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

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ABSTRACT

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 homologs, 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 the wild-type strain and the Δ*recA* mutant strain after exposure to the DNA damaging agent mitomycinC. In agreement with studies in other bacteria, we identified an imperfect palindrome AATAAGAACATATGTTCGTTT 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; Δ*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. Subsequently, stress survival studies showed Δ*recA* mutant cells to be less resistant to heat, H_2O_2, 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.

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 at a wide pH range (pH 4.6 to pH 9), at high salt concentrations (up to 13 %), and over a wide temperature range (-0.4 to 46 ºC) (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 in induction of genetic variation in stressed and stationary phase cells (Schlacher & Goodman, 2007). It is regulated by LexA and RecA. LexA is an autoregulatory repressor which binds to the CGAACATATGTTCG consensus sequence in the promoter region of the SOS response genes as determined for *Bacillus subtilis* (Au *et al.*, 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 between 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 *Bacillus 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 Δ*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

Strain *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 Hearth Infusion (BHI) broth (Difco) containing 15% sterile glycerol (BDH) at -80 ºC. Single colonies were inoculated in BHI broth and grown at 37 ºC and 200 rpm (New Brunswick type C24KC). Antibiotics were added to the medium to maintain plasmids (10 $\mu g \cdot ml^{-1}$ erythromycin [Sigma] or 50 $\mu g \cdot ml^{-1}$ kanamycin [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 Δ*recA* and Δ*yneA* following the protocol described previously (Wouters *et al.*, 2005). The primers for amplification of the flanking regions (recA-A to D for Δ*recA* and yneA-A to D for Δ*yneA*) are listed in Table 2. This resulted in a 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 (Leiden, The Netherlands). The sequence of this gene (Appendix 1) 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 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 Δ*recA* strain were grown in 50 ml BHI broth (250 ml conical flasks, 37 ºC, 200 rpm,) until an absorbance (OD_{600}) of approximately 0.5 was obtained. At that moment 1 mg/L MMC (Sigma) was added to the cultures. Ten ml samples were taken before exposure to MMC and one hour afterwards, and dissolved in 20 ml RNAprotect (Qiagen). The mixtures were incubated for 5 min at room temperature, centrifuged for 10 min at 4300 rpm (Heraeus type megafuse 1.0R), and the pellets were stored at -80 ºC. 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 % sodium dodecyl sulfate (Sigma) and treated for 30 min at 37 ºC in a shaker (350 rpm; Eppendorf Thermomixer Comfort) with 200 µl 50 mM Tris-HCl (pH 6.5) containing 50 mg/ml lysozyme (Merck), 2 mg/ml Proteinase K (Ambion), 2.5 U/ml mutanolysin (Ambion), and 4 U/ml SUPERase (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 analyzed on a 2100 Bioanalyzer (Agilent Technologies) and quantified on a ND-1000 spectrophotometer (NanoDrop Technologies).

cDNA synthesis and labeling and micro-array hybridization, washing, scanning, and analyzing

Five µg of total RNA of each sample was used for cDNA synthesis and labeling with both cyanine 3 (Cy3) and cyanine 5 (Cy5) dyes. The CyScribe cDNA post-labeling kit (RPN5660; GE Healthcare) was used according to the manufacturer's protocol. Aliquots of 0.3 µg labeled cDNA were used for hybridization on custom-made *L. monocytogenes* EGD-e micro-arrays (Agilent Technologies). These arrays (8 x 15K format) contained i*n situ* synthesized 60-mer oligomers with a theoretical melting temperature of approximately 82 ºC (following nearest neighbor calculations (Peyret *et al.*, 1999) using 1 M Na^+ and 10^{-12} M oligo-nucleotides). The *L. monocytogenes* genes were represented on the array by 1 probe for 36 genes, 2 probes for 94 genes, 3 probes for 2701 genes, or 6 probes for 1 gene and a total of 23 genes was not represented on the arrays because no unique probe could be selected. The labeled cDNA samples were hybridized on 16 arrays for 17 hours at 60 ºC following a dye swap triple loop design. The micro-arrays were washed, scanned, and analyzed according to the protocol described extensively by Saulnier et al. (Saulnier *et al.*, 2007). The micro-array data are available at GEO (http://www.ncbi.nlm.nih.gov/geo) using accession number GSE12634.

158 **Quantitative real-time PCR**

159 One μg of total RNA from each sample was used for cDNA synthesis using Superscript
160 III Reverse transcriptase (Invitrogen) following manufacturers protocol. Q-PCR reactions
161 were performed using 10 μl of 2 x Sybr Green PCR Master Mix (Applied Biosystems), 2
162 μl of diluted cDNA, and 200 nM primers in a total volume of 20 μl. The reactions were
163 run on the 7000 PCR System (Applied Biosystems) with initial steps of 2 min at 50 ºC
164 and 10 min at 95 ºC, and 40 cycles of 15 sec at 95 ºC and 1 min at 60 ºC. A dissociation
165 curve was added to verify single product formation. Forward and reverse primers for
166 *recA, lexA, yneA, dinB*, and *bilEA* (Table 2) were designed with an amplicon length of
167 about 100 bp and a melting temperature of 60 ºC. For each primer set a calibration curve
168 was generated to calculate the efficiency of the PCR reactions. Three housekeeping genes
169 (tpi, rpoB, and 16S rRNA) were included for normalization of the samples.
170
171 **Prediction of SOS-response genes**
172 The promoter region of putative SOS-response genes (300 bp) was collected. These genes
173 were selected based on the following criteria: 1) significant up-regulation (fold-
174 change>1.5 and $p<0.05$) in the wild-type strain after MMC exposure, 2) no significant
175 up-regulation (fold-change<1.5 or $p>0.05$) in the Δ*recA* mutant strain after MMC
176 exposure, and 3) the MMC treatment resulted in significant higher up-regulation (fold-
177 change>1.5 and $p<0.05$) in the wild-type strain compared with the Δ*recA* mutant strain.
178 The promoter regions were analyzed for conserved motifs by the MEME program (Bailey
179 & Elkan, 1994). The MEME search criteria were set at a minimal length of the motif of 8
180 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 x 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 x magnification. The images were analyzed in eight-bit type after adjusting the threshold to black and white using the ImageJ program (http://rbs.info.nih.gov/ij/download.html). Distribution graphs of cell sizes were constructed in Excel (Microsoft) from a minimum of 500 cells of three independent experiments.

