Agr system of Listeria monocytogenes EGD-e: role in adherence and differential expression pattern.

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Running title: *agr* mutation in biofilm formation of *L. monocytogenes*

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In this study, we investigated the $agrBDCA$ operon in the pathogenic bacterium *Listeria monocytogenes* EGD-e. In-frame deletion of $agrA$ and $agrD$ resulted in an altered adherence and biofilm formation on abiotic surfaces, suggesting the involvement of the $agr$ system of *L. monocytogenes* during the early stages of biofilm formation. Real time PCR experiments indicated that the transcript levels of $agrBDCA$ depended on the stage of biofilm development, since it was lower after initial attachment period than during biofilm growth. Whereas, during planktonic growth, transcription was not growth-phase dependent. The mRNA quantification data also suggested that the $agr$ system was autoregulated and pointed to a differential expression of the $agr$ genes during sessile and planktonic growth. Although the RT-PCR experiments revealed that the four genes were transcribed as a single messenger, chemical half-life and 5’ RACE experiments indicated that the full size transcript underwent cleavage followed by degradation of the $agrC$ and $agrA$ transcripts, which supposes a complex regulation of $agr$ transcription.
INTRODUCTION

Listeria monocytogenes is a Gram-positive human pathogenic bacterium; it is the causative agent of listeriosis, a serious infection characterized by high mortality rates, in immunocompromised individuals and pregnant women (19). This pathogenic bacterium is widely spread in the environment (soil, vegetation, animals, farm environment). In connexion with these extended reservoirs, L. monocytogenes is also a contaminant of the food industry. Its presence on working surfaces in food-processing plants is a major problem as a source of food contamination (1, 32). As most bacteria, L. monocytogenes is able to colonize surfaces and form biofilms (sessile growth) while, in natural environments, free floating cells (planktonic growth) are transitory (28). Several steps can be identified during biofilm development: after an initial step of reversible then irreversible adherence, bacteria grow as microcolonies and spread on the surface; finally, biofilms develop as complex, three-dimensional structures during the maturation step (17). Biofilm development and maturation requires complex cellular mechanisms in which cell-cell communication is involved (14, 30). To date, three major signalling systems have been identified; to regulate these systems, bacterial extracellular signalling molecules called autoinducers are produced (8). The acylhomoserine lactones (AHL) have been identified as autoinducers in Gram-negative bacteria (3, 13, 27, 46). The autoinducer 2 (AI-2) is found in both Gram-negative and Gram-positive bacteria (5, 7, 11, 35, 36, 54). Finally, peptide-mediated signalling pathways have been characterized in Gram-positive bacteria. Among these, the agr system has been described initially in Staphylococcus aureus (41); the production of many of its virulence factors (toxins, enzymes and cell surface proteins) is regulated by this system (4). The role of the agr system during S. aureus biofilm development is complex (57). It depends on the hydrodynamic conditions of the experimental set up; under static conditions, agr expression
reduces the attachment of the cells to the surface (52, 55) and under turbulent dynamic conditions, agr expression may affect biofilm maturation (55). Orthologs of the agr system have also been described in Enterococcus faecalis (fsr) (45), Lactobacillus plantarum (lam) (48) and L. monocytogenes (agr) (6). In E. faecalis, expression of a gelatinase (GelE) is fsr dependent (45). In L. plantarum, the lam system plays a role during biofilm development (48).

In L. monocytogenes, the four genes (agrB, agrD, agrC and agrA) of the agr locus are organized as an operon (Fig. 1A). They encode the two-component histidine kinase AgrC and response regulator AgrA, a precursor peptide AgrD and AgrB, a protein that is involved in the processing of AgrD into a matured autoinducing peptide. Limited data concerning the role of the agr locus on the physiology of L. monocytogenes is available. Williams et al. (53) showed that among sixteen putative response regulator genes of L. monocytogenes EGD-e, in-frame deletion in agrA did not affect the growth in BHI medium at various temperatures (20°C, 37°C and 43°C), in the presence of 9% NaCl, 5% ethanol or 0.025% H₂O₂. Swimming motility was not affected either. No alteration of virulence could be identified during in vitro infection of cell cultures nor in vivo after intravenous infection of BALB/c mice. In a previous study, Autret et al. (6) reported a moderate attenuation of the virulence in Swiss mice after insertion of Tn1545 in the L. monocytogenes EGD-e agrA gene.

To further elucidate the role of the agr system, we first of all examined its involvement during attachment to abiotic surfaces and biofilm growth. agrA and agrD in-frame deletion mutants were compared to the parental strain EGD-e during sessile growth. Secondly, we determined the expression pattern of the four genes of the agr operon during planktonic and sessile growth and we evidenced post-transcriptional events during expression of this operon.
MATERIALS AND METHODS

Bacterial strains and growth media

The bacterial strains and plasmids used in this study and their characteristics are shown in Table 1. L. monocytogenes EGD-e, isolated from a rabbit listeriosis outbreak (39), and two mutants, derivatives of L. monocytogenes EGD-e, L. monocytogenes DG119D (ΔagrD) and L. monocytogenes DG125A (ΔagrA) were grown in tryptic soy broth (TSB, Biokar diagnostics, Pantin, France) at 25°C for biofilm and planktonic cultures and in brain heart infusion broth (BHI, Biokar diagnostics) at 40°C for mutant construction. Escherichia coli TOP10 and Match1 (Invitrogen, Cergy Pontoise, France) were grown aerobically in Luria-Bertani broth (LB, Biokar diagnostics) at 37°C. When appropriate, antibiotics (Sigma, St Quentin Fallavier, France) were added as follows: kanamycin, 50 µg.ml⁻¹ (E. coli); ampicillin, 200 µg.ml⁻¹ (E. coli); chloramphenicol, 10 µg.ml⁻¹ (L. monocytogenes) (Table 1).

