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Contribution of *Listeria monocytogenes* RecA to acid and bile survival and invasion of human intestinal Caco-2 cells

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ABSTRACT

The food-borne pathogen *Listeria monocytogenes* is able to colonize the human gastro-intestinal tract and subsequently cross the intestinal barrier. Thus, for *L. monocytogenes* to become virulent, it must survive the low pH of the stomach, high bile concentrations in the small intestine, and invade the epithelial cells. In this study, we show that RecA, which is an important factor in DNA repair and the activator of the SOS response, contributes to the resistance against acid and bile and to the ability of *L. monocytogenes* to adhere and invade human intestine epithelial cells. Activation of *recA* was shown with a promoter reporter after exposure to low pH and high bile concentrations and during adhesion and invasion of Caco-2 intestinal epithelial cells. Furthermore, an in-frame *recA* deletion mutant showed reduced survival after exposure to low pH and high bile concentrations. This mutant also showed a deficiency in adhesion and invasion of Caco-2 cells. These results suggest that RecA may contribute to the colonization of the human gastro-intestinal tract and crossing of the intestinal barrier.

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Introduction

The food-borne pathogen *Listeria monocytogenes* is the causative agent of listeriosis, which is generally manifested as meningitis, encephalitis, sepsis, spontaneous abortion, or gastro-enteritis, and the affected population generally consists of elderly or immuno-compromised persons (Vazquez-Boland et al., 2001). The number of listeriosis cases is relatively low compared with other foodborne pathogens, but the mortality rate is extremely high (Mead et al., 1999). Furthermore, the incidence of listeriosis has been increasing in many European countries, making it a great concern for public health (Denny and McLauchlin, 2008). It has previously been estimated that 99% of the listeriosis cases are caused by contaminated food products (Mead et al., 1999). For *L. monocytogenes* to cause an infection following transmission through contaminated foods, it must survive the stressful environments in the gastro-intestinal (GI) tract, such as acid conditions in the stomach and high bile concentrations in the small intestine, and subsequently cross the intestinal barrier. The specific location of intestinal translocation remains controversial. It has been demonstrated that *L. monocytogenes* invades the basolateral surface of polarized, differentiated enterocytes in the intestinal epithelium, for which the specific mechanisms and factors involved have been investigated in great detail (reviewed in Cossart et al., 2003; Vazquez-Boland et al., 2001). However, the possibility that *L. monocytogenes* also penetrates M cells and subsequently translocates through the Peyer's patches and the mechanisms that might be involved in this process remain to be elucidated (Barbuddhe and Chakraborty, 2009).

The ability of *L. monocytogenes* to survive the acidic environment of the stomach is essential in order to colonize host organisms. *L. monocytogenes* contains several mechanisms that attribute to its acid survival capacity. *L. monocytogenes* contains glutamate decarboxylase (GAD) systems that consume intracellular protons by converting glutamate to γ-aminobutyrate (Cotter et al., 2001), an arginine deiminase (ADI) system that converts arginine to ornithine, carbon dioxide, and ammonia, thereby increasing the cytoplasmic pH (Ryan et al., 2009), and a proton-extruding F_1F_0-ATPase that has been shown to be important for pH homeostasis (Cotter et al., 2000). *L. monocytogenes* must subsequently survive the bactericidal activities of bile in the small intestine. Three specific bile resistance systems have been described for *L. monocytogenes* thus far, namely a bile salt hydrolase (BSH) (Dussurget et al., 2002), a bile acid dehydratase (BtlB) (Begley et al., 2005), and a bile exclusion system (BilE) (Sleator et al., 2005). Interestingly, a role for UvrA, which is an important component of the nucleotide excision repair pathway, was shown for *L. monocytogenes* during growth under acidic conditions and in the presence of bile (Kim et al., 2006). It has previously been shown in several organisms that both acid and bile exposure could result in DNA damage (Jeong et al., 2008; Merritt and Donaldson, 2009; Prieto et al., 2006), which could explain the role of UvrA in acid and bile resistance. In *L. monocytogenes*, *uvrA* is

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a member of the SOS response regulon (van der Veen et al., 2010), which is a pathway that is involved in DNA repair and restart of stalled or collapsed replication forks (Lusetti and Cox, 2002).