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

Mutagenesis in wild-type and Δ*recA* strains

To investigate the role of the SOS response in introducing mutations, exponentially growing cultures of wild-type and Δ*recA* mutant cells were plated on 0.05 µg/ml rifampicin or 75 µg/ml streptomycin. Cultures of the wild-type strain and the Δ*recA* mutant strain were grown in 10 ml BHI broth in 100 ml conical flasks at 37 ºC and 200 rpm. When an absorbance (OD_{600}) of 0.5-0.7 was reached, the cells were collected (10 min, 3720 x g, room temperature; Heraeus type megafuse 1.0R) and dissolved in 1 ml 1 x phosphate buffered saline (PBS; Sigma). The cell suspensions were serially diluted in 1 x

PBS and appropriate dilutions were plated on BHI agar and BHI agar containing 75 μg/ml streptomycin (Sigma) or 0.05 μg/ml rifampicin (Sigma). The plates were incubated at 37 ºC for 3 days and colonies were enumerated. The complete experiment was performed in triplicate.

Stress resistance of wild-type and Δ*recA* strains

Cultures of the wild-type strain and Δ*recA* mutant strain were grown in 10 ml BHI broth at 37 ºC and 200 rpm in 100 ml conical flasks until an absorbance (OD_{600}) of approximately 0.3 was obtained. At this moment 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 ºC, the oxidative stress resistance was tested by addition of 60 mM H_2O_2 (Merck), and the acid resistance was tested by dissolving the collected cultures (10 min, 3720 x 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 hour 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 ºC. Experiments were performed in triplicate.

RESULTS

Identification of SOS response genes

To identify genes belonging to the SOS response, transcriptional profiles of the wild-type and Δ*recA* strains were compared before and after exposure to MMC. The Δ*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 cfu counts for both wild-type and Δ*recA* mutant strain, which was on average 1.40-fold for the wild-type strain and 0.85-fold for the Δ*recA* strain. However, longer exposure to MMC resulted in a rapid decrease in viability for the Δ*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 up-regulation (fold-change >1.5 and $p<0.05$) in the wild-type strain after MMC exposure while no significant up-regulation was found in the Δ*recA* mutant strain. Furthermore, the MMC treatment resulted in significant higher up-regulation of these genes in the wild-type strain compared to the Δ*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=$2.2e^{-13}$) (Fig. 4), representing 29 genes (Table 3). For five of these genes (*recA*, *yneA*, *lexA*, *bilEA*, and *dinB*) the micro-array results were verified using Q-PCR (Table 3). Very good correlation between micro-array and Q-PCR results was observed (R^2=0.97). The SOS response of *L. monocytogenes* consist 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.

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 Δ*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 Δ*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.

RecA dependent mutagenesis

Exponentially growing cultures of wild-type and Δ*recA* mutant cells were plated on 0.05 μg/ml rifampicin or 75 μg/ml streptomycin. 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 $1.25 \cdot 10^{-7}$, which was 14 times higher than the resistant fraction of the Δ*recA* cultures (Fig. 6). Furthermore, the Δ*recA* cultures did not show a resistant fraction to streptomycin ($<10^{-9}$), while a resistant fraction for the wild-type strain of $1.33 \cdot 10^{-8}$ 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 Δ*recA* strain to verify that the observed differences between the wild-type and Δ*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 *Escherichia coli* host strains DH5α, DH10β, 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 Δ*recA* strains to heat (55 ºC), 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 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 and 7B). Furthermore, the wild-type strain showed higher resistance to these stresses than the Δ*recA* strain (Fig. 7C). In particular, high sensitivity of the Δ*recA* strain to heat and oxidative stress was observed under the conditions used. The Δ*recA* mutant strains showed approximately 3 log higher reductions in cell counts after 1 hour exposure to 55 ºC 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 Δ*recA* 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 consists 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 5 SOS genes are commonly present in the bacteria analyzed 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 contains 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 mid-cell, and is a complex process involving many proteins. For several bacteria the products of a number of SOS response genes were found to inhibit this process. Such genes include *sulA* for *Escherichia coli* (Huisman *et al.*, 1984), *yneA* for *Bacillus subtilis* (Kawai *et al.*, 2003), Rv2719c for *Mycobacterium tuberculosis* (Chauhan

