytogenes is a Gram-positive facultative intracellular bacterium responsible for food-borne infections in humans and animals. Upon infection, this pathogen crosses the intestinal, blood–brain and placental

Abbreviations: RT-PCR, reverse transcriptase PCR; SNPs, simple nucleotide polymorphisms.

Three supplementary figures and three supplementary tables are available with the online version of this paper.

barriers leading to gastroenteritis, meningoencephalitis and materno-fetal infections in humans with a fatality rate estimated at 20–30 % of infected individuals (Cossart & Toledo-Arana, 2008; Hamon *et al.*, 2006; Vázquez-Boland *et al.*, 2001). Several surface proteins of this pathogen display distinct modes of association with the cell wall and direct key steps of the infection process, including bacterial adhesion to host cells, pathogen internalization and subversion of host cell cytoskeleton dynamics (Bierne & Cossart, 2007). A remarkable feature shared by all species of the *Listeria* genus is the high content of genes encoding surface proteins which bear an LPXTG sorting motif (Bierne & Cossart, 2007; Doumith *et al.*, 2004; Glaser *et al.*, 2001; Hain *et al.*, 2007). This motif is present in more than 40 surface proteins of these bacteria and is recognized by sortases, specialized enzymes that mediate their covalent anchorage to the peptidoglycan (Marraffini *et al.*, 2006; Spirig *et al.*, 2011). How bacteria regulate the production of the set of LPXTG proteins is at present unknown, although recent data obtained in *L. monocytogenes* suggest that sortase A may be a contributing factor (Mariscotti *et al.*, 2012). Coordinate regulation has been proposed for the production of different cell wall polymers, including peptidoglycan, teichoic acids, lipoteichoic acids and the capsule (Hanson & Neely, 2012). Whether such a type of regulation occurs at the level of surface proteins that interact with the cell wall is at present unknown.

In this study, we report a cell wall proteome analysis of *L. monocytogenes* mutants lacking distinct LPXTG proteins such as InlA, InlF, InlG, InlH, InlJ, LapB and Vip, previously characterized for their contribution to virulence and to pathogen–host cell interactions (Cabanes *et al.*, 2005; Guzman *et al.*, 1995; Kirchner & Higgins, 2008; Lingnau *et al.*, 1995; Raffelsbauer *et al.*, 1998; Reis *et al.*, 2010; Sabet *et al.*, 2005). Two of these mutants, Δ*inlG* and Δ*vip*, inadvertently exhibited a defective proteome characteristic of a deficiency in LPXTG proteins regulated positively by the stress-related alternate sigma factor SigB. This sigma factor plays a central role in promoting adaptation to different types of stress and virulence in *L. monocytogenes* as well as other Gram-positive bacteria e.g. *Bacillus subtilis* and *Staphylococcus aureus* (Abram *et al.*, 2008a; Chaturongakul & Boor, 2006; Giotis *et al.*, 2008; Kazmierczak *et al.*, 2006; Palmer *et al.*, 2009; Raengpradub *et al.*, 2008; Swaminathan & Gerner-Smidt, 2007). Further studies uncovered the presence of secondary mutations causing loss of function in regulatory elements required to activate SigB, such as RsbS, RsbU and RsbV. These proteins form part of a complex regulatory cascade starting in the membrane-bound stressosome protein complex, which triggers a signal transduction upon perception of the stress stimuli. Such a signal culminates with the phosphorylation of the anti-sigma protein RsbW and the release of SigB from the sigma/anti-sigma complex (O'Byrne & Karatzas, 2008). Appearance of mutations affecting SigB activity was observed in independent Δ*inlG* and Δ*vip* mutants generated in different laboratories. Considering that SigB is an important factor mediating stress survival and virulence in Gram-positive bacterial pathogens (Cheung *et al.*, 2004; Hecker *et al.*, 2007; Novick, 2003; OByrne & Karatzas, 2008), the results reported here highlight the value of monitoring activity of the regulon controlled by this protein when performing genetic manipulations in *L. monocytogenes*.

METHODS

Bacterial strains and culture conditions. The *L. monocytogenes* strain EGDe (Glaser *et al.*, 2001) of serotype 1/2a was used as parental strain. The isogenic mutants lacking LPXTG proteins used in this study are listed in Table 1. Unless otherwise indicated, bacteria were grown in brain heart infusion (BHI) medium at 37 °C in shaking aerobic conditions.

Complementation of *inlG* and *vip* mutations. For complementation, *inlG* and *vip* alleles were PCR-amplified from chromosomal DNA of wt strain EGDe and cloned in the *Sac*I and *Sph*I restriction sites of the pP1 plasmid (Dramsi *et al.*, 1995) using primers listed in Table S1 (available in Microbiology Online). The final constructs were verified by sequencing and the relative expression of the transgene estimated by reverse transcriptase PCR (RT-PCR) using oligonucleotides primers listed in Table S1.

Preparation of cell wall extract for proteomic analysis. Peptidoglycan was purified from wt and isogenic mutants lacking LPXTG proteins grown in BHI medium to exponential phase, as previously described (Calvo *et al.*, 2005). Purified peptidoglycan was digested with modified trypsin (sequencing grade, Promega), as described previously (Calvo *et al.*, 2005). The resulting peptide mixture was lyophilized and kept at −20 °C. Protein identification by liquid chromatography–tandem mass spectrometry was performed as described previously (García-del Portillo *et al.*, 2011).

Western blot analyses of cell surface proteins bound to peptidoglycan. Cell wall extracts were prepared from bacteria grown at 37 °C in BHI medium under non-shaking conditions, as described previously (Pucciarelli *et al.*, 2005). To release covalently bound LPXTG proteins from cell walls, whole bacteria were incubated with mutanolysin to digest peptidoglycan (Pucciarelli *et al.*, 2005). Polyclonal rabbit anti-Lmo0160, anti-Lmo0171, anti-InlG, anti-InlH, anti-Vip, anti-Lmo0610, anti-Lmo0880 and anti-Lmo2085 immune sera were generated using full-length His-tagged recombinant proteins as antigens. Mouse monoclonal anti-InlA (Mengaud *et al.*, 1996) was used as control for cell wall fractions. Goat anti-mouse and goat anti-rabbit antibodies conjugated to horseradish peroxidase (Bio-Rad) were used as secondary antibodies. Proteins were visualized by chemoluminescence using luciferin–luminol reagents.

