agr System of *Listeria monocytogenes* EGD-e: Role in Adherence and Differential Expression Pattern[∇]

Aurélie Rieu,¹ Stéphanie Weidmann,² Dominique Garmyn,¹ Pascal Piveteau,^{1*} and Jean Guzzo²

UMR 1229 Microbiologie du Sol et de l'Environnement, Université de Bourgogne, INRA, F-21000 Dijon, France,¹ and Laboratoire REVV, Université de Bourgogne, IUVV, F-21000 Dijon, France²

Received 16 March 2007/Accepted 27 July 2007

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 the levels were lower after the initial attachment period than during biofilm growth, whereas transcription during planktonic growth 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 reverse transcription-PCR experiments revealed that the four genes were transcribed as a single messenger, chemical half-life and 5' RACE (rapid amplification of cDNA ends) experiments indicated that the full size transcript underwent cleavage followed by degradation of the *agrC* and *agrA* transcripts, which suggests a complex regulation of *agr* transcription.

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, etc.). In connection 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). Like 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 and 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 signaling systems have been identified; to regulate these systems, bacterial extracellular signaling molecules called autoinducers are produced (8). The acylhomoserine lactones have been identified as autoinducers in gram-negative bacteria (3, 13, 27, 46). The autoinducer 2 is found in both gram-negative and gram-positive bacteria (5, 7, 11, 35, 36, 54). Finally, peptide-mediated signaling 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

* Corresponding author. Mailing address: UMR 1229 Microbiologie du Sol et de l'Environnement, Université de Bourgogne, INRA, F-21000 Dijon, France. Phone: 33 3 80 39 68 93. Fax: 33 3 80 39 39 55. E-mail: piveteau@u-bourgogne.fr. development is complex (57). It depends on the hydrodynamic conditions of the experimental setup. 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), *Lactoba-cillus 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 are available. Williams et al. (53) showed that among 16 putative response regulator genes of L. monocytogenes EGD-e, inframe deletion in agrA did not affect the growth in brain heart infusion (BHI) medium at various temperatures (20, 37, and 43°C), in the presence of 9% NaCl, 5% ethanol, or 0.025% H₂O₂. Swimming motility was also not affected. 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. Second, we determined the expression pattern of the four genes of the *agr* operon during planktonic and sessile growth, and we evidenced posttranscriptional events during expression of this operon.

^v Published ahead of print on 3 August 2007.



	aa	ago	ago	tat	cqq	aac	ctatgaaaaatatgaataaatcagttggtaa												
			42	9	1		Μ	K	Ν	М	N	K	S	V	G	K	F		
C)	ct	ttc	tag	act	tgo	tto	atg	ttt	gta	tac	gaa	icca	aaa	agt	CC a	ttt	.gt g		
	L	S	R	L	A	S	C	L	Y	Т	Ν	0	K	V	Н	L	* *		

FIG. 1. (A) Schematic diagram of L. monocytogenes agr operon. The gray 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), and a (AF2; AR2). The black lines indicate the probes used for Northern blotting (NB, NC, and NA). The position of the transcription initiation site and the transcription termination site are indicated, respectively, by the bent arrow and the gray dot. (B and C) DNA and deduced amino acid sequences of agrA gene containing a 106-bp deletion in DG125A ($\Delta agrA$) mutant (B) and of agrD gene containing a 49-bp deletion in DG119D ($\Delta agrD$) mutant (C). The position of the deletion is represented by an inverted black triangle, the nucleotides before and after the deletion are mentioned, the ribosome binding sites are underlined, the start codons are boxed, and the stop codon is represented by three asterisks.

MATERIALS AND METHODS

Bacterial strains and growth media. The bacterial strains and plasmids used in the present 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 ($\Delta agrD$) and

L. monocytogenes DG125A ($\Delta agrA$) 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 (*L. conic*); ampicillin, 200 µg ml⁻¹ (*L. coli*); and 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. monocyto-genes* EGD-e by using a two-step integration/excision procedure (10) that 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 600-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 *E. coli* TOP10. Plasmid pGID118 was digested with Nhel/EcoRI and the resulting 500-bp fragment containing an internal part of *agrA* was ligated into pGID121 restricted with XbaI/EcoRI to obtain pGID125. This plasmid was electroporated into *L. monocytogenes* EGD-e.

Second, a similar strategy was used for the construction of an *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 *agrD*. The PCR product was cloned into pGF-EM (33) to obtain pGID112. Primers D3 and D4 (Table 2) were used to amplify a 600-bp DNA fragment including the 3' end of *agrD* and the 5' end of *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 *L. monocytogenes* EGD-e.

Finally, transformants were selected on BHI agar plates (Biokar Diagnostics) containing 10 μ g of chloramphenicol (Sigma) ml⁻¹. 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 ($\Delta agrA$) and DG119D ($\Delta agrD$). The deletion of 106 bp in

Bacterial strain or plasmid	Relevant property ^a	Source or reference		
Strains				
E. coli TOP10	Cloning host	Invitrogen		
E. coli Match1	Cloning host	Invitrogen		
L. monocytogenes EGD-e	Wild type of serotype $1/2a$ for which the genome sequence is available	39		
L. monocytogenes DG125A	L. monocytogenes EGD-e with in-frame deletion of the agrA gene	This work		
L. monocytogenes DG119D	L. monocytogenes EGD-e with in-frame deletion of the agrD gene	This work		
Plasmids				
pGF-EM	Cm ^r Am ^r ; 9.4-kb derivative of pCON-1 containing the 0.8-kb <i>gfp</i> gene of pKV111 and the <i>erm</i> gene of Tn917	33		
pGID109	Km ^r Am ^r ; 4.4-kb derivative of pCR21-TOPO containing the 0.6-kp 3' end of <i>agrD</i> and the 5' end of <i>agrC</i>	This work		
pGID112	Cm ^r Am ^r ; 10.4-kb derivative of pGF-EM containing the 0.4-kb 5' end of agrD	This work		
pGID118	Km ^r Am ^r ; 4.3-kb derivative of pCR21-TOPO containing the 0.5-kp internal region of <i>agrA</i>	This work		
pGID119	Cm ^r Am ^r ; 9,4-kb derivative of pGID112 containing the 3' end of <i>agrD</i> inserted into the XbaI/EcoRI site of pGID109	This work		
pGID121	Cm ^r Am ^r ; 10.6-kb derivative of pGF-EM containing the 0.6-kb 3' end of <i>agrC</i> and the ATG of <i>agrA</i>	This work		
pGID125	Cm ^r Am ^r ; 9.3-kb derivative of pGID121 containing the internal region of <i>agrA</i> inserted into the XbaI/EcoRI site of pGID118	This work		

TABLE 1. Bacterial strains and plasmids used in this study

^a Km^r, kanamycin resistant; Am^r, ampicillin resistant; Cm^r, chloramphenicol resistant.

