

A study on the effects of some laboratory derived genetic mutations on biofilm formation by *Listeria monocytogenes*

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Abstract

Biofilms formed by the human pathogen *Listeria monocytogenes* in food processing environments can be a potential source of contamination. In this study, we investigated the ability of *L. monocytogenes* wild type and its laboratory derived isogenic mutants in *cwhA*, *prfA*, *agrA*, *flaA*, *degU*, *ami* and *sigB* to adhere to and form biofilms on abiotic surfaces. The results suggest that inactivation of the two component regulatory system *degU* completely abolished biofilm formation, while inactivation of the flagellar gene *flaA*, two component response regulator *agrA* and the autolysin-adhesin gene *ami* lead to severe impairment of initial attachment and the subsequent development of a mature biofilm by *L. monocytogenes*. Mutants in global regulator of virulence *prfA* and the alternative sigma factor *sigB* were unaffected and formed biofilms similar to the wild type *L. monocytogenes*.

Key words *Listeria monocytogenes* . Biofilm . *ami* . *agrA* . electron microscopy

Introduction

Listeria monocytogenes is a Gram-positive facultative intracellular pathogen capable of causing fatal infections in susceptible populations. These infections are more common in the newborn, pregnant women and immunocompromised individuals, with symptoms such as meningitis, encephalitis and septicemia. *L. monocytogenes* has been associated with several outbreaks involving ready to eat food products (Lianou and Sofos 2007). The fatality rate associated with *L. monocytogenes* infections is as high as 30%, making it one of the most important food-borne pathogens. The organism is widely distributed in the environment with the ability to survive adverse conditions.

Many bacteria have the ability to attach to abiotic surfaces and form sessile communities known as biofilms (Davey and O'Toole 2000). Biofilms consist of bacteria embedded in an extracellular polymeric substance, and the biofilm bacteria are resistant to antimicrobials such as plant disinfectants, UV light and drying. Biofilm formation in the food processing environment by pathogenic bacteria is of great concern, since such bacteria can contribute to post process contamination. Several studies have shown that *Listeria* is widely present in the processing environs. In many studies, contamination of finished products with *L. monocytogenes* has been attributed to the presence of this organism in food processing equipment. Though the studies indicate that *L. monocytogenes* is capable of forming biofilms, strain to strain variations have been observed (Djordjevic et al. 2002; Borucki et al. 2003). Recent studies have described the roles of alternative sigma factor sigma B (σ^B), flagellin, LuxS, DegU and the Agr systems of *L. monocytogenes* in surface attachment and biofilm formation (Vatanyoopaisarn et al. 2000; Schwab et al. 2005; Sela et al. 2006; Tresse et al. 2006; Rieu et al. 2007). In the study described here, we compared the biofilms formed by wild type *L. monocytogenes* and its mutant strains in known virulence genes by microtitre plate assay, fluorescence and scanning electron microscopy. While some important virulence genes were found to have no role, others had varying effects from moderate to severe, on attachment and biofilm formation by *L. monocytogenes*.

Materials and methods

Bacterial strains and media

Two wild type (WT) strains EGDe (BAA-679, serotype 1/2a) and its derivative EGD (ATCC 35152, serotype 1/2a) were used. The isogenic deletion mutants in *cwhA*, *agrA*, *lmo1799*, *prfA* and *sigB* were derived from *L. Monocytogenes* EGDe (Pilgrim et al. 2003; Joseph and Goebel 2007). The *degU* deletion mutant was derived from the *L.*

monocytogenes strain EGD (Williams et al. 2005). The wild type and the mutant strains were obtained from the *Listeria* strain collection of the Department of Microbiology, University of Wurzburg, Germany.

Insertional mutants of *L. monocytogenes* EGD in the flagellin gene *flaA* and the autolysin-adhesin gene *ami* were derived in this study using pLSV101 mutagenesis vector (Joseph et al. 2006). The target genes were amplified using primers Ami-SF: cgcggatcctttattggcacctccgctac, Ami-SR: cgcaagcttgaagcaaaggacttgctgt and FlaA-F: cgcggatccagcgcagaacgtagcat, FlaA-R: cgcaagcttgagcagcctgtagcagtttg containing the BamHI and HindIII restriction sites (underlined) in forward and reverse primers respectively. The PCR products were digested with restriction enzymes, ligated into the similarly digested plasmid pLSV101 and transformed into *L. monocytogenes* EGD by electroporation. The mutants were selected on erythromycin plates (5 µg/ml) by incubating at 42 °C. The integration of recombinant plasmids into the target sites was confirmed by PCR and sequencing.