The *L. monocytogenes* SOS response regulon consists of 29 genes encoding proteins with functions in DNA repair and translesion DNA synthesis and also includes the bile exclusion system BilE (van der Veen et al., 2010). The SOS response is regulated by the RecA activator and the LexA repressor (Butala et al., 2009; Cox, 2007b). LexA is an autoregulatory repressor that binds to the imperfect palindrome AATAAGAACATATGTTCGTTT in the promoter of the SOS response genes of *L. monocytogenes* (van der Veen et al., 2010). Accumulation of single-stranded DNA in the cell results in the formation of RecA nucleoprotein filaments that facilitate autocleavage of LexA and induction of the SOS response (Arenson et al., 1999). Besides activating the SOS response, RecA is directly involved in several DNA repair and recombination processes (reviewed in Cox, 2007a,b). For *L. monocytogenes*, a role for RecA in acid resistance was identified recently (van der Veen et al., 2010). In that study, a deficiency in acid resistance was shown for a Δ*recA* deletion mutant after exposure of exponentially growing cells to pH 3.4. However, the human gastric pH can reach values down to pH 2 (Audia et al., 2001), and the role of RecA in the resistance of *L. monocytogenes* to such low pH conditions remains to be determined. Furthermore, it has been shown that *recA* is activated during growth of *L. monocytogenes* in a murine macrophage cell line (Chatterjee et al., 2006), indicating that RecA might be involved in the pathogenicity of this organism.

In this study, we investigated the role of RecA in acid and bile resistance and in adhesion and invasion of Caco-2 cells, to obtain insight in the possible role of RecA during colonization and infection of the human host.

Materials and methods

Strains and growth conditions

L. monocytogenes EGD-e (Glaser et al., 2001), its isogenic in-frame Δ*recA* deletion mutant (van der Veen et al., 2010), and its *recA* promoter reporter mutant EGD-e:PrecA-EGFP (enhanced GFP) (van der Veen et al., 2010) were stored in brain heart infusion (BHI) broth (Becton Dickinson, Le Pont de Claix, France) containing 15% sterile glycerol (Fluka, Buchs, Switzerland) at −80 °C. Single colonies were inoculated in 10 ml BHI broth in 50 ml polypropylene tubes (Greiner Bio-One, Frickenhausen, Germany) and grown overnight at 37 °C and 200 rpm (New Brunswick type Innova 4000). Overnight-grown cultures were inoculated (1%) in 10 ml BHI in 100 ml shake flasks and grown at 37 °C and 200 rpm until an absorbance (OD_{600}) of approximately 0.5 was obtained for an exponential-phase culture or for 24 h for a stationary-phase culture.

Acid and bile resistance of wild type and ΔrecA strains

Exponential- and stationary-phase cultures of the wild type and Δ*recA* mutant strain were centrifuged (10 min, 5000 × *g*, 24 °C, Eppendorf type 5804 R), and pellets were dissolved in 10 ml pre-warmed (37 °C) BHI adjusted with 37% HCl (Merck, Darmstadt, Germany) to pH 1.8, pH 2.6, pH 3.4, or pH 7.4 (negative control) for acid exposure or pre-warmed (37 °C) BHI containing 0% (negative control) or 20% oxbile (Oxoid, Basingstoke, UK) for bile exposure. Cultures were incubated at 37 °C, and samples of 0.1 ml were collected up to 1 h for acid-exposed cultures and up to 20 min for bile-exposed cultures. Samples were neutralized by diluting (1/10) in D/E neutralizing broth (Becton Dickinson, Le Pont de Claix, France), serially diluted in phosphate buffered saline (PBS) (Merck, Darmstadt, Germany), and appropriate dilutions were plated on BHI agar. Plates were incubated at 37 °C for 3–5 days, and colonies were enumerated. Stress experiments were performed in 3 biological independent replicates on different days.

