Identification and disruption of btlA, a locus involved in bile tolerance and general stress resistance in *Listeria monocytogenes*

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Abstract

A transposon Tn917 mutant of *Listeria monocytogenes* L028 was isolated on the basis of reduced growth on agar adjusted to pH 5.5. The disrupted gene, designated *btlA* (bile tolerance locus), encodes a putative secondary transporter of the major facilitator superfamily, which has significant homology to *yxiO* in *Bacillus subtilis* (lmo1417 in *L. monocytogenes* EGDe). The mutant demonstrated decreased growth rates relative to the wild-type when grown in sub-lethal levels of various stressors (acid, salt, ethanol, bile, SDS, ampicillin and phosphomycin). The mutant was also more sensitive to lethal levels of bile. A pOR119 insertion mutant demonstrated similar phenotypes. Murine virulence studies indicated that disruption of *btlA* does not influence virulence potential. BtlA therefore represents a membrane protein essential for the maintenance of homeostasis under stress conditions, but is not involved in pathogenicity.

Keywords: *Listeria monocytogenes*; Transposon mutagenesis; Environmental stress; Bile tolerance

1. Introduction

*Listeria monocytogenes* is a food-borne pathogen that is the causative agent of human listeriosis, an opportunistic infection that primarily infects pregnant women and immunologically compromised individuals [1]. The disease is characterised by septicemia, leading to infection of the foetus and abortion in pregnant women, or life-threatening meningitis in non-pregnant individuals. The infection is a significant cause of mortality due to food-borne disease and mortality rates of common-source outbreaks often approach 30% [2].

The ability of the organism to sense and respond to sudden changes in its environment is crucial to its pathogenesis. Not only does the pathogen endure potentially lethal environmental extremes during food production, but it must also encounter numerous environmental insults during infection of the host. A number of genetic loci have been identified that play a role in survival of *L. monocytogenes* under conditions experienced both during food production and host colonisation [3–5]. It has been demonstrated that numerous systems are involved in adaptation to individual stresses and that some general stress systems play a role in survival under a variety of extreme conditions [3,4,6].

The recent publication of the genome sequences of both *L. monocytogenes* EGDe and the non-pathogenic species *L. innocua* is a major step towards the identification and characterisation of genetic loci involved in stress adaptation and virulence in *L. monocytogenes* [7]. However, functional genomics approaches are now required to identify stress responsive systems from the large number (35.3%) of open reading frames (ORFs) for which no function is currently known. We have implemented a transposon-based approach to identify genes involved in resistance to low pH in *L. monocytogenes* LO28. A mutant was isolated from a bank of Tn917 insertional mutants based upon impaired growth on agar plates adjusted to pH 5.5. This study describes the molecular and physiological characterisation of the transposon mutant, the creation of a second mutant using a plasmid integration system and examination of the virulence potential of both mutants.
2. Materials and methods

2.1. Media, chemicals and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *L. monocytogenes* strains were grown in brain heart infusion (BHI) broth. *Escherichia coli* was grown in Luria–Bertani (LB) medium. All media was supplied by Oxoid. Defined medium (DM) was prepared as described by Premaratne et al. [8] and the glucose minimal media as described by Yoshida et al. [9]. Erythromycin and chloramphenicol were made up as concentrated stocks, and added to media at the required levels. For solid media, agar was added to 1.5%. All *L. monocytogenes* cultures were incubated at 37°C with shaking. Under these conditions, cultures entered exponential phase at an optical density at 600 nm of approximately 0.15. Viable plate counts were performed by serial dilution in one-quarter strength Ringer’s solution, and enumeration on BHI. Nisin was obtained from Sigma Chemical as a 2.5% nisin solution in sodium chloride containing denatured milk solids. The nisin concentrations are given in micrograms of total solids per millilitre. Stock solutions were made by suspending 100 mg of nisin per ml in 0.05% acetic acid and then diluted 10-fold with dimethylsulfoxide. Bovine bile (oxgall B-3883) and bile acids (sodium cholate and sodium deoxycholate) were obtained from Sigma. Bile was solubilised in water and added to media as filter sterilised stocks after autoclaving. Antibiotic disks (ampicillin, cefaclor, cefotaxime, cefuroxime, cephalaxin, cephradine, chloramphenicol, clindamycin, colistin sulfate, erythromycin, fusidic acid, gentamicin, kanamycin, minocycline, nalidixic acid, novobiocin, oxytetracycline, penicillin, phosphomycin, polymyxin B, rifampicin, spectinomycin, streptomycin, tetracycline, trimethoprim and vancomycin) were obtained from Oxoid. Heavy metals assayed were As, Cd, Co, Cu, Cr, Zn in the forms of NaAsO2, CdSO4·8H2O, CoCl2·6H2O, CuCl2·2H2O, K2CrO4 and ZnSO4·7H2O, respectively.

