HrcA and DnaK are important for static and continuous-flow biofilm formation and disinfectant resistance in *Listeria monocytogenes*

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The food-borne pathogen *Listeria monocytogenes* is able to form biofilms in food processing environments. Since biofilms are generally difficult to eradicate during clean-up procedures, they pose a major risk for the food industry. Stress resistance mechanisms involved in *L. monocytogenes* biofilm formation and disinfectant resistance have, to our knowledge, not been identified thus far. In this study, we investigated the role of *hrcA*, which encodes the transcriptional regulator of the class I heat-shock response, and *dnaK*, which encodes a class I heat-shock response chaperone protein, in static and continuous-flow biofilm formation and resistance against benzalkonium chloride and peracetic acid. Induction of both *hrcA* and *dnaK* during continuous-flow biofilm formation was observed using quantitative real-time PCR and promoter reporters. Furthermore, in-frame deletion and complementation mutants of *hrcA* and *dnaK* revealed that HrcA and DnaK are required to reach wild-type levels of both static and continuous-flow biofilms. Finally, disinfection treatments of planktonic-grown cells and suspended static and continuous-flow biofilm cells of wild-type and mutants showed that HrcA and DnaK are important for resistance against benzalkonium chloride and peracetic acid. In conclusion, our study revealed that HrcA and DnaK are important for *L. monocytogenes* biofilm formation and disinfectant resistance.

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INTRODUCTION

The food-borne pathogen *Listeria monocytogenes* is frequently encountered in food processing facilities (Chasseignaux *et al.*, 2002; Pritchard *et al.*, 1995; Tompkin, 2002), where it most likely survives in the form of biofilms. Biofilms are generally considered to be more resistant to antimicrobial agents and disinfectants than planktonic cells (Lewis, 2001; Mah & O'Toole, 2001; Robbins *et al.*, 2005), which poses a major threat for the food industry. Some studies have shown that *L. monocytogenes* biofilms are more resistant to disinfectants than are planktonic-grown cells (Berrang *et al.*, 2008; Folsom & Frank, 2006; Pan *et al.*, 2006). Several mechanisms have been proposed to explain the increased resistance of biofilms against disinfectants, such as the restricted penetration of the biofilm, the slow growth rate of biofilm cells and the induction of resistance mechanisms in the biofilm (Lewis, 2001). It has been suggested that biofilms contain many microniches in which bacteria are stressed and consequently induce stress resistance mechanisms (Costerton *et al.*, 1995). However, it has also been shown previously that detached biofilms are not more resistant to disinfectants than

planktonic cells (Kastbjerg & Gram, 2009; Stopforth *et al.*, 2002), which might indicate that resistance is dependent on the restricted penetration of the biofilm.

For *L. monocytogenes* biofilms, two specific morphotypes have been described thus far. Static biofilms generally consist of small rod-shaped cells, which form microcolonies or homogeneous layers (Kalmokoff *et al.*, 2001; Rodriguez *et al.*, 2008). In contrast, continuous-flow biofilms consist of a network of elongated cells that form knitted chains and surround ball-shaped microcolonies (Rieu *et al.*, 2008). An important role for motility was shown in these types of biofilms. Although loss of motility initially decreased surface attachment, in later stages the formation of hyperbiofilms was observed (Todhanakasem & Young, 2008). Furthermore, continuous-flow biofilm formation is dependent on the activation of the SOS response (van der Veen & Abee, 2010). So far, little is known about the function and expression of other stress response mechanisms during static and continuous-flow biofilm formation and their impact on the resistance of biofilms to disinfectants.

In this study, we investigated the role of the class I heat-shock response in *L. monocytogenes* biofilm formation and

Abbreviation: EGFP, enhanced GFP.