RNA extraction and semiquantitative RT-PCR. RNA was purified from bacteria grown in 10 ml of BHI at exponential phase (OD_{600}=0.2) using the TRIzol reagent method (Invitrogen), as described previously (Toledo-Arana *et al.*, 2009). RNA integrity and concentration were assessed by agarose-Tris-acetate-EDTA electrophoresis and absorbance at 260 nm after treatment with DNase I for 30 min at 37 °C (Turbo DNA-free kit, Ambion/Applied Biosystems). RT-PCR was performed using a one-step RT-PCR kit (Qiagen). Briefly, RT-PCR was carried out in a final volume of 25 µl consisting of 5 µl buffer (5×), 1 µl of dNTP (10 mM), 3 µl each of the forward and reverse primers (5 mM), 1 µl of RT-PCR enzyme mix, 40 ng of RNA and RNase-free water. RT-PCR cycling conditions were as follows: 50 °C for 35 min and 95 °C for 15 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and then an

Table 1. *Listeria monocytogenes* strains used in this study

Strain	Relevant genotype	Source or reference
EGDe	wt	Glaser *et al.* (2001)
MD1297	Δ*inlG(1)*	This work
MD3415	Δ*inlG(2)*	This work
MD1660	Δ*inlF*	This work
MD1658	Δ*sigB*	This work
MD2306	Δ*inlA*	This work
DC220	Δ*lmo0320 (vip) (1)*	This work
DC431	Δ*lmo0320 (vip) (2)*	This work
BUG2235	Δ*lmo0320 (vip)*::km	Cabanes *et al.* (2005)
MD2701	Δ*lmo0320 (vip) (1)*pP1*lmo320*	This work
MD2708	Δ*lmo0320 (vip) (1)*pP1*rsbV*	This work
MD2709	Δ*lmo0320 (vip)*::km pP1*rsbS*	This work
MD2711	EGD-epP1*lmo320*	This work
MD2712	Δ*inlG(1)*pP1*inlG*	This work
DC109	Δ*lapB*	Reis *et al.* (2010)
BUG2569	Δ*inlH*	Personnic *et al.* (2010)
BUG2159	Δ*inlJ*	Sabet *et al.* (2005)

extra elongation step at 72 °C for 10 min. Oligonucleotides used in these RT-PCR assays were designed using the software Primer Express v3.0 (Applied Biosystems) and are listed in Table S1.

Nucleotide sequencing of *rsbRSTU* and *rsbVW-sigB-rsbX* operons in Δ*inlG*, Δ*vip* and Δ*vip*::km mutants. Sequencing of the *rsbRSTU* and *rsbVW-sigB-rsbX* operons was performed upon PCR amplification of 500–600 bp fragments from chromosomal DNA isolated from wt EGDe strain, the original mutant Δ*vip*::km (Cabanes *et al.*, 2005), and the Δ*vip(1)* and Δ*inlG(1)* deletion mutants constructed for this study. Oligonucleotides used are listed in Table S1.

Whole-genome sequencing. Chromosomal DNA from *L. monocytogenes* EGDe, Δ*vip*::km (Cabanes *et al.*, 2005), Δ*vip* and Δ*inlG* strains was extracted using cetyl-trimethyl-ammonium bromide and alkaline lysis, as described previously (Wilson, 2001). Bacteria were previously treated with 20 mg ml^{-1} lysozyme for 20 min at 37 °C in Tris-EDTA-sucrose buffer. Samples were further treated with 0.5 mg ml^{-1} RNase A (bovine pancreas, Roche) for 30 min at 37 °C and the same volume of phenol was added. Chromosomal DNA was finally precipitated with 2-propanol, washed in 70 % (v/v) ethanol and air-dried before suspending in MiliQ-filtered water. Absence of contaminating RNA was tested in 0.8 % agarose gels and by measurement of the A_{260}/A_{280} absorbance ratio, which was in all samples approx. 2.0. Whole-genome sequencing of strains were performed with a single-reads sequencing technology. Illumina library preparation and sequencing followed standard protocols developed by the supplier. Single reads of 100 cycles were collected on a HiSeq 2000 (Illumina). After sequencing was complete, image analysis, base calling and error estimation were performed using Illumina Analysis Pipeline version 1.7.

Construction of a new collection of *L. monocytogenes* Δ*inlG* and Δ*vip* deletion mutants. To construct the Δ*inlG* mutant strain, fragments containing ~500 bp DNA flanking the ORFs of *inlG* (*lmo0262*) were amplified by PCR using chromosomal DNA of *L. monocytogenes* strain EGD-e and cloned into the suicide integrative vector pAUL-A, as previously described (Chakraborty *et al.*, 1992; Dussurget *et al.*, 2002). Oligonucleotide primers used are listed in Table S1. To construct the Δ*vip* mutant strain, we used a modified version of pDC4 (Cabanes *et al.*, 2005). The vector was digested with *Kpn*I and *Xba*I to remove the kanamycin resistance cassette, treated with T4 DNA polymerase, religated and transformed into *E. coli* XL1-Blue to yield pDC218. Gene deletion by double recombination was performed as described previously (Arnaud *et al.*, 2004; Cabanes *et al.*, 2004; Wouters *et al.*, 2005). These original mutants lacking the ORFs of *inlG* and *vip* were referred as series '1', i.e. Δ*inlG(1)*and Δ*vip(1)*. A different Δ*vip* deletion mutant, named Δ*vip(2)*, was also generated leaving 63 nt in the 5′ and 30 nt in the 3′ terminal ends of the *vip* gene. Fragments containing ~1000 bp DNA flanking the ORFs of *vip* were amplified by PCR using chromosomal DNA of *L. monocytogenes* strain EGDe. Oligonucleotide primers used are listed in Table S1. These 5′ and 3′ flanking ~1000 bp fragments were digested with *Sal*I/*Mlu*I and *Mlu*I/*Nco*I respectively and cloned into the suicide integrative vector pMAD to yield pDC425 (Arnaud *et al.*, 2004). Gene deletion by double recombination was performed as described previously (Boneca *et al.*, 2007). Likewise, a second Δ*inlG(2)* was also generated by gene splicing by overlap extension (gene SOEing) and a deletion protocol based in the pAUL-A. Gene replacement was performed as described previously (Arnaud *et al.*, 2004; Cabanes *et al.*, 2004; Wouters *et al.*, 2005) but the non-permissive temperature was changed to 42 °C. The Δ*inlG(1)*, Δ*inlG(2)*, Δ*vip(1)* and Δ*vip(2)* deletions were verified by PCR using the primers listed in Table S1. The comparison among the protocols used is shown in Table S2.