The attachment and surface growth on polystyrene microtitre plate was studied using two minimal media, modified Welshimers broth (MWB) (Premaratne et al. 1991) and Hsiang-Ning Tsai medium (HTM) (Tsai and Hodgson 2003).

Biofilm assay using polystyrene microtitre plates

The ability of wild and mutant strains of *L. monocytogenes* to attach to polystyrene surfaces was evaluated using microtitre plate assay previously described (Djordjevic et al. 2002). Briefly, the strains were grown in tryptic soy broth containing 0.6% added yeast extract (TSB-YE) overnight without shaking. A 1:40 dilution of the broth cultures was prepared by diluting the overnight cultures in minimal media. The diluted culture was vortexed briefly and a 100 µl was dispensed into each of 6 wells of a microtitre plate. Fresh medium was added as blanks. The plates were incubated at 30 °C for 40 h. After incubation, the microplate wells were washed 5 times with sterile distilled water and air dried for 30 min. The biofilm bacteria were stained with 1% freshly prepared crystal violet solution for 30 min at room temperature. The wells were washed with 150 µl sterile distilled water 5 times, air dried and destained with 200 µl of 95% ethanol at 8 °C for 45 min. A 100 µl volume of the mixture was transferred into wells of a fresh microtitre plate and the O.D was measured at 595 nm.

Fluorescence and electron microscopy

For microscopy, the strains were grown overnight in TSB-YE and 5 ml of 1:40 dilution of the overnight culture was prepared with minimal media in 50 ml conical tubes. Sterile borosilicate glass coverslips were immersed in diluted bacterial culture and incubated at 30 °C for 2 days and 8 days in

separate experiments. For fluorescence microscopy, the coverslips were rinsed in sterile distilled water to remove loosely adherent bacteria, stained with 4', 6-Diamidino-2-phenylindole (DAPI) for 10 min in the dark, rinsed with distilled water and air-dried in cold. Biofilms were observed using Leica, DMR fluorescence microscope equipped with the MetaMorph image analysis software (Universal Imaging Co., Pa.), under the oil immersion objective.

For electron microscopy, the cover slips containing attached bacteria were rinsed and fixed in 2.5% buffered glutaraldehyde [50 mM cacodylate pH 7.2, 50 mM KCl, 2.5 mM MgCl₂]. Cells attached to coverslips were stepwise dehydrated in acetone and critical-point dried with CO₂. Photographs were taken with a Zeiss scanning electron microscope (DSM 962; Zeiss, Oberkochen, Germany). Several fields were scanned prior to photographing a representative field in order to draw conclusions on the biofilm structure.

Statistical analysis

The mean and standard error were calculated for the O.D₅₉₅ values obtained and compared using Mann-Whitney U test (n=3, P<0.05).

Results and discussion

Biofilm formation by pathogenic microorganisms is of immense significance to food processing industries. *L. monocytogenes* is one such important food-borne pathogen capable of colonizing food processing environments, thus acting as a constant source of contamination. We carried out this study to determine if certain well-known genetic determinants of virulence in *L. monocytogenes* play any role in biofilm formation.

The biofilm forming potential of *L. monocytogenes* was evaluated by microtitre plate technique. The two minimal mediums used in this study supported biofilm formation, but the density of biofilm formed in MWB was higher than that observed in HTM (Fig. 1). Nevertheless, the trend in biofilm formations was similar in both media. The mutant strains deficient in biofilm formation in MWB were equally deficient in HTM. However, there was no obvious difference in the rate of growth in these two media after 48 h incubation at 30 °C (data not shown). Microscopic analysis of biofilms revealed that biofilms formed in HTM composed of uniformly adherent cells in the form of a monolayer. In contrast, the biofilms formed in MWB were thick with masses of cell aggregates. The basic difference between these two media is that HTM lacks aminoacids histidine, tryptophan, leucine, isoleucine, valine, arginine and glutamine. It needs to be determined if one or more amino acids among these play any role in enhancing biofilm potentials of *L. monocytogenes*.