Adhesion and invasion of Caco-2 intestine epithelial cells

Caco-2 human intestine epithelial cells (ATCC number HTB37) were seeded at a density of 5×10^5 cells/well in 12-well tissue culture plates (Cornig, Schiphol-Rijk, The Netherlands) containing 1 ml/well tissue culture medium (TCM), which is composed of Dulbecco's Modified Eagle's Medium (DMEM) with addition of 25 mM Hepes, 4.5 g/l glucose, 10% heat-inactivated fetal bovine serum (30 min at 56 °C), 1% MEM non-essential amino acids, 20 mM L-glutamine, and 50 μg/ml gentamycin (all components obtained from Invitrogen, Breda, The Netherlands). Cells were incubated for 21 days at 37 °C in 5% CO_2 to grow to a confluent monolayer of differentiated cells. Cells at passage 44–47 were used for all experiments. One hour prior to the adhesion and invasion experiments, the medium was replaced with pre-warmed TCM without gentamycin. Exponential- and stationary-phase bacteria were centrifuged (0.5 ml, 2 min, 5000 × *g*, 24 °C, Heraeus type Fresco 17), and pellets were washed 3 times with an equal volume of TCM without gentamycin, diluted in pre-warmed TCM without gentamycin, and added to the Caco-2 monolayers with a multiplicity of infection (MOI) of 100. The bacterial suspension was removed after 1 h incubation at 37 °C in 5% CO_2. For adhesion experiments, the Caco-2 monolayers were washed 3 times with 1 ml pre-warmed PBS. Caco-2 cells were lysed with 1 ml of 1% Triton-X100 (Sigma–Aldrich, Steinheim, Germany) in PBS and serial diluted in PBS. Appropriate dilutions were plated on BHI agar, and bacteria were enumerated after 3 days incubation at 37 °C. For invasion experiments, the TCM was replaced with 1 ml TCM containing 300 μg/ml gentamycin, and the cells were incubated for 3 h at 37 °C in 5% CO_2. The Caco-2 monolayers were washed 3 times with 1 ml pre-warmed PBS and lysed with 1 ml of 1% Triton-X100 in PBS. Cells were serial diluted in PBS, and appropriate dilutions were plated on BHI agar. Bacteria were enumerated after 3 days incubation at 37 °C. All adhesion and invasion experiments were performed in 3 biological independent replicates.

Microscopy

Phase contrast and fluorescence microscopy experiments were performed on a BX41 microscope (Olympus, Zoeterwoude, The Netherlands) using the EGD-e:PrecA-EGFP mutant and following the procedures described above. Images were acquired using a XC30 camera (Olympus) and Olympus Cell^B software. Neutralized bacterial samples of the stress experiments were centrifuged (1 ml, 2 min, 5000 × *g*, 24 °C, Heraeus type Fresco 17), and pellets were dissolved in PBS. Samples (5 μl) were loaded on microscope slides (76 mm × 26 mm), and square cover glasses (18 mm) were placed on top of the samples. EGFP was visualized using the MNIBA3 filter (Olympus). To visualize activation of EGFP expression during adhesion and invasion of the EGD-e:PrecA-EGFP mutant to Caco-2 cells, the cells were washed with PBS, and square cover glasses were placed on top. Furthermore, a control experiment was performed by incubating the EGD-e:PrecA-EGFP mutant for 1 or 4 h in TCM without gentamycin. Samples were centrifuged (1 ml, 2 min, 5000 × *g*, 24 °C, Heraeus type Fresco 17), and pellets were dissolved in PBS. Five microlitres of the samples were loaded on microscope slides and covered with cover glasses before visualization of EGFP expression.

Statistical analyses

Significant differences in acid resistance, bile resistance, and adhesion and invasion of Caco-2 cells were identified using the 2-tailed homoscedastic Student's *t*-test ($p < 0.05$) in Excel.