Constructions pGID121 AAAGCTTCGCGTTTTTCGTCATGATT HindIII C13 pGID112 AICTAGCATAAATTCATCCCCATTCT Xbal A12 pGID118 AGCTAGCAACAGGAGATCCCCATTGAG Nhel A15 pGID112 CTAACGTTACATCCACATT E2 pGID109 CTATGAGTCCGCAACATTICATGG Xbal D3 pGID109 CTATGAGTCTCACACTTICATG Xbal D3 qRT-PCR drm GCGGAAAAACCACTTGCTATG DRMP2 qRT-PCR drm CGGCAAAAGCTGTTATATGGG DRMP2 qRT-PCR agrB CGGCAGACACAGAAAGGTGTTGTCATGTATACG BF2 qRT-PCR agrD AAATCAGTTGGTTAATACG BR2 qRT-PCR agrC CGGGGGCAATCGCAGGTTTTG DR qRT-PCR agrA GCAAGCAGAGAAGGATTTTCCTTCTAG DR2 qRT-PCR agrA GCAAGCAGAGAGAGATTCCCAAGGTT DR2 qRT-PCR agrA GCGCAGCAAAAACCAGGATTTCCT DR2 qRT-PCR agrA GCGACAATCGCAGGATTCCTTC CR2 qRT-PCR agrA GCGACGACAGAAGATTCCCTTC DR2 qRT-PCR agrA GCAAGCGGGATTCT DR RT-PCR agrD CAAGCGGGATTCGTCGAACT HindIII D1 RT-PC	Description and application ^a	Sequence $(5' \rightarrow 3')^b$	Created restriction site	Primer
pGID121AAGCTTCGCGTTTTCGTCATGATTHindIIIC13ATCTCAGCATAAATTTCATCCCCATTCTXbalA12pGID118AGCTAGCAACAGGAGATCCGTTTGAGNhelA15pGID112CAAGCTTTTACTGCAAACAHindIIIID1pGID109CTATGAGTCATAGATTCCACTTD2pGID109CTATGAGTCATGAGTTGCTCATGXbalD3AACAAATCCGCCAACATTCCD4D3RNA analysisGCGGAAAAACCACTTGCTTACTDRMF2gRT-PCR agrBCGGCAAAAATCCGCCAACATTCGDRMF2qRT-PCR agrDAAACAAATCGGTGTGTCAATGTATAGGDR2qRT-PCR agrCCGGGAACGGTGTGTCAATGCAACGBR2qRT-PCR agrCCGGGAACGGTTTTGGTTGGTAACADR2qRT-PCR agrACGGCAAGCACGAAGATTGCR2qRT-PCR agrACGCAGCCACACACAGATTGGTTGGTTACCACR2qRT-PCR agrACGCAGCCAGAAGAACGATTTCCAAR2qRT-PCR agrACGCATGTGTGGTGGTGCGTAACCAAR2qRT-PCR agrACGCAGCAGAAAACGAGTTTCCR2qRT-PCR agrACGCAGCAGAAAACGATTAR2qRT-PCR agrACGCAGCAGAAGAACGATTCDRRT-PCR agrACGCATTTCACAAATGCGCGATTCDRRT-PCR agrACGCATTTCACAAAACAAACAATACAATACCCATGAAAGTGCCGGATTCCART-PCR agrACGCATTCCATAAACCAGCGCATTCCATDRRT-PCR agrACGCAGCAGCAGAAGTGCTGTGCAAG2RT-PCR agrACGCATTCATGCAAACGCGCATTCCATDRRT-PCR agrACGCAGCAGCATTCCATDRRT-PCR agrACGCAGCAGCATTCCATCDRRT-PCR agrACGCCTCAGAAAGGAGTGCTGGGACC35RT-PCR agrA <td>Constructions</td> <td></td> <td></td> <td></td>	Constructions			
ATCTAGACATAAATTCATCCCCATTCTXbalA12pGID118AGCTAGCAACAGGAGATCCGTTTGAGNhelA15cTTACCATAAATTCCACATTE2pGID112CAAAGCTTTACTGCAAACAHindIIIpGID109CTATGAGTCCACACTTCATGD2pGID109CTATGAGTCTACAGTGCTTCATGXbalgRT-PCR agrBCGGGAAAAACCACTTGCTACTDRME2qRT-PCR agrBCGGCAGACACAGAACGAGTTGCAATGTATTAGGBR2qRT-PCR agrCGGGAAAAACCACTTGCTTCTTGDRqRT-PCR agrCGGGCAGACCAGAAAGTGTGTGATACAACGBR2qRT-PCR agrCGGGCAGACCAGAAAACCACTTGCTTCTDR2qRT-PCR agrCGGGCAGACCAGAAAACCACTTGCTTCTGDR2qRT-PCR agrCGGGCAGACCAGAAAACCACTTGCTTCTGDR2qRT-PCR agrCGGGCGCAACCAGAAAACCACTTGCTTACADR2qRT-PCR agrCGGGCGCTCATCCGCGGTACR2qRT-PCR agrAGCAAGCCAGAAGACGATTTCCCAAHindIIIqRT-PCR agrAGCAAGCCAGAAGACGATTTCDF2qRT-PCR agrDCAAGCCTTACCAAAAGCAGTTTCDFqRT-PCR agrDCCCATGAAGTGTGCGAACAHindIIIDIDIDIRT-PCR agrDACAAGCCATTGCGCAACAHindIIIDRDF2CAAAGGAAGGTCGTTGCCAACAHindIIIRT-PCR agrDACAAGCACTTCCCAAAAACAACAATCACCATCATCATGCCGCATCCAART-PCR agrDACCATCATGTCGCGCAACACAHindIIIDRDF2CAAAGCACTTCATCGTTGGCAAACAHindIIIRT-PCR agrDACCATCATGTGCGCAACACAHindIIIRT-PCR agrDACCATCATCATGTCTCGCGCAAACACACCATCATCATCATCCTCAAAACAACAATCAATACCC	pGID121	AAAGCTTCGCGTTTTTCGTCATGATT	HindIII	C13