In-frame deletion of agrD and agrA genes

The mutant strains listed in Table 1, carrying an in-frame deletion in the response regulator agrA or in the precursor peptide agrD genes, were constructed from the parental strain L. monocytogenes EGD-e using a two step integration/excision procedure (10) which is based on the mutagenesis plasmid pGF-EM (33).

First, for the construction of an agrA in–frame deletion mutant, primers C13 and A12 (Table 2) were used to amplify a 6000-bp DNA fragment including the 3’ end of agrC and the ATG of agrA. The PCR product was cloned into pGF-EM (33) after digesting this PCR product and vector with HindIII/XbaI. The resulting plasmid, pGID121 was transformed into chemically competent E. coli Match1 as recommended by the manufacturer (Invitrogen). Primers A15 and E2 (Table 2) were used to amplify a 500-bp internal fragment of agrA. The resulting fragment was cloned into pCR2.1 TOPO vector (Invitrogen) to obtain plasmid pGID118. This
vector was transferred into \textit{E. coli} TOP10. Plasmid pGID118 was digested with \textit{NheI/EcoRI} and the resulting 500-bp fragment containing an internal part of \textit{agrA} was ligated into pGID121 restricted with XbaI/EcoRI to obtain pGID125. This plasmid was electroporated into \textit{L. monocytogenes} EGD-e.

Secondly, a similar strategy was used for the construction of an \textit{agrD} in-frame deletion mutant. Primers D1 and D2 (Table 2) were used to amplify a 400-bp DNA fragment including the 5’ end of \textit{agrD}. The PCR product was cloned into pGF-EM (33) to obtain pGID112. Primers D3 and D4 (Table 2) were used to amplify a 6000-bp DNA fragment including the 3’ end of \textit{agrD} and the 5’ end of \textit{agrC}. The resulting fragment was cloned into pCR2.1 TOPO vector (Invitrogen) to obtain plasmid pGID109. After digestion, the plasmid pGID109 was ligated into digested plasmid pGID112 to obtain pGID119. This plasmid was electroporated into \textit{L. monocytogenes} EGD-e.

Finally, transformants were selected on BHI agar plates (Biokar diagnostics) containing 10 \(\mu\text{g.mL}^{-1}\) chloramphenicol (Sigma). A transformant was serially subcultured in BHI at 40°C to direct chromosomal integration of the plasmid by homologous recombination. Chromosomal integration was confirmed by PCR and a single colony, with a chromosomal integration, was serially subcultured in BHI at 40°C and screened for loss of chloramphenicol resistance. Allelic exchange mutagenesis was confirmed by PCR amplification and direct sequencing of the PCR product (GENOME express, Meylan, France). The mutants selected were named DG125A (\(\DeltaagrA\)) and DG119D (\(\DeltaagrD\)). The deletion of 106 bp in strain DG125A (\(\DeltaagrA\)) (Fig. 1B) and the deletion of 49 bp in strain DG119D (\(\DeltaagrD\)) (Fig. 1C) resulted in a frameshift causing the premature appearance of a stop codon and thus the premature stop of translation.

\textbf{Adhesion on glass slide}
An overnight culture of the bacterium in TSB was used to inoculate (1/100, vol/vol) fresh TSB and was grown at 25°C to an optical density at 600 nm of 0.1. Five microliters of the culture to be tested was then deposited on a glass slide, and the glass slide was incubated for 2 hours at 25°C. After incubation, the glass slide was washed twice in distilled water and the adhering cells were stained with a 0.1% (wt/vol) aqueous crystal violet solution for one minute and washed with distilled water. Adhering cells were observed using a ZEISS Axioplan 2 imaging microscope. For each experiment, 12 replicates resulting from three independent inocula were analysed.

Biofilm formation

(i) on polystyrene microplate An overnight culture of the bacterium in TSB at 25°C was used to inoculate (1/100, vol/vol) fresh TSB. One hundred microliters per well were dispensed on a 96 well microtiter plate (Nunc, Dominique Dutscher S.A., Brumath, France) which was incubated at 25°C for 16 to 72 h. Cells attached to the well walls were quantified as previously described (16) with some modifications. After incubation, the medium was removed from each well, and the plates were washed twice using a microtiter plate washer (Cellwash; Thermolabsystems, Cergy Pontoise, France) with 100 µl of 150 mM NaCl solution in order to remove loosely attached cells. The plates were then stained with a 0.05% (wt/vol) aqueous crystal violet solution for 45 min and washed three times. In order to quantitatively assess biofilm production, 100 µl of 96% ethanol (vol/vol) were added to each well, and the optical density at 595 nm was determined. For each experiment, 15 replicates resulting from three independent inocula were analysed. Each microtiter plate included eight wells with sterile TSB as control.