Evaluation of SigB function by chitinase activity. The functional status of the SigB regulon was tested by monitoring chitinase activity in bacteria grown on solid medium containing tryptone (10 g l^{-1}), yeast extract (5 g l^{-1}), NaCl (10 g l^{-1}), agar (15 g l^{-1}) and phosphate buffer, pH 6.9 (Oxoid). This medium was supplemented with acid hydrolysed chitin (3.0 g l^{-1}) as described previously (Larsen *et al.*, 2010), omitting the freeze-drying steps. The plates were incubated under aerobic conditions at 30 °C and scored for chitinase activity (clearing zones) for 5 days.

RESULTS

Cell wall proteomics of *L. monocytogenes* mutants deficient in LPXTG surface proteins

Cooperative regulation is known to exist among cell wall components of Gram-positive bacteria such as peptidoglycan,

capsule, lipoteichoic and teichoic acids (Hanson & Neely, 2012). Thus, the absence of one of these cell wall components can affect the production, stability or modification of the others. For example, disruption of teichoic acid production in *S. aureus* alters peptidoglycan cross-linkage and the activity of some autolysins (Atilano *et al.*, 2010; Schlag *et al.*, 2010). Based on these observations, we reasoned that some members of the family of LPXTG surface proteins of *L. monocytogenes* could contribute to cell wall homeostasis and influence the relative amount of other cell wall components, including surface proteins strongly bound to peptidoglycan. To test this hypothesis, we used high-resolution MS to analyse the protein content of cell wall extracts prepared from *L. monocytogenes* EGDe-derived mutants lacking LPXTG proteins. As most of the *L. monocytogenes* genes encoding LPXTG proteins are still uncharacterized, we examined previously reported mutants lacking LPXTG proteins involved in virulence and pathogen–host cell interactions. These mutants included Δ*inlA*, Δ*inlF*, Δ*inlG*, Δ*inlH*, Δ*inlJ*, Δ*lapB* and Δ*vip* (Cabanes *et al.*, 2005; Guzman *et al.*, 1995; Kirchner & Higgins, 2008; Lingnau *et al.*, 1995; Personnic *et al.*, 2010; Raffelsbauer *et al.*, 1998; Reis *et al.*, 2010; Sabet *et al.*, 2005). High-resolution MS analysis revealed that the cell wall proteome of most of these mutants remained essentially unchanged with respect to the content of LPXTG proteins. Some minor changes were observed for Lmo0171, an LPXTG protein identified with an average of four unique peptides, except in the Δ*inlF* and Δ*inlH* strains (Table 2); and InlG, identified with an average of six peptides, except in the Δ*inlA* strain (Table 2). These differences were, however, not confirmed by Western blotting using cell wall extracts of these strains and specific anti-Lmo171 and anti-InlG antibodies (Fig. S1). This finding was indicative of the difficulty of identifying minor changes in the relative amount of low-abundance surface proteins. In sharp contrast to the wt strain, no peptides corresponding to the LPXTG proteins Lmo0263 (InlH), Lmo0610, Lmo0880 and Lmo2085 were detected in the cell wall of the Δ*inlG* and Δ*vip* mutants (Table 2). Of interest, the genes encoding these four LPXTG proteins are regulated by the stress-related sigma factor SigB (Hain *et al.*, 2008; Loh *et al.*, 2006). This observation led us to postulate that InlG and Vip could contribute to cell wall homeostasis and that these two LPXTG proteins could work with SigB for the correct sensing of stresses linked to cell wall alterations. Alternatively, the absence on the bacterial surface of InlG or Vip could disrupt cell wall homeostasis and, as a consequence, generate a stress that could overstimulate SigB resulting in a deleterious effect to the cell. In this latter scenario, loss-of-function mutations abrogating SigB function were expected to be selected.

Validation of proteomic data uncovers the presence of additional mutations affecting SigB function in Δ*inlG* and Δ*vip* mutants