In order to substantiate the results obtained with microplate biofilm assay, biofilms formed on borosilicate coverslips were subjected to fluorescence microscopy at 15 min, 6 h and 2 days. The DAPI stained coverslips revealed that bacteria attach to cover slips as early as 15 min. Long chains of bacteria were seen after 6 h on the surface of glass cover slip (data not shown). After 2 days, *L. monocytogenes* EGDe wild-type formed a thick and confluent biofilm on coverslip.

Electron microscopy revealed that *L. monocytogenes* EGDe wild-type strain formed a biofilm that was maintained for up to 8 days in MWB (Fig. 2). The biofilm architecture of *L. monocytogenes* appeared to be reinforced by strands of a presumably polymeric material. Previous studies on *L. monocytogenes* biofilms have evidenced the production of an extracellular polysaccharide substance (Borucki et al. 2003). We observed that the extracellular polymeric substance was secreted in larger quantity in MWB compared to in HTM. The high O.D observed in MWB microtitre plate assay was perhaps due to the production of exopolysaccharides (EPS). The factors triggering the secretion of EPS could not be determined in this study

In the present study, we tested *L. monocytogenes* EGDe strain with deletions in *cwhA*, *prfA*, *lmo1799* and *sigB* genes. *cwhA* (cell wall hydrolase A) encodes an extracellular protein p60 (Pilgrim et al. 2003). PrfA encoded by *prfA* gene is a positive regulator of expression of many virulence genes in *L. monocytogenes*, while *lmo1799* encodes a putative peptidoglycan bound protein with LPXTG motif (Joseph and Goebel 2007). The alternative sigma factor SigB in *L. monocytogenes* contributes to viability during carbon starvation, resistance to oxidative stress, adaptation to cold temperature and acid stress (Ferreira et al. 2001). The results of microplate assay indicated that $\Delta cwhA$, $\Delta prfA$, $\Delta lmo1799$, and $\Delta sigB$ mutants of EGDe were unaffected in their ability to surface-attach and formed biofilms of similar intensity to WT strain. The O.D₅₉₅ values of crystal violet stained biofilms of these strains were not significantly different from wild type parent strain EGDe (P<0.05) (Fig. 1).

Disruptions of two component regulatory system *agrA* and autolysin-adhesin gene *ami* resulted in decrease in the biofilm forming ability of *L. monocytogenes* as evidenced by the low O.D₅₉₅ values and the diffused adherence phenotype observed by electron microscopy (Fig. 3a, 3b). Recently, Rieu et al. (2007) reported the involvement of *agrA* and *agrD* in the early phase of development of biofilm by *L. monocytogenes*. The molecular mechanisms by which the *agr* locus participates in or influences the biofilm formation by *L. monocytogenes* remains to be elucidated.

The 917 amino acid autolytic amidase encoded by *ami* is known to participate in the attachment of *L. monocytogenes* to eukaryotic cells (McLaughlan and Foster 1998). The N-terminal 179 amino acid sequence of Ami has high similarity with AtlE of *S. aureus* reported to be

responsible for the attachment of *S. aureus* to polystyrene surfaces and a biofilm negative phenotype was later reported in an *atl* deletion mutant of *S. aureus* (Biswas et al. 2006). The impaired biofilm formation by *ami* mutant in our study suggests a possible role for Ami in the attachment of *L. monocytogenes* to abiotic surfaces.

Inactivation of the two component response regulator *degU* resulted in a non-motile mutant of *L. monocytogenes* that was biofilm negative (Fig. 3c). To further prove that the inability to attach and form biofilm by $\Delta degU$ may not be due to the absence flagella alone, we created a non-motile flagellin mutant of *L. monocytogenes* by insertional inactivation of *flaA* gene. This mutant strain could form a biofilm on the polystyrene microtitre plate surface and on glass coverslips. However, the biofilm formed by the *flaA* mutant strain was less dense when compared to its wild type parent strains as revealed by microtitre plate readings and electron microscopy (Fig. 3d). In this milieu, our results compel us to conclude that it is not merely the motility, but some changes in cell surface structures or unknown biochemical pathways might be responsible for biofilm deficient phenotype of *degU* mutant. This hypothesis needs further investigation, since *degU* appears to be important for biofilm formation in *L. monocytogenes*.