pGID118 AGCTAGCAACAGGAGATCCGTTTGAG Nhel A15 pGID112 CTAAGCAATAAATTCCACTT E2 pGID109 CTATCGATGAATAATTCCACTG D2 pGID109 CTATGAGTIGATGATTCATG D3 AAGAATCGCGCAACATTCC D4 RNA analysis gRT-PCR dm GGGGAAAAACCACTTGCTTACT DRMF2 gRT-PCR dm GGGGAAAAACCACTTGCTTACT BR2 gRT-PCR agrB CGGGAAAGGTGTGTCAATGTATAGG DRMR2 gRT-PCR agrB CGGGGACGACACAGAAAGTTTG BR2 gRT-PCR agrD AAATCAGTTGGTAATTCCTTCTAG DF2 qRT-PCR agrD AAATCAGTTGGTGATATCCACG BR2 qRT-PCR agrD CGGGGTCAATCGCAGGTTTG CF2 qRT-PCR agrA GCAAGCAGAAGAACGATTTGCTAACA HindIII qRT-PCR agrA GCAAGCAGAAGAACGATTTCCA AF2 qRT-PCR agrA GCAAGCAGAAGAACGATTCCA BR2 gRT-PCR agrA CGCGTCTCAAAAAACAAGAATCA GR2 RT-PCR agrA CCAAGCGGAATCTGTC DF RT-PCR agrBD CAAAGCGTTTACCAAATGGCGATTCT DR RT-PCR agrCA GGACATCACACAATCAATCA HindIII DI RT-PCR agrCA GGCACTTCACAAAAACAAATCAATCA C10 RT-PCR agrCA CGGCGCACTTCCAAAAAACAAATCAATCA C10 RT-PCR agrCA GG		ATCTAGACATAAATTCATCCCCATTCT	XbaI	A12
$\begin{array}{cccc} CTTACCATAAAATTCCACTT & E2 \\ pGID112 & CAAAGCTTTACGCAACA & HindIII & D1 \\ AAGAATCCGCAACTTTCATGG & HindIII & D1 \\ AAGAATCCGCAACTTTCATGG & XbaI & D3 \\ AACAAATTCGCCAACATTCC & XbaI & D4 \\ \hline \\ RNA analysis & & & & & & & & & & & & & & & & & & $	pGID118	AGCTAGCAACAGGAGATCCGTTTGAG	NheI	A15
pGID112 CAAGACTTTACTGCAAACA HindIII D1 AGAATCCGCAACCTTTAGG D3 pGID109 CTATGAGTCGCAACATTTCG Xbal D3 RNA analysis gRT-PCR drm CGGGAAAAACCACTTGCTTACT DRMP2 qRT-PCR drm GCGGAAAGGTGTGTCAATGTATAGG DRMR2 qRT-PCR drm CGGCAAGGTGTGTCAATGTATAGG DRMR2 qRT-PCR agrB CGGCAGACACAGAAAGTTG BP2 qRT-PCR agrD AAATCAGTTGGTAATATCCTTCTAG DP2 qRT-PCR agrD AAATCAGTTGGTGAATTCCTTTCTAG DP2 qRT-PCR agrC GGGGTCAATCGCAGGTTTG CF2 qRT-PCR agrA GGCAAGCAGAAGAACGATTTCCAA AR2 qRT-PCR agrA CGCTGTCGTAAACACAAGAATAT AR2 qRT-PCR agrA CGCAGTCGCAGAAGAACGATTCCAA AR2 qRT-PCR agrA CGCAGTCTCAAAAAACAAGAATAT AR2 RT-PCR agrA CGCAAGCAGGAAAGCGAATTCAT DF RT-PCR agrA CGCAATTGTGCCGGATTTG DF RT-PCR agrC CGAAGCAGAAGACGGAATTCAT DR RT-PCR agrC GGAATTGTGCCGGAATTCAT DF RT-PCR agrC GGACATTCAAAAACAATCAATCA C10 RT-PCR agrC CGCAGTTACCAAAAACCAATTCAT C10 RT-PCR agrA CGCTGCGAATTCAT C10 RT-PCR agrC GGGACTTACAAAAA	1	CTTACCATAAAATTCCACTT		E2
pGID109 AAGAATCCGCAACTTTCATGG D2 pGID109 CTATGAGTCGAGATTGCTTCATG Xbal D3 qRT-PCR dm GCGGAAAAACCACTTGCTTACT DRMP2 qRT-PCR dm CGGCAAAAGTGTGTGCAATCATTAGG DRMP2 qRT-PCR agrB CGGCAGACACAGAAAGTTTG BF2 qRT-PCR agrD AATCAATTGGTGTCAATCTTCTTCTAG DF2 qRT-PCR agrD AAATCAGTTGGTATTAGCAACG BR2 qRT-PCR agrD CAATCGATCGTAGTTGTGCGTATCACA DR2 qRT-PCR agrA GCGCACACGCAGAGAACACAGTAT BR2 qRT-PCR agrA GCACTTACGCAGGTTTTGGC CF2 qRT-PCR agrA GCAAGCAGAGAACACAGTATA AR2 qRT-PCR agrD CAAGCTTTTACTGCCAGACTTT DR TTGCGATTTCACACAAATGGACCATT DR R2 RT-PCR agrD CAAGCTTTTACTGCCAGACTA HindIII RT-PCR agrD CAAGCTTTTCATGCGCAACA HindIII RT-PCR agrD CAAGCATTCTACAAAAACAACAATCAATAC C35 RT-PCR agrD CCATGCAAAGTGGCGCATTCT DR RT-PCR agrD CAAGCATTGCGCGCATTCT C11 RT-PCR agrC GGAAATGTACCACATCGCGCATATCAT C10 RT-PCR agrCA CCAAGCACATCAACATCATCAT C11 RT-PCR agrB CCCATGAAAGTGTACATCAT C11 RT-PCR agrB CCATGGTAACATCGCGC	pGID112	CAAAGCTTTTACTGCAAACA	HindIII	D1