(ii) on stainless steel chips AISI 304 stainless steel chips of 8 cm diameter (Goodfellow SARL, Lille, France) were inserted in Petri dishes containing 20 mL of inoculated TSB (1/100, vol/vol) and incubated at 25°C for 2 h to allow adhesion. For biofilm development,
the medium was removed and 20 mL of fresh TSB was added. Biofilms were grown during
24 h and 72 h at 25°C. Following incubation, the medium was removed and 10 ml of saline
solution (150 mM NaCl) were softly poured twice onto coupons and agitated 1 min on an
orbital shaker at 240 rpm (IKA®KS 130 basic) to remove loosely adhering bacteria. Sessile
cells were detached from coupons in 10 ml saline solution by scraping with a sterile
disposable cell lifter (TPP, Dominique Dutscher S.A.).

**RNA extraction and cDNA preparation**

Cells were harvested by centrifugation (10000g 5 min), resuspended in 1 ml of Tri-reagent
(Sigma) and disrupted with glass beads (106 µm) in a FastPrep FP120 Instrument (Thermo
Savant-BIO101) at 4°C for 8x30s at 6000g. Nucleic acids were extracted twice in 0.2 volume
of chloroform and purified by precipitation in 1 volume of isopropanol. RNA pellets were
dried and resuspended in 80 µl of RNAse-free water. Nucleic acid concentrations were
calculated by measuring absorbance at 260 nm using a SmartSpec Plus Spectrophotometer
(Bio-Rad, Marnes La Coquette, France).

Before reverse transcription, 2 µg of total RNA were treated with 2 U of DNAse (Invitrogen)
as described by the manufacturer. The absence of chromosomal DNA contamination was
checked by real-time PCR. cDNA were then synthesized using an iScript cDNA synthesis kit
(Bio-Rad) as recommended.

**Real time PCR experiments**

Real time PCR as described by Desroche et al. (15) was used to quantify mRNA levels. Gene-
specific primers (Table 2, Fig. 1A) were designed to amplify cDNAs of the transcripts of
*agrB, agrD, agrC* and *agrA* with the Bio-Rad SYBR Green kit in a Bio-Rad I-Cycler. These
gene-specific primers were designed outside zones of deletion allowing the determination of
the transcript levels of the *agrB, agrD, agrC* and *agrA* genes in the parental strain and in both
mutants DG125A (*ΔagrA*) and DG119D (*ΔagrD*). This method was used to analyse their
mRNA level during planktonic growth at early exponential phase (6.5 \(10^7\) cfu.ml\(^{-1}\); OD\(_{600}\) of 0.1), mid-exponential phase (2.5 \(10^8\) cfu.ml\(^{-1}\); OD\(_{600}\) of 0.4) and stationary phase (4 \(10^8\) cfu.ml\(^{-1}\); OD\(_{600}\) of 0.6) and after 2 h, 24 h and 72 h of biofilm formation on AISI 304 stainless steel chips. The specificity of each primer pair was controlled by melt curves, and the mean of efficiencies for the five primer pairs was 98±9%. Results were analysed using the comparative critical threshold method (\(\Delta\Delta C_T\)) in which the amount of targeted mRNA was first of all normalised using a specific mRNA standard, then compared to a calibrator condition (15). drm, a gene coding for a phosphopentomutase was selected as internal standard since drm transcript levels were stable in the conditions tested. For our work, the calibrator condition corresponded to agrA mRNA level at OD\(_{600}\) of 0.1. The relative level of agrA mRNA at OD\(_{600}\) of 0.1 thus corresponded to 1. mRNA quantification was performed in triplicate from total RNA extracted from three independent cultures.

**RT-PCR**

RT-PCR were performed with cDNA synthesized with total RNA extracted from three different mid-exponential phase cultures (OD\(_{600}\) of 0.4) as described above. Specific primers (Table 2) were designed to amplify transcripts of the four genes and the two intergenic regions. PCR products were separated by electrophoresis on a 1% agarose gel (Invitrogen). Samples were checked for DNA contamination by performing PCR prior reverse transcription.

**Northern blotting**

Northern blot analysis were performed by fractionation of RNA samples on 1% (wt/vol) agarose gel containing 20% (wt/vol) formaldehyde. 30 \(\mu\)g of total RNA were loaded in each well of the gel. Transfer to a nylon membrane (Hybond-N, Amersham, Orsay, France) was performed as recommended. Internal probes (Fig. 1A) of the agrB, agrC and agrA genes were generated by PCR using primers described in Table 2. 10 ng of PCR products were labelled
with [α-\textsuperscript{32}P]dATP (Amersham) by the random priming method according to the manufacturer instructions (Invitrogen) to produce specific RNA probes. Sizes were determined with a RNA ladder (Invitrogen) as molecular weight standard.

**mRNA chemical half-life determination**

The chemical half-life of mRNA of the 4 genes of the *agr* operon was determined from mid-exponential phase cultures (OD\textsubscript{600} of 0.4). Total RNA was isolated as described above from samples taken 1, 4, 7 and 10 min after rifampicin treatment (250 µg.ml\textsuperscript{-1}, Sigma). The half-life was determined for each gene by real-time PCR using the time at which rifampicin was added as calibrator and 16S rRNA transcript levels as internal standard.