Western blots were performed to validate the proteomic data obtained for the Δ*inlG* and Δ*vip* mutants by an alternative method. A Δ*sigB* mutant was also included as control. These experiments confirmed that the lack of InlG or Vip correlated with a dramatic decrease of SigB-regulated proteins such as InlH, Lmo0610 and Lmo0880 (Fig. 1a) in the cell wall. This effect was specific since it was not observed for other LPXTG proteins, such as Lmo0160 or surface proteins strongly bound to the peptidoglycan by the LysM domain, e.g. the autolysin P60 (Fig. 1a). To determine whether these changes reflected a failure of the entire SigB regulon, we monitored the expression of genes positively regulated by SigB but not related to cell wall such as *bsh* and *lmo2695*, which encode a bile salt hydrolase and a subunit of the dihydroxyacetone kinase enzyme, respectively (Abram *et al.*, 2008b; Begley *et al.*, 2005). Reverse transcriptase PCR (RT-PCR) assays confirmed that, like the phenotype observed in the Δ*sigB* mutant, expression of *bsh* and *lmo2695* was reduced in the Δ*inlG* and Δ*vip* mutants (Fig. 1b). Decreased amounts of *sigB* transcript and SigB protein were evident in the *inlG* and Δ*vip* mutants (Fig. 1b), which was consistent with the lower activity of the SigB regulon in these two mutants and the positive auto-regulation that SigB exerts over the *rsbV-rsbW-sigB-rsbX* regulon (O'Byrne & Karatzas, 2008). Similar findings were obtained with a previously reported Δ*vip*::km mutant (Cabanes *et al.*, 2005), which also turned out to be defective in the production of LPXTG proteins regulated positively by SigB (data not shown). Furthermore, all the three mutants, Δ*inlG*, Δ*vip* and Δ*vip*::km, displayed sensitivity to acid stress in a similar fashion to the Δ*sigB* mutant (Fig. S2). Taken together, these observations indicated that SigB function was dramatically impaired in the *L. monocytogenes* lacking the LPXTG proteins InlG or Vip. Unexpectedly, complementation assays with wt $inlG^+$ and vip^+ alleles did not result in suppression of any of the phenotypes related to the loss-of-function in SigB. As a representative example, recovery in the production of the SigB-regulated LPXTG protein Lmo0880 was not observed in the complemented strains (Fig. 1c). These strains were also found to be impaired in the production of other SigB-regulated LPXTG proteins, as InlH and Lmo0610 (data not shown). Expression of the wt vip^+ allele in the Δ*vip*::km mutant also failed to restore SigB functionality (data not shown). Altogether, these findings supported the existence of additional secondary mutations present in the Δ*inlG* and Δ*vip* mutants that were affecting SigB functionality. Since the SigB protein was detected at its predicted molecular mass in the Δ*inlG* and Δ*vip* mutants (Fig. 1b), such loss of function could be tentatively be assigned to point mutations in the *sigB* gene resulting in a non-functional protein. Additionally, there are proteins that form the regulatory cascade that modulate SigB's activity (O'Byrne & Karatzas, 2008). SigB function is primarily regulated at the level of free protein that remains unbound from its anti-sigma factor, RsbW. Formation of the SigB-RsbW complex depends on the phosphorylation status of RsbW (O'Byrne & Karatzas, 2008). Thus, RsbW acquires auto-kinase activity upon binding to RsbV, a process that results in phosphorylated RsbW that dissociates from SigB. Mutations affecting either RsbW or upstream elements of the regulatory cascade that determine

Table 2. Surface proteins covalently bound to peptidoglycan identified by high resolution MS in the cell wall of *L. monocytogenes* mutants lacking LPXTG proteins involved in virulence

Surface protein†	Function	Unique peptides identified per protein and mutant*							
		EGDe (wt)	Δ*inlA*	Δ*inlF*	Δ*inlG*	Δ*inlH*	Δ*inlJ*	Δ*lapB*	Δ*vip*
Lmo2185	Unknown, sortase–B substrate	41	31	38	32	42	45	44	48
Lmo2186	Unknown, sortase–B substrate	9	8	10	10	11	9	13	13
Lmo2714	Peptidoglycan anchored protein (LPXTG motif)	17	14	17	18	16	15	25	17
Lmo0130	Similar to 5–nucleotidase, putative peptidoglycan bound protein (LPXTG motif)	31	24	39	35	31	32	29	35
Lmo0880	**Similar to cell surface proteins (LPXTG motif)**	**18**	**9**	**15**	**–**	**20**	**17**	**20**	**–**
Lmo1666 (LapB)	LPXTG protein adhesin required for entry into eukaryotic cells	20	15	23	21	25	23	(‡)	22
Lmo0160	Putative peptidoglycan bound protein (LPXTG motif)	19	15	22	15	19	17	18	18
Lmo0433 (InlA)	Internalin A (InlA); invasin	19	(‡)	13	10	18	20	18	10
Lmo0263 (InlH)	**Internalin H (InlH)**	**10**	**8**	**11**	**–**	(‡)	**13**	**16**	**–**
Lmo2178	Putative peptidoglycan bound protein (LPXTG motif)	10	9	8	9	8	15	16	12
Lmo0514	Similar to internalin proteins, putative peptidoglycan bound protein (LPXTG motif)	10	7	10	6	5	9	13	7
Lmo0842	Putative peptidoglycan bound protein (LPXTG motif)	10	9	13	4	8	8	9	10
Lmo0327	Similar to cell surface proteins (LPXTG motif)	8	6	11	15	6	9	6	11
Lmo0610	**Similar to internalin proteins, putative peptidoglycan bound protein (LPXTG motif)**	**7**	**4**	**11**	**–**	**9**	**7**	**6**	**–**
Lmo0262 (InlG)	Internalin G (InlG)	5	–	3	‡	6	7	7	5
Lmo1413	Similar to internalin, putative peptidoglycan bound protein (LPXTG motif)	4	6	–	2	3	3	4	3
Lmo2085	**Putative peptidoglycan bound protein (LPXTG motif)**	**2**	**2**	**6**	**–**	**8**	**6**	**7**	**–**
Lmo0171	Similar to internalin proteins, putative peptidoglycan bound protein (LPXTG motif)	5	4	–	4	–	4	7	3
Lmo2396	Similar to internalin proteins, putative peptidoglycan bound protein (LPXTG motif)	1	1	2	3	2	4	3	2
Lmo0835	Putative peptidoglycan bound protein (LPXTG motif)	–	–	9	3	–	1	–	1
Lmo2026	Putative peptidoglycan bound protein (LPXTG motif)	2	2	–	2	2	2	1	2
Lmo0159	Putative peptidoglycan bound protein (LPXTG motif)	3	2	–	2	5	2	4	3
Lmo0331	Similar to internalin, putative peptidoglycan bound protein (LPXTG motif)	1	–	–	2	–	2	3	1
Lmo1290	Similar to internalin, putative peptidoglycan bound protein (LPXTG motif)	2	–	–	–	2	2	1	1
Lmo2821 (InlJ)	Internalin J (InlJ)	1	–	–	2	–	(‡)	–	2
Lmo0333	Putative peptidoglycan bound protein (LPXTG motif)	1	–	–	1	–	–	–	–
Lmo1136	Similar to internalin, putative peptidoglycan bound protein (LPXTG motif)	–	1	–	–	–	–	–	–

Table 2. cont.