2.2. Construction and screening of a Tn917 mutant bank

An *L. monocytogenes* LO28 Tn917 bank was created using the temperature-sensitive plasmid pTV1-OK [10]. Transformants were grown overnight in TSB–YE containing kanamycin (50 μg ml−1) at 30°C, sub-cultured (1%) into TSB–YE containing erythromycin (0.04 μg ml−1) at 42°C, and selected for kanamycin-sensitive Tn917 integrants on TSA–YE containing 10 μg ml−1 erythromycin. Erythromycin-resistant colonies from the bank were scored for acid sensitivity by patching them onto BHI pH 7 and BHI adjusted to pH 5.5 with 3 M lactic acid. One isolate showed impaired ability to grow on acidic plates and was designated an acid-sensitive mutant (MB5).

2.3. Genetic manipulations and sequence analysis

An inverse PCR approach was used to elucidate the point of transposon insertion in MB5. Genomic DNA was digested with *Hpa*I for upstream sequence and *Hind*III for downstream sequence. Following self-ligation, inverse PCRs were performed using primers 4784 (5’CATGAGTTGTCCGAGAGTG3’) and 4785 (5’CTCACTCAATAGAGATGTCACCGTC3’) for *Hpa*I digested DNA, and primers 4292 (5’CAAAGCCTAGTAATGCGGTCA TTCC3’) and 4293 (5’CTTGGAGAGTATAAACTTGACTTG3’) for *Hind*III digested DNA. The PCR products were purified with the Qiaex II gel extraction kit (Qiagen, Hilden, Germany) and cloned into the pCR2.1 vector using the TOPO TA cloning kit (Invitrogen, San Diego, CA, USA). Plasmids were isolated with the Qiagen QIAprep Spin Miniprep Kit. Nucleotide sequence determination was performed on an ABI 373 automated sequencer. Restriction enzymes, T4 DNA ligase and PCR reagents were purchased from Boehinger Mannheim, Germany and used according to the manufacturers recommendations. A Hybaid (Middlesex, UK) PCR express system was used. Colony PCR was carried out following lysis of cells with IGEPAL CA-630 (Sigma). Var-

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2.4. Construction of pORI19 mutant

A mutant (designated MB10) was constructed by gene disruption followed by a single crossover event, as described by Law et al. [11]. A 290-bp fragment of \textit{btlA} was generated by PCR with primers 5'CAGCAAGTATTCCGGATGGGATAAAGGTG3' and 5'CTAATGTGGCCTTCTTAGAATGA3'), which were modiﬁed to contain the restriction sites \textit{EcoRI} and \textit{XhoI}, respectively. The PCR product was cut with \textit{EcoRI} and \textit{XhoI} and ligated into similarly digested pORI19. The resulting plasmid was transformed into \textit{E. coli} EC101 by standard methods and then into \textit{L. monocytogenes} harbouring pVE6007 using the protocol outlined by Park and Stewart [12]. One transformant was grown overnight in 10 ml BHI broth prewarmed to 42°C (the non-permissi

2.5. RNA procedures

Total RNA was extracted from exponential phase cultures (optical density at 600 nm of approximately 0.2) by using a hot-acid-phenol protocol as previously described [13]. Samples were treated with DNase, which was subsequently inactivated by heating to 65°C. For reverse transcriptase PCR (RT-PCR) analysis, samples were serially diluted in DEPC-treated water, and cDNA was synthesized from 2 μl of each dilution. Primers 1(5'ATGATGCGGTTTTATTATATTGAGTA3'), 2 (5'CCATCTAGTGAAGAATAAAGG3'), 16SF (5'TTAGCTAGTTGGTAGG3' and 16SR (5'AATCCGGACACGTGGTC3') were used for primer extension and PCR amplification of cDNA. PCRs were carried out for 16, 22 or 30 cycles to allow optimal quantitation of products. cDNA template was added to PCR reactions at levels that gave similar band intensities for 16S RNA (control) reactions.