342 *et al.*, 2006), and *divS* for *Corynebacterium glutamicum* (Ogino *et al.*, 2008). These
343 studies reported the occurrence of cell elongation as a consequence of this process. In a
344 previous study, we found that *yneA* was up-regulated during heat-shock and that YneA
345 had a potential role in cell elongation and cell division (van der Veen *et al.*, 2007). This
346 role of YneA was confirmed in this study. Induction of the SOS response by MMC
347 exposure resulted in cell elongation of wild type cells, while this was not observed in the
348 Δ*yneA* strain. Notably, cells of the latter mutant appeared to be more sensitive to heat-
349 inactivation than the wild-type strain (results not shown). The parameters involved in
350 sensitization of the Δ*yneA* mutant to heat remain to be elucidated. However, we anticipate
351 that it might be related to prevention of transection of the genome during replication fork
352 stalling after heat exposure. This process allows bacteria to rescue their genome by re-
353 initiation of chromosomal replication and segregation due to RecA-dependent activation
354 of specific SOS response genes.
355
356 One of the major functions of RecA is the activation of translesion DNA synthesis
357 polymerases and DNA repair mechanisms (Courcelle & Hanawalt, 2003; Harfe & Jinks-
358 Robertson, 2000). Therefore, we investigated these specific functions of RecA in *L.*
359 *monocytogenes*. RecA-dependent mutagenesis in *E. coli* is dependent on the derepression
360 of genes encoding any of the translesion DNA polymerases Pol II (*polB*), Pol IV (*dinB*),
361 or Pol V (*umuDC*) (Goodman, 2000; Napolitano *et al.*, 2000). For *B. subtilis*, an
362 additional polymerase, DnaE, was required (Duigou *et al.*, 2004; Sung *et al.*, 2003). The
363 *L. monocytogenes* SOS response contains homologs of these genes, except for *polB*,
364 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 Δ*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), or *Streptococcus uberis* (Varhimo *et al.*, 2007). The frequency of rifampicin resistant mutants in the Δ*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 Δ*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. (Duwat *et al.*, 1995) showed that RecA of *L. 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 Δ*recA* mutant was much less resistant to these stresses than the wild-type strain. Whether the observed stress sensitivity of the Δ*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 persistence of *L. monocytogenes* in a range of environments.

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TABLES

Table 1. Bacterial strains and plasmids used in this study

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

606 Table 2. PCR primers used in this study

Primer	Sequence (5'-3')[a]
recA-A	GTGGGGATCCCTGCTGATTTAAACGATTTG
recA-B	GTGGGCGGCCGCACGATCATTCACATTGTTGC
recA-C	GTGGGCGGCCGCACACACAGATATTCGTGATGAG
recA-D	GTGGGTCGACCGGTTTTCTGATTCTTTGAC
recA-E	GTGGGAGCTCAGTATTAGACGAACGCTGGA
recA-F	GTGGCCATGGTGTTGCCTCCTTCAATAAAT
yneA-A	GTGGGGATCCATCCAAGGGAAGTCAGTTCT
yneA-B	GTGGGCGGCCGCTTTTAAAGTCATTAATAATCCCTC
yneA-C	GTGGGCGGCCGCGCAAATCAGTAAGGTCGATTTAG
yneA-D	GTGGGTCGACTAAAAGCATTGAGCCGTGT
yneA-E	GTGGGAGCTCGTATAGCAAAAGGGAAACCA
yneA-F	GTGGCTGCAGCATGGTTATTCTCCTTTTCC
yneA-G	GTGGCCATGGATAATCCCTCCAAAAAGAATG
recA-fwd	TAAGACGTGCGGAACAACTG
recA-rev	CACCTTCACGCGAAATACCT
yneA-fwd	GCGACTATTCAGAGGTGAACG
yneA-rev	GCTAACAAAGTCCGCTTTCG
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	GTTCACGAACCACACGTTCC
16S-rRNA-fwd	GATGCATAGCCGACCTGAGA
16S-rRNA-rev	TGCTCCGTCAGACTTTCGTC

607 [a] Nucleotides introduced to create restriction sites are underlined

608 Table 3. Genes belonging to the *L. monocytogenes* SOS response. The putative LexA binding sites and $\log_2$ expression ratios between

609 the wild-type (wt) and Δ*recA* mutant after MMC exposure are given.

				MMC treatment		**Δ*recA*/wt**		
Gene[a]	**Gene designation**	**Putative LexA binding site**	**Description product**	**wt**	**Δ*recA***	**Untreated**	**MMC treated**	**MMC treatment**
		GTTGCGAACGT						
lmo0157		AGGTTCTGTG	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
		AAAAAGAATGT		1.74	0.25	-1.58	-3.06	-1.48
lmo1302	*lexA*	ATGTTCGCTT	Transcription repressor of SOS response	(1.97)[b]	(0.30)	(-2.03)	(-3.70)	(-1.67)
		AAAGCGAACAT						
		ACATTCTTTT,						
		TGTACGAACGG		3.95	-0.06	-4.21	-8.22	-4.01
lmo1303	*yneA*	TTGTTCTATA	Similar to *B. subtilis* YneA protein	(3.38)	(-0.59)	(-4.30)	(-8.27)	(-3.97)
		AATACGAATAA		2.58				
lmo1398	*recA*	ATGTTCGCTT	Transcription activator of SOS response	(2.03)	0.50	-2.90	-4.98	-2.08
		ATATAGAACAT	Osmoprotectant transport system ATP-	1.13	0.30	-1.55	-2.38	-0.83
lmo1421	*bilEA*	ACATTCGATT	binding protein, bile resistance	(1.12)	(-0.64)	(-1.82)	(-3.57)	(-1.76)
			Osmoprotectant transport system permease					
lmo1422	*bilEB*		protein, bile resistance	1.21	0.17	-1.23	-2.27	-1.04
		AACACGAACAC						
lmo1574	*dnaE*	ACTTTCTTTT	DNA polymerase III alpha subunit	1.37	0.03	-1.18	-2.53	-1.34
		AAACAGAACAT						
lmo1640		ATGTTTTATC	Hypothetical protein	1.90	-0.52	-1.58	-4.00	-2.42
			DNA-3-methyladenine glycosidase, base					
lmo1639			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
		AATAAGAACAA						
lmo1759	*pcrA*	ATGTTTGTAT	ATP-dependent DNA helicase	0.93	-0.20	-0.14	-1.27	-1.13
lmo1758	*ligA*		NAD-dependent DNA ligase	0.65	-0.20	-0.16	-1.01	-0.85
		AATAAGAACGC		3.08	-0.34	1.88	-1.54	-3.41
lmo1975	*dinB*	TTGTTCGTTT	DNA polymerase IV	(2.73)	(-0.03)	(1.09)	(-1.67)	(-2.76)
lmo2222		AATAAGAACGT	Predicted DNA repair exonuclease	1.95	-0.48	-0.26	-2.69	-2.42