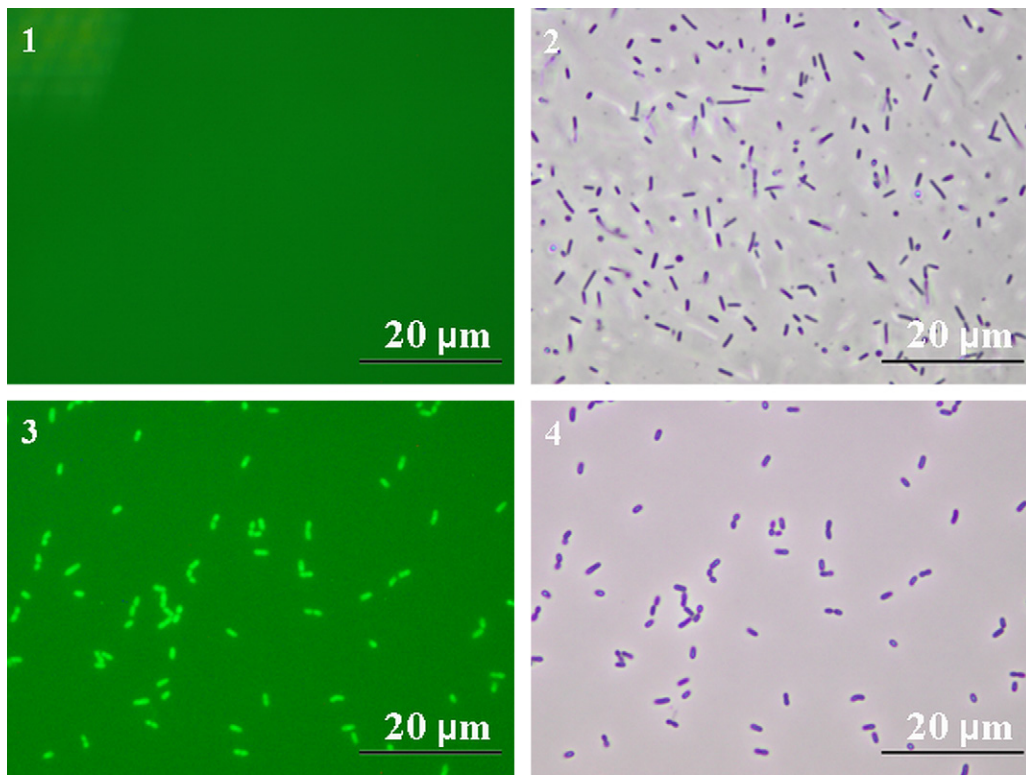

Fig. 1. Activation of *recA* in exponential-phase cells after exposure for 1 h to BHI at pH 7.4 (1 and 2) or pH 1.8 (3 and 4). Micrographs show fluorescence (1 and 3) and phase contrast (2 and 4) pictures of cells expressing EGFP from the *recA* promoter.

Results

RecA-dependent acid resistance

The role of *recA* in acid resistance was investigated by exposing exponential- and stationary-phase cells of the wild type strain, the Δ*recA* mutant, and the EGD-e:PrecA-EGFP mutant to pH 1.8, pH 2.6, pH 3.4, and pH 7.4. No significant difference was observed in growth at 37 °C in BHI between the wild type strain and the Δ*recA* mutant ($p < 0.05$, *t*-test) (Supplemental Fig. 1). Exposure of the EGD-e:PrecA-EGFP mutant for 1 h to the acid conditions resulted in visible expression of EGFP in both exponential- and stationary-phase cells, while no EGFP expression was observed during exposure at pH 7.4 (Fig. 1 and Supplemental Fig. 2). Furthermore, both exponential- and stationary-phase cells of the wild type strain showed higher resistance against acid exposure than exponential- and stationary-phase cells of the Δ*recA* mutant (Fig. 2). No inactivation during exposure to pH 7.4 was observed for both wild type and Δ*recA* mutant strains (results not shown). Exponential-phase cells of the wild type strain showed higher resistance against acid exposure at pH 1.8, pH 2.6, and pH 3.4 compared with exponential-phase cells of the Δ*recA* mutant (Fig. 2A), while a difference in acid resistance of stationary-phase cells of the wild type and the Δ*recA* mutant strain was in particular observed at pH 1.8 (Fig. 2B). A difference between the wild type and the Δ*recA* mutant was also observed at pH 2.6, but only after 60 min exposure. Stationary-phase cells of both the wild type and the Δ*recA* mutant strains showed higher resistance to acid exposure compared with exponential-phase cells, but survival of the wild type was significantly higher at the lower pH values tested. These promoter reporter and inactivation experiments show that *recA* is activated after exposure to acidic conditions and that RecA is an important factor for acid survival.

RecA-dependent bile resistance

To investigate the role of RecA in bile resistance of *L. monocytogenes*, exponential- and stationary-phase cells of the wild type strain, the Δ*recA* mutant, and the EGD-e:PrecA-EGFP mutant were exposed for 20 min to 20% oxbile. Exposure of exponential- and stationary-phase cells of the EGD-e:PrecA-EGFP mutant to 20% oxbile resulted in visible expression of EGFP, while no expression was observed during exposure to 0% oxbile (Fig. 3 and Supplemental Fig. 3). Also, exponential- and stationary-phase cells of the Δ*recA* mutant showed lower survival after exposure to 20% oxbile compared with cells from the wild type strain (Fig. 4), while no inactivation during incubation in 0% oxbile was observed for both strains (results not shown). In particular, exponential-phase cells of the Δ*recA* mutant appeared to be very sensitive to bile exposure. Rapid 5–6 log inactivation was observed for exponential-phase cells of the Δ*recA* mutant within 5 min exposure. Also exponential- and stationary-phase cells of the wild type strain and stationary-phase cells of the Δ*recA* mutant showed 1–2 log inactivation within 5 min exposure and a tail afterwards, pointing to the presence of a resistant fraction. These inactivation experiments show that RecA is an important component for the resistance of cells against bile exposure.