2.6. Growth curves and stress challenge assays

For growth experiments, overnight cultures were inoculated (3%) into BHI supplemented with sub-lethal levels of various stressors (pH 5.5, 7% NaCl, 5% ethanol, 200 ng ml\(^{-1}\) ampicillin, 0.05% SDS, 20 μg ml\(^{-1}\) phosphomycin or 100 μg ml\(^{-1}\) nisin) and incubated at 37°C with shaking. Cell growth was measured spectrophotometrically by determining the optical density at 600 nm. For bile tolerance assays, cultures were incubated anaerobically at 37°C without shaking and viable cell counts were performed at intervals. For challenge assays, stationary or exponential phase cultures grown at pH 7 were centrifuged (12000×g for 6 min) and subjected to various stressors. The ability to survive high salt concentrations was examined by inoculating sodium phosphate buffer containing 5 M NaCl at 22°C. Bacteria were exposed to thermal stress by inoculation in PBS at 54°C and to ethanol stress by inoculation in BHI with 15% ethanol. Survival at low pH was examined in BHI broth adjusted to pH 3.5 with lactic acid. For bile adaptation and challenge assays, cells were adapted by resuspending cell pellets in BHI containing sub-lethal levels of bile salts (0.08% sodium cholate, sodium deoxycholate [1:1]) or left unadapted (BHI alone). After 30 min, cells were centrifuged and then challenged with BHI containing 0.3% (w/v) bile salts. Viable plate counts were performed at intervals. Where results are presented as percentage survival, this was calculated as viable cell counts after challenge, expressed as a percentage of viable cell counts at time zero, i.e. immediately prior to treatment.

2.7. Mouse Virulence Assay

For intraperitoneal inoculations, BALB/c mice were infected with overnight cultures of the LO28 parent and mutant (pORI19) strains suspended in 0.2 ml of phosphate-buffered saline to a final concentration of 1.5×10^6 bacteria. For peroral inoculations, wild-type and mutant strains were mixed at a ratio of 1:1 in buffered saline with gelatin (0.85% NaCl, 0.01% gelatin, 2.2 mM K\(_2\)HPO\(_4\) and 4.2 mM Na\(_2\)HPO\(_4\)). Mice were infected with approximately 10^10 cells using a micropipette tip placed immediately behind the incisors. Bacterial counts were performed on spleens and livers three days post-infection.

3. Results

3.1. Isolation of an acid-sensitive Tn917 mutant, and identification and sequence analysis of the disrupted gene

A bank of 3×10^4 Tn917 transposon mutants was screened by replica plating onto BHI agar plates at pH 7.0 and BHI plates adjusted to pH 5.5 with lactic acid. A mutant (designated MB5) was isolated that grew well at pH 7.0 but reproducibly failed to display normal growth at pH 5.5. A combination of PCR assays performed with Tn917-specific primers and Southern analysis confirmed that the mutant harboured only one copy of the transposon (data not shown). Sequencing of inverse PCR products (see Materials and methods) determined that the transposon had inserted into a gene with significant homology to \textit{yxiO} in \textit{Bacillus subtilis}. The complete ORF
(1245 nt) was sequenced in *L. monocytogenes* LO28 and has been submitted to GenBank (accession number AY151087). Subsequent analysis of the completed *Listeria* genome sequences [7] showed that the ORF in strain LO28 has 99% homology to lmo1417 in *L. monocytogenes* strain EGDe and 85% homology to the lin1456 gene in *L. innocua*. A putative ribosomal binding site was found 15 bp upstream of the start codon, but no consensus promoter sequence could be identified. A probable Rho-independent terminator was located 21 bp downstream of the stop codon. This was composed of a 34-nt symmetric sequence which can form a 13-bp stem loop structure, followed by a 4-nt poly (U) tail, which is known to be an additional stabilising feature. In silico analyses suggest that the disrupted gene, designated *btlA* (bile tolerance locus; see below), encodes a putative 414 amino acid membrane transporter containing two blocks of six putative transmembrane domains, separated by a hydrophilic loop (Fig. 1B). This topology is characteristic of secondary transporters of the major facilitator superfamily (MFS) [14]. The MFS permease signature motifs between transmembrane segments 2 and 3, and transmembrane segments 8 and 9, which are thought to be important for structural and functional aspects of the permeases, were found in corresponding positions in BtLA [15,16]. Also, the Conserved Domain Search service identified a sugar transporter domain (pfam00083), a characteristic of most families in the MFS.

### 3.2. Transcriptional analysis

Analysis of *yxiO* in *Bacillus subtilis* demonstrated that transcription of the gene is repressed under nutrient limiting conditions in defined nutrient sporulation medium (DSM) but is induced in glucose minimal medium (MM) [9,17]. The growth rates of both *btlA* mutants were similar to the parent in each of these media. We used RT-PCR to determine whether *btlA* was transcriptionally induced in this MM, BHI pH 7 or *Listeria*-defined medium. DSM was not used as this is a sporulation medium and lack of a carbon source does not permit the growth of the non-sporulating *L. monocytogenes*. Fig. 1C shows that a product of equal intensity was amplified for all three media after 30 PCR cycles, suggesting constitutive expression of *btlA* under the conditions tested. Furthermore, both RT-PCR analysis and β-galactosidase fusion assays demonstrated that no change in transcription occurs following exposure to mild stress conditions (data not shown).