disinfectant resistance. The class I heat-shock response contains molecular chaperones that are activated under conditions that result in the accumulation of damaged, denatured or aggregated proteins and provides protection or reactivates these proteins (Narberhaus, 1999). We selected to study *hrcA*, which encodes the regulator of the class I heat-shock response, and *dnaK*, which encodes a molecular chaperone that is important for protein protection and disaggregation of denatured proteins (Diamant *et al.*, 2000). Maintenance of protein quality is essential during exposure to stress conditions (Liberek *et al.*, 2008) and *dnaK* may therefore be a very important factor during biofilm formation and disinfection resistance. The class I heat-shock response is controlled by the auto-regulatory HrcA transcriptional repressor, which was first shown in *Bacillus subtilis* to recognize the CIRCE operator sequence (TTAGCACTC-N₉-GAGTGCTAA) located in front of these genes (Schulz & Schumann, 1996). In *L. monocytogenes*, CIRCE operator sequences were later also identified in front of the class I heat-shock genes (Gahan *et al.*, 2001; Hanawa *et al.*, 2000), and HrcA-dependent regulation of these genes has been confirmed with transcription and mutant analyses (Hu *et al.*, 2007). *hrcA* and *dnaK* are located within the *hrcA-grpE-dnaK* operon, which contains both a SigB and a SigA promoter (Hu *et al.*, 2007). A second SigB promoter is located upstream of the *dnaK* coding region. Comparison of the transcription profiles of a wild-type strain and its isogenic $\Delta hrcA$ mutant

showed that HrcA is also indirectly involved in the induction and repression of 56 other genes encoding proteins with functions in resistance to heat, cold, acid and oxidative stress (Hu *et al.*, 2007). In the current study, we investigated the role of HrcA and its regulon member DnaK in *L. monocytogenes* biofilm formation and disinfection resistance.

METHODS

Strains, media and plasmids. *L. monocytogenes* strains EGD-e and mutants thereof (Table 1) were grown in brain heart infusion (BHI) broth (Becton Dickinson). The temperature-sensitive suicide plasmid pSvS26 was constructed by cloning the *XhoI*-*ScaI*-digested origin of replication fragment from vector pGH9:ISS1 in the *NheI*-*ScaI*-digested plasmid pNZ5319. The $\Delta hrcA$ and $\Delta dnaK$ deletion mutants were constructed with plasmids pSvS38 and pSvS34, which contain the flanking regions of these genes, and the primers *hrcA*-1 to *hrcA*-4 for $\Delta hrcA$ and *dnaK*-1 to *dnaK*-4 for $\Delta dnaK$ (Table 2) following the protocol described previously (Lambert *et al.*, 2007). This resulted in a 1014 bp internal in-frame deletion for *hrcA* and a 1818 bp internal in-frame deletion for *dnaK*. Both deletions were verified by sequencing. Genomic expressed promoter reporter strains were constructed using the site-specific integration plasmids pIMK-Pr.*hrcA*-EGFP and pIMK-Pr.*dnaK*-EGFP. These plasmids are derivatives of plasmid pIMK2-EGFP. The promoter regions of *hrcA*, containing both SigA and SigB promoters, and of *dnaK*, including the specific SigB promoter located in front of *dnaK*, were amplified using primers *hrcA*-5, *hrcA*-7, *dnaK*-5 and *dnaK*-7 and cloned into pIMK2-EGFP as a *SacI*-*NcoI* fragment, which replaced the constitutive active