Surface protein†	Function	Unique peptides identified per protein and mutant*							
		EGDe (wt)	Δ*inlA*	Δ*inlF*	Δ*inlG*	Δ*inlH*	Δ*inlJ*	Δ*lapB*	Δ*vip*
Lmo1289	Similar to internalin, putative peptidoglycan bound protein (LPXTG motif)	–	–	–	–	–	–	2	–
Lmo0409 (InlF)	Similar to internalin, putative peptidoglycan bound protein (LPXTG motif)	–	–	(‡)	–	–	–	2	–

*Δ*inlG* and Δ*vip* mutants refer to the original Δ*inlG(1)* and Δ*vip(1)* strains (see text for details).
†LPXTG–proteins in bold are known to be regulated by SigB and are absent in the cell wall of Δ*inlG* and Δ*vip* mutants.
‡Protein encoded by the gene that was deleted in that concrete mutant strain.

the ratio of phosphorylated to non-phosphorylated RsbW could also have similar consequences. For example, a null mutation in RsbV could result in a shift of all RsbW molecules to the non-phosphorylated form and, as a consequence, a long-term state of SigB in its inactive state trapped with the anti-sigma factor.

Mapping of the mutations carried by Δ*inlG* and Δ*vip* mutants affecting SigB function

To discern the type of mutations carried by the Δ*inlG*, Δ*vip* and Δ*vip*::*km* mutants that were impairing SigB function, we first considered their presence in *sigB* itself or some of the genes encoding elements forming the regulatory cascade that controls RsbW phosphorylation. These elements, together with RsbW and SigB itself, are encoded in the *rsbRSTU* and *rsbVW-sigB-rsbX* operons. Specific oligonucleotides were designed to obtain the complete nucleotide sequence of all ORFs in the two operons and their respective promoter regions (Table S1). This sequencing showed the presence of loss-of-function mutations in each of the Δ*inlG*, Δ*vip*::km and Δ*vip* mutants affecting distinct elements of the regulatory cascade (Fig. 2a). More precisely, the Δ*inlG* mutant harbours an A → –/A frame shift in the *rsbU* coding sequence that disrupts the protein from amino acid residue 195, while the Δ*vip*::km mutant contains a nonsense mutation CAG (Gln) → TAG (stop) of the *rsbS* coding region corresponding to codon of amino acid residue 17. In the Δ*vip* mutant, herein referred to as Δ*vip(1)* to distinguish it from the published Δ*vip*::km mutant (Cabanes *et al.*, 2005), a nonsense mutation GAA (Glu) → TAA (stop) was found in the coding region of *rsbV* corresponding to codon of amino acid residue 9 (Fig. 2a). To further confirm that these mutations were responsible for the lack of SigB function, we selected the Δ*vip*::km and Δ*vip* mutants for complementation assays with the respective $rsbS^+$ and $rsbV^+$ wild-type alleles. Expression of the $rsbV^+$ allele in the Δ*vip(1)* mutant restored the production of SigB-regulated LPXTG proteins such as InlH, Lmo0880 or Lmo0610 (Fig. 2b). However, no such effect was observed for the Δ*vip*::km/$rsbS^+$ complemented strain (Fig. 2b). Unlike RsbV, which is a cytosolic protein, RsbS is part of the stressosome complex. This complex remains uncharacterized in *L. monocytogenes* but is believed to function as in *B. subtilis*. The stressosome acts in the envelope to perceive external environmental signals that stimulate a signal transduction cascade which activates SigB upon release of its cognate anti-sigma factor (O'Byrne & Karatzas, 2008). Interestingly, RsbS is phosphorylated upon signal perception. Based on this, it is possible that RsbS overexpression occurring in our complemented Δ*vip*::km/$rsbS^+$ strain may lead to changes in the stoichiometry of the stressosome affecting the phosphorylation step. Likewise, our findings did not allow us to discard the possibility that the Δ*vip*::km mutant could carry additional mutations besides that found in *rsbS* (Fig. 2a). Similar negative results were obtained when attempting to complement the Δ*inlG* mutant with an

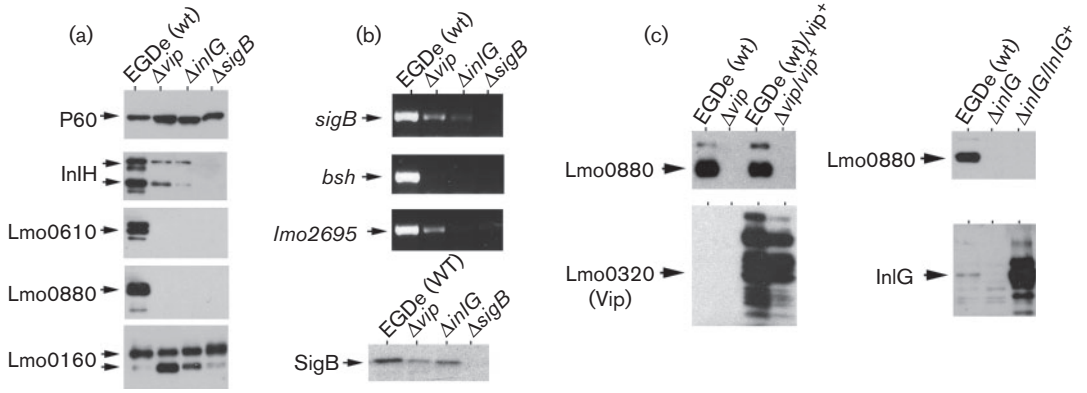
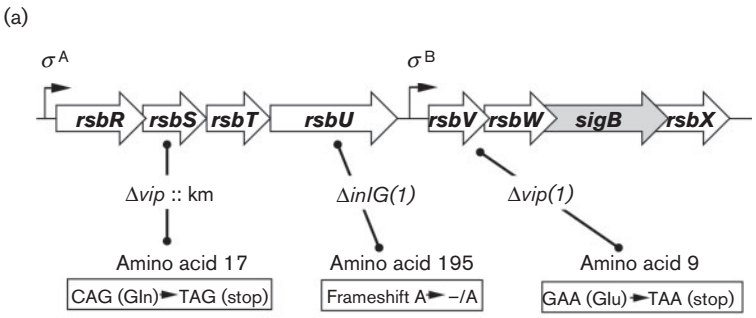