### 3.3. Physiological analysis

The growth potential of the transposon mutant MB5 was clearly impaired at pH 5.5 on solid media. A pOR119 insertion mutant, designated MB10, also demonstrated an inability to grow on agar at pH 5.5, confirming a role for this gene in growth under these conditions.

Mutants were unaffected in carbohydrate utilisation (as determined by API CH30) or listeriolysin O production on blood agar plates. No apparent differences in the microscopic morphology, aspect of colonies nor motility was observed. BLAST searches revealed that BtLA showed significant homology to a number of antibiotic and heavy metal efflux pumps. Disk diffusion assays were employed to examine antibiotic resistance and the minimal inhibitory concentrations of various heavy metals were determined using the broth dilution method (Materials and methods). Mutants exhibited identical resistance to the parent strain (not shown). As the closest functionally characterised homologues are tetracycline efflux proteins, resistance to this antibiotic was examined as described by Cheng et al. [18]. However, the mutants did not show impaired resistance. Transposon or plasmid mutants in *btlA* were able to grow as well as the wild-type under normal laboratory growth conditions; however, further analysis demonstrated that an intact gene is required for optimal growth of *L. monocytogenes* under a variety of stress conditions. Growth of mutants was significantly impaired in exponential phase growth rates (*P* = 0.05) in the

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Fig. 1. A: Site of Tn917 insertion in *L. monocytogenes* MB5. The NCBI annotation number (lmo1417) for the corresponding gene in *L. monocytogenes* EGDe is given. The gene disrupted in LO28, designated *btlA*, is indicated in black. Adjacent open reading frames in EGDe are in white. B: Secondary structure prediction of BtLA. This figure was constructed based on results from the www site http://www.bioinformatics.org/s岁資/net "biotools.html. The 12 transmembrane segments, which traverse the membrane in a zigzag fashion, are grouped into two blocks of six, separated by a hydrophilic loop, a topology characteristic of MFS permeases. C: Analysis of transcription of *L. monocytogenes btlA* using RT-PCR. Total bacterial RNA was isolated from cultures growing in BHI pH 7 (lane 2), *Listeria* defined media (lane 3) or *B. subtilis* minimal media (lane 4). Identical concentrations of RNA were analysed for specific *btlA* RNA. The picture represents 30 cycles of PCR. Similar results were obtained for mild stress conditions.
presence of acid (pH 5.5 with lactic acid), ethanol (5%), bile (20%), SDS (0.05%), ampicillin (200 ng ml\(^{-1}\)) (Fig. 2), salt (7% NaCl), and phosphomycin (20 μg ml\(^{-1}\)) (data not shown). Growth of mutants was not impaired at low or high temperatures (4°C or 42°C), at alkaline pH (pH 9 with NaOH), under carbon starvation conditions (DM with 0.1% glucose) or oxidative stress (H\(_2\)O\(_2\)) (data not shown). However, both mutants demonstrated slightly improved growth rates in the presence of the bacteriocin nisin. Mutations in the gene did not affect survival at lethal levels of most environmental stresses examined. Exponential phase and stationary phase cells of both mutant strains were killed by acid stress (pH 3.5) (Fig. 3A), ethanol stress (15% ethanol) and salt stress (5 M NaCl) at the same rate as the wild-type (data not shown). Mutants were also capable of adapting to sub-lethal levels of these stresses in a manner similar to the parent strain (data not shown). However, significant differences were observed for survival in bile (Fig. 3B,C) and, as enhanced sensitivity to bile seemed to be the strongest phenotype associated with \(\text{btlA}\), this stress was examined in more detail.