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristics	Reference
<i>L. monocytogenes</i>		
EGD-e	Wild-type serotype 1/2a strain	Glaser <i>et al.</i> (2001)
$\Delta hrcA$	EGD-e $\Delta hrcA$	This study
$\Delta dnaK$	EGD-e $\Delta dnaK$	This study
EGD-e:Pr. <i>hrcA</i> -EGFP	EGD-e, genomic expression of EGFP from the <i>hrcA</i> promoter using plasmid pIMK-Pr. <i>hrcA</i> -EGFP	This study
EGD-e:Pr. <i>dnaK</i> -EGFP	EGD-e, genomic expression of EGFP from the <i>dnaK</i> promoter using plasmid pIMK-Pr. <i>dnaK</i> -EGFP	This study
<i>hrcA</i> -c	EGD-e $\Delta hrcA$: genomic complementation of <i>hrcA</i> using plasmid pIMK- <i>hrcA</i>	This study
<i>dnaK</i> -c	EGD-e $\Delta dnaK$: genomic complementation of <i>dnaK</i> using plasmid pIMK- <i>dnaK</i>	This study
Plasmids		
pNZ5319	Cm ^r Em ^r ; cloning plasmid for gene replacements in Gram-positive bacteria	Lambert <i>et al.</i> (2007)
pGHost9:ISS1	Em ^r ; plasmid for random mutagenesis of Gram-positive bacteria	Maguin <i>et al.</i> (1996)
pSvS26	Cm ^r Em ^r ; pNZ5319 derivative containing the temperature-sensitive origin of replication of plasmid pGHost9:ISS1	This study
pSvS38	Cm ^r Em ^r ; pSvS26 derivative containing homologous regions up- and downstream of EGD-e <i>hrcA</i>	This study
pSvS34	Cm ^r Em ^r ; pSvS26 derivative containing homologous regions up- and downstream of EGD-e <i>dnaK</i>	This study
pIMK	Kan ^r ; site-specific listerial integrative vector	Monk <i>et al.</i> (2008)
pIMK2-EGFP	Kan ^r ; pIMK2 derivative containing EGFP	van der Veen <i>et al.</i> (2010)
pIMK-Pr. <i>hrcA</i> -EGFP	Kan ^r ; pIMK2-EGFP derivative containing the <i>hrcA</i> promoter in front of EGFP	This study
pIMK-Pr. <i>dnaK</i> -EGFP	Kan ^r ; pIMK2-EGFP derivative containing the <i>dnaK</i> promoter in front of EGFP	This study
pIMK- <i>hrcA</i>	Kan ^r ; pIMK2-EGFP derivative containing the <i>hrcA</i> gene	This study
pIMK- <i>dnaK</i>	Kan ^r ; pIMK2-EGFP derivative containing the <i>dnaK</i> gene	This study

Table 2. PCR primers used in this study

Primer	Sequence (5'–3')*
hrcA-1	AACCCTCGAGCTACAAGCAATGCAAGATCAC
hrcA-2	TTCTGTAAACATAGCAATCACC
hrcA-3	AACCAAAATTAGGAAGGCTG
hrcA-4	AACCAGATCTGTGCATCATTGAAGTAAGCTG
hrcA-5	GTGGGAGCTCGCAAGATAGTGATGAAAATGC
hrcA-6	GTGGGTCGACTGTAACATTTGAAAGAGATGTTG
hrcA-7	GTGGCCATGGTTATTTCTCCTGTTATTTCAG
hrcA-fwd	TTCCCGATGGTCTTCAAGTG
hrcA-rev	AGTACGATACCGCCAACACG
dnaK-1	AACCCTCGAGGATGCTTCATTTGATGGAAG
dnaK-2	AATTTTGCTCATTGTTATTTCC
dnaK-3	GAAAACAAATAATCTTTTAAAGG
dnaK-4	AACCAGATCTCTTGAATTTTCAAACGAAC
dnaK-5	GTGGGAGCTCAATTCCTCTAAAACCGCACT
dnaK-6	GTGGCCCGGGCTTATGCTTTTTCAGCCTTC
dnaK-7	GTGGCCATGGCAATCACCTCTTTAGCAC
dnaK-fwd	AGAAGGCGCACGTACAACAC
dnaK-rev	GCAGCACGTTTCGCTACTTC
tpi-fwd	AACACGGCATGACACCAATC
tpi-rev	CACGGATTTGACCACGTACC
rpoB-fwd	CGTCGTCTTCGTTCTGTTGG
rpoB-rev	GTTACGAACCACACGTTCC
16S-rRNA-fwd	GATGCATAGCCGACCTGAGA
16S-rRNA-rev	TGCTCCGTCAGACTTTTCGTC

*Nucleotides introduced to create restriction sites are underlined.

P_{hrcA} promoter in front of enhanced GFP (EGFP). The *hrcA* and *dnaK* genomic complementation mutants were constructed with the plasmids pIMK-*hrcA* and pIMK-*dnaK*. These plasmids were made by replacing the P_{hrcA} promoter and EGFP gene of plasmid pIMK2-EGFP with the genes *hrcA* and *dnaK*. These genes, including promoter regions and a terminator for *dnaK*, were amplified using the primers hrcA-5, hrcA-6, dnaK-5 and dnaK-6 and cloned as *SacI*–*SmaI* and *SacI*–*SalI* fragments, respectively, in pIMK2-EGFP. The plasmid pIMK-*hrcA* still contained the terminator present behind the EGFP, while for plasmid pIMK-*dnaK*, this terminator was replaced with the native terminator of *dnaK*.