		ATATTCGGTT						
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
		AATAAAAACAT						
lmo2268	*addB*	ATGTTCGGTG	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
		TTCAAGAACGT						
lmo2271		TTGTTCGTAT	Bacteriophage A118 protein	3.40	-0.65	1.34	-2.72	-4.05
		AAAAAGAACGT	Site-specific DNA recombinase, integrase					
lmo2332	*int*	ATGTGCGAAA	(Bacteriophage A118)	1.58	0.05	0.50	-1.03	-1.53
		AATGCGAAAAT						
lmo2489	*uvrB*	ATGTTCGGTT	Excinuclease ABC (subunit B)	2.76	0.10	1.40	-1.26	-2.66
lmo2488	*uvrA*		Excinuclease ABC (subunit A)	2.63	0.10	1.08	-1.45	-2.53
		AATAAGAACAT						
lmo2675	*umuD*	TTGTTCGTAT	DNA polymerase V	2.93	-0.51	0.52	-2.92	-3.44
lmo2676	*umuC*		DNA polymerase V	2.14	-0.30	0.19	-2.26	-2.44
		TTTAAGAACGT	Predicted equivalent to the UmuD subunit of					
lmo2828		TTGTTCGTAT	polymerase V from gram-negative bacteria	4.56	0.13	2.67	-1.76	-4.43

610 [a]The first gene of a putative operon is given in bold.

611 [b]Q-PCR results are shown between brackets.

Legends to the figures

Fig.1. MMC survival curve of the wild-type and Δ*recA* mutant strains. The graph shows the surviving fraction of exponentially growing wild-type (diamonds) and Δ*recA* (triangles) cells in BHI broth after exposure to 1 mg/L MMC.

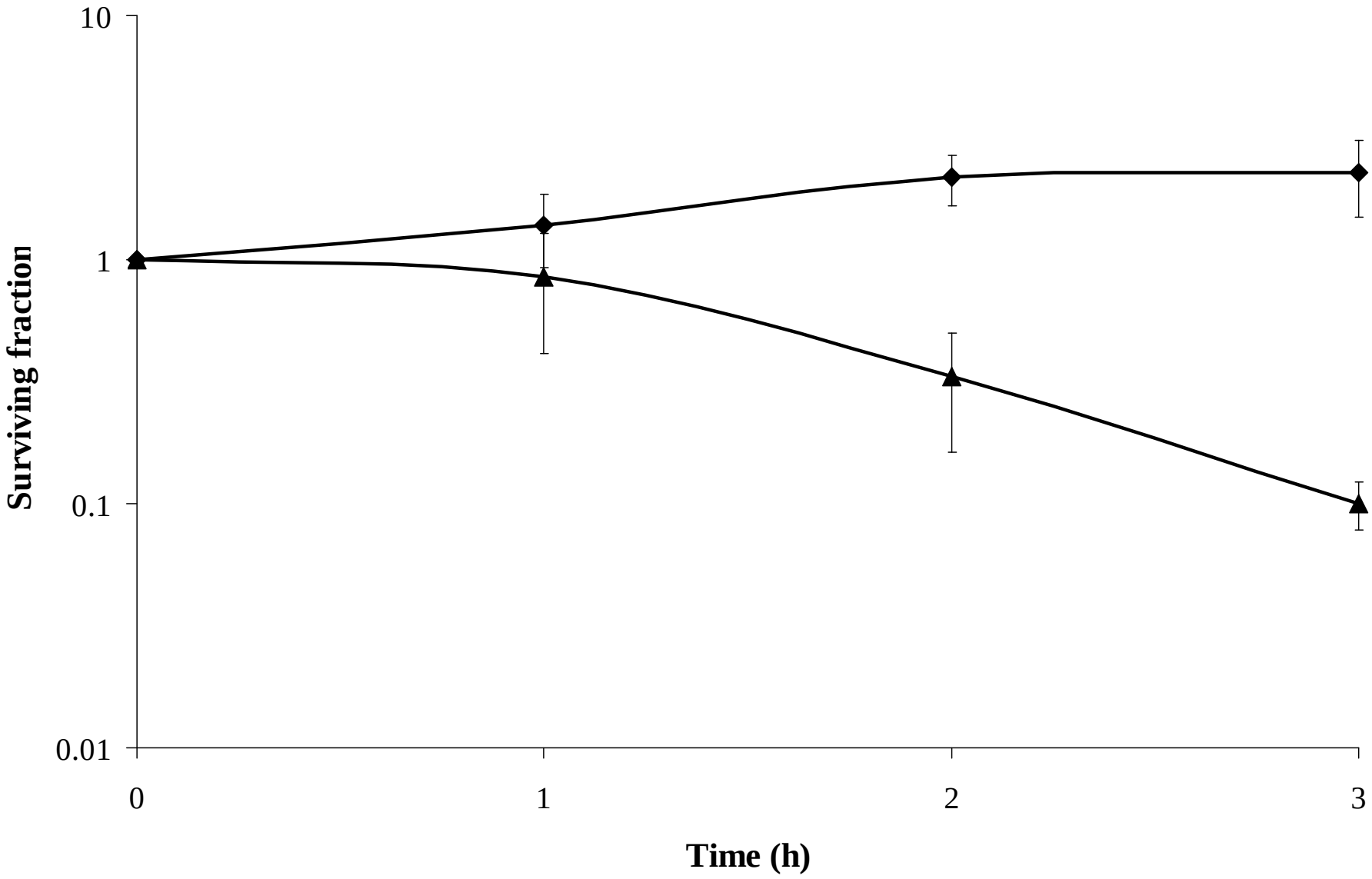
Fig. 2. Activation of *recA* and the SOS response after exposure to MMC. Fluorescence (1 and 3) and phase contrast (2 and 4) microscopy pictures of cells expressing EGFP from the *recA* (A) and *yneA* (B) promoters. Microscopy pictures were taken before (1 and 2) and after (3 and 4) exposure for 1 h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