**Analysis of the 5’ end of *agr* mRNA**

Primer extensions were performed by incubating 5 µg of RNA isolated from *L. monocytogenes* cells in planktonic growth, 20 pmol of oligonucleotide, 92 GBq of [α-\textsuperscript{32}P]dATP (Perkin-Elmer, Courtaboeuf, France) and 100 U of SuperScriptII reverse transcriptase (Invitrogen). The oligonucleotides used in this experiment are described in Table 2. The corresponding DNA-sequencing reactions were carried out using the same primers and Cycle Reader\textsuperscript{TM} DNA Sequencing Kit as recommended by the manufacturer (Fermentas, Euromedex, Souffel Weyersheim, France).

A capping assay was used to distinguish 5’ triphosphate (indicating initiation points) from 5’ monophosphate (indicating processed products) according to Bensing *et al.* (9), using the 5’ RACE kit (Ambion, Applied Biosystems, Courtaboeuf, France). Only 5’ monophosphate are selectively ligated to an RNA oligonucleotide. The primary 5’ end may be ligated only after removal of a 5’ pyrophosphate through Tobacco Acid Pyrophosphatase (TAP) activity. The oligonucleotides used in this experiment are described in Table 2. Sequencing of 5’-RACE products obtained from TAP-treated and non-treated RNA was performed to differentiate cleavage products from primary transcripts. Primers used for PCR amplification between
adaptor and the gene of interest are described in Table 2. PCR products were sequenced by GENOME express.

**Statistical analysis**

A one-way analysis of variance (ANOVA) was performed using the SigmaStat® Version 3.0.1 software (SPSS Inc.) in order to test the significance of the differences (i) in gene expression during planktonic growth and biofilm formation using ΔCₜ value and (ii) in biofilm quantity. When one-way ANOVA was significant, the Holm-Sidak test (n=3, P<0.05) was used to locate significant differences.

**Nucleotide sequence accession number**

The DNA sequence data of the mutant strains described in this work have been deposited in the GenBank/EMBL/DDBJ under the following accession numbers: AM412557 and AM412558.
RESULTS

The agr operon is involved during the sessile growth of *L. monocytogenes* EGD-e

In order to study the involvement of the putative transcriptional regulator AgrA during sessile growth, an *agrA* in-frame deletion mutant of *L. monocytogenes* EGD-e was constructed. As expected, this mutation did not alter cell or colony morphology nor planktonic growth (data not shown). In contrast, mutant DG125A (ΔagrA) was affected on glass slide adherence. Indeed, the number of adhered cells of DG125A was significantly reduced (n=3, P<0.05) by 62% compared to the parental strain EGD-e (Fig. 2A). As cell-cell communication affects biofilm formation in many bacterial species, an *agrD* in-frame deletion was designed to generate a mutant unable to produce the putative autoinducer peptide processed from AgrD. As demonstrated in mutant DG125A (ΔagrA), no pleiotropic effect was observed in mutant DG119D (ΔagrD) (data not shown). Moreover, the adhesion phenotype of DG119D (ΔagrD) was similar to that of DG125A (ΔagrA) (Fig. 2A). Micrographs of the microscopic field of adhering cells on glass slides confirmed that the quantity of adhering cells with *L. monocytogenes* DG119D (ΔagrD) and DG125A (ΔagrA) was less compared to the parental strain EGD-e (Fig. 2B). These results suggested the involvement of the *agr* system during adhesion of *L. monocytogenes*, the first step in biofilm development.

The ability of *L. monocytogenes* EGD-e to develop biofilms on polystyrene was also affected by the deletion of *agrA* and *agrD* (Fig. 3). Indeed, there was significantly less biofilm (n=3, P<0.05) produced within the first 24 h of incubation. The differences were no longer significant during the later stages of biofilm formation, namely, at 48 h and 72 h.

In light of the sessile growth alteration observed in the mutants with deletion in the genes encoding the putative transcriptional regulator AgrA and the putative autoinducer peptide processed from AgrD, we therefore decided to investigate the expression of the *agr* operon...
Relative expression and transcriptional autoregulation of the genes of the *agr* operon

The transcription of the four genes of the *agr* operon was studied during sessile and planktonic growth using real time PCR experiments with each of the four primer sets (Fig. 1A, Table 2; b [BF2-BR2]; d [DF2-DR2]; c [CF2-CR2]; a [AF2-AR2]). Analysis of the relative expression levels indicated that, during sessile growth, the levels of transcripts of *agrB*, *agrD* and *agrC* were significantly lower (n=3, P<0.05) after 2 h of adhesion than after 24 h and 72 h of sessile growth (Fig. 4A). For each gene, the differences of relative expression observed between 24 h and 72 h of biofilm growth were not significant. The levels of *agrA* transcripts were similar at 2 h, 24 h and 72 h. Furthermore, during sessile growth, for each condition, the levels of transcripts of *agrB*, *agrD* and *agrC* were never significantly different. In contrast, the relative expression levels of *agrA*, for each condition, were significantly lower (n=3, P<0.05) than those of *agrB*, *agrD* and *agrC*.

During planktonic growth, the relative expression levels of each gene was not affected by the phase of growth. The levels of transcripts of *agrB* and *agrD*, for each condition, were never significantly different. In contrast, the relative expression levels of *agrC* and *agrA*, for each condition, were significantly lower (n=3, P<0.05) than those of *agrB* and *agrD*. For example, under our experimental conditions, at mid exponential growth phase (OD$_{600nm}$ of 0.4) *agrC* and *agrA* transcript levels were respectively of 13 and 24 fold lower than the *agrB* transcript level (Fig. 4A).