Biofilm formation

Static biofilm formation. Overnight-grown cultures (18 h, 20 °C, BHI) were used for inoculation (1%) of 12-well polystyrene microtitre plates (Greiner Bio-One) containing 3 ml BHI broth. After 48 h static incubation at 20 °C, the medium was removed and biofilms were washed three times with PBS (Merck). Biofilms were dispersed in 1 ml PBS by pipetting rigorously. Dispersal of the biofilms into single cells was microscopically verified. The empty wells were stained with 0.1% crystal violet (Merck) to verify complete removal of the biofilm. Dispersed biofilm cells were serially diluted in PBS and plated on BHI agar. After 2 days incubation at 30 °C, colonies were enumerated. Three independent biological experiments using two replicates each were performed for static biofilm formation experiments.

Continuous-flow biofilm formation. Overnight-grown cultures (18 h, 20 °C, BHI) were diluted in BHI (1%) and used for inoculation of a flow cell (BST FC 281; Biosurface Technologies). Bacteria were left to adhere for 1 h and BHI broth (20 °C) was pumped through the flow

cell with a flow of 10 ml h^{−1}. Biofilms were harvested after 48 h and dispersed in 10 ml PBS. Dispersal of the biofilms into single cells was microscopically verified. Dispersed biofilm cells were serially diluted in PBS and plated on BHI agar. Agar plates were incubated for 2 days at 30 °C and colonies were enumerated. Three independent biological experiments using two replicates each were performed for continuous-flow biofilm formation experiments.

Quantitative real-time PCR. RNeasy Protect (Qiagen) was used according to the manufacturer's protocol to quench planktonic cells and biofilms. RNA extraction and cDNA synthesis was performed according to the protocols described previously (van der Veen *et al.*, 2010). Quantitative real-time PCR was performed on a ABI Prism 7000 Sequence Detection System (Applied Biosystems) using 2 × Sybr Green PCR Master Mix (Applied Biosystems) and 200 nM primers (Table 2) in a total volume of 20 µl. Expression levels were normalized using the average expression of the housekeeping genes *tpi*, *rpoB* and *16S rRNA*.

Microscopy. A BX41 microscope and MNIBA3 filter (Olympus) were used for phase-contrast and fluorescence microscopy, respectively.

Disinfection treatments. One millilitre of dispersed static and continuous-flow biofilms (48 h, 20 °C, BHI) and 1 ml of planktonic-grown cells (24 h, 20 °C, BHI) were centrifuged for 2 min at 5000 g and resuspended in 1 ml sterile water (20 °C). Benzalkonium chloride (Merck) or peracetic acid (Sigma-Aldrich) was added to these cell suspensions to a final concentration of 20 µg ml^{−1}. Samples were taken in a time series up to 15 min and diluted (1/10) in neutralizing liquid [3 g lecithin l^{−1} (VWR International), 3% (v/v) Tween-80 (Merck), 5 g sodium thiosulphate l^{−1} (VWR International), 1 g L-histidine l^{−1} (Sigma-Aldrich), 0.34 g potassium dihydrogen phosphate l^{−1} (Sigma-Aldrich)].

Fig. 1. Expression of *hrcA* and *dnaK* during biofilm formation. Differential expression between 48 h planktonic cultures (black), 48 h static biofilms (light grey) and 48 h continuous-flow biofilms (dark grey) grown at 20 °C in BHI is shown relative to expression in planktonic cultures, which was set at 1. The bars represent the mean $\pm$ SD of three independent biological experiments. *Significant difference compared with planktonic cultures ($P < 0.05$, *t*-test).

(Merck)]. After subsequent serial dilution in PBS, samples were plated on BHI agar. After 3–5 days incubation at 30 °C, colonies were enumerated. Three independent biological replicates were performed for all disinfection experiments.

Data analyses. The reparameterized Gompertz model (Zwietering *et al.*, 1990) was used to fit the inactivation curves of the disinfectant treatments with the following equation:

$$\log_{10} N_t = \log_{10} N_0 + A \exp \left\{ -\exp \left[\frac{ke}{-A} (t_s - t) + 1 \right] \right\}$$

where *A* is the difference between the surviving population and the initial population ($\log_{10}$ c.f.u. per well), *k* is the maximum specific inactivation rate ($\log_{10} \text{ min}^{-1}$) and *t_s* is the duration of the shoulder (min). The inactivation curves were fitted in Microsoft Excel by minimizing the residual sum of squares using the Excel Solver add-in.