Fig. 3. Comparison of differentially expressed genes after MMC treatment between the wild-type and Δ*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).

Fig. 5. Microscopic image analysis of the wild-type, Δ*yneA*, and yneA-c strains before and after exposure for 1 hour to MMC. The graphs show the distribution of cell sizes; for details see Materials and Methods.

635 Fig. 6. RecA dependent mutagenesis. The graph shows the resistant fractions of
636 exponentially growing wild-type and Δ*recA* mutant cultures after exposure to 0.05 μg/ml
637 rifampicin (dark grey) and 75 μg/ml streptomycin (light grey).
638
639 Fig. 7. The role of RecA and the SOS response in stress resistance. A) and B)
640 Fluorescence (1, 3, and 5) and phase contrast (2, 4, and 6) microscopy pictures of cells
641 expressing EGFP from the *recA* (A) and *yneA* (B) promoters. Activation of *recA* and
642 *yneA* was monitored after 30 min exposure to 55 ºC (1 and 2), 60 mM H_2O_2 (3 and 4),
643 and pH 3.4 (5 and 6). C) Surviving fraction of the wild-type (dark grey) and Δ*recA* (light
644 grey) strain in BHI broth after 1 hour exposure at 55 ºC, 60 mM H_2O_2, or pH 3.4. Cell
645 counts were made after 3 days incubation at 30 ºC.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0.01
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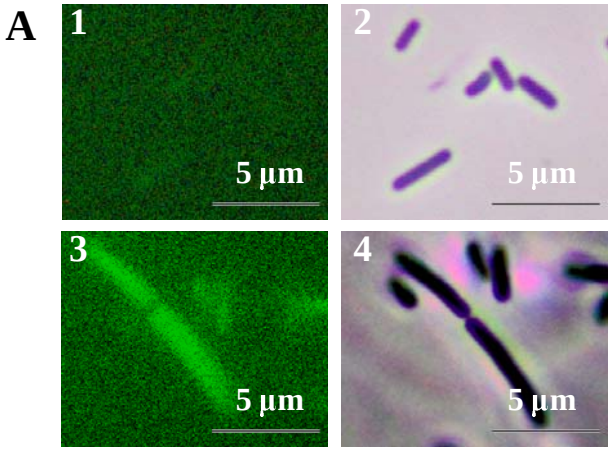
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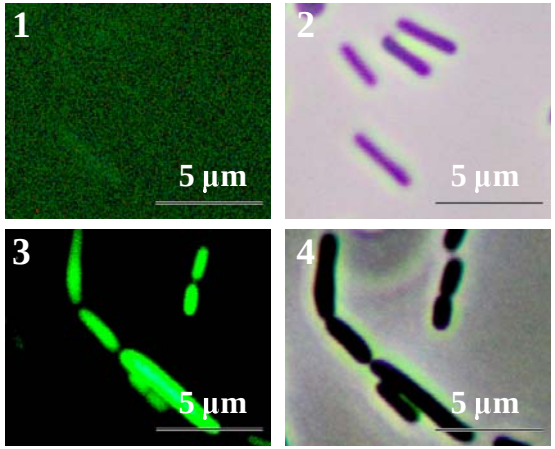