Based on the above observations, we conclude that at 30 °C, the *L. monocytogenes* wild type strains, $\Delta lmo1799$, $\Delta prfA$, *cwhA* and *sigB* mutants could surface attach and form biofilms on polystyrene surfaces, while $\Delta degU$, Δagr , *flaA* and *ami* mutants were partially or totally impaired in this ability. Future studies will concentrate on determining the physiological roles of these gene products responsible for impaired biofilm formation by *L. monocytogenes*.

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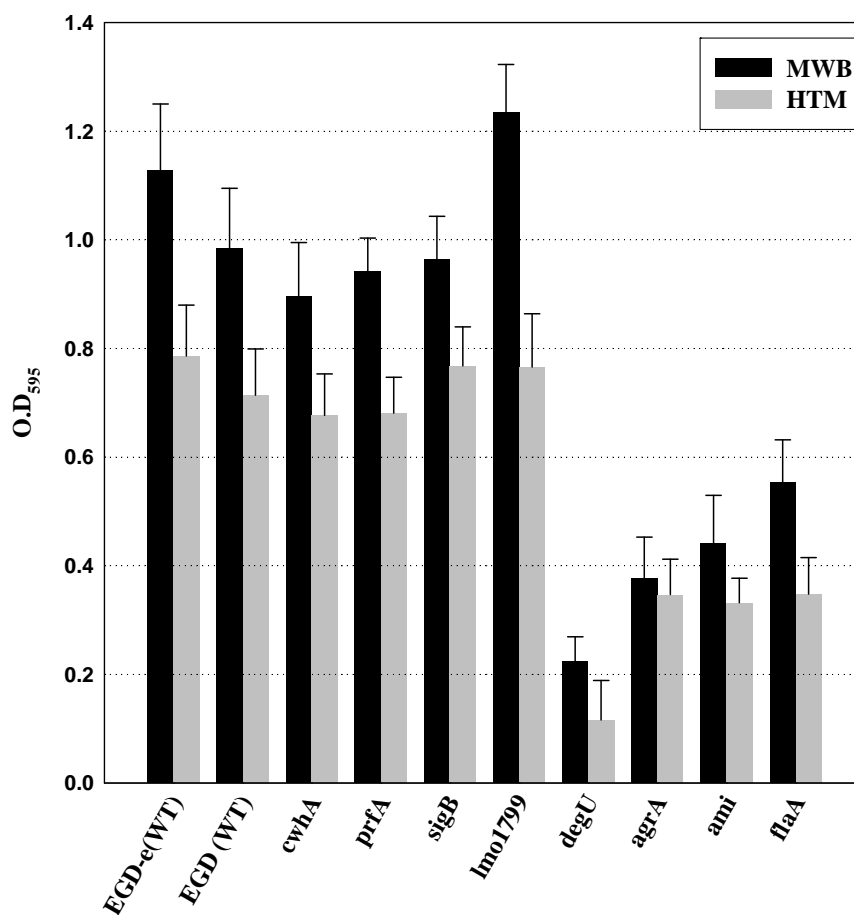


Fig. 1 Quantification of biofilms formed by *L. monocytogenes* wild-type strains and mutants on a polystyrene surface after 40 h of incubation at 30 °C in minimal media MWB and HTM. Quantification of surface adherent cells was done by crystal violet destaining method. The bars represent means of 3 independent experiments, with each strain grown in triplicate wells

