The SOS response of *Listeria monocytogenes* is involved in stress resistance and mutagenesis

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The SOS response is a conserved pathway that is activated under certain stress conditions and is regulated by the repressor LexA and the activator RecA. The food-borne pathogen *Listeria monocytogenes* contains RecA and LexA homologues, but their roles in *Listeria* have not been established. In this study, we identified the SOS regulon in *L. monocytogenes* by comparing the transcription profiles of a wild-type strain and a $\Delta recA$ mutant strain after exposure to the DNA-damaging agent mitomycin C. In agreement with studies in other bacteria, we identified an imperfect palindrome AATAAGAACATATGTTTCGTTT as the SOS operator sequence. The SOS regulon of *L. monocytogenes* consists of 29 genes in 16 LexA-regulated operons, encoding proteins with functions in translesion DNA synthesis and DNA repair. We furthermore identified a role for the product of the LexA-regulated gene *yneA* in cell elongation and inhibition of cell division. As anticipated, RecA of *L. monocytogenes* plays a role in mutagenesis; $\Delta recA$ cultures showed considerably lower rifampicin- and streptomycin-resistant fractions than the wild-type cultures. The SOS response is activated after stress exposure as shown by *recA*- and *yneA*-promoter reporter studies. Stress-survival studies showed $\Delta recA$ mutant cells to be less resistant to heat, H₂O₂ and acid exposure than wild-type cells. Our results indicate that the SOS response of *L. monocytogenes* contributes to survival upon exposure to a range of stresses, thereby likely contributing to its persistence in the environment and in the host.

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INTRODUCTION

Listeriosis is a food-borne infection caused by *Listeria monocytogenes*. The disease has a high case-mortality rate and is therefore of great concern to public health (Mead *et al.*, 1999). *L. monocytogenes* can grow and survive during exposure to severe environmental stresses. It has the ability to grow over a wide pH range (pH 4.6–9), at high salt concentrations (up to 13%), and over a wide temperature range (−0.4 to 46 °C) (Kallipolitis & Ingmer, 2001; van der Veen *et al.*, 2008). Exposure of *L. monocytogenes* to stress can lead to stress adaptation, due to the transcriptional activation of stress-response genes (Foster, 2007). Several classes of stress-response genes have been described in *L. monocytogenes*, which are activated during exposure to

specific or general stresses. In particular, the class I and class III heat-shock and the SigB response have been investigated thoroughly (Derre *et al.*, 1999; Hanawa *et al.*, 1999; Kazmierczak *et al.*, 2003). Recently, it has been shown that the SOS response of *L. monocytogenes* is activated upon mild heat exposure (van der Veen *et al.*, 2007).

Certain environmental insults lead to undesirable DNA damage that requires repair, while under other circumstances increased mutation rates are needed to maximize chances of survival. The SOS response is an inducible pathway involved in DNA repair, restart of stalled replication forks (Cox *et al.*, 2000; Maul & Sutton, 2005), and induction of genetic variation in stressed and stationary-phase cells (Schlachter & Goodman, 2007). It is regulated by LexA and RecA. LexA is an autoregulatory repressor that binds to the CGAACATATGTTTCG consensus sequence in the promoter region of the SOS-response genes as determined for *Bacillus subtilis* (Au *et al.*,

Abbreviations: MMC, mitomycin C; Q-PCR, quantitative real-time PCR.

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A supplementary figure is available with the online version of this paper.

2005), thereby repressing transcription. A consensus LexA-binding motif for *L. monocytogenes* has not been identified thus far. Generally, the SOS response is induced under circumstances in which single-stranded DNA accumulates in the cell. This results in activation of RecA, which in turn stimulates cleavage of LexA, and ultimately in the induction of the SOS response (Schlacher *et al.*, 2006).

For an increasing number of bacteria it has been shown that the SOS response is activated during stress exposure (Cirz *et al.*, 2007; DiCapua *et al.*, 1990) or during pathogenesis (Justice *et al.*, 2006; Kelley, 2006). A comparative analysis of the SOS regulon of *B. subtilis* and *Escherichia coli* showed a surprisingly small overlap (eight genes), while the regulons in each of these species contain over 30 genes (Kelley, 2006). The SOS regulon and its role in *L. monocytogenes* has not been established, but activation of the SOS response was previously observed during heat shock (van der Veen *et al.*, 2007). Induction of the SOS regulon was postulated to suppress cell division, thereby preventing transection of the genome after replication fork stalling (van der Veen *et al.*, 2007). This effect of interruption of Z-ring formation in the vicinity of the nucleoid is called 'nucleoid occlusion' (Rothfield *et al.*, 2005). For *B. subtilis*, activation of the SOS-response gene *yneA* leads to accumulation of YneA at the midcell, thereby preventing septum formation, which results in cell elongation (Kawai *et al.*, 2003). Whether YneA has a similar function in *L. monocytogenes* remains to be elucidated.