Fig. 1. *L. monocytogenes* mutants defective in the LPXTG surface proteins InlG or Vip display deficient function of the SigB regulon. (a) Levels of individual surface proteins detected in cell wall extracts prepared from the indicated strains. Among the proteins shown, InlH, Lmo0610, and Lmo0880 are regulated by SigB. (b) Lack of either InlG or Vip affects the entire SigB regulon as shown by the reduced expression of SigB-regulated genes not related to cell wall metabolism, such as *bsh* and *lmo2695*. (c) Expression *in trans* of wt *inlG*$^+$ and *vip*$^+$ alleles does not restore SigB functionality in the Δ*inlG* and Δ*vip* mutants.

rsbU$^+$ allele (data not shown). Complementation based on exchange of the *rsbU* and *rsbV* mutant alleles by wild-type sequences could have proved that these were the only causative mutations for the loss of activity in the SigB regulon. However, allelic exchange methods involve genetic manipulations that include in some cases integration and excision of suicide plasmids into the chromosome. Given the nature of our study, we avoided this subsequent



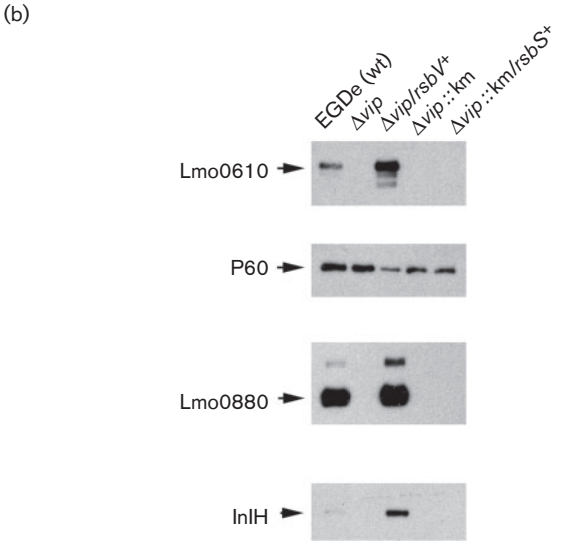


Fig. 2. Independent Δ*inlG* and Δ*vip* mutants counter-selected SigB functionality by mutations in genes encoding elements of the SigB regulatory cascade. (a) Mutations uncovered by sequencing of the *rsbRSTU* and *rsbVW-sigB-rsbX* operons with specific oligonucleotides. Note that in each case the indicated gene products are lost by either nonsense mutations or a frameshift in the respective ORFs. (b) Recovery of SigB function in the Δ*vip* mutant upon expression of the *rsbV*$^+$ allele. Such complementation was not successful in the case of the Δ*vip*::km/*rsbS*$^+$ strain (see text for details).

procedure to minimize the possibility of introducing new and unknown selective pressures in the original Δ*vip* and Δ*inlG* mutants. To discern whether additional mutations besides those found in the SigB-related operons could be present in these mutants, we accomplished complete genome sequencing of EGDe (wild-type), Δ*inlG*, Δ*vip*::km and Δ*vip(1)* strains. This procedure was facilitated by the known genome sequence of the EGDe strain (Glaser *et al.*, 2001). A few discrepancies in the reads were found in *lmo1799* and *lmo0412*, two genes having a large number of internal nucleotide repeats and encoding an LPXTG protein and a protein of unknown function, respectively. Besides these ambiguities, genome comparison confirmed the deletions generated in the *inlG* and *vip* genes and each of the point mutations in *rsb* genes previously identified by sequencing of the *rsbRSTU* and *rsbVW-sigB-rsbX* operons (see above and Table S3). A few simple nucleotide polymorphisms (SNPs) were also found in intergenic regions and as missense mutations in coding regions of the *lmo0247, lmo0319* and *lmo2576* genes. Additional changes of the type Pro → Ala and Ala → Ser were found in the *trpS* and *uvrB* genes of the Δ*inlG* mutant as well as a nucleotide change of the type G → T in the Δ*inlG*, Δ*vip*::km, Δ*vip(1)* mutants that was mapping in the terminator region of the *rpmH* gene encoding the 50S ribosomal protein L34. These changes are summarized in Table S3. A closer analysis of these findings indicated that no genuine changes could be assigned to the Δ*vip*::km mutant, which could not be complemented by the expression *in trans* of the $rsbS^+$ gene, in comparison to the Δ*vip(1)* mutant, which recovered SigB functionality upon complementation with the $rsbV^+$ allele (Fig. 2b). Therefore, it was concluded that these defective mutants carried no additional changes affecting SigB functionality besides those mutations mapping in the *rsb* genes. Based on this, we reasoned that in those cases in which a lack of complementation was observed it could be due to the excess of protein expressed from the plasmid impairing proper signalling through the SigB regulatory cascade.

Population study of the negative selection of SigB activity upon deletion of *inlG* or *vip* genes

The Δ*vip*::km, Δ*vip(1)* and Δ*inlG* mutant strains described in previous experiments were constructed independently in three different laboratories. To further confirm that SigB function was counter-selected in bacteria lacking either InlG or Vip proteins, we generated two new Δ*vip* and Δ*inlG* deletion mutants using similar genetic procedures involving a suicide integrative plasmid carrying flanking regions of the gene to be deleted (see Methods for details and Table S2). These new mutants were designated Δ*vip(2)* and Δ*inlG(2)*. Strikingly, Western assays revealed that Δ*vip(2)* and Δ*inlG(2)* strains produced SigB-regulated LPXTG proteins InlH, Lmo0610, Lmo0880 and Lmo2085 at levels comparable to those of the wt strain (Fig. S3a, b). These observations raised the possibility that the original mutants Δ*vip*::km, Δ*vip(1)* and Δ*inlG* [hereafter named Δ*inlG(1)*] were subjected to an unknown type of pressure during subculturing in different host laboratories or during the genetic manipulation that could result in counter-selection of SigB function. Alternatively, some side effects related to the distinct plasmids used in the different genetic procedures could be responsible for the appearance of mutations abrogating SigB function. Finally, we considered the possibility that mutations affecting SigB function might occur stochastically and that the few Δ*vip* and Δ*inlG* mutants examined so far were not representative on a population basis. To test this, we generated a large collection of new Δ*vip* and Δ*inlG* mutants using the same plasmids that were used originally for constructing the Δ*vip(1)*, Δ*vip(2)* and Δ*inlG(2)* strains (see Table S2). A total of 80, 136 and 54 new clones were generated after the recombination events with distinct suicide plasmids (Table 3). PCR assays confirmed that 23, 23 and 15 mutants of each of these series were genuine Δ*vip* and Δ*inlG* deletion mutants (Table 3). To assess SigB functionality in these clones, we applied a rapid screening on plates based on monitoring of chitinase activity. Chitinase production is regulated positively by SigB (Larsen *et al.*, 2010) and can be visualized as a halo surrounding bacteria growing on solid