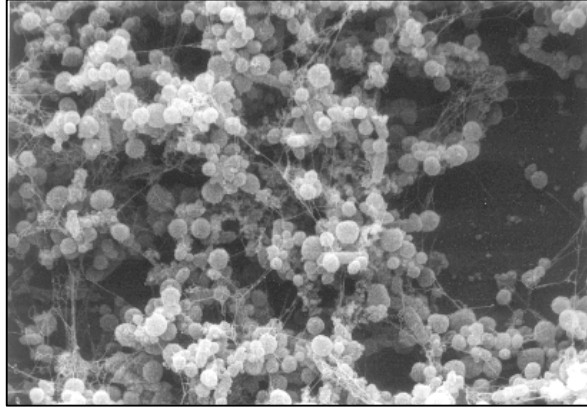


Fig. 2 Scanning electron micrograph of 8-day old biofilm of *L. monocytogenes* EGDe WT grown in MWB (magnification=5000×). The biofilm cells are seen enmeshed in a polymeric substance

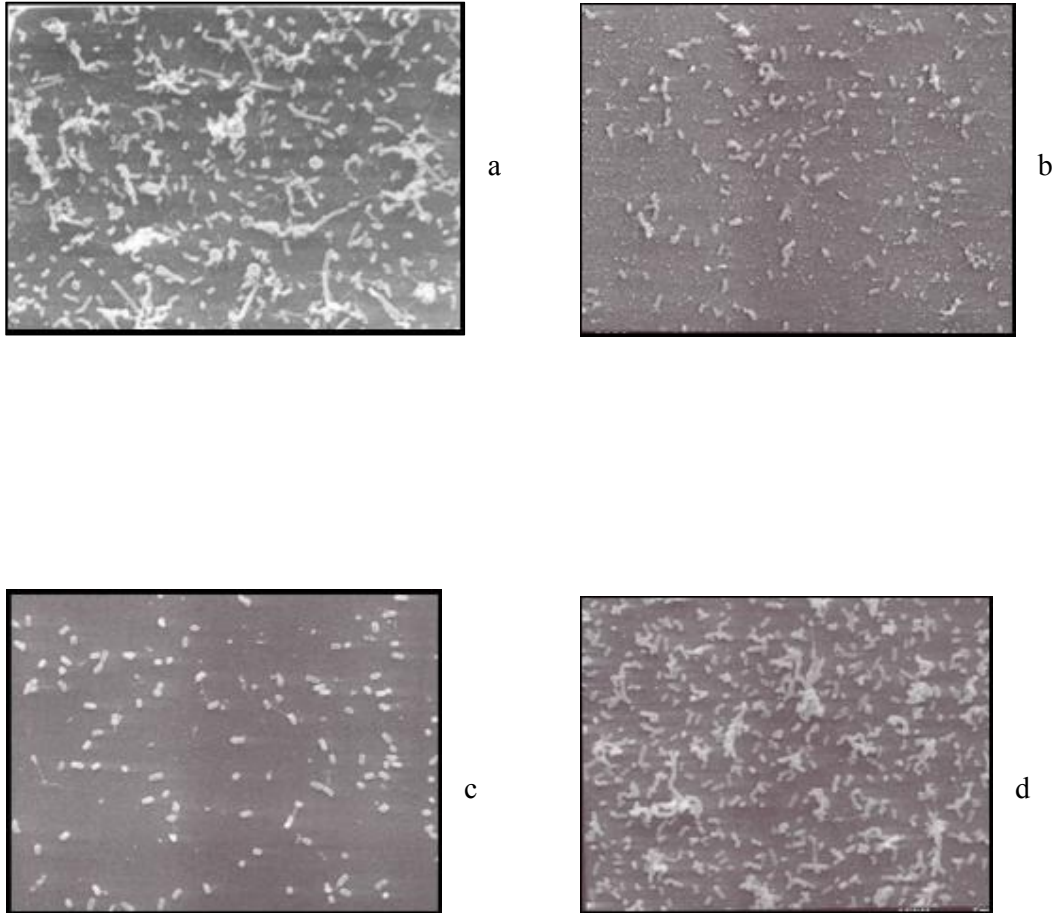


Fig. 3 Scanning electron micrograph showing severely impaired attachment by mutant strains of *L. monocytogenes* grown in MWB. (a) $\Delta agrA$ mutant (b) *ami* mutant (c) $\Delta degU$ mutant (d) *flaA* mutant (magnification =3000 \times)