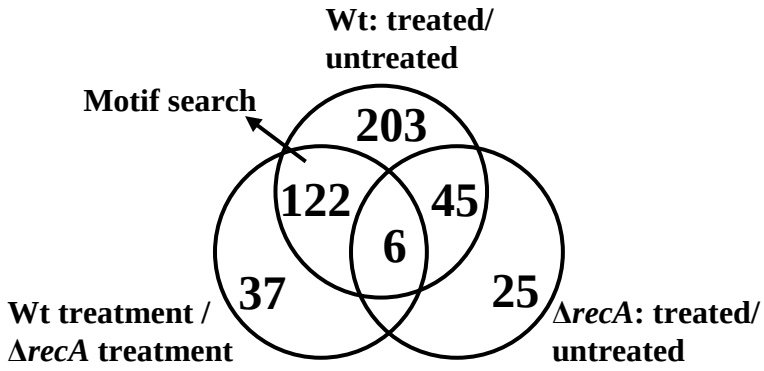
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Motif search
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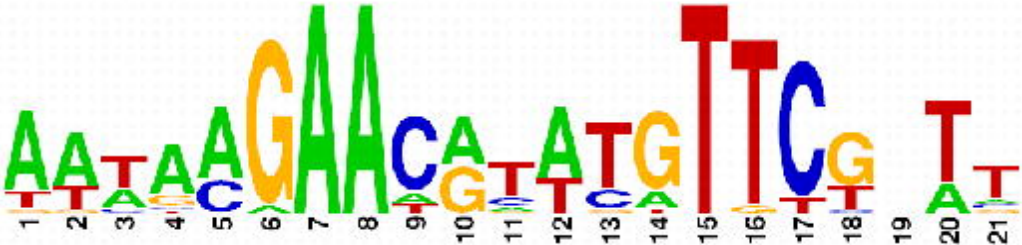