In this study, we established the regulon of the SOS response in *L. monocytogenes* by comparing whole-genome expression profiles of a $\Delta recA$ strain and the isogenic wild-type strain before and after exposure to the DNA-damaging

agent mitomycin C (MMC). Furthermore, we demonstrated that RecA-controlled functions of *L. monocytogenes* are involved in mutagenesis and stress survival, and that the *L. monocytogenes* SOS-response gene *yneA* is involved in cell elongation.

METHODS

Strains, media and plasmids. *L. monocytogenes* EGD-e (Glaser *et al.*, 2001) was the wild-type parent strain in this study. This strain and its mutants (Table 1) were stored in Brain Heart Infusion (BHI) broth (Difco) containing 15% sterile glycerol (BDH) at -80°C . Single colonies were inoculated in BHI broth and grown at 37°C and 200 r.p.m. (New Brunswick type C24KC). Antibiotics were added to the medium to maintain plasmids [$10\text{ }\mu\text{g}$ erythromycin ml^{-1} (Sigma) or $50\text{ }\mu\text{g}$ kanamycin ml^{-1} (Sigma)]. Standard protocols were performed for recombinant DNA techniques (Sambrook *et al.*, 1989). The temperature-sensitive suicide plasmid pAULa (Chakraborty *et al.*, 1992) was used for construction of the $\Delta recA$ and $\Delta yneA$ mutants following the protocol described previously (Wouters *et al.*, 2005). The primers for amplification of the flanking regions (*recA*-A to D for $\Delta recA$ and *yneA*-A to D for $\Delta yneA$) are listed in Table 2. This resulted in 915 bp and 306 bp internal deletions for *recA* and *yneA*, respectively. Vector pIMK2 (Monk *et al.*, 2008), containing the PSA phage integrase system, was used for construction of the *yneA* complementation mutant. Primers *yneA*-E and *yneA*-F (Table 2) were used for amplification of *yneA* and its promoter region, and the amplified fragment was cloned into pIMK2 as a *SacI*-*PstI* fragment resulting in vector pIMK-*yneA*. Vector pIMK2 was also used to make promoter reporter fusion constructs with the *yneA* and *recA* promoters. A gene expressing the enhanced green fluorescent protein EGFP was synthesized by the company BaseClear. The sequence of this gene (Supplementary Fig. S1, available with the online version of this paper) was modified to replace codons that are infrequently encountered in *L. monocytogenes* (threshold of 10%) by codons that are more frequently used. EGFP was cloned into pIMK2 as a *NcoI*-*PstI* fragment, resulting in vector pIMK2-EGFP. The promoter regions of *recA* and *yneA* were amplified using primers

Table 1. Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant genotype or characteristics	Reference
<i>L. monocytogenes</i>		
EGD-e	Wild-type serotype 1/2a strain	Glaser <i>et al.</i> (2001)
$\Delta recA$	EGD-e $\Delta recA$	This study
$\Delta yneA$	EGD-e $\Delta yneA$	This study
$\Delta yneA$: <i>yneA</i> -c	EGD-e $\Delta yneA$ complemented for <i>yneA</i> using vector pIMK- <i>yneA</i>	This study
EGD-e: <i>PreCA</i> -EGFP	EGD-e expressing EGFP from the <i>recA</i> promoter	This study
EGD-e: <i>PyneA</i> -EGFP	EGD-e expressing EGFP from the <i>yneA</i> promoter	This study
Plasmids		
pAULa	Em^r ; cloning plasmid for gene replacements in Gram-positive bacteria	Chakraborty <i>et al.</i> (1992)
pAULa- $\Delta recA$	Em^r ; pAULa derivative containing homologous regions up- and downstream of EGD-e <i>recA</i>	This study
pAULa- $\Delta yneA$	Em^r ; pAULa derivative containing homologous regions up- and downstream of EGD-e <i>yneA</i>	This study
pIMK2	Kan^r ; site-specific listerial integrative vector	Monk <i>et al.</i> (2008)
pIMK- <i>yneA</i>	Kan^r ; pIMK2 derivative containing the <i>yneA</i> gene	This study
pIMK2-EGFP	Kan^r ; pIMK2 derivative containing EGFP	This study
pIMK- <i>PreCA</i> -EGFP	Kan^r ; pIMK2-EGFP derivative containing the <i>recA</i> promoter in front of EGFP	This study
pIMK- <i>PyneA</i> -EGFP	Kan^r ; pIMK2-EGFP derivative containing the <i>yneA</i> promoter in front of EGFP	This study