Table 3. Series of new *L. monocytogenes* mutants defective in the LPXTG surface proteins Vip or InlG that were constructed and analysed for SigB function

PCR result in region of interest*	Number of new clones obtained upon applying gene deletion procedures in the *L. monocytogenes* genes *vip* and *inlG*		
	Series Δ*vip(3)*†	Series Δ*vip(4)*‡	Series Δ*inlG(3)*§
wt	57	113	39
Deletion mutation	23	23	15
Total	80	136	54

*See Fig. 3 for representative examples.

†Plasmid pDC218 (pKSV7 derivative), used originally to construct Δ*vip(1)*, was used to generate series Δ*vip(3)*.

‡Plasmid pDC425 (pMAD derivative), used originally to construct Δ*vip(2)*, was used to generate series Δ*vip(4)*.

§Plasmid pAUL-A, used originally to construct Δ*inlG(2)*, was used to generate series Δ*inlG(3)*.

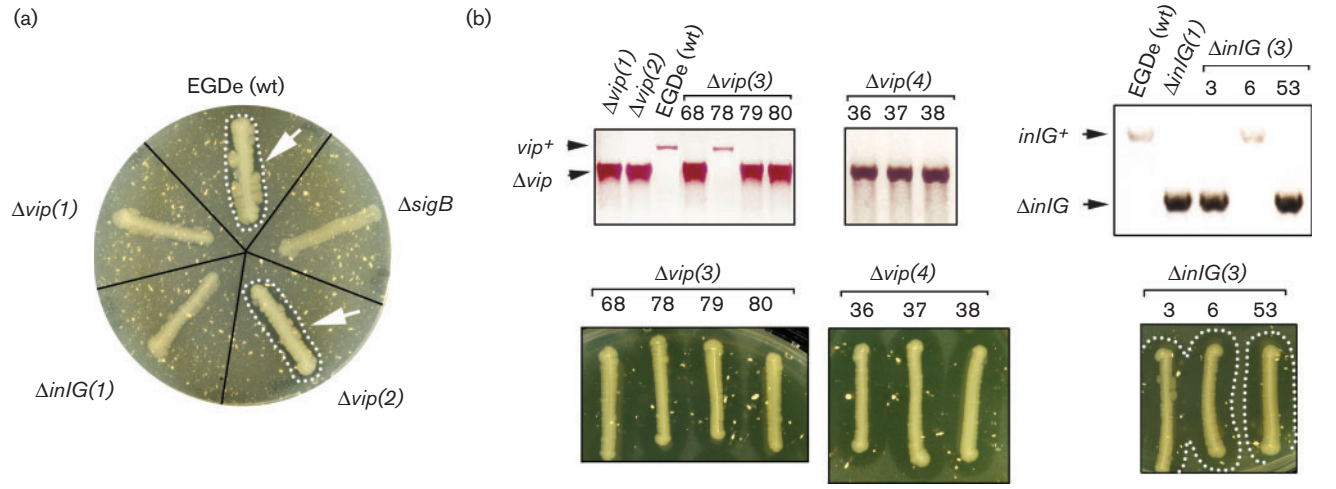


Fig. 3. Counter-selection of SigB function is not reproduced in a new series of Δ*inlG* and Δ*vip* deletion mutants generated with the same plasmid as the original mutant strains. (a) Screening of chitinase activity differentiates defective strains with no SigB function, such as Δ*inlG(1)*, Δ*vip(1)* and Δ*sigB*. Although not shown, the Δ*vip*::km mutant also exhibited no chitinase activity. Note that the second Δ*vip(2)* mutant generated for this study behaved as wt bacterium concerning chitinase activity. Arrows and dotted lines indicate the clearing zones denoting such enzymatic activity. (b) Representative cases of clones generated upon usage of the suicide plasmids pAUL-A, pOD23 and pMAD (see Table 3 for details). Clones shown were obtained after resolution of the plasmids and tested by PCR. Note that regardless of the presence/absence of the corresponding *inlG* or *vip* genes, all clones displayed chitinase activity and therefore had a functional SigB regulon. The new series of mutants were named Δ*inlG(3)*, Δ*vip(3)* and Δ*vip(4)* to differentiate them from the individual mutants tested previously (see text and Table 3 for details). Numbers identify individual clones of the three series.

media containing chitin. Control experiments showed that, unlike the wt bacteria and the Δ*vip(2)* mutant, the Δ*vip(1)*, Δ*inlG(1)* and Δ*sigB* mutants were unable to degrade chitin (Fig. 3a). This result was consistent with the previous biochemical and proteomic analysis that unravelled the loss of SigB function. Surprisingly, none of the 61 deletion mutants generated ad hoc for this study (23+23=46 Δ*vip* and 15 Δ*inlG*) was impaired in chitinase production. Some representative examples are shown in Fig. 3b. Additional phenotypic assays, such as sensitivity to an acidic environment (pH 2.5), confirmed that the newly generated series of Δ*vip* and Δ*inlG* mutants were all phenotypically $SigB^+$ (data not shown). Considering that this population study was performed on a significant number of Δ*vip* and Δ*inlG* mutants, it seems probable that the counter-selection of SigB function in the original mutants lacking the LPXTG surface proteins InlG or Vip was an event that occurred inadvertently.