RecA-dependent adhesion and invasion of Caco-2 cells

To investigate the role of RecA in *L. monocytogenes* adhesion and invasion of human intestine epithelial cells, Caco-2 adhesion and invasion assays were performed with exponential- and stationary-phase cells of the wild type strain, the Δ*recA* mutant, and the EGD-e:PrecA-EGFP mutant. Adhesion and invasion assays with exponential- and stationary-phase cells of the EGD-e:PrecA-EGFP mutant resulted in visible expression of EGFP, while no EGFP

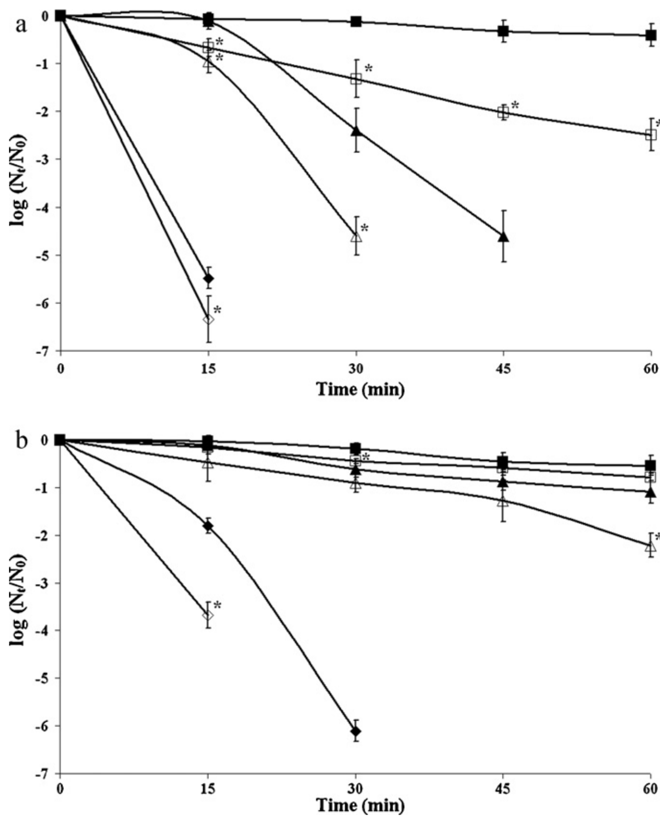

Fig. 2. RecA is involved in the survival of acid exposure. The graphs present the average and standard deviation of the survival of exponential- (A) phase and stationary- (B) phase cells of the wild type strain (closed symbols) and the Δ*recA* mutant (open symbols) after exposure for 1 h to pH 1.8 (diamonds), pH 2.6 (triangles), and pH 3.4 (squares). Data points below the detection limit [$\log(N_t/N_0) \approx -6.5$] are not shown in the graphs. *Data points significantly different from the wild type strain ($p < 0.05$, *t*-test).

expression was observed during incubation for 1 or 4 h in TCM without gentamycin (Fig. 5 and Supplemental Fig. 4). These results suggest that *recA* is specifically activated during adhesion and invasion of Caco-2 human intestine epithelial cells. Furthermore, both exponential- and stationary-phase cells of the Δ*recA* mutant showed a reduction in adhesion and invasion of Caco-2 cells compared with the wild type strain (Fig. 6). These results demonstrate that RecA is required to reach wild type levels of adhesion and invasion of Caco-2 human intestinal epithelial cells.

Discussion

In this study, the contribution of RecA to acid and bile resistance and adhesion and invasion of Caco-2 intestine epithelial cells was assessed in *L. monocytogenes*. The ability of pathogens such as *L. monocytogenes* to adapt to and survive the stressful environments encountered during colonization of the human host is considered one of the important attributes of their success. *L. monocytogenes* must withstand the extreme low pH in the stomach and subsequently the high bile concentrations in the small intestine. Several specific acid and bile resistance mechanisms have been identified in *L. monocytogenes* that actively prevents their damaging activity, such as GAD, ADI, F_1F_0-ATPase, BSH, BilE, and BtlB (Begley et al., 2005; Cotter et al., 2000, 2001; Dussurget et al., 2002; Ryan et al., 2009; Sleator et al., 2005). However, less attention has been given to the role of systems that repair the possible damage of cellular components by acid and bile when these specific resistance mechanisms turn out not to be sufficient for complete resistance. It has previously been shown for several organisms that acid and bile exposure can result in DNA damage (Bernstein et al., 1999; Jeong et al., 2008; Merritt and Donaldson, 2009; Prieto et al., 2004, 2006). In some of these studies a role for RecA or other SOS response components in DNA repair after damage caused by acid or bile was shown. These results are in line with our study that shows that RecA contributes to the resistance after exposure