Table 2. PCR primers used in this study

Primer	Sequence (5'–3')*
recA-A	GTGGGGATCCCTGCTGATTAAACGATTG
recA-B	GTGGGCGGCCGCACGATCATTACATTGTTGC
recA-C	GTGGGCGGCCGCACACACAGATATTCGTGATGAG
recA-D	GTGGGTCGACCGGTTTCTGATTCTTTGAC
recA-E	GTGGGAGCTCAGTATTAGACGAACGCTGGA
recA-F	GTGGCCATGGTGTTCCTCCTTCAATAAAT
yneA-A	GTGGGGATCCATCCAAGGGAAGTCAGTTCT
yneA-B	GTGGGCGGCCGCTTTTAAAGTCATTAATAATCCCTC
yneA-C	GTGGGCGGCCGCGCAAATCAGTAAGGTCGATTAG
yneA-D	GTGGGTCGACTAAAAGCATTGAGCCGTGT
yneA-E	GTGGGAGCTCGTATAGCAAAAGGGAACCA
yneA-F	GTGGCTGCAGCATGGTTATTCTCCTTTTCC
yneA-G	GTGGCCATGGATAATCCCTCCAAAAGAATG
recA-fwd	TAAGACGTGCGGAACAACCTG
recA-rev	CACCTTCACGCGAAATACCT
yneA-fwd	GCGACTATTCAGAGGTGAACG
yneA-rev	GCTAACAAAGTCCGCTTTTCG
lexA-fwd	AAGTTCCGCAATCAATGGAG
lexA-rev	AAAGCATCATTTTCGGGTTG
bilEA-fwd	CGGCGAAATTGTCCAAGTAG
bilEA-rev	GGCGTATTGAAAGCATGTCC
dinB-fwd	TTCCGAGGGAAGCCACTTAT
dinB-rev	GCTGAATGCACACCGAATTT
tpi-fwd	AACACGGCATGACACCAATC
tpi-rev	CACGGATTTGACCACGTACC
rpoB-fwd	CGTCGTCTTCGTTCTGTTGG
rpoB-rev	GTTACGAACCACACGTTCC
16S-rRNA-fwd	GATGCATAGCCGACCTGAGA
16S-rRNA-rev	TGCTCCGTCAGACTTTCGTC

*Nucleotides introduced to create restriction sites are underlined.

recA-E, recA-F, yneA-E and yneA-G (Table 2) and cloned into vector pIMK2-EGFP as *SacI*–*NcoI* fragments, thereby replacing the constitutive active *Phelp* promoter, which resulted in vectors pIMK-PrecA-EGFP and pIMK-PyneA-EGFP.

Sample collection and RNA isolation. In three independent replicates, cultures of the wild-type and $\Delta recA$ strain were grown in 50 ml BHI broth (250 ml conical flasks, 37 °C, 200 r.p.m.) until an OD₆₀₀ of approximately 0.5 was obtained. At that point 1 mg MMC l⁻¹ (Sigma) was added to the cultures. Ten-millilitre samples were taken before exposure to MMC and 1 h afterwards, and dissolved in 20 ml RNeasy lysis buffer (Qiagen). The mixtures were incubated for 5 min at room temperature, centrifuged for 10 min at 4300 r.p.m. (Heraeus type megafuse 1.0R), and the pellets were stored at –80 °C. The cell pellets were washed in 400 µl SET buffer [50 mM NaCl (Sigma), 5 mM EDTA (Sigma) and 30 mM Tris/HCl (pH 7.0; Sigma)] containing 10% SDS (Sigma) and treated for 30 min at 37 °C in a shaker (350 r.p.m.; Eppendorf Thermomixer Comfort) with 200 µl 50 mM Tris/HCl (pH 6.5) containing 50 mg lysozyme ml⁻¹ (Merck), 2 mg Proteinase K ml⁻¹ (Ambion), 2.5 U mutanolysin ml⁻¹ (Ambion) and 4 U SUPERase ml⁻¹ (Ambion). Total RNA was extracted using the RNeasy mini kit (Qiagen) with an on-column DNase treatment according to the manufacturer's protocol. The quality of the RNA was analysed on a 2100 Bioanalyser (Agilent Technologies) and quantified in an ND-1000 spectrophotometer (NanoDrop Technologies).

cDNA synthesis and labelling and micro-array hybridization, washing, scanning and analysing.

Five micrograms of total RNA of each sample was used for cDNA synthesis and labelling with both cyanine 3 (Cy3) and cyanine 5 (Cy5) dyes. The CyScribe cDNA post-labelling kit (RPN5660; GE Healthcare) was used according to the manufacturer's protocol. Aliquots of 0.3 µg labelled cDNA were used for hybridization on custom-made *L. monocytogenes* EGD-e micro-arrays (Agilent Technologies). These arrays (8 × 15K format) contained *in situ*-synthesized 60-mer oligomers with a theoretical melting temperature of approximately 82 °C [following nearest-neighbour calculations (Peyret *et al.*, 1999) using 1 M Na⁺ and 10⁻¹² M oligonucleotides]. The *L. monocytogenes* genes were represented on the array by one probe for 36 genes, two probes for 94 genes, three probes for 2701 genes, or six probes for one gene; in total, 23 genes were not represented on the arrays because no unique probe could be selected. The labelled cDNA samples were hybridized on 16 arrays for 17 h at 60 °C following a dye-swap triple-loop design. The micro-arrays were washed, scanned and analysed according to the protocol described extensively by Saulnier *et al.* (2007). The micro-array data are available at GEO (<http://www.ncbi.nlm.nih.gov/geo>) under accession number GSE12634.