pGID109CTATGAGTTGACATTGCTTCATGXbalD3 D4RNA analysis	1 -	AAGAATCCGCAACTTTCATGG		D2
AACAAATTCGCCAACATTCC D4 RNA analysis (RT-PCR agrB GCGGAAAGAACCACTTGCTTACT DRME2 qRT-PCR agrB CGGCAAGGTGTGTCAATGTATAGG DRME2 qRT-PCR agrD AAATCAGTTGGTAAATGCAACG BR2 qRT-PCR agrC GGGGTCAATGGTATTAGCAACG DR2 qRT-PCR agrA AAATCAGTTGGTAAATTCCTTTCTAG DF2 qRT-PCR agrA GCGACTGCGTATTAGCAACG BR2 qRT-PCR agrA GCGAAGCACGAAAACAATTCCTTAGCAACG DR2 qRT-PCR agrA GCAAGCAGAAGAACGATTTCGAA AR2 qRT-PCR agrBD CAAGCAGAAGAACAATTCCATA AR2 RT-PCR agrDA CGCTGTCTCAAAAAACAAGATTA AR2 RT-PCR agrDA CAAGCAGAAACAATTCAATA AR2 RT-PCR agrDA CCATGAAAGTGGCGATTTC DR RT-PCR agrDA CCATGAAAACCAGCGAATCAATA AR2 RT-PCR agrDA CCATGAAAACCAGCGCATTCAT DR RT-PCR agrCA CCAAGAAGAACTCGCGCAATTCAT DF GGCACTTACAAAACCAGCGCGCAATTCAT C10 C11 RT-PCR agrCA CGAAAGCAGAAGTCCGTTGGTAAATCA C10 RT-PCR agrA ACCATTCATGTCCGGCTGCC A46 Northern blot agrA ACCATTCATGTCCGTGTGAAAATCA Neol S'RACE agrA TGCGAATGCATTCAATGTTTGGTG BR2 S'RACE agrA TGCGAAT	pGID109	CTATGAGTCTAGATTGCTTCATG	XbaI	D3
RNA analysis GCGGAAAACCACTTGCTTACT DRMF2 qRT-PCR agrB CGGCAGACAGGAAAGTTGG DRMR2 qRT-PCR agrB CGGCAGACAGAAAGTTTG DF2 qRT-PCR agrD AATCAGTTGGTAATGTATAGCA DR2 qRT-PCR agrC GGGGTCAATCGCAGGTTTTG DF2 qRT-PCR agrC GGGGTCAATCGCAGGTTTTG DR2 qRT-PCR agrC GGGGTCAATCGCAGGTTTTG CR2 qRT-PCR agrA GCAACCAGAAGAACGATTTCCAA AF2 qRT-PCR agrA GCAAGCAGAAGAACGATTTCCAA AF2 qRT-PCR agrA GCAAGCAGAAGAACGATTTCCAA AF2 qRT-PCR agrD CAAAGCTTTTACTGCAAAAACAAGATTA AR2 RT-PCR agrD CAAAGCTTTTCACAAAACAAGATTA AF2 GGCACTTACCAAAATCGAACTC CGS CTTGCATTTCACAAAACAATCAATAC CG RT-PCR agrDC CCATGAAGTGTGGCGAATTCCT DF GGCACTTCCAAAAACAATCAATAC C35 RT-PCR agrC and Northern blot agrC GGCACTTCCAAATCAATAC C35 C11 CTTCAAATCGTCGCGCTGCC A66 Northern blot agrB ACCATGGGTAATTCATTGTGTGTGTGTGAA R62 C11 DR Northern blot agrA GCGCACTGCCCCC A66 A66 A67	F	AACAAATTCGCCAACATTCC		D4
qRT-PCR dmGCGGAAAAACCACTTGCTTACTDRMF2qRT-PCR agrBCGGCAAGACAAAAACCAATTAGGDRMR2qRT-PCR agrDAAATCACGTGGTATTAGCAACGBR2qRT-PCR agrDAAATCACGTGGTAAATTCCTTTCTAGDR2qRT-PCR agrCGGGGCAATCGCACGGAAGGTTTGCF2qRT-PCR agrAGCAAGCAGAAAACTTCCTTCTAGDR2qRT-PCR agrAGCAAGCAGAAAACCACTTTTGCF2qRT-PCR agrAGCAAGCAGAAGAACGATTTCCAAHindIIIDITTGCATTTCCAAAAACAAGATATAR2RT-PCR agrDCAAAGCTTTTCCACAAAACAAGATATAR2RT-PCR agrDCCATGAAAACTGCGCATTCTDRRT-PCR agrDCCATGAAAACAATGGAACTTDRRT-PCR agrDCCATGAAAACAATTGCACACAHindIIIDITTGCATTTCCAAAAACAAGATATAR2RT-PCR agrDCCATGAAAACTGGCGCATTCTDFGGCACTTACAAAAACCAATCAATACC10C11RT-PCR agrC and Northern blot agrCGGAATGTIGGCGAATTGTTC10RT-PCR agrCACAAAGCATCAGTGCCGCCA46Northern blot agrBACCATGGGTAAATTCGTTGTAAAATANcoIB17CTGCAATCAATGTCCGCCTTCAAAAACGAGGAACGGCTGGTGGGBRS'RACE agrBTGCGAATGGAAAACAATCCACTTE25'RACE agrATGCGAATGGTATGAGGCATTCGTGGGA44ACCATTCATGTCCGGCGGCA46CTCAAAGCACTCATGAGGCATTCGTGA44ACCATTCATAAAAACAATCCAGGA44ACCATTCATGTCCGGCGGCA46CTCAATTATTCGTTAGCAGGGCTAC355'RACE agrATTGATGTGGTGAGGCATTCGTGGA44ACCATTCATTATCGTTAGCGCGGCA46CTCAATTATTCGTTAGGCAGGCGCTTCGTGA46CCGCGCG	RNA analysis			