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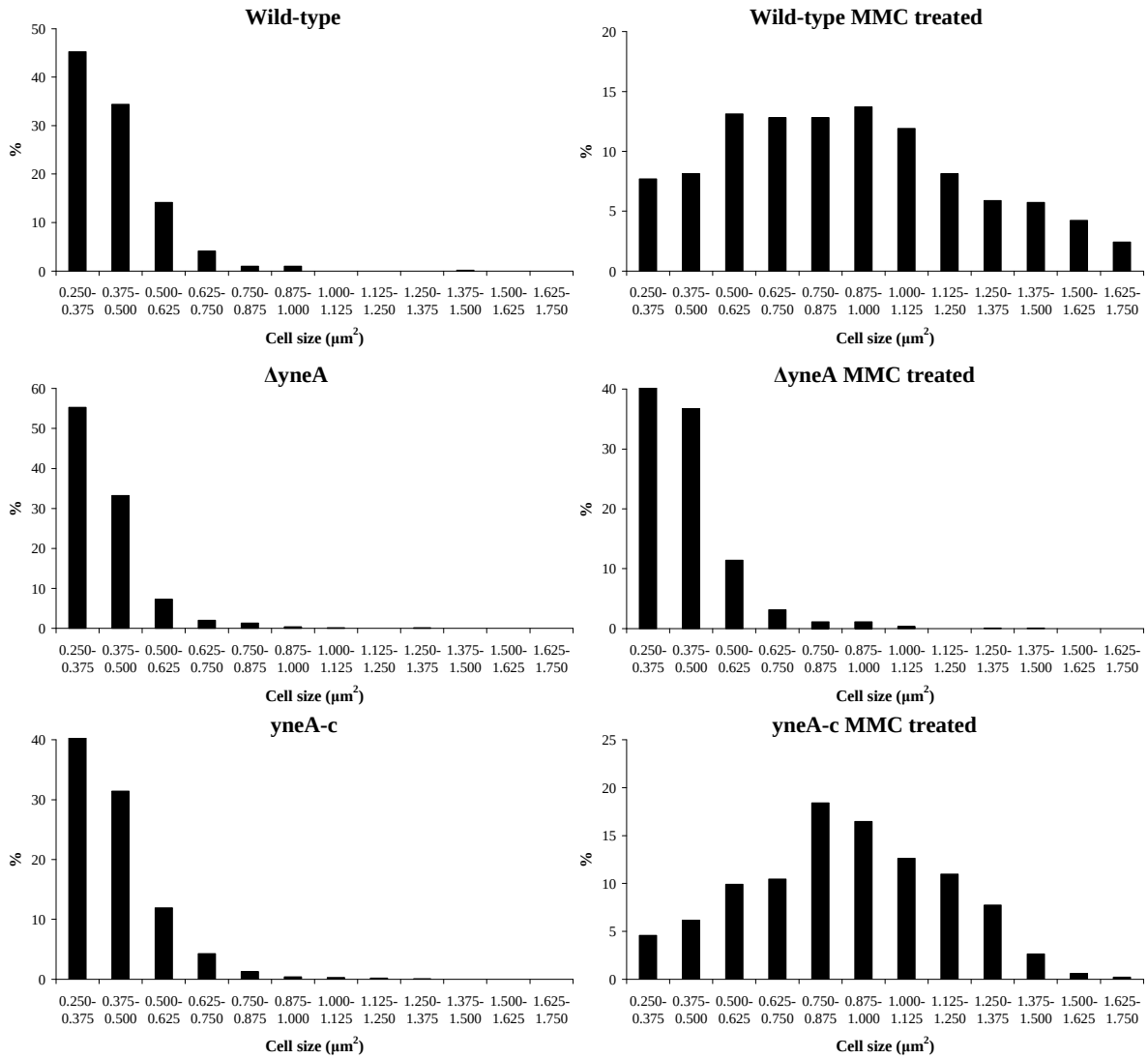
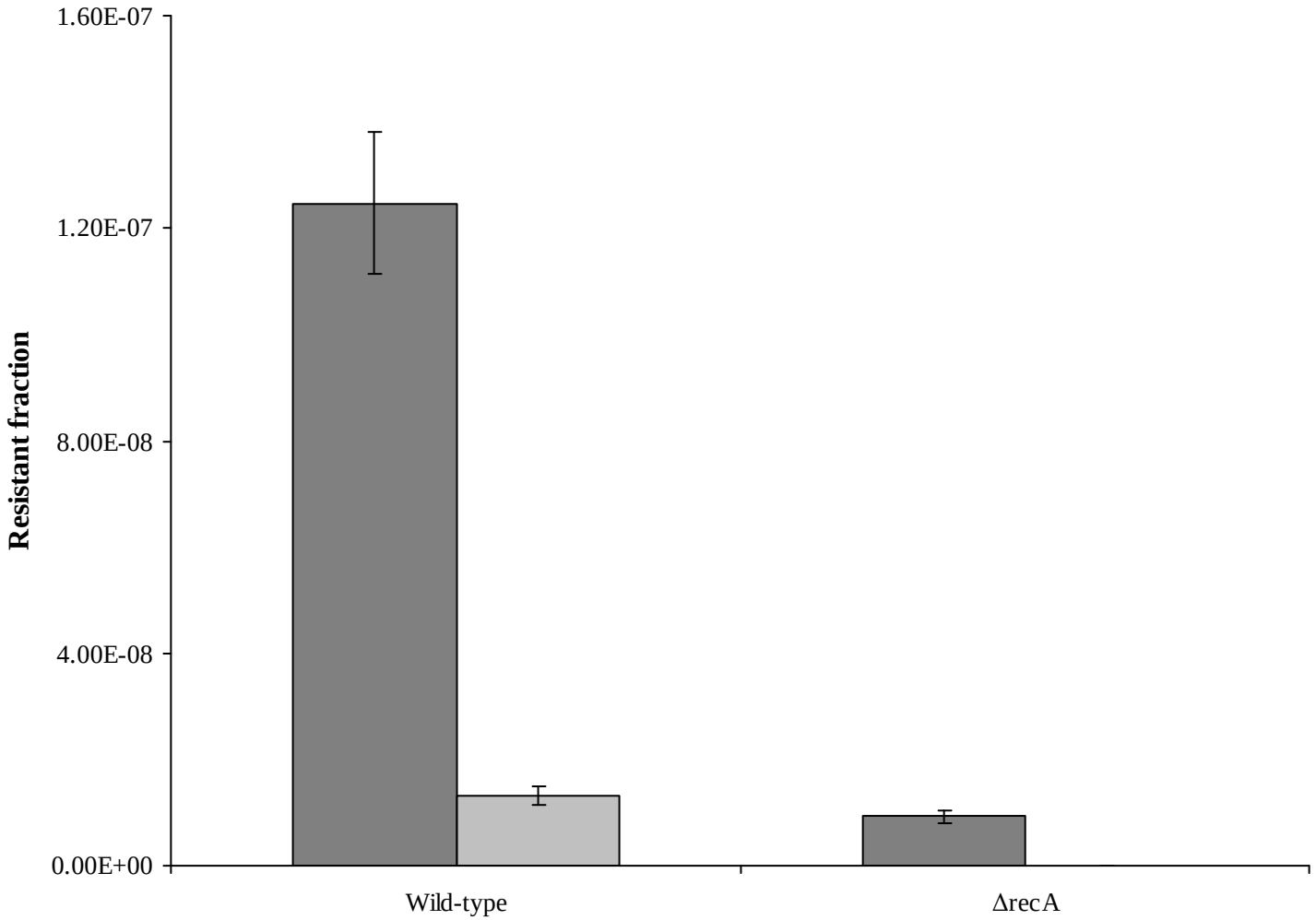
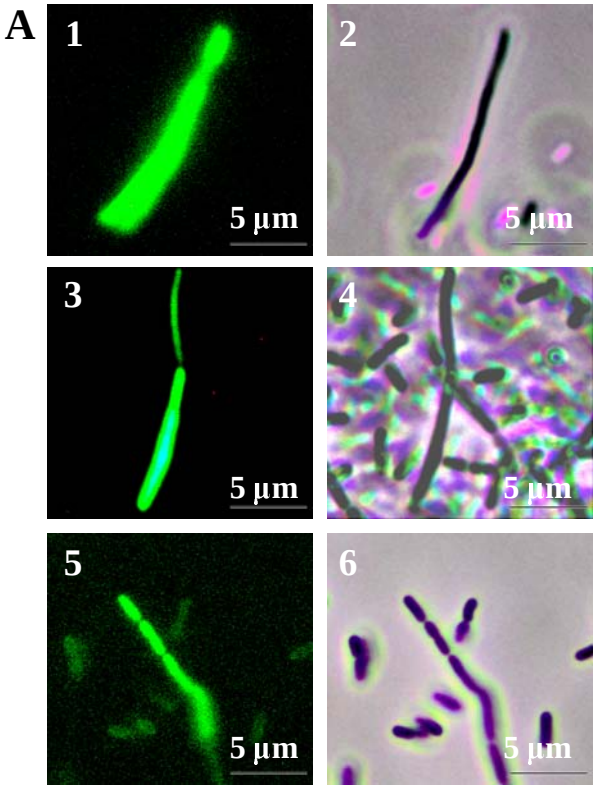
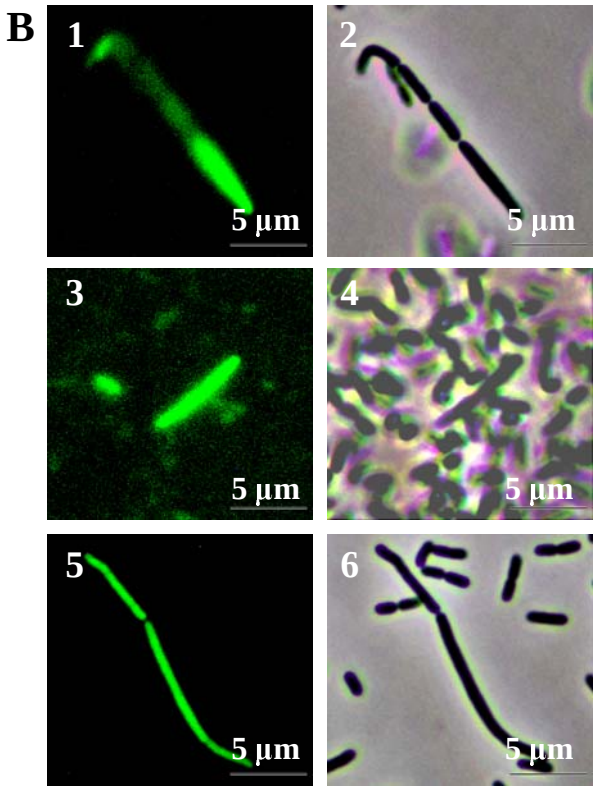
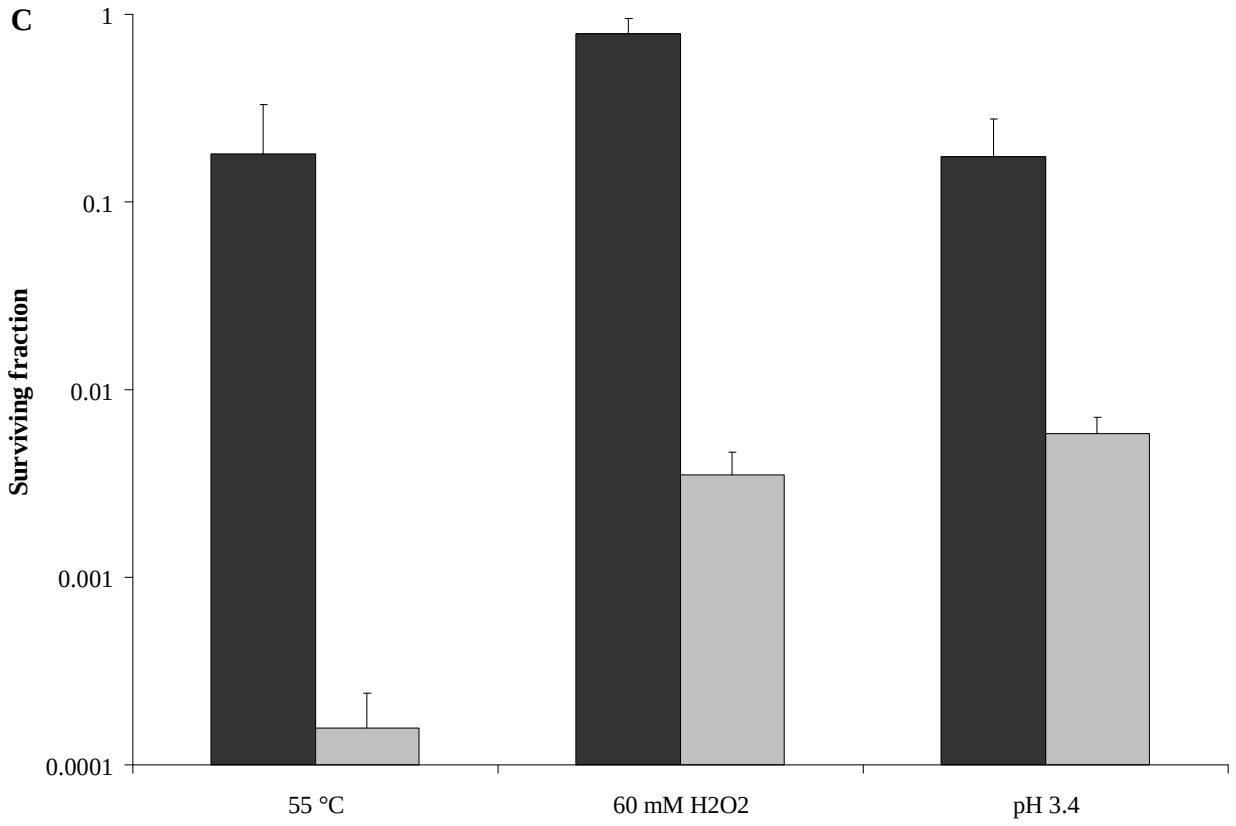