Quantitative real-time PCR (Q-PCR). One microgram of total RNA from each sample was used for cDNA synthesis using Superscript III Reverse transcriptase (Invitrogen) following the manufacturer's protocol. Q-PCRs were performed using 10 µl 2 ×

Sybr Green PCR Master Mix (Applied Biosystems), 2 µl diluted cDNA and 200 nM primers in a total volume of 20 µl. The reactions were run on the 7000 PCR System (Applied Biosystems) with initial steps of 2 min at 50 °C and 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. A dissociation curve was added to verify single-product formation. Forward and reverse primers for *recA*, *lexA*, *yneA*, *dinB* and *bileA* (Table 2) were designed with an amplicon length of about 100 bp and a melting temperature of 60 °C. For each primer set a calibration curve was generated to calculate the efficiency of the PCRs. Three housekeeping genes (*tpi*, *rpoB* and 16S rRNA) were included for normalization of the samples.

Prediction of SOS-response genes. The promoter region of putative SOS-response genes (300 bp) was collected. These genes were selected based on the following criteria: (1) significant upregulation (fold-change >1.5 and $P < 0.05$) in the wild-type strain after MMC exposure; (2) no significant upregulation (fold-change <1.5 or $P > 0.05$) in the $\Delta recA$ mutant strain after MMC exposure; and (3) the MMC treatment resulted in significant higher upregulation (fold-change >1.5 and $P < 0.05$) in the wild-type strain compared with the $\Delta recA$ mutant strain. The promoter regions were analysed for conserved motifs by the MEME program (Bailey & Elkan, 1994). The MEME search criteria were set at a minimal length of the motif of 8 nt and a maximal length of 40 nt. The consensus *L. monocytogenes* LexA-binding motif in the putative SOS-response genes was visualized using the WebLogo tool (Crooks *et al.*, 2004).

Microscopic image analysis. Cell sizes were determined using microscopic image analysis. Culture samples of 100 µl were collected at 5000 g for 1 min (Eppendorf type 5417R). The pellets were dissolved in nigrosin solution (Sigma) and 5 µl of a cell suspension was dried on glass slides. A Dialux 20 microscope (Leica) was used to make images of the cells at 100× magnification. The images were analysed in eight-bit type after adjusting the threshold to black and white using the ImageJ program (<http://rsbweb.nih.gov/ij/>). Distribution graphs of cell sizes were constructed in Excel (Microsoft) from a minimum of 500 cells of three independent experiments.

Fluorescence microscopy was performed with a BX41 microscope (Olympus) using the U-MNIBA3 filter (Olympus).

Mutagenesis in wild-type and $\Delta recA$ strains. To investigate the role of the SOS response in introducing mutations, exponentially growing cultures of wild-type and $\Delta recA$ mutant cells were plated on medium containing 0.05 µg rifampicin ml⁻¹ or 75 µg streptomycin ml⁻¹. Cultures of the wild-type strain and the $\Delta recA$ mutant strain were grown in 10 ml BHI broth in 100 ml conical flasks at 37 °C and 200 r.p.m. When an OD₆₀₀ of 0.5–0.7 was reached, the cells were collected (10 min, 3720 g, room temperature; Heraeus type megafuse 1.0R) and dissolved in 1 ml 1× PBS (Sigma). The cell suspensions were serially diluted in 1× PBS and appropriate dilutions were plated on BHI agar and BHI agar containing 75 µg streptomycin ml⁻¹ (Sigma) or 0.05 µg rifampicin ml⁻¹ (Sigma). The plates were incubated at 37 °C for 3 days and colonies were enumerated. The complete experiment was performed in triplicate.

Stress resistance of wild-type and $\Delta recA$ strains. Cultures of the wild-type strain and $\Delta recA$ mutant strain were grown in 10 ml BHI broth at 37 °C and 200 r.p.m. in 100 ml conical flasks until an OD₆₀₀ of approximately 0.3 was obtained. At this point the cultures were exposed to different stresses. The heat resistance was tested by transferring the cultures to a shaking water bath (60 % shaking speed; GFL type 1083) set at 55 °C, the oxidative stress resistance was tested by addition of 60 mM H₂O₂ (Merck), and the acid resistance was tested by dissolving the collected cultures (10 min, 3720 g, room temperature; Heraeus type megafuse 1.0R) in 10 ml BHI (pH 3.4;

adjusted with 10 % HCl) in 100 ml conical flasks. Samples were taken before stress exposure and 1 h after stress exposure and serially diluted in PBS. Dilutions were plated on BHI agar and colonies were enumerated after 3–5 days incubation at 30 °C. Experiments were performed in triplicate.