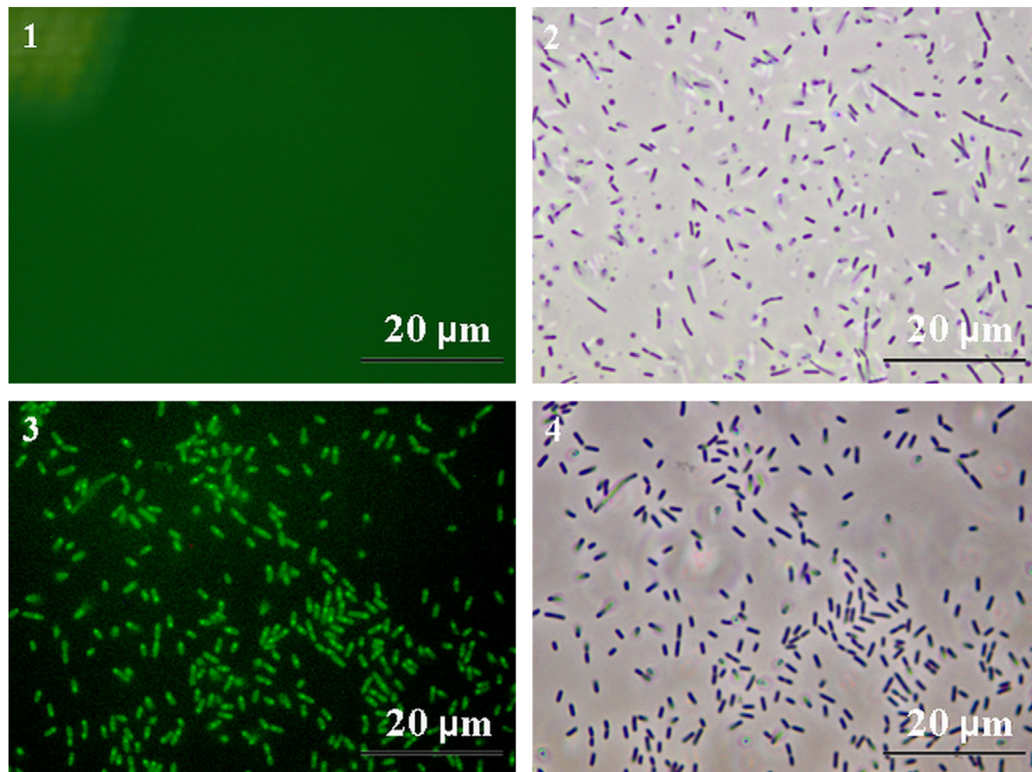

Fig. 3. Activation of *recA* in exponential-phase cells after exposure for 20 min to BHI containing 0% (1 and 2) or 20% (3 and 4) oxbile. Micrographs show fluorescence (1 and 3) and phase contrast (2 and 4) pictures of cells expressing EGFP from the *recA* promoter.

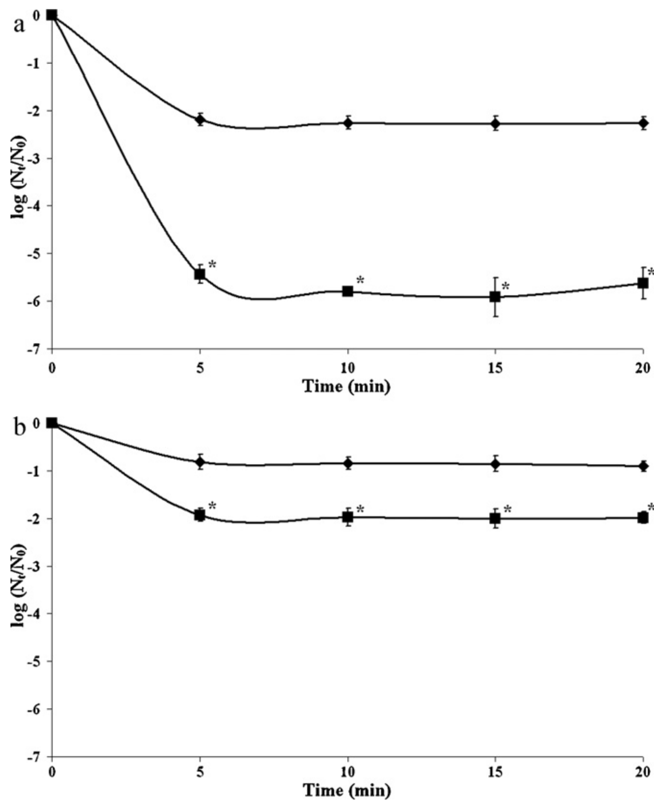

Fig. 4. RecA is involved in the survival of oxbile. The graphs present the average and standard deviation of the survival of exponential- (A) phase and stationary- (B) phase cells of the wild type strain (diamonds) and the Δ*recA* mutant (squares) after exposure for 20 min to 20% oxbile. *Data points significantly different from the wild type strain ($p < 0.05$, *t*-test).