Student's *t*-test ($P < 0.05$) was used to identify significant differences in resistance against inactivation treatments, biofilm formation and gene expression.

RESULTS

Expression of *hrcA* and *dnaK* during biofilm formation

To investigate whether *hrcA* and *dnaK* are activated during *L. monocytogenes* biofilm formation, quantitative real-time PCR experiments were performed (Fig. 1). Compared with planktonic cells, expression of *dnaK* in static biofilms was induced (1.5-fold), while no difference in *hrcA* expression was observed. However, expression of *hrcA* and *dnaK* was highly induced in continuous-flow biofilms. The expression of *hrcA* was 3.3-fold higher in continuous-flow biofilms compared with planktonic-grown cells and the expression of *dnaK* was 4-fold higher. Promoter activities of *hrcA* and *dnaK* in biofilms were further analysed with promoter reporters. Continuous-flow biofilm formation resulted in high expression of EGFP for both promoter reporters (Fig. 2), while no expression for the *hrcA* promoter or very faint expression for the *dnaK* promoter was observed during static biofilm formation (data not shown). These results show that *hrcA* and *dnaK* are activated during continuous-flow biofilm formation in particular.

The impact of HrcA and DnaK on biofilm formation

To assess the role of HrcA and DnaK in static and continuous-flow biofilm formation, differences in biofilm formation between the wild-type strain and in-frame deletion and complementation mutants were determined (Fig. 3). The wild-type and mutant strains showed no

Fig. 2. Activation of the *hrcA* (a) and *dnaK* (b) promoter during continuous-flow biofilm formation. Micrographs show fluorescence (1) and phase-contrast (2) pictures of cells expressing EGFP from the *hrcA* and *dnaK* promoter after 48 h biofilm formation in BHI at 20 °C.

differences in planktonic growth (data not shown). The $\Delta hrcA$ mutant showed increased biofilm formation compared with the wild-type strain under static-biofilm-forming conditions, indicating that constitutive activation of the class I heat-shock response in this mutant is beneficial for static biofilm formation. The importance of the class I heat-shock response in static biofilm formation was furthermore emphasized by the deficiency in static biofilm formation by the $\Delta dnaK$ mutant. Static biofilm formation was restored to wild-type levels in the $hrcA$ and $dnaK$ complementation mutants. Compared with the wild-type strain, a deficiency in continuous-flow biofilm formation was also observed for the $\Delta dnaK$ mutant. Continuous-flow biofilm formation was restored to wild-type levels in the $dnaK$ complementation mutant, which shows that the presence of DnaK is important for continuous-flow biofilm formation. However, continuous-flow biofilm formation was also reduced with the $\Delta hrcA$ mutant. Furthermore, continuous-flow biofilm formation was not completely restored to wild-type levels using the $hrcA$ complementation mutant, although a significantly higher amount of biofilm was produced with this strain compared with the $\Delta hrcA$ mutant ($P < 0.05$, t -test). These results might indicate that a constitutive highly expressed class I heat-shock response is not beneficial for continuous-flow biofilm formation. On the other hand, HrcA was previously shown to activate a range of stress proteins (Hu *et al.*, 2007), and it remains to be elucidated whether these contribute to continuous-flow biofilm formation.

Fig. 3. Comparative analysis of static and continuous-flow biofilm formation between wild-type, $\Delta hrcA$, $hrcA$ -c, $\Delta dnaK$ and $dnaK$ -c strains. Biofilm formation in BHI at 20 °C after 48 h under static (light grey) and continuous-flow (dark grey) conditions is shown. The bars represent the mean $\pm$ SD of three independent biological experiments. *Significant difference compared with wild-type ($P < 0.05$, t -test); #significant difference compared with the complementation strain ($P < 0.05$, t -test).