RESULTS

Identification of SOS-response genes

To identify genes associated with the SOS response, transcriptional profiles of the wild-type and $\Delta recA$ strains were compared before and after exposure to MMC. The $\Delta recA$ strain appeared to be more sensitive to MMC than the wild-type strain (Fig. 1). Exposure for 1 h to MMC resulted in a small change in c.f.u. counts for both the wild-type and the $\Delta recA$ mutant, which was on average 1.40-fold for the wild-type strain and 0.85-fold for the $\Delta recA$ strain. However, longer exposure to MMC resulted in a rapid decrease in viability for the $\Delta recA$ strain. To verify activation of the SOS response after 1 h exposure to MMC, promoter reporter studies were performed using the promoters of two already-described SOS-response genes in *L. monocytogenes*, namely *recA* and *yneA*. Activation of both genes was observed after 1 h exposure to MMC, which was indicated by the cells showing EGFP expression (Fig. 2). For unexposed exponentially growing cells, no EGFP expression was observed. A LexA-motif search was carried out for 122 selected genes that showed significant upregulation (fold-change >1.5 and $P < 0.05$) in the wild-type strain after MMC exposure while no significant upregulation was found in the $\Delta recA$ mutant strain. Furthermore, the MMC treatment resulted in significantly higher upregulation of these genes in the wild-type strain compared to the $\Delta recA$ mutant (Fig. 3). The upstream regions of these 122 genes were collected and compared for similar motifs. A consensus motif was identified in 16 promoter regions (E -value = 2.2×10^{-13}) (Fig. 4), representing 29 genes (Table 3). For five of these genes (*recA*, *yneA*, *lexA*, *bileA* and *dinB*) the micro-array results were

Fig. 1. MMC survival curve of the wild-type (◇) and $\Delta recA$ mutant (▲). The graph shows the surviving fraction of exponentially growing cells in BHI broth after exposure to 1 mg MMC l⁻¹.

Fig. 2. Activation of *recA* and the SOS response after exposure to MMC. Fluorescence (1 and 3) and phase-contrast (2 and 4) microscopy pictures of cells expressing EGFP from the *recA* (a) and *yneA* (b) promoters. Microscopy pictures were taken before (1 and 2) and after (3 and 4) exposure to 1 mg MMC l⁻¹ for 1 h.

verified using Q-PCR (Table 3). Very good correlation between micro-array and Q-PCR results was observed ($R^2=0.97$). The SOS regulon of *L. monocytogenes* consists of genes encoding the specific regulators of the SOS response RecA and LexA, the translesion DNA polymerases DinB and UmuDC, the excinuclease UvrBA, and the cell division inhibitor YneA. These SOS-response genes were recently also found to be induced by heat stress (van der Veen *et al.*, 2007). The newly identified SOS-response genes encode (predicted) helicase systems (lmo0157–lmo0158, lmo1759–lmo1758 and lmo2268–lmo2264), translesion DNA polymerases (lmo1574 and lmo2828), and exo/excinuclease systems (lmo1640–lmo1638 and lmo2222–lmo2220). These results show that the majority of the SOS-response genes of *L. monocytogenes* encode DNA-repair systems and translesion DNA polymerases that help during replication fork stalling. Furthermore, two of the SOS-response genes are part of a bile-resistance system

(lmo1421–lmo1422). Since bile exposure may result in DNA damage (Prieto *et al.*, 2004), activation of this system as part of the SOS response may provide additional protection of cellular DNA. Lastly, the first and the last gene of the *comK* integrated bacteriophage A118 (lmo2271 and lmo2332) are LexA controlled.

Role of YneA in cell elongation

To investigate the role of the SOS-response gene *yneA* in cell elongation, cell size distribution graphs were constructed from the wild-type strain, the $\Delta yneA$ strain and the *yneA*-complemented strain before and after triggering the SOS response by MMC exposure. Exposure to MMC resulted in a significant increase in cell size for the wild-type strain compared with the unexposed cells, while the $\Delta yneA$ mutant strain did not show an increase in cell size compared with unexposed cells (Fig. 5). The results for the *yneA*-complemented strain were similar to the wild-type strain. Similar results were obtained after triggering the SOS response by exposure to 48 °C for 40 min (as in van der Veen *et al.*, 2007), although cell elongation was less pronounced (data not shown). These results show that YneA activity is associated with cell elongation after triggering of the SOS response.

Fig. 3. Comparison of differentially expressed genes after MMC treatment between the wild-type and $\Delta recA$ mutant strains. The number of differentially expressed genes is indicated in the circles and the overlapping areas indicate that the same genes were differentially expressed. The arrow indicates the group of genes on which the LexA-motif search was performed.

Fig. 4. Consensus sequence of the LexA-binding motif of the putative SOS-response genes (Table 3) in *L. monocytogenes*, visualized with WebLogo (Crooks *et al.*, 2004).

Table 3. Genes belonging to the *L. monocytogenes* SOS regulon

The putative LexA-binding sites and log₂ expression ratios between the wild-type (Wt) and $\Delta recA$ mutant after MMC exposure are given.