qrift for andCGGAAAGGTGTGTCAATGTAAGGDRMR2qRT-PCR agrBCGGCAATGGTATTGGCAATGTAAGGBF2qRT-PCR agrDAAATCAGTTGGTAATGTAACADF2qRT-PCR agrCGGGGTCAATCGCAGGTTTTGCF2qRT-PCR agrAGCAAGCAGAAGAACATTTTGGTTCGTATACADR2qRT-PCR agrAGCAAGCAGAAGAACAATTTCAAGGATTTCCAAAF2qRT-PCR agrACCAAGCAGAAGAACAACAATTTCCAAGACAAHindIIID1D1D1RT-PCR agrBDCAAAGCTTTTCCAAAAACAAGATATAR2RT-PCR agrCCCATGAAAGTTGCGGGATTCTDRRT-PCR agrACCAAGCAGAAGAACAATGGACTTDRRT-PCR agrCCCATGAAAGTTGCGGGATTCTDFGGCACTTACAAAAACAATCAATCAC11RT-PCR agrCCCAAAGGTGTGGGGAC23ACCATTCATGTCGGCGCCA46Northern blot agrCGGAATCGTTGTTGAAAATTANcolRT-PCR agrCACCAAAGGGAGAGTCGTGGAC23ACCATTCATGTCCGGCTGCCA46Northern blot agrAAGCTAGCAACAGGAGATCCGTTGTAAAATANcolS'RACE agrBCGCAATGGAATCATTCTTTGGBRS'RACE agrCGGCAATGGTATTAGCAACGB84S'RACE agrCCGAATGGTATAAATACCACCGCCATB34S'RACE agrATTGATTACATAAAAACAATCCGCCA44ACCATTCATGTCCGGCTGCCA46CTTCAATATATTTCCGTTAACATACC35S'RACE agrATGGAATGGTATTACATCGTCGGCATCCGTGA44ACCATTCATGTCCGGCGCCA46CTCCATTAAAAAAGGGACTTCGTGGAC36S'RACE agrATTGATTTACCAAAAACCATCCGCCATB34S'RACE agrATTGATGTGAGACATCCGTGGA	aRT-PCR drm	GCGGAAAAACCACTTGCTTACT		DRMF2
qRT-PCR agrBCGGCAGACACAGAAAGTTTGBF2qRT-PCR agrDAAATCAGTGTGATAAAATCCTTTCTAGDF2qRT-PCR agrCGGGGTCAATCGCAGGTTTTGGTTGCCGTACF2qRT-PCR agrAGCAAGCAGAAAGAACGATTTCCAAQR2qRT-PCR agrAGCAAGCAGAAGAACGATTTCCAAAR2qRT-PCR agrDCGAAGCAGAAGAACGATTTCCAAAR2qRT-PCR agrAGCAAGCAGAAGAACGATTTCCAAHindIIID1TTGCATTTTCACCAAACAHindIIIRT-PCR agrDCCATGAAGAGATCCAATCGAACAHindIIIRT-PCR agrDCCCATGCATTTCCACAAAACAATCCAATCACG5RT-PCR agrDCCGGCATTTCACAAAAACAATCAATCAATCACG5RT-PCR agrCGGAATGTTGCGGCATTCTTDRRT-PCR agrCACGAAGCTGGGGAACTCCATCATC10RT-PCR agrCACAAAGGAGAGAGCTCGTGGAC23Northern blot agrCGGAATGTTGCGGCTCGCCA46Northern blot agrAACCATTCATGTCCGGCTGCCA46Northern blot agrAAGCTAGCGACACAGGAGATCCTTTGGB17CGTGCAATTCAATGTCCAGCTGBRNorthern blot agrAAGCTAGCAACAGGAGATCCACTTB345'RACE agrATGCGAATGGTATTACACACTCCGCCATB345'RACE agrATGCGAATGGTATTAGCAACAATCACACTCGGCTGCCA46CCTCCATACAATCAACAACCATCCGGCTGCCA46CCTCCATACAATCATCTGTCGGCTGCCA46CCTCCATACAATATTCGTTAACAATACC355'RACE agrATTGCATTCATGTCGGCATCCTGA48CTTCAATATATTTCGTTAACCTA62Primer extension agrBGACAAGGGACTTTGTCGCGCCA46CTTCCTCATCTCTCCACGGCTGCCA48CTTCCTCATCTTCTCCACGGCTGC	qitti i oitumi	CGGAAAGGTGTGTCAATGTATAGG		DRMR2
qrift Ockag/BCOCONTRACTIONBR2qRT-PCR agrDAAATCACTTGGTAAATTCCTTTCTAGDF2qRT-PCR agrCGGGGTCAATCGCAGGTTTTGCF2qRT-PCR agrAGCAAGCAGAAGAACGATTTCCAAAR2qRT-PCR agrACCAAGCAGAAGAACGATTTCCAAAR2RT-PCR agrBDCAAAGCTTTTACTGCAAACAAHindIIID1D1RT-PCR agrBDCAAGCAGAAGAAACAATCGCAAACAAHindIIIIRT-PCR agrBDCCATGAAAGTGCGCAATTCCAADR2RT-PCR agrCGGCACTTTTCACAAAAACAAGCATHindIIIIRT-PCR agrCCCATGAAAGTTGCGGAATTCTDRRT-PCR agrCACGAATGTTGGCGCAATTCATC11RT-PCR agrCACGAATGTTGGCCGCAATTCATC10RT-PCR agrCACAAAAGGACAAGGCGTGGGAC23RT-PCR agrCACAAAAGGACAAGGCGTGGGAC23Northern blot agrBACCATTCATGTCCGGCTGCCA46Northern blot agrAAGCTAGGTAAATTCGTTTGAAAATANcoIB17S'RACE agrBCGCAATGCAATGCAATCCATTCE2S'RACE