Fig. 4. Disinfection treatment of planktonic and biofilm cells with benzalkonium chloride. Inactivation of 24 h planktonic cells (a), cells from a 48 h dispersed static biofilm (b) and cells from a 48 h dispersed continuous-flow biofilm (c) treated with 20 μg benzalkonium chloride ml^{-1} for 15 min at 20 °C of wild-type (◆), $\Delta hrcA$ (■), $hrcA$ -c (□), $\Delta dnaK$ (▲) and $dnaK$ -c (△) strains is shown. The graphs represent the mean $\pm$ SD of three independent biological experiments. Data points below the detection limit [$\log_{10}(N_t/N_0) \approx -6.5$] are not shown.

The role of HrcA and DnaK in resistance against disinfectants

The disinfectants benzalkonium chloride (Fig. 4) and peracetic acid (Fig. 5) were used to investigate the role of HrcA and DnaK in disinfection resistance of planktonic cells and static and continuous-flow biofilm cells. Biofilms were detached and dispersed to exclude the possible influence of biofilm matrix components on the resistance of biofilm cells, thereby focusing on the intrinsic resistance of cells grown in biofilms. Parameter estimates were determined after modelling the inactivation curves of wild-type and mutant strains (Table 3). Differences in disinfectant resistance are reflected in the surviving population (A), the maximum specific inactivation rate (k) or the duration of the shoulder (t_s). Differences in disinfectant resistance between wild-type and mutant strains and between planktonic and biofilm cells are considered significant when any of the three parameters A , k or t_s of the inactivation curve is significantly different. Compared with planktonic-grown cells, increased resistance of dispersed static biofilm cells was observed for the wild-type strain after exposure to benzalkonium chloride (Fig. 4 and Table 3) and peracetic acid (Fig. 5 and Table 3). In contrast, resistance of dispersed continuous-flow biofilm cells was only increased after peracetic acid treatments. Furthermore, a role for HrcA and DnaK in the resistance against disinfectants was identified. Planktonic cells and dispersed static and continuous-flow biofilm cells of the $\Delta hrcA$ mutant showed lower resistance against benzalkonium chloride compared with the wild-type strain (Fig. 4 and Table 3). Resistance of planktonic cells and dispersed static and continuous-flow biofilm cells of the $\Delta hrcA$ mutant was also lower after peracetic acid treatment (Fig. 5 and Table 3). Furthermore, planktonic cells and dispersed static and continuous-flow biofilm cells of the $\Delta dnaK$ mutant were less resistant to benzalkonium chloride (Fig. 4 and Table 3) and peracetic acid (Fig. 5 and Table 3) treatment compared with the wild-type strain. Complementation of the $\Delta hrcA$ and $\Delta dnaK$ mutant completely or partly restored the disinfectant sensitive phenotype of the mutants (Fig. 4, Fig. 5 and Table 3). These results revealed that HrcA and DnaK play an important role in the resistance of *L. monocytogenes* planktonic and biofilm cells against disinfectants.

DISCUSSION

So far, not much is known about the activation and requirement of stress response genes in *L. monocytogenes* biofilm formation and subsequently in the resistance of biofilms against disinfectants. Therefore, we chose to investigate the involvement of the class I heat-shock response, which is one of the most important stress resistance mechanisms of *L. monocytogenes*, in biofilm formation and disinfectant resistance by expression and mutant analyses. We selected HrcA and DnaK, which are

Fig. 5. Disinfection treatment of planktonic and biofilm cells with peracetic acid. Inactivation of 24 h planktonic cells (a), cells from a 48 h dispersed static biofilm (b) and cells from a 48 h dispersed continuous-flow biofilm (c) treated with 20 µg peracetic acid ml⁻¹ for 15 min at 20 °C of wild-type (◆), $\Delta hrcA$ (■), $hrcA$ -c (□), $\Delta dnaK$ (▲) and $dnaK$ -c (△) strains is shown. The graphs represent the mean $\pm$ SD of three independent biological experiments. Data points below the detection limit ($\log_{10}(N_t/N_0) \approx -6.5$) are not shown.