Gene*	Gene designation	Putative LexA-binding site	Description of product	MMC treatment†		$\Delta recA$ /Wt†		
				Wt	$\Delta recA$	Untreated	MMC treated	MMC treatment
lmo0157		GTTGCGAACGTAGGTTCTGTG	Predicted ATP-dependent helicase	1.61	-0.13	0.99	-0.75	-1.74
lmo0158			Predicted hydrolase	1.18	0.21	0.85	-0.11	-0.96
lmo1302	<i>lexA</i>	AAAAAGAATGTATGTTTCGCTT	Transcription repressor of SOS response	1.74 (1.97)	0.25 (0.30)	-1.58 (-2.03)	-3.06 (-3.70)	-1.48 (-1.67)
lmo1303	<i>yneA</i>	AAAGCGAACATACATTCTTTT, TGTACGAACGGTTGTTCTATA	Similar to <i>B. subtilis</i> YneA protein	3.95 (3.38)	-0.06 (-0.59)	-4.21 (-4.30)	-8.22 (-8.27)	-4.01 (-3.97)
lmo1398	<i>recA</i>	AATACGAATAAATGTTTCGCTT	Transcription activator of SOS response	2.58 (2.03)	0.50	-2.90	-4.98	-2.08
lmo1421	<i>bileA</i>	ATATAGAACATACATTCGATT	Osmoprotectant transport system	1.13 (1.12)	0.30 (-0.64)	-1.55 (-1.82)	-2.38 (-3.57)	-0.83 (-1.76)
lmo1422	<i>bileB</i>		ATP-binding protein, bile resistance					
			Osmoprotectant transport system	1.21	0.17	-1.23	-2.27	-1.04
			permease protein, bile resistance					
lmo1574	<i>dnaE</i>	AACACGAACACACTTTCTTTT	DNA polymerase III alpha subunit	1.37	0.03	-1.18	-2.53	-1.34
lmo1640		AAACAGAACATATGTTTATC	Hypothetical protein	1.90	-0.52	-1.58	-4.00	-2.42
lmo1639			DNA-3-methyladenine glycosidase, base excision repair	2.24	-0.53	-1.54	-4.31	-2.77
lmo1638			Predicted peptidase	2.22	-0.24	-1.47	-3.94	-2.47
lmo1759	<i>pcrA</i>	AATAAGAACAAATGTTTGAT	ATP-dependent DNA helicase	0.93	-0.20	-0.14	-1.27	-1.13
lmo1758	<i>ligA</i>		NAD-dependent DNA ligase	0.65	-0.20	-0.16	-1.01	-0.85
lmo1975	<i>dinB</i>	AATAAGAACGCTTGTTTCGTTT	DNA polymerase IV	3.08 (2.73)	-0.34 (-0.03)	1.88 (1.09)	-1.54 (-1.67)	-3.41 (-2.76)
lmo2222		AATAAGAACGTATATTCGGTT	Predicted DNA repair exonuclease	1.95	-0.48	-0.26	-2.69	-2.42
lmo2221			Hypothetical protein	2.38	-0.21	-0.15	-2.75	-2.60
lmo2220			Predicted exonuclease	1.52	0.16	0.17	-1.19	-1.36
lmo2268	<i>addB</i>	AATAAAAAACATATGTTTCGGTG	Predicted ATP-dependent helicase	1.59	-0.15	0.47	-1.26	-1.73
lmo2267			Predicted ATP-dependent helicase	1.74	0.09	0.05	-1.61	-1.65
lmo2266			Predicted hydrolase	1.56	-0.06	0.11	-1.52	-1.62
lmo2265			Hypothetical protein	1.61	0.06	-0.04	-1.59	-1.55
lmo2264			Hypothetical protein	1.28	-0.39	0.26	-1.42	-1.67
lmo2271		TTCAAGAACGTTTGTTTCGTAT	Bacteriophage A118 protein	3.40	-0.65	1.34	-2.72	-4.05
lmo2332	<i>int</i>	AAAAAGAACGTATGTGCGAAA	Site-specific DNA recombinase, integrase (Bacteriophage A118)	1.58	0.05	0.50	-1.03	-1.53
lmo2489	<i>uvrB</i>	AATGCGAAAATATGTTTCGGTT	Excinuclease ABC (subunit B)	2.76	0.10	1.40	-1.26	-2.66
lmo2488	<i>uvrA</i>		Excinuclease ABC (subunit A)	2.63	0.10	1.08	-1.45	-2.53
lmo2675	<i>umuD</i>	AATAAGAACATTGTTTCGTAT	DNA polymerase V	2.93	-0.51	0.52	-2.92	-3.44
lmo2676	<i>umuC</i>		DNA polymerase V	2.14	-0.30	0.19	-2.26	-2.44
lmo2828		TTTAAGAACGTTTGTTTCGTAT	Predicted equivalent to the UmuD subunit of polymerase V from Gram-negative bacteria	4.56	0.13	2.67	-1.76	-4.43

*The first gene of a putative operon is given in bold.

†Q-PCR results are shown in parentheses.

Fig. 5. Microscopic image analysis of the wild-type, $\Delta yneA$ and $yneA$ -complemented ($yneA$ -c) strains before and after exposure for 1 h to MMC. The graphs show the distribution of cell sizes; for details see Methods.