Wild-type
%
0
10
20
30
40
50
0.250-0.375
0.375-0.500
0.500-0.625
0.625-0.750
0.750-0.875
0.875-1.000
1.000-1.125
1.125-1.250
1.250-1.375
1.375-1.500
1.500-1.625
1.625-1.750
Cell size (µm2)
Wild-type MMC treated
%
0
5
10
15
20
0.250-0.375
0.375-0.500
0.500-0.625
0.625-0.750
0.750-0.875
0.875-1.000
1.000-1.125
1.125-1.250
1.250-1.375
1.375-1.500
1.500-1.625
1.625-1.750
Cell size (µm2)
ΔyneA
%
0
10
20
30
40
50
60
0.250-0.375
0.375-0.500
0.500-0.625
0.625-0.750
0.750-0.875
0.875-1.000
1.000-1.125
1.125-1.250
1.250-1.375
1.375-1.500
1.500-1.625
1.625-1.750
Cell size (µm2)
ΔyneA MMC treated
%
0
10
20
30
40
0.250-0.375
0.375-0.500
0.500-0.625
0.625-0.750
0.750-0.875
0.875-1.000
1.000-1.125
1.125-1.250
1.250-1.375
1.375-1.500
1.500-1.625
1.625-1.750
Cell size (µm2)
yneA-c
%
0
10
20
30
40
0.250-0.375
0.375-0.500
0.500-0.625
0.625-0.750
0.750-0.875
0.875-1.000
1.000-1.125
1.125-1.250
1.250-1.375
1.375-1.500
1.500-1.625
1.625-1.750
Cell size (µm2)
yneA-c MMC treated
%
0
5
10
15
20
25
0.250-0.375
0.375-0.500
0.500-0.625
0.625-0.750
0.750-0.875
0.875-1.000
1.000-1.125
1.125-1.250
1.250-1.375
1.375-1.500
1.500-1.625
1.625-1.750
Cell size (µm2)


1.60E-07
1.20E-07
8.00E-08
4.00E-08
0.00E+00
Resistant fraction
Wild-type
ΔrecA


A
1
5 µm
2
5 µm
3
5 µm
4
5 µm
5
5 µm
6
5 µm


B
1
5 µm
2
5 µm
3
5 µm
4
5 µm
5
5 µm
6
5 µm


C
1
0.1
0.01
0.001
0.0001
Surviving fraction
55 °C
60 mM H2O2
pH 3.4