the repressor and a chaperone protein, respectively, of the class I heat-shock response. The class I heat-shock response is, together with the class III heat-shock response (Nair *et al.*, 2000), responsible for protein maintenance and turnover in many bacteria. A role for class I heat-shock response genes in biofilm formation has not been established in *L. monocytogenes* thus far, although a role in resistance to heat, cold, acid and ethanol has been established (Gahan *et al.*, 2001; Hanawa *et al.*, 1999; Liu *et al.*, 2002). Our results show that *hrcA* and *dnaK* are specifically induced during continuous-flow biofilm formation. Furthermore, mutant analyses indicate that HrcA and DnaK are involved in both static and continuous-flow biofilm formation. The impact of the class I or class III heat-shock response on the biofilm-forming behaviour of *Streptococcus mutans* has been shown previously. Forced downregulation of *dnaK* in *S. mutans* resulted in a reduced static-biofilm-forming capacity (Lemos *et al.*, 2007). In this organism, the capacity to form biofilms was also reduced in a *clpP* mutant, ClpP being an ATP-dependent protease of the class III heat-shock response (Lemos & Burne, 2002). A mutant of *clpP* in *Pseudomonas fluorescens* also showed a reduced biofilm-forming capacity (O'Toole & Kolter, 1998). Our results show that DnaK is important for both static and continuous-flow biofilm formation in *L. monocytogenes*, indicating that active protein quality maintenance influences the biofilm-forming capacity. This was furthermore highlighted with the induced static-biofilm-forming capacity of the *hrcA* mutant, which shows induced *dnaK* expression. However, the *hrcA* mutant showed reduced

biofilm formation during continuous-flow biofilm formation. For *L. monocytogenes*, it was shown previously that continuous-flow biofilm formation was dependent on the activation of the SOS response factor YneA, which results in the formation of knitted chains composed of elongated cells (van der Veen & Abee, 2010). It might be possible that the induction of the class I heat-shock response in this mutant, or the differential expression of numerous other indirectly regulated genes (Hu *et al.*, 2007), affects continuous-flow biofilm formation by preventing the activation of the SOS response.

Biofilms generally show higher resistance against disinfectants than planktonic-grown cells. However, the genetic mechanisms responsible for this increased resistance are not known. Therefore, we investigated the role of HrcA and DnaK in the resistance of static and continuous-flow biofilms against the disinfectants benzalkonium chloride and peracetic acid, which are two of the most commonly used disinfectants in the food industry (McDonnell & Russell, 1999). Previously, it has been shown that *L. monocytogenes* biofilms are more resistant against benzalkonium chloride and peracetic acid than planktonic-grown cells (Romanova *et al.*, 2007; Stopforth *et al.*, 2002), although detached and suspended biofilm cells appeared to be equally sensitive to peracetic acid. Benzalkonium chloride and peracetic acid use different modes of action to kill bacteria: benzalkonium chloride disrupts the cell membrane, which results in leakage of intracellular material, while peracetic acid is an oxidizing agent that is expected to attack essential cellular components such as DNA and proteins (McDonnell & Russell, 1999).

Table 3. Parameter estimates of the inactivation curves after treatment with disinfectants

Values shown are the mean $\pm$ SD.