RecA-dependent mutagenesis

Exponentially growing cultures of wild-type and $\Delta recA$ mutant cells were plated on BHI medium containing $0.05 \mu\text{g}$ rifampicin ml^{-1} or $75 \mu\text{g}$ streptomycin ml^{-1} . These concentrations of antibiotics were the minimal inhibitory concentration (MIC) for the wild-type strain (data not shown). The rifampicin-resistant fraction of the wild-type cultures was 1.25×10^{-7} , which was 14 times higher than the resistant fraction of the $\Delta recA$ cultures (Fig. 6). Furthermore, the $\Delta recA$ cultures did not show a streptomycin-resistant fraction ($<10^{-9}$), while for the wild-type strain a resistant fraction of 1.33×10^{-8} was observed. These results indicate that in the absence of RecA, mutation rates in the cell are lower due to the inability of LexA cleavage and derepression of the SOS response. Numerous attempts were performed to construct

a complementation mutant for the $\Delta recA$ strain to verify that the observed differences between the wild-type and $\Delta recA$ strains were completely dependent on the absence of RecA. However, we did not succeed in the construction of a $recA$ complementation vector in any of the *E. coli* host strains DH5 α , DH10 β or TOP10, most likely due to constitutive high activity of the $recA$ promoter in these *E. coli* strains.

RecA-dependent stress resistance

The role of $recA$ in stress resistance was investigated by exposing the wild-type and $\Delta recA$ strains to heat (55°C), oxidative stress (60 mM H_2O_2) and acid (pH 3.4). To investigate possible activation of the $recA$ and the SOS response after exposure to these stresses, promoter reporter

Fig. 6. RecA-dependent mutagenesis. The graph shows the resistant fractions of exponentially growing wild-type and ΔrecA mutant cultures after exposure to 0.05 μg rifampicin ml^{-1} (dark grey) and 75 μg streptomycin ml^{-1} (light grey).

studies were performed using the promoters for *recA* and *yneA*. Stress exposure for 30 min resulted in visible expression of EGFP for both promoters (Fig. 7a, b). Furthermore, the wild-type strain showed higher resistance to these stresses than the ΔrecA strain (Fig. 7c). In particular, high sensitivity of the ΔrecA strain to heat and oxidative stress was observed under the conditions used. The ΔrecA mutant strains showed approximately 3 log higher reductions in cell counts after 1 h exposure to 55 °C and 60 mM H_2O_2 than the wild-type strain. These results indicate that *recA* and the SOS response are activated after exposure to various stresses and that RecA and possibly other SOS response factors are important for stress survival.

DISCUSSION

In this study, the SOS regulon of *L. monocytogenes* was characterized and its role in mutagenesis and stress resistance was assessed by comparative transcriptome and phenotype analysis of the EGD-e wild-type and its *recA* deletion mutant. A consensus motif for LexA binding was identified upstream of the differentially expressed genes. Sixteen putative binding sites were found, controlling the expression of 29 genes with roles in DNA repair and translesion DNA synthesis. The SOS-response gene *yneA* was shown to be involved in cell elongation or inhibition of cell division. Furthermore, a role for RecA in the introduction of mutations and in the resistance to stress was established by antibiotic resistance assays, promoter reporter studies and stress-resistance tests.

The regulon of the SOS response in *L. monocytogenes* was determined by comparing the transcription profiles of wild-type and an SOS-deficient ΔrecA strain after exposure to a DNA-damaging agent. This approach was previously used to identify the SOS regulons of *E. coli* (Courcelle *et al.*, 2001), *B. subtilis* (Au *et al.*, 2005; Goranov *et al.*, 2006), *Staphylococcus aureus* (Cirz *et al.*, 2007) and *Pseudomonas aeruginosa* (Cirz *et al.*, 2006). The complete SOS regulon

has furthermore been determined for *Caulobacter crescentus* (da Rocha *et al.*, 2008) and *Pseudomonas fluorescens* (Jin *et al.*, 2007). The various SOS regulons in these bacteria consist of 57 genes in *E. coli*, 63 genes in *B. subtilis*, 15 genes in *P. aeruginosa*, 37 genes in *C. crescentus*, 17 genes in *P. fluorescens* and 16 genes in *S. aureus*, and here we identified 29 genes in *L. monocytogenes*. Only five SOS genes are commonly present in the bacteria analysed thus far, namely *lexA*, *recA*, *uvrBA* and *dinB*. In *L. monocytogenes* the other SOS-response genes encode proteins involved in DNA repair (excinucleases, helicases and recombinases) or translesion DNA synthesis (translesion DNA polymerases). A number of these proteins have been investigated in other bacteria as part of their specific SOS response (for a review see Erill *et al.*, 2007). The *L. monocytogenes* SOS response also includes a LexA-regulated bile exclusion system (BilE). BilE has been shown to play a role in *L. monocytogenes* bile resistance and virulence (Sleator *et al.*, 2005). The role of the SOS response and more specifically that of BilE in *L. monocytogenes* stress resistance and virulence remains to be elucidated.