The relative expression levels of the four genes of the *agr* operon were determined at mid-exponential phase during planktonic growth (OD$_{600}$ of 0.4) in the parental and mutant (*ΔagrA*, *ΔagrD*) backgrounds. In mutant DG125A (*ΔagrA*), the relative expression levels of *agrB*, *agrD* and *agrC* were significantly lower (n=3, P<0.05) than those of the parental strain EGD-e (Fig. 4B). Indeed, the relative transcript levels for *agrB*, *agrD* and *agrC* were respectively
of 56, 68 and 3 fold lower than those measured with the parental strain. A similar pattern of
gene expression was observed for mutant DG119D (ΔagrD) (Fig. 4B). In terms of the relative
expression levels of agrA, no significant differences were observed between the mutants
(DG119D and DG125A) and parental strains. These results suggest an autoregulation of the
transcription of agrB, agrD and agrC and a low constitutive expression of the putative
transcriptional regulator AgrA.

The mRNA quantification data suggested that the agr system was autoregulated and pointed
to a differential expression of the agr genes during sessile and planktonic growth. Either post
transcriptional processing or transcription from another promoter region, not yet identified,
could account for these results.

**Processing of the mRNA and identification of the 5’ end of the mRNA agr operon**

In order to further investigate the hypothesis of a processing, RT-PCR, Northern blotting and
mRNA chemical half-life were carried out. The four genes and the two intergenic regions
were detected by RT-PCR (Fig. 5), indicating cotranscription of the complete agrBDCA
operon and the presence of a full-size transcript. PCR on the RNA samples before reverse
transcription gave no amplification signals, confirming that there was no contamination by
genomic DNA (data not shown). However, a polycistronic mRNA was never detected by
Northern blotting but only small size products (data not shown). The mRNA chemical half-
life was determined using real time PCR experiments with the same primer sets as above (Fig.
1A, Table 2). Results indicated that the chemical half-life of agrB and agrD transcripts was
7.4 and 6.8 min respectively, while it was lower for agrC and agrA (4.3 min and 4.1 min
respectively) (Fig. 6). To pinpoint these differences in chemical half-life and to highlight the
differential expression pattern observed by real time PCR, 5’RACE experiments were carried
out to search for transcription initiation points or cleavages within agrC and agrA transcripts.

Regardless of the treatment with TAP, multiple PCR products were detected, confirming
degradation after cleavage through RNAse activity. After amplification with adequate primers (Table 2), four 5’ ends were identified among the *agrC* fragments sequenced (Fig. 7). Similarly, five 5’ ends were observed among the *agrA* fragments sequenced (Fig. 7). From two hypotheses formulated, data analysis confirmed post-transcriptional cleavage and degradation.

5’ RACE was also used to characterize the 5’ end of the mRNA *agr* operon. PCR amplification was observed in TAP-treated and untreated samples, suggesting a processing event at the 5’ end, mapped by a “T” (Fig. 7). As expected, primer extension analysis with the three specific primers B34, B26 and B18 (Table 2) revealed two signals. They were separated by one nucleotide and corresponded to a “G” and the previously described “T”. This finding suggested that the 5’ end of the *agr* transcript was located 15 or 14 nucleotides upstream from the putative start codon (Fig. 7). Similar results were obtained in samples harvested at OD$_{600}$ of 0.1, 0.4 and 0.6. Moreover, two hexanucleotides (TGGTTA and TAAAAT) separated by 18 nucleotides were detected upstream. They presented similarities to the consensus –35 and –10 sequences of several promoters of housekeeping genes from Gram positive bacteria as well as from *E. coli* (21, 23). Sequence conservation is higher in the –10 region than in the –35 region, and a TGn extension was observed upstream of the –10 region. Similar features were found in several promoters of Gram positive bacteria (20, 38).
DISCUSSION

Orthologs of the *agr* system, initially described in *S. aureus*, have been reported in *L. plantarum, E. faecalis* and *L. monocytogenes* (6, 44, 48, 56). So far, the role of the *agr* system has not been clearly described in the pathogenic bacterium *L. monocytogenes*. In this study, we investigated the role of *agrA* and *agrD* in the sessile growth of *L. monocytogenes* and we focused on the molecular characterization of the transcription of the *agr* operon. In-frame deletions of *agrA*, that encodes a transcriptional regulator, or in-frame deletions of *agrD* encoding a propeptide affected adhesion and the early stages of biofilm formation on glass and polystyrene surfaces within the first 24 h of incubation. No significant differences were observed afterwards. These results are in accordance with those obtained by Sturme *et al.* (48) with a *lamA* mutant of *L. plantarum* WCFS1. Indeed, the *lamA* mutant was impaired in its ability to adhere to glass surfaces. It showed a 1.5-fold and 1.7-fold decrease in glass adherence compared to the parental strain after 24 and 48 h respectively. In contrast, Vuong *et al.* (52), working with *S. aureus* RN6390 and 601055, demonstrated that their *agr−* genotype led to a pronounced attachment to polystyrene, 1.8-fold and 2.5-fold increase respectively compared to that of the isogenic *agr+* wild type after 24 h. Biofilm-associated infections have special clinical relevance and in staphylococcal infections, these diseases include endocarditis, osteomyelitis, implanted device-related infections, and even some skin infections (54). Indeed, the *agr−* biofilm phenotype may have important consequences. For example, the dysfunction of *agr* is correlated with persistent bacteremia in *S. aureus* (49) and mutation of *S. aureus agr* system increased bacterial persistence (52), suggesting that interference with cell-cell communication would enhance rather than suppress this important type of staphylococcal disease (42).