3.4. Growth and survival in bile

We have recently reported the ability of \(L.\) monocyto-genes LO28 to survive concentrations of bile exceeding those encountered in vivo [19]. Fig. 2C compares the bile tolerance of \(\text{btlA}\) mutants to the parent strain. As expected, the parent strain was capable of growth in 20% oxgall bile after a small initial reduction in numbers. In comparison, growth of the \(\text{btlA}\) mutants was significantly impaired. After 10 h in bile, numbers of mutants were almost 1.5 log lower than the wild-type. A more dramatic difference was observed for higher concentrations of bile (not shown). The response of exponential phase cells to lethal concentrations of unconjugated bile acids (sodium cholate:sodium deoxycholate [1:1]) was also examined. As expected, killing of the parent was almost instantaneous in that there was almost a 5-log reduction in numbers after exposure to only 15 s of bile (Fig. 3B). The mutant was more sensitive, showing almost a 6-log reduction in numbers. A more dramatic difference was observed for stationary phase cultures with the mutant being approximately 2.5 log more sensitive than the parent (0.01% vs. 3.7% survival; Fig. 3C). For both parent and mutants, adaptation to sub-lethal levels of bile provided significant cross-protection to lethal levels (37% vs. 0.01%) (Fig. 3B,C).

3.5. Virulence

As there is strong evidence of the importance of stress-induced responses for a successful infection of \(L.\) monocytogenes [4,5,20–23], a mouse model was used to examine the effects of disrupting \(\text{btlA}\) on virulence. Fig. 4 shows that while the numbers of wild-type recovered from the liver and spleen three days post intraperitoneal infection were slightly higher than the pORI19 mutant, there was no significant difference. Similar results were obtained following intragastric inoculation (Fig. 4). This shows that loss of \(\text{btlA}\) does not significantly affect the virulence potential of the organism, at least in this model system.
4. Discussion

The recent publication of the complete genome sequences of *L. monocytogenes* and *L. innocua* has necessitated the implementation of functional genomics approaches in order to assign gene function to putative ORFs. We have instigated a transposon-based approach to identify genes involved in stress resistance in *L. monocytogenes*. In this study a gene (designated *btlA*) was isolated which is required for growth on agar plates adjusted to pH 5.5 with lactic acid. A mutant with pORI19 integrated in the same gene displayed a similar phenotype, confirming that this locus was responsible for the observed phenotype. Both mutants were able to grow as well as the wild-type under normal laboratory growth conditions, indicating that the disrupted gene is not required for normal cell function.

The data also suggest that the presence of a transposon or plasmid in the genome of *L. monocytogenes* does not overtly interfere with bacterial physiology. Further analyses demonstrated that an intact *btlA* gene is required for optimal growth of *L. monocytogenes* under a variety of sub-optimal stress conditions that may be encountered in food-processing environments (low pH, salt, ethanol), sanitising treatments (SDS) or in clinical situations (ampicillin). By contrast, disruption of the gene confers slightly increased resistance to the bacteriocin nisin. Interestingly, all of these physiological stimuli exert their effects on the cell envelope. It is possible that production of BtlA may directly/indirectly alter membrane structure or permeability and the barrier function may be compromised when the associated gene is disrupted.

RT-PCR analysis showed that *btlA* is constitutively expressed and transcription is not altered under the various stress conditions examined. This constitutive expression indicates that the gene would not have been identified using strategies that rely upon transcriptional up-regulation, such as microarrays or reporter-based approaches. Subsequent examination of the gene sequence revealed that the putative initiation codon is UUG rather than AUG; this may serve to limit expression of the gene at the level of translation [24].

Overall, the data suggest that BtlA represents a membrane protein that is present in readiness for encountering stressful conditions and, directly or indirectly through the maintenance of membrane integrity, permits growth. The intact *btlA* gene seems to be of particular importance in the listerial response to bile stress and is required for both growth and survival. As exposure to bile represents a major challenge in the intestinal tract the ability of the pathogen to tolerate bile is likely to be important for survival and colonisation. Presumably this ability to tolerate bile...
requires a wide array of proteins, including many which govern cell envelope architecture. However, to date, relatively little information is available concerning genes involved. Recently, we identified five loci required for tolerance to bile by screening a separate transposon Tn917 bank. Interestingly, an identical btlA mutant was also isolated in that study [19]. Discovery of this mutant during two separate screens for stress genes highlights the importance of this locus to L. monocytogenes LO28 stress responses. The closest functionally characterised homologues are drug efflux pumps. These pumps are known to play an important role in the bile resistance of Gram-negative microorganisms, for example, the constitutively expressed acrAB multidrug efflux pump of E. coli plays a significant role in bile acid efflux [25]. Disruption of btlA did not significantly affect the virulence potential of the organism. It is possible that proteins with overlapping functions may compensate for loss of the gene in vivo.

In conclusion, although btlA appears to be non-essential under standard laboratory conditions and is not involved in pathogenicity, we have shown that this protein represents a membrane locus in L. monocytogenes LO28 that is important in its stress responses, particularly bile. At present, the substrate conveyed by this putative transporter remains to be determined, but is currently under investigation in our laboratory.

Acknowledgements

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References