to acid and bile. Furthermore, it has previously been shown that the specific bile exclusion system, BilE, is a member of the RecA-regulated SOS response regulon of *L. monocytogenes* (van der Veen et al., 2010). Therefore, the reduced resistance of the Δ*recA* mutant against a bile treatment might be explained by the reduced expression of *bilE* in this mutant. Interestingly, stationary-phase cells of both wild type and Δ*recA* mutant strains showed higher resistance against acid and bile exposure compared with exponential-phase cells. This might be related with the SigB-dependent induction of several of the specific *L. monocytogenes* acid and bile resistance mechanisms upon entry in the stationary phase (Ferreira et al., 2003; Kazmierczak et al., 2006). SigB is an autoregulatory alternative sigma factor that regulates the expression of the class II stress genes (Becker et al., 1998), which include *gad*, *bsh*, and the RecA-regulated operon *bilE* (Sue et al., 2003; van der Veen et al., 2010; Wemekamp-Kamphuis et al., 2004).

Caco-2 human intestinal epithelial cells were used to explore the contribution of RecA to translocation of *L. monocytogenes* from the GI tract and crossing of the intestinal barrier. *L. monocytogenes* is internalized in the intestinal epithelium through the interactions of its internalin proteins InlA and InlB with the host receptors E-cadherin and c-Met, respectively, in the multicellular adherens junctions of intestinal epithelial cells (Bonazzi et al., 2008; Pentecost et al., 2010). Other virulence genes that are involved in the intracellular survival of *L. monocytogenes* and subsequent escape and cell-to-cell spread include the genes of the LIPI-1 pathogenicity island, which encode the master virulence regulator PrfA, the phospholipase C proteins PlcA and PlcB, the pore-forming toxin listeriolysin O (LLO), the metalloprotease mpl, and actin-based motility mediator ActA (reviewed in Hamon et al., 2006; Vazquez-Boland et al., 2001). Our results now show that expression of *recA* is induced during adhesion and invasion of Caco-2 cells and that a Δ*recA* mutant is impaired in these capabilities, which suggests that RecA may contribute to adhesion and inva-