In our experimental conditions, growth phase-dependent transcriptional regulation was not
observed during planktonic growth. This is in agreement with a previous report which showed that during exponential and stationary phases, the amount of *agrB* and *agrC* mRNAs was not significantly different (6), although a twofold difference in the quantity of *agrA* transcripts was recorded by these authors. In contrast, the transcription of the orthologous *agr*, *lam* and *fsr* operons from *S. aureus* MN NJ, *L. plantarum* WCFS1 and *E. faecalis* OG1RF respectively, increased from early to mid-exponential phase, suggesting growth phase-dependent transcription up-regulation (44, 48, 56).

During sessile growth, the transcription of the *agr* genes depended on the stage of biofilm development, suggesting that this system is important during biofilm development. Interestingly, a significant decrease in the transcript levels of *agrB*, *agrD* and *agrC* was observed following initial attachment. At first glance, this may seem surprising as initial attachment is the step where *agr* impairment is most detrimental to biofilm formation; this points out that developmental regulations involved during sessile growth are complex (22). In *L. monocytogenes*, *agr*-dependent regulation may be transitory during biofilm formation as was observed in other systems. For example, two communication systems (*las* and *rhl*) play a role in transitional episodes in *Pseudomonas aeruginosa* biofilm development. The Las regulon is involved in early biofilm development but not in later stages; on the opposite, the Rhl regulon plays a role in maturation of the biofilm (14, 46). The *agr* system of *L. monocytogenes* could also regulate the expression of proteins necessary for the ability to attach to abiotic surfaces without being involved once the cells attach to the surface. Indeed, in *S. aureus* and *S. epidermidis*, *agr*-dependent regulation of the expression of several adhesion proteins has been demonstrated (12, 34).

Significant differences were measured in the relative quantities of the transcripts of *agrB*, *agrD*, *agrC* and *agrA*, while the differences between the transcripts of *agrB* and *agrD* were never significantly different. This observation suggested either post transcriptional processing...
of the full-size *agr* transcript of *L. monocytogenes* or the presence of another promoter region between *agrD* and *agrC*. Determination of 5’ ends corresponding to cleavage, and not to an initiation transcription points, supported the post transcriptional events hypothesis. Indeed, several 5’ ends were detected within *agrC* and *agrA* transcripts. This suggested also that most of the transcripts quantified by real time PCR were degradation products generated after cleavage of the full-length transcript, probably within the intergenic region or at the 5’ end of *agrC* and *agrA*. These post-transcriptional events may have regulatory functions resulting in a differential stability and a rapid processing of the mRNA. This could account for a fine tuning of the expression of the individual genes of the *agr* operon as has previously been suggested for the pattern of expression of other loci of Gram-negative and Gram-positive bacteria (2, 24, 25, 37, 40). Moreover, our work indicated that the *agr* operon of *L. monocytogenes* was autoregulated in a positive way since the deletion of *agrA* or *agrD* reduced the transcription of *agrB*, *agrD* and *agrC*, although *agrA* transcription was not *agr* dependent. A similar pattern has been described in the orthologous *fsr* system of *E. faecalis* in which expression of *fsrB*, *fsrD* and *fsrC* are *fsr* dependent, while expression of the *fsrA* is weak, constitutive and *fsr*-independent (29, 45). It is proposed, in one hand, that the regulator is constitutively expressed in order to provide a basal amount of regulator ready to respond to the presence of the signal in the environment of the cell; on the other hand, when the signal is high in the environment of the cell, it would induce the expression of the transmembrane protein, the propeptide and the sensor; this in turn would enable the transfer of the signal to the neighbouring cells and prepare the cell to the monitoring of an amplified signal (18, 26).

Two 5’ ends of the *agr* mRNA were determined. One was mapped to a “G” and located at an appropriate distance downstream of the putative promoter region (43), leading us to consider it as the apparent start site for the promoter of the *agr* operon. The second 5’ end identified was located one base downstream of the 5’ end of the primary transcript. Such cleavage of
one nucleotide downstream of the initiation site may derive from a modification of the primary transcript by a phosphatase or pyrophosphatase, or from endonucleolytic cleavage (31, 47, 50, 51). The significance of such commonly observed processing remains to be clarified.

In conclusion, this study is the first description of the involvement of cell-cell communication on adherence of *L. monocytogenes*. Our data showed that impairment of the response regulator or the propeptide resulted in a similarly altered adhesion and biofilm phenotype. The *agr* system of *L. monocytogenes* differed from the homologous systems previously described as post transcriptional processing occurred at the site of initiation of transcription and within the full-length transcript. It will be of interest to investigate the significance of such a processing in the expression of the *agr* system and in the physiology of *L. monocytogenes*. 
ACKNOWLEDGEMENTS

This work was supported by the Ministère de l’Education Nationale de la Recherche et de la Technologie, the Université de Bourgogne and the Institut National de la Recherche Agronomique. We thank Dr. Sofia Kathariou for providing the pGF-EM vector, Dr. Philippe Gaudu for his critical reading of this manuscript and Mary Boulay for her reading of the English text.
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biofilm formation with biological, and physical environment on quorum sensing in structured microbial communities. Anal. 499
subtilis
Listeria monocytogenes biofilm formation in but does not contribute to low-temperature transcription. J. Bacteriol.
biogenesis in the Lactobacillus plantarum


### TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Relevant property</th>
<th>Reference or source</th>
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<td><em>E. coli</em> TOP10</td>
<td>Cloning host</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>E. coli</em> Match1</td>
<td>Cloning host</td>
<td>Invitrogen</td>
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<td><em>L. monocytogenes</em> EGD-e</td>
<td>Wild type of serotype 1/2a for which the genome sequence is available</td>
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<td><em>L. monocytogenes</em> DG125A</td>
<td><em>L. monocytogenes</em> EGD-e with in-frame deletion of <em>agrA</em> gene</td>
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<td>Km(^r), Am(^r), 4.4-kb derivative of pCR21-TOPO containing 0.6-kb 3’ end of <em>agrD</em> and 5’ end of <em>agrC</em></td>
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<td>Cm(^r), Am(^r); 10.4-kb derivative of pGF-EM containing 0.4-kb 5’ end of <em>agrD</em></td>
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<td>Km(^r), Am(^r), 4.3-kb derivative of pCR21-TOPO containing 0.5-kb internal region of <em>agrA</em></td>
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<td>pGID119</td>
<td>Cm(^r), Am(^r); 9.4-kb derivative of pGID112 containing 3’ end of <em>agrD</em> inserted into XbaI/EcoRI site of pGID109</td>
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*a* Km\(^r\), kanamycin resistant; Am\(^r\), ampicillin resistant; Cm\(^r\), chloramphenicol resistant.
TABLE 2. Oligonucleotides used in this study

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<td></td>
<td>CGAAATCACACATTCCGCC</td>
<td></td>
<td>B34</td>
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</table>

<sup>a</sup> Specific restriction sites are underlined and extra nucleotides added to include restriction sites to the PCR product are shown in bold type.
FIGURE LEGENDS

FIG. 1. (A) Schematic diagram of *L. monocytogenes* agr operon. The grey arrows indicate the orientation and the size in base pairs of the four genes. Numbers between parentheses indicate the size in base pairs of the two intergenic regions. Arrowheads indicate the positions of the oligonucleotides used for real time PCR: b (BR2 ; BF2), d (DF2 ; DR2), c (CF2 ; CR2), a (AF2 ; AR2) and black lines indicate the probes used for Northern blotting (NB ; NC ; NA). The position of the transcription initiation site and the transcription termination site are indicated respectively by the bent arrow and the grey dot.

DNA and deduced amino acid sequence of (B) *agrA* gene containing a 106-bp deletion in DG125A (*ΔagrA*) mutant and of (C) *agrD* gene containing a 49-bp deletion in DG119D (*ΔagrD*) mutant. The position of the deletion is represented by an inverted black triangle, the nucleotides before and after the deletion are mentioned, the RBS are underlined, the start codons are boxed and the stop codon is represented by three stars.

FIG. 2. (A) *L. monocytogenes* EGD-e ■, DG125A (*ΔagrA*) □, and DG119D (*ΔagrD*) □ cell adhesion after 2 hours incubation at 25°C on glass slides. Histograms represent the number of adhered cells counted per microscopic field. Each bar indicates the mean of three independent experiments with four microscopic fields per experiment. (B) Micrographs of microscopic fields showing *L. monocytogenes* EGD-e, DG125A (*ΔagrA*) and DG119D (*ΔagrD*) cells adhering to glass slides after 2 hours incubation at 25°C. Magnification x 63.

FIG. 3. Biofilm formation by *L. monocytogenes* EGD-e ■, DG125A (*ΔagrA*) □, and DG119D (*ΔagrD*) □ in batch conditions in polystyrene 96-well plates after 16, 24, 48 and 72
hours incubation at 25°C. Each histogram indicates the mean of three independent
experiments with five measurements for each point.

FIG. 4. Semilogarithmic representation of (A) relative expression levels of the agrB, agrD, agrC and agrA genes of the parental strain L. monocytogenes EGD-e determined during biofilm formation (2 hours □, 24 hours ☐ and 72 hours ◇) and planktonic growth (early exponential phase OD$_{600}$ of 0.1 ◆, mid exponential phase OD$_{600}$ of 0.4 ●, stationary phase OD$_{600}$ of 0.6 ▲). (B) relative expression levels of the agrB, agrD, agrC and agrA genes of L. monocytogenes determined during mid exponential phase OD$_{600}$ of 0.4 (EGD-e strain ■, DG125A (ΔagrA) strain □, and DG119D (ΔagrD) strain ◻). For both graphs, gene expression was quantified using real time PCR and the comparative critical threshold (ΔΔC$_T$) method. The drm gene was used as the internal standard, and expression of the agrA gene in the early exponential phase (OD$_{600}$ of 0.1) was used as the calibrator. Three independent experiments were performed; histograms indicate standard deviations.

FIG. 5. RT-PCR analysis of RNA from L. monocytogenes cells at mid exponential phase (OD$_{600}$ of 0.4). (A) The dotted lines enclosed by arrows indicate the positions of the primers and PCR products. (B) The amplified products, numbered 1 to 4, were separated by electrophoresis on 1% agarose gel and corresponded respectively to the expected size of 567, 388, 435 and 353 base pairs. Sizes of the DNA marker fragments are notified in base pairs.