agrCGGCACTTACAAAAACAATCCATTCGTTGAGNheIA15S'RACE agrATGCGATGCAATCAATCCGGCTGCCA44ACCATTCATGTCAGGCATTCCATCC35S'RACE agrATGGTGTAAATTCGTTGCGGCTGCCA44ACCATTCATGATCAGGCATTCCGTGA44ACCATTCATGTCGGCCCCA44ACCATTCATGTCGGCTGCCA46CGTCCAAAGGGACTTTGCAGGCATTCCGTGA44ACCATTCATGTCGGCCCCCA46CGTCCATTCATGTCGGCTGCCA46CGTCCATCATCTTTCCAA48CTTCATATATATTCGTTAACCTA50Primer extension agrB <td>aBT-PCB agrB</td> <td>CGGCAGACACAGAAAGTTTG</td> <td></td> <td>BF2</td>	aBT-PCB agrB	CGGCAGACACAGAAAGTTTG		BF2
qRT-PCR agrDAAATCAGTTGGTAAATTCCTTTCTAGDF2qRT-PCR agrCGGGTCAATCGCAGGTTTTGDR2qRT-PCR agrAGCAATCGCAGGTTGGTGCCGTACR2qRT-PCR agrAGCAAGCAGAAGAACGATTCCAAAF2CGTGTCAAAAACAAGATATAR2RT-PCR agrDCCAAAGCTTTTCACAAAAGAGACTTDRRT-PCR agrDCCCATGAAGTGTGGTGGCGGATTCCTDFGGCACTTACAAAAGCAGACTTDRRT-PCR agrDCCCATGAAGTGGCGAATTCCTDFGGCACTTACAAAAGCAGCGCGCATATCATC11RT-PCR agrCACAAAGGAGAGAGAGGCTGGGAC23RT-PCR agrCACAAAGGGAGAGGCGGCGTGGGAC23Northern blot agrCGGGACTTCATGTCGGCGCCA46Northern blot agrBACCATTCGGCGAATTCGTTGTAAAATANcoINorthern blot agrACGTGCAATTCAATGTTTTGGBRNorthern blot agrAGGCACTTACAAAAGGAGAACCGTTGTGAGBR2S'RACE agrBTGCGAATGCATTACAACCCGGCCATB345'RACE agrCGGAATCAACACACCGGCCATTCAATCCC35S'RACE agrATGCGGAATTCAATGTATGGTGTGA44ACCATTCAGGCTGCCA46CGTGCCATTACAAAAACAATCAATACC35S'RACE agrATGCGAATGAACACTCGGCTGCCA46CGTGCCATTCAGTAAAATAAAGGGAAGGCTAC35S'RACE agrATTGATGTGTGGGCATTCTGTTACATACCC35Primer extension agrBGACAAAGGACTTTGCTTGCAGCB18CTTCTCATATATTTCGTTAACCTTTCCAGCGCA46CTTCTCATATCTTTCGTTACGCTGCA46CTTCTCATCTTTCGTTACGCCGCCA46CTTCTCATCTTTCGTTACGCTTTCCAGCGTB18CTTCTCATCTTTCGTTACGCTTTCGCGCB26 </td <td>qitti i eit<i>ugib</i></td> <td>TGCGAATGGTATTAGCAACG</td> <td></td> <td>BR2</td>	qitti i eit <i>ugib</i>	TGCGAATGGTATTAGCAACG		BR2
qRTPCK agrDAATGGACTTTGGTTGGTTGGTTACADR2qRT-PCR agrCGGGGTCAATCGCAGGTTTGGCF2qRT-PCR agrAGCAAGCAGAAGCAGTTTGGCTGTACR2qRT-PCR agrAGCAAGCAGAAGCAGTTTCCAAAR2RT-PCR agrBDCAAGCGTTTTACTGCAAACAAGATATAR2RT-PCR agrDCCCATGAAAGTGCGGATTCTDFGGCACTTACAAATGGACTTCIRT-PCR agrC and Northern blot agrCGGAATGTGGCGGATTTGTCIRT-PCR agrCACAAAGGTGCGGAATTGTTCIRT-PCR agrCACAAAGGGTAAATCCGGCATTTGTCIRT-PCR agrCACAAAAGGAGAGAGGTCGTGGAC23RT-PCR agrCACAAAAGGAGAAGGTCGTGGAAC23Northern blot agrBACCATGGGTAAATCGTGTGTAAAATANcoINorthern blot agrACGTGCAATCAATGCGTTGTGAAAATCABI7CGTGCAATCAATCACCGGCATTE25'RACE agrCCGAATCAACAATCAATCACCTTE25'RACE agrCCGAATCAACAATCACATCGGCCATB345'RACE agrCCGCAATCAACAATCACATCACATACCC355'RACE agrATGCGAATGATATAAAAAAAAAAAACAATCAATACC365'RACE agrATGACGAATGAGCATCCGCCCA44ACCATCATGTGTGGAGGAAGGCTAC365'RACE agrATGATGTGTGGAGCATTCGGCCA44ACCATCATCATGTCGGCCA44ACCATCATGTGTGGAGGATCTCTCTA48CTTCAATATATCCGTCGGCCA46CGCTGCATTCAAAAGGGAAGGCTAC365'RACE agrATGATGTGTGGGGCCA46CGCTGCATCTCATCATCTCTCTCACCGCCA46CGCTGCATCTCATCATCTCTCTCACGCCA48CTTCATCATCATCTCTCTCACGCCA48CTTCATC	aRT-PCR agrD	AAATCAGTTGGTAAATTCCTTTCTAG		DE2
qRT-PCR agrCGGGGTCAATCGCAGGTTTTGCCF2qRT-PCR agrAGCAAGCAGAAGAACGATTTCCAAAF2qRT-PCR agrAGCAAGCAGAAGAACGATTTCCAAAF2RT-PCR agrBDCAAAGCTTTTACTGCAAACAHindIIIDITTGCATTTTCACAAATGGACTTDRRT-PCR agrDCCCATGAAAGTGTGCGGATTCCDFGGCACTTACAAAAACAACAATCAATACC35RT-PCR agrCAGGAATGTTGCGGCATTTGTC10RT-PCR agrCACCATGAAAGTGTGCGGCATATCATC10RT-PCR agrCACAAAGGAGAAGGTCGTGGGAC23ACCATTCATGTCCGGCGTGCCA46Northern blot agrBACCATTCATGTTCGGGCTGCCA46Northern blot agrAAGCAAGGAAAGATCCATTANcoIS'RACE agrCGGCACTTACAAAAACCAATCAATCAB17S'RACE agrCGGCACTTACAAAAACCAATCAATCAB24S'RACE agrCGGCACTTACAAAAACCAATCAATCAB34S'RACE agrATGCGAATGGTATTAGCAACGC35S'RACE agrATGCGAATGACAGGCCCA46CTTACACATAAATTCGTCGTGGCCA46CTTACACATAAAATCAACCATCCGCCATB34S'RACE agrATGCGAATGGTATTAGCGAACGC35S'RACE agrATGGAAGTGACGCCA46CTTCAATATTTCCGCGCCGCCA46CGCTGCATTCTGTTGTTAACCTC35Primer extension agrBGACAAAGGGACTTTGCGGCGCCA46CTTCATCATCATCGTTGCGGCGGCA46CTTCATCATCATCGTTGTTAACCTA50Primer extension agrBGACAAAGGGACTTTGCGGCGCGCA46CTTCATCATCATCGTTGCGGCGGCGB18CTTCATCATCATCGTTGCGGCGGCGCB26CTTCCTCATCATCGTGTGTGTGCGCC <td>qitti i eit<i>ugi b</i></td> <td>AATGGACTTTTTTGGTTCGTATACA</td> <td></td> <td>DR^2</td>	qitti i eit <i>ugi b</i>	AATGGACTTTTTTGGTTCGTATACA		DR^2
qRTPCR agrACTTTAAGTTCGTTGGTTGCCGTACR2qRT-PCR agrAGCAAGCAGAAGAACAGATTTCCAAAF2CGCTGTCCAAAAAACAAGATATAR2CGCTGTCCAAAAAACAAGATATAR2RT-PCR agrBDCAAAGCTTTTACTGCAAACAHindIIIDITTGCATTTTCACAAATGGACTTDRRT-PCR agrDCCCATGAAAAGCAATCAATACC35RT-PCR agrCa and Northern blot agrCGGAATGTTGGCGAATTCATC11CTTCAAAACCCGGCAATTCATC11C11RT-PCR agrCACAAAAGGAGAGGTCGTGGGAC23ACCATTCATGTCCGGCTGCCA46Northern blot agrBACCATGGGTAAATTCGTTGTAAAAATANcoICTTACCATAAAACCAGGAGATTCCGTTGTAAAATANcoIB17CGTGCAATTCAATGTTTTGGBRNorthern blot agrAAGCTAGGAATTCAATGCCGTTGTGAGNheIAGCTAGCAATCAACAGGAGATCCGTTTGAGNheIA15S'RACE agrBTGCGAATGGTATTAGCAACGC35S'RACE agrATGGGAATGTACACATCCGCCCATB34S'RACE agrATGGTGAAACAATCCAACATCCGCCCATA44ACCATTCATGTCTGGTAGAAGGAAGGCTAC35S'RACE agrATGGTGAAGGCATTCGTGGCCA46Primer extension agrBGACAAAGGGACTTTGTTAACCTTA48CTTCATCATCATCATCGTGCCA46CTTCATCATCATCATCATCATCATCATCATCATCATCATC	aRT-PCR agrC	GGGGTCAATCGCAGGTTTTG		CE2
qRT-PCR agrAGCAAGCAGAAGAACGATTTCCAAAR2RT-PCR agrBDCGAAGCATTTCAAAAACAAGATATAR2RT-PCR agrBDCAAAGCTTTTACTGCAAACGHindIIIDITTGGCATTTTCACAAATGGACTTDRRT-PCR agrCCCATGAAAGTTGCGGATTCTDFGGCACTTACAAAAGCAATCAATACC35RT-PCR agrC and Northern blot agrCGGAATGTTGCGGAATTGTTC10RT-PCR agrCACAAAAGGAGAAGGTCGTGGGAC23ACCATGGGTAAATTCATCATCATC10RT-PCR agrBCCATGGGTAATTCATCATC10RT-PCR agrCACAAAAGGAGAAGGTCGTGGGAC23Northern blot agrBACCATGGGTAATTCATGTTGTGAAAATANcoINorthern blot agrAAGCTAGCAACAGGAGATCCGTTTGAGNheIA15CTTACCATAACAATCACTE25'RACE agrBGGCACTTACAAAACAATCACTCC355'RACE agrATGGGAATGTATAGGCAATTCATGC355'RACE agrATGGATGTGTAGGCATTCATGCTGGA44ACCATTCATGTCCGGCTGCCA46CGTACATTACATATATTCGTGTGTAAAATAAAGGAAGGCTAC365'RACE agrATGGATGTGTAGGCATTCGTGA44ACCATTCATGTCCGGCTGCCA46CGCTGCATTCCATGTCCGGCTGCCA46ACCATTCATGTCCGGCTGCCA46ACCATTCATGTCCGGCTGCCA46ACCATTCATGTCCGGCTGCCA46ACCATTCATGTCCGGCTGCCA46ACCATTCATGTCCGGCTGCCA46ACCATTCATGTCCGGCTGCCA46ACCATTCATGTCCGGCTGCCA46ACCATTCATGTTGCTGTAACCTA50Primer extension agrBGACAAGGGACTTTTCCAGGCB18CTTCTTCATCATGTTTCCAGCGGB18 <td>qitti i eit ugi e</td> <td>CTTTAAGTTCGTTGGTTGCCGTA</td> <td></td> <td>CR2</td>	qitti i eit ugi e	CTTTAAGTTCGTTGGTTGCCGTA		CR2
qRTPCR agrBDCGATGCTCAAAAAACAAGATATAR2RT-PCR agrBDCAAAGCTTTTACTGCAAACAHindIIID1RT-PCR agrDCCCATGAAAGATGCGGATTCTDRGGCACTTACAAAAAGTGCGGGATTCTGGCACTTACAAAAACAATCAATACC35RT-PCR agrC and Northern blot agrCGGAATGTTGCGGAATTGTTC11RT-PCR agrCACAAAAGGAGAAGGTCGTGGAC23Northern blot agrBCGTGCATTCATGTCGGGATTCGTTGTAAAATANcoINorthern blot agrACGTGCAATTCATGTCGGGAATTCGTTGTGAAAATANcoIS'RACE agrBTGCGAATGGTATTCCATTTTGGBRS'RACE agrCGGCACTTACAAAAACAAGGAGAAGGTCCGTTTGAGNheIA15'STACE agrATGCGAATGGTATTAGCAACGB82S'RACE agrATGCGAATGCGAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	aRT-PCR agr4	GCAAGCAGAAGAACGATTTCCAA		AE2
RT-PCR agrBDCAAAGCTITTACTGCAAACAHindIIID1RT-PCR agrDCCCATGAAAGTIGCGGATTCTDRRT-PCR agrC and Northern blot agrCGGAATGTTGGCGAATCATACC35RT-PCR agrCACGAATGTTGGCGAATTCGTTC11CTTCAAACCCGGCATATCATC10RT-PCR agrCACAAAAGGAGAAGGTCGTGGGAC23Northern blot agrBACCATTCATGTCCGGCTGCCA46Northern blot agrAACCATGGGTAAATTCGTTGTTAAAAATANcoIB17CGTGCAATTCATGTTCGGCAATTCGTTGTGAAAATANcoIB17S'RACE agrBTGCGAATGGTATTGCACGCTTTE25'RACE agrATGCGAATGGTATATCAACGCBR25'RACE agrATIGATGTTACAAAAACAATCCAATACC355'RACE agrATIGATGATAAATAAAGGAGATCCGGCTGCA44ACCATTCATGTCCGGCTGCCA46CGTCATTACAAAAACAATCCAATACC365'RACE agrATIGATGTTGTAGCAATCGGCTGCA46CGCTGCATTCTGTAGGCATTCGTGGA44ACCATTCATGTCCGGCTGCCA44ACCATTCATGTCCGGCTGCCA46CGCTGCATTCTGTTATCTTCAA48CTTCCATCATCATGTTCCGGCTGCCA46CGCTGCATTCTGTAACCTA50Primer extension agrBGACAAAGGGACTTTGCTGCGCTGCA46CTTCTTCATCATCATCTTTCCAGCGCB18CTTCTTCATCATCTTCCATCATCTTCCAGCGB18CTTCTTCATCATCTTCTCCAGCGCB26CTTCTTCATCATCTTCTCCAGCGCB26CTTCTTCATCATCTTCTCCAGCGCB26CTTCTTCATCATCTTTCCCAGCGCB26CTTCTTCATCATCTTCTCCGCGCTGCC10CTTCTTCATCATCTTCTCCAGCGCC10CTTCTTCATCATCTTCTCCAGCGC	qitti i eiteugizi	CGCTGTCTCAAAAACAAGATAT		AR2
INTEGRATION CALCENTRICACIANT CONTROLInternDRRT-PCR agrDCCCATGAAAGTTGCGGGATTCTDFGGCACTTACAAAAACAATCAATACC35RT-PCR agrC and Northern blot agrCGGAATGTTGGCGGCATATCATC10RT-PCR agrCACAAAAGGAGAAGGTCGTGGAC23ACCATTCATGTCCGGCTGCCA46Northern blot agrBACCATTCATGTCCGGCTGCCA46Northern blot agrAAGCTAGCAACAGGAGATTTAGCAACGBRNorthern blot agrAAGCTAGCAACAGGAGATCCGTTTGAGNheIA155'RACE agrBTGCGAATGGTATTAGCAACGBR25'RACE agrCGGCACTTACAAAACAATCCATTCC355'RACE agrATTGATGTGTAGGCATTCGTGA44ACCATTCCATGTGTGAGGCATTCGTGA44ACCATTCCATGTGTGAGGCATTCGTGA44ACCATTCCATGTGTGAGGCATTCGTGA44ACCATTCCATGTAGGCATTCGTGA44ACCATTCGTGTAGGCATTCGTGA46CTTCAATATATTCCGTTACCCTA50Primer extension agrBGACAAAGGGACTTTTGCAGGTB18CTTCATCATCATCATCATCCTCATTCAGGCGB26COLCTTCATCATCTTTCCAGCGCB26	RT-PCR agrBD	CAAGCTTTTACTGCAAACA	HindIII	D1
RT-PCR agrDCCCATGAAAGTTGCCGGATTCTDFGGCACTTACAAAAACAATCAATACC35RT-PCR agrC and Northern blot agrCGGAATGTTGCGGCAATTGTTC11CTTCAAACCGGCGCATATCATC10RT-PCR agrCACAAAAGGAGAAGGTCGTGGAC23ACCATTCATGTCCGGCTGCCA46Northern blot agrBACCATGCAATGCTAATGTTTTGGBRCGTGCAATTCATGTCCGGCTGCCA46Northern blot agrAAGCTAGCAACAGGAGAAGGTCGTTGGAB17CTTACCATAAAATTCATTGTTTGGANheIA15CTTACCATAAAATTCCACTTE25'