DISCUSSION

This study was designed to dissect whether LPXTG surface proteins could contribute to maintenance of cell wall homeostasis in *L. monocytogenes*. The proteomic analysis uncovered an unexpected functional association between the LPXTG proteins InlG and Vip and the stress-related sigma factor SigB. Among the LPXTG protein-defective mutants generated for this study, those lacking InlG or Vip exhibited a marked decrease in the activity of the entire SigB operon. This finding was corroborated with another *vip* mutant previously reported to be affected in virulence (Cabanes *et al.*, 2005). Surprisingly, counter-selection of SigB function following the deletion of *inlG* or *vip* genes was not confirmed in subsequent experiments in which a large number of new mutants were constructed. Unfortunately, no other mutant clones were kept in collections from the original knock-out experiments, so we are uncertain of the ultimate reasons why such mutations causing loss-of-function in SigB emerged in those initial genetic manipulations. The possibility that such counter-selection occurred inadvertently during subculturing in different host laboratories should also be considered. Although spontaneous mutations affecting sigma factors have been previously described in the literature, this report is the first that correlates such negative selection to the probable generation of stress linked to the alteration of cell wall-associated proteins. In Gram-negative bacteria like *Escherichia coli*, stress resistance varies between strains, suggesting that mutations favouring increased stress resistance may be counter-selected in certain environments (Ferenci, 2008). Of interest, no correlation has been found between counter-selection of the alternative sigma factor RpoS, required to cope with nutritional stress, and its expression level in different environments (King *et al.*, 2006). Based on this, it has been suggested that mutational *rpoS* sweeps could be

affected by yet-unknown complex physiological and regulatory variables (King *et al.*, 2006). Competition between the alternate sigma factors RpoS and RpoD for binding to the RNA polymerase core has also been proposed to favour the appearance of mutations in *rpoS* (Farewell *et al.*, 1998). These mutations affect the affinity of binding and are generally loss-of-function with little or no residual RpoS protein (Ferenci, 2008). No mutations were, however, found in *sigB* for any of the *L. monocytogenes* mutants analysed in our study. This suggests that selection pressure(s) may exist favouring loss-of-function mutations in elements of the SigB regulatory cascade rather than in the sigma factor itself.

Nucleotide polymorphisms have been associated with virulence defects in all *L. monocytogenes* lineages (Orsi *et al.*, 2011). Indeed, while premature stop codons seem to be common in *L. monocytogenes* food isolates, these mutations are rare among human clinical isolates (Orsi *et al.*, 2011). On the other hand, recent evidence shows that *L. monocytogenes* can adapt through selective mutations during *in vitro* and intra-host growth. Thus, *L. monocytogenes* possesses the ability to express growth advantage in the stationary phase through acquisition of mutations that optimize fitness during long-term stationary growth without negatively impacting virulence (Bruno & Freitag, 2011). Regarding *in vivo* adaptation, intra-host environment(s) were reported to stimulate *rsbW* mutations that impair SigB-dependent acid resistance together with invasion and growth of *L. monocytogenes* within macrophages and epithelial cells (Asakura *et al.*, 2012). The identity of the selective pressures favouring these mutations is unknown. Recent studies have also provided genetic evidence for naturally occurring mutations in the *sigB* operon of *S. aureus*. Thus, natural *S. aureus* human isolates selected for high production of proteases and α-haemolysin exhibit a SigB-deficient phenotype (Karlsson-Kanth *et al.*, 2006). Nucleotide sequencing revealed that two of these strains had stop codons in *rsbU* and *sigB* while the other strain had an insertion sequence element in *rsbU*. Adaptive evolution of *S. aureus* during chronic infection of a cystic fibrosis patient was also linked to a high mutation rate in loci associated with *sigB* (McAdam *et al.*, 2011). As mentioned above, mutations in *sigB* seem, however, less favoured in *L. monocytogenes*. Our findings agree with recent microevolution analyses involving 195 *L. monocytogenes* isolates which revealed that *sigB* function is highly conserved (76 synonymous and 3 non-synonymous mutations) compared to *actA* (59 synonymous and 70 non-synonymous mutations) or *inlA* (64 synonymous and 44 non-synonymous mutations) (den Bakker *et al.*, 2008). Further studies should examine the ultimate basis of the apparent conservation of the *sigB* gene in *L. monocytogenes*, which seems not to be targeted by mutation at the same rate as in *S. aureus*. Notably, none of these studies has yet provided clues about the ultimate causative step(s) responsible for the emergence of these mutations in this important alternative sigma factor.

To our knowledge, this is the first report showing counter-selection of SigB during mutant construction in *L. monocytogenes*. The similarity found between mutants in distinct LPXTG proteins (Δ*vip*, Δ*vip*::km and Δ*inlG*) and some human isolates regarding the emergence of mutations in *sigB*-related genes suggests that common evolutionary pathways could be shared and that yet-unknown selective pressures could act on these loci resulting in a beneficial phenotype. Generation of deletion mutants is a common genetic procedure but, as demonstrated here for *L. monocytogenes*, it can inadvertently lead to inactivation of the SigB operon. A rapid chitinase-based test following the generation of any mutant may avoid subsequent work with strains carrying undesired mutations affecting SigB.

ACKNOWLEDGEMENTS

We thank Diana Barroso, Pablo García and Catarina Correia for their technical assistance. This work was supported by the following grants: from the Spanish Ministry of Economy and Competitiveness (Listress ERANet-Pathogenomics PIM2010EPA-00714; to F. G. P.); from the Spanish Ministry of Economy and Competitiveness (BIO2010-18962; to M. G. P.); from the Fundação para a Ciência e Tecnologia of Portugal, COMPETE and FEDER programmes (PTDC/SAU-MIC/111581/2009FCOMP-FEDER and ERANet-Pathogenomics Listress ERA-PTG/0003/2010; to D. C.); European Reseach Council advanced grant ERC-233348, Fondation Le Roch les Mousquetaires and Listress ERANet-Pathogenomics (ANR 2010-PATH-001-01) (to P. C.); and Listress ERANet-Pathogenomics Project from the German Federal Ministry of Education and Research (to T. C.). L. B. M. and F. C. were supported by doctoral fellowships from FPU Program (Spanish Ministry of Education, Culture and Sports) and Fundação para a Ciência e Tecnologia of Portugal (SFRH/BD/61825/2009), respectively.

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Edited by: H. Ingmer