Strain	Growth condition	Benzalkonium chloride			Peracetic acid		
		A	k	t_s	A	k	t_s
Wild-type	Planktonic	-4.45 ± 0.41	0.72 ± 0.04	0.41 ± 0.27	-6.37 ± 0.15	1.19 ± 0.02	1.56 ± 0.16
	Static biofilm	$-1.29 \pm 0.37^*$	$0.12 \pm 0.01^*$	$2.20 \pm 0.50^*$	$-4.14 \pm 0.34^*$	$0.39 \pm 0.00^*$	$3.89 \pm 0.97^*$
	Continuous-flow biofilm	-4.15 ± 0.16	0.61 ± 0.07	0.26 ± 0.39	$-5.38 \pm 0.15^*$	$0.56 \pm 0.04^*$	1.97 ± 0.34
$\Delta hrcA$	Planktonic	$-5.54 \pm 0.36^\dagger$	1.00 ± 0.19	0.48 ± 0.18	$-7.97 \pm 0.79^\dagger$	$1.89 \pm 0.03^\dagger$	$2.06 \pm 0.06^\dagger$
	Static biofilm	$-5.08 \pm 0.20^\dagger$	$0.90 \pm 0.17^\dagger$	$0.40 \pm 0.34^\dagger$	$-8.01 \pm 1.75^\dagger$	$0.55 \pm 0.03^{*\dagger}$	$1.01 \pm 0.76^{*\dagger}$
	Continuous-flow biofilm	$-5.73 \pm 0.49^\dagger$	$2.82 \pm 0.24^{*\dagger}$	$0.84 \pm 0.09^*$	$-6.13 \pm 0.50^*$	$0.80 \pm 0.06^{*\dagger}$	$0.81 \pm 0.44^{*\dagger}$
<i>hrcA-c</i>	Planktonic	-3.74 ± 0.19	0.62 ± 0.08	0.55 ± 0.37	-6.62 ± 0.49	$0.76 \pm 0.07^\dagger$	1.50 ± 0.20
	Static biofilm	$-0.68 \pm 0.09^*$	$0.06 \pm 0.04^*$	1.95 ± 1.70	$-5.38 \pm 0.19^{*\dagger}$	$0.36 \pm 0.01^{*\dagger}$	$2.25 \pm 0.42^*$
	Continuous-flow biofilm	$-5.09 \pm 0.38^{*\dagger}$	$0.89 \pm 0.03^{*\dagger}$	0.19 ± 0.14	-5.90 ± 0.38	$0.58 \pm 0.07^*$	$1.06 \pm 0.12^{*\dagger}$
$\Delta dnaK$	Planktonic	$-5.52 \pm 0.22^\dagger$	$1.49 \pm 0.24^\dagger$	0.27 ± 0.08	-7.01 ± 0.57	$1.82 \pm 0.06^\dagger$	1.51 ± 0.05
	Static biofilm	$-4.25 \pm 0.13^{*\dagger}$	$0.53 \pm 0.12^{*\dagger}$	$0.40 \pm 0.45^\dagger$	$-8.17 \pm 0.67^\dagger$	$0.54 \pm 0.09^{*\dagger}$	2.81 ± 1.32
	Continuous-flow biofilm	$-6.22 \pm 0.18^{*\dagger}$	$3.37 \pm 0.15^{*\dagger}$	$0.85 \pm 0.07^*$	-6.70 ± 1.00	$0.89 \pm 0.04^{*\dagger}$	$0.87 \pm 0.11^{*\dagger}$
<i>dnaK-c</i>	Planktonic	-4.56 ± 0.41	0.62 ± 0.16	0.05 ± 0.14	-6.05 ± 0.32	$1.07 \pm 0.04^\dagger$	1.05 ± 0.33
	Static biofilm	$-2.86 \pm 0.50^{*\dagger}$	$0.26 \pm 0.01^{*\dagger}$	$2.59 \pm 1.02^*$	$-5.27 \pm 0.07^{*\dagger}$	$0.32 \pm 0.02^{*\dagger}$	2.14 ± 0.73
	Continuous-flow biofilm	$-6.24 \pm 0.43^{*\dagger}$	$1.55 \pm 0.14^{*\dagger}$	$0.50 \pm 0.06^*$	$-6.90 \pm 0.34^{*\dagger}$	$0.71 \pm 0.01^{*\dagger}$	1.55 ± 0.51

*Parameter estimate significantly different from planktonic cells ($P < 0.05$; t -test).

†Parameter estimate significantly different from the wild-type strain ($P < 0.05$; t -test).

Resistance to benzalkonium chloride has previously been related to the fatty acid composition of the cell membrane and the induction of an efflux pump (Romanova *et al.*, 2006; To *et al.*, 2002), while no peracetic acid resistance mechanism has been reported thus far. The reduced disinfectant resistance of biofilm cells and planktonic cells of the $\Delta dnaK$ mutant indicates that protein maintenance is an important factor contributing to resistance. This result is in line with the hypothesis that peracetic acid disrupts cellular proteins.

Therefore, it is somewhat surprising that the $\Delta hrcA$ mutant, which shows induced expression of *dnaK* and other class I heat-shock genes that are involved in protein maintenance, is more sensitive to disinfectants than the wild-type strain. However, it has been shown previously that HrcA is also indirectly involved in the up- and down-regulation of 56 other genes, including genes encoding proteins with functions in oxidative, acid, heat and cold stress resistance (Hu *et al.*, 2007), and this may offer an explanation for the observed disinfection sensitivity of the $\Delta hrcA$ mutant.

In conclusion, our study showed that HrcA and DnaK are important for *L. monocytogenes* static and continuous-flow biofilm formation and disinfection resistance. The specific HrcA-regulated genes that are involved in disinfectant resistance remain to be elucidated in future studies.

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