FIG. 6. Chemical half-life of the transcripts of the agr operon. Semilogarithmic plots of mRNA decay corresponding to agrB ■agrD ◆agrC ◻ and agrA ▲ genes. Total RNA was prepared 0, 1, 4, 7 and 10 minutes after treatment with rifampicine (250 µg.mL$^{-1}$). Results were obtained by real-time PCR analysis and normalized using 16S mRNA amounts. The
correlation coefficient ($R^2$) and half life ($T_{1/2}$) were determined for each regression analysis. Three independent experiments were carried out.

FIG. 7. Analysis of the 5' end of $agrB$, $agrC$ and $agrA$ transcripts using total RNAs isolated from EGD-e cells collected during the exponential growth phase (OD$_{600}$ of 0.4). # indicates the 5' end identified by primer extension and * indicates processing sites identified by 5' RACE. The putative –10 with TGn extension and –35 sequences are double underlined, the RBS are underlined, the start codons are boxed and the stop codon of $agrC$ is represented by three stars.
Fig. 1

\[
\begin{align*}
&\text{NB} & &\text{NC} & &\text{NA} \\
&\text{agdB} & &\text{agdD} & &\text{agdC} \\
&612 & &563 & &1293 \\
&\text{agdI} & & & &726 \\
&3\mu\text{m} & &\text{500 bp} & &
\end{align*}
\]

Fig. 2

(A) Cells per microscopic field

(B) EGD-e DG119D DG125A

(C) EGD-e DG125A DG119D

Fig. 3

OD 595 nm

Time (h)

0 0.2 0.4 0.6 0.8 1.0

0 0.2 0.4 0.6 0.8 1.0

0 0.2 0.4 0.6 0.8 1.0

0 0.2 0.4 0.6 0.8 1.0

0 0.2 0.4 0.6 0.8 1.0
Fig. 4

Fig. 5

Fig. 6
**Fig. 7**

**agrB**

\[
\text{ttatgggtaaattcgttgtaaaat} \\
\text{attagtggaggt} \\
\text{gaattagttgagtaattttactgcaaaagtccctttgtca}
\]

M S N F T A K V P L S

\[
\text{tacatttttggtta}
\]

**agrC**

\[
\text{ttatgggtaaattcgttgtaaaat} \\
\text{attagtggaggt} \\
\text{gaattagttgagtaattttactgcaaaagtccctttgtca}
\]

M S N F T A K V P L S

\[
\text{agaaggcaaacttacatatatttaaacatagatattttagacagtggagg}
\]

M F S I L M A I I Q I T

\[
\text{attaatatgtttagtattttgatggcaattatacagataacg}
\]

G I F I A I Q I L T N K V F S I K E G L V T I A I A M L A F

**agrA**

\[
\text{agaaggcaaacttacatatatttaaacatagatattttagacagtggagg}
\]

M F S I L M A I I Q I T

\[
\text{attaatatgtttagtattttgatggcaattatacagataacg}
\]

G I F I A I Q I L T N K V F S I K E G L V T I A I A M L A F

\[
\text{agaaggcaaacttacatatatttaaacatagatattttagacagtggagg}
\]

M F S I L M A I I Q I T

\[
\text{attaatatgtttagtattttgatggcaattatacagataacg}
\]

G I F I A I Q I L T N K V F S I K E G L V T I A I A M L A F

\[
\text{agaaggcaaacttacatatatttaaacatagatattttagacagtggagg}
\]

M F S I L M A I I Q I T

\[
\text{attaatatgtttagtattttgatggcaattatacagataacg}
\]

G I F I A I Q I L T N K V F S I K E G L V T I A I A M L A F

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M F S I L M A I I Q I T

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M F S I L M A I I Q I T

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G I F I A I Q I L T N K V F S I K E G L V T I A I A M L A F

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M F S I L M A I I Q I T

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\]

G I F I A I Q I L T N K V F S I K E G L V T I A I A M L A F

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\]

M F S I L M A I I Q I T

\[
\text{attaatatgtttagtattttgatggcaattatacagataacg}
\]

G I F I A I Q I L T N K V F S I K E G L V T I A I A M L A F

\[
\text{agaaggcaaacttacatatatttaaacatagatattttagacagtggagg}
\]

M F S I L M A I I Q I T

\[
\text{attaatatgtttagtattttgatggcaattatacagataacg}
\]

G I F I A I Q I L T N K V F S I K E G L V T I A I A M L A F

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G I F I A I Q I L T N K V F S I K E G L V T I A I A M L A F

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\]

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\[
\text{attaatatgtttagtattttgatggcaattatacagataacg}
\]

G I F I A I Q I L T N K V F S I K E G L V T I A I A M L A F

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M F S I L M A I I Q I T

\[
\text{attaatatgtttagtattttgatggcaattatacagataacg}
\]

G I F I A I Q I L T N K V F S I K E G L V T I A I A M L A F

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\]

M F S I L M A I I Q I T

\[
\text{attaatatgtttagtattttgatggcaattatacagataacg}
\]

G I F I A I Q I L T N K V F S I K E G L V T I A I A M L A F

\[
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\]

M F S I L M A I I Q I T

\[
\text{attaatatgtttagtattttgatggcaattatacagataacg}
\]