Inhibition of cell division is a common phenomenon that has been associated with activation of the SOS response. Cell division in bacteria is initiated by accumulation of FtsZ at the midcell, and is a complex process involving many proteins. For several bacteria the products of a number of SOS-response genes have been found to inhibit this process. Such genes include *sulA* for *E. coli* (Huisman *et al.*, 1984), *yneA* for *B. subtilis* (Kawai *et al.*, 2003), Rv2719c for *Mycobacterium tuberculosis* (Chauhan *et al.*, 2006), and *divS* for *Corynebacterium glutamicum* (Ogino *et al.*, 2008). These studies reported the occurrence of cell elongation as a consequence of the SOS response. In a previous study, we found that *yneA* was upregulated during heat-shock and that YneA had a potential role in cell elongation and cell division (van der Veen *et al.*, 2007). This role of YneA was confirmed in this study. Induction of the SOS response by MMC exposure resulted in elongation of wild-type cells, while this was not observed in the ΔyneA strain. Notably, cells of the latter mutant appeared to be more sensitive to heat-inactivation than the wild-type strain (results not shown). The parameters involved in sensitization of the ΔyneA mutant to heat remain to be elucidated. However, we anticipate that this sensitivity might be related to prevention of transection of the genome during replication fork stalling after heat exposure. This process allows bacteria to rescue their genome by reinitiation of chromosomal replication and segregation due to RecA-dependent activation of specific SOS-response genes.

One of the major functions of RecA is the activation of translesion DNA synthesis polymerases and DNA-repair mechanisms (Courcelle & Hanawalt, 2003; Harfe & Jinks-Robertson, 2000). Therefore, we investigated these specific functions of RecA in *L. monocytogenes*. RecA-dependent mutagenesis in *E. coli* is dependent on the derepression of genes encoding any of the translesion DNA polymerases

Fig. 7. Role of RecA and the SOS response in stress resistance. (a, b) Fluorescence (1, 3 and 5) and phase-contrast (2, 4 and 6) microscopy pictures of cells expressing EGFP from the *recA* (a) and *yneA* (b) promoters. Activation of *recA* and *yneA* was monitored after 30 min exposure to 55 °C (1 and 2), 60 mM H₂O₂ (3 and 4) and pH 3.4 (5 and 6). (c) Surviving fraction of the wild-type (dark grey) and $\Delta recA$ (light grey) strain in BHI broth after 1 h exposure at 55 °C, 60 mM H₂O₂ or pH 3.4. Cell counts were made after 3 days incubation at 30 °C.

Pol II (*polB*), Pol IV (*dinB*) or Pol V (*umuDC*) (Goodman, 2000; Napolitano *et al.*, 2000). For *B. subtilis*, an additional polymerase, DnaE, was required (Duigou *et al.*, 2004; Sung *et al.*, 2003). The *L. monocytogenes* SOS regulon contains homologues of these genes, except for *polB*, suggesting that mechanisms involved in RecA-dependent mutagenesis are similar. Our results confirmed that RecA performs an important function in mutagenesis, as shown by the rifampicin- and streptomycin-resistant fractions of wild-type and $\Delta recA$ cultures. In the presence of RecA,

rifampicin-resistant mutants arose with a frequency of 10^{-7} , which was similar to the frequencies that were reported in previous studies for *L. monocytogenes* (Boisivon *et al.*, 1990), *E. coli* (Salmelin & Vilpo, 2002) and *Streptococcus uberis* (Varhimo *et al.*, 2007). The frequency of rifampicin-resistant mutants in the $\Delta recA$ mutant was 14-fold lower than in the wild-type strain. Streptomycin-resistant mutants were found with a frequency of 10^{-8} in the wild-type strain, while no resistant mutants were detected in the $\Delta recA$ mutant strain. Streptomycin-resistant

mutants were found at 10-fold lower frequencies than rifampicin-resistant mutants. This lower frequency might be related to the occurrence of specific mutations in the *L. monocytogenes* genes *rpoB* and *rpsL*, which are required for resistance to the antibiotics rifampicin and streptomycin, respectively (Hosoya *et al.*, 1998; Morse *et al.*, 1999).

A variety of stresses can induce DNA damage (oxidative stress) or replication fork stalling (heat stress), indicating that RecA may play an important role in survival during stress exposure. Duwat *et al.* (1995) showed that RecA of *Lactococcus lactis* is involved in survival of oxidative and heat stress. Furthermore, it was shown for *E. coli* that exposure to acidic pH could activate the SOS response (Sousa *et al.*, 2006), indicating a potential function of the SOS response in acid resistance. Our promoter reporter study revealed that *recA* and *yneA* of *L. monocytogenes* are indeed activated after 30 min exposure to heat, oxidative and acid stress, pointing to an important role for the SOS response during stress exposure. This role was further substantiated by our finding that the $\Delta recA$ mutant was much less resistant to these stresses than the wild-type strain. Whether the observed stress sensitivity of the $\Delta recA$ mutant completely depends on the absence of RecA or whether the inability of this mutant to activate the SOS response contributes to this phenomenon remains to be elucidated in future studies.

In conclusion, the SOS regulon of *L. monocytogenes* was characterized and shown to contain genes encoding translesion DNA polymerases, DNA-repair proteins and a bile resistance system. Furthermore, our results showed that RecA of *L. monocytogenes* plays an important role in stress survival and mutagenesis. These results indicate an important role for the SOS response in the persistence of *L. monocytogenes* in a range of environments.

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