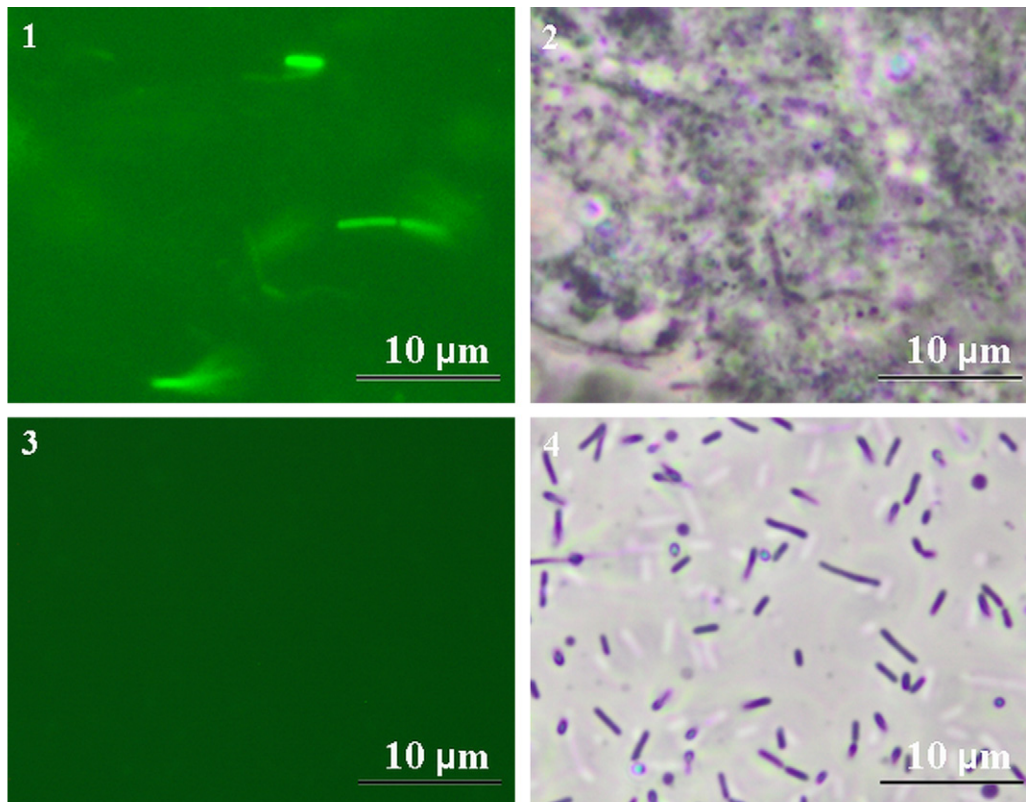

Fig. 5. Activation of *recA* during adhesion of exponential-phase cells to Caco-2 cells. Micrographs show fluorescence (1 and 3) and phase contrast (2 and 4) pictures of cells expressing EGFP from the *recA* promoter after adhesion to Caco-2 cells (1 h) (1 and 2) and after exposure for 1 h to TCM without gentamycin (3 and 4).

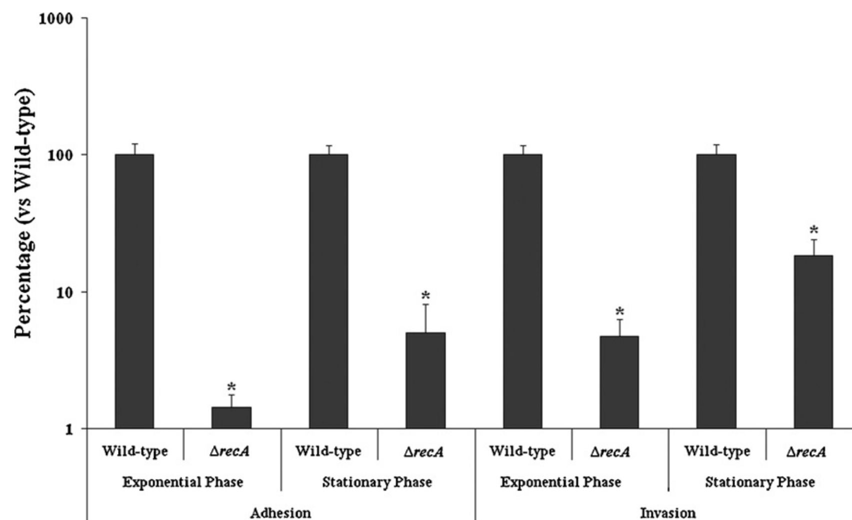

Fig. 6. RecA is involved in the adhesion and invasion of Caco-2 cells. The graph shows the average and standard deviation of the adhesion and invasion of exponential- and stationary-phase cells of the wild type strain and the Δ*recA* mutant to Caco-2 cells. All values are relative to the wild type strain, which was set at 100% for each condition. *Significantly different from the wild type strain ($p < 0.05$, *t*-test).

sion of intestine epithelial cells. It has been reported that other stress response genes could also play a role in adhesion and invasion of Caco-2 cells. The CtsR-regulated class III heat-shock Clp-ATPase ClpC was shown to positively affect the expression of *actA* and *inlAB*, and a Δ*clpC* mutant showed a deficiency in adhesion and invasion of Caco-2 cells (Nair et al., 2000). Furthermore, it has also been shown that the class II stress response regulator SigB contributes to the invasion of Caco-2 cells, which was related to its control of the expression of the genes encoding the internalins InlA and InlB (Kim et al., 2004, 2005). Crosstalk and coregulation between SigB and the major virulence regulator PrfA have also been shown previously (Kazmierczak et al., 2003; Milohanic et al., 2003; Nadon et al., 2002). The mechanisms underlying the contribution of RecA to *L. monocytogenes* adhesion and invasion of Caco-2 human intestinal epithelial cells remain to be investigated.

In conclusion, this study shows that RecA contributes to acid and bile resistance and to adhesion and invasion of Caco-2 cells. While additional studies are necessary to investigate the specific mechanisms in RecA-dependent stress resistance and interaction with intestine epithelial cells, our results suggest that RecA may contribute to *L. monocytogenes* colonization of the GI tract and translocation across the intestinal barrier.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijmm.2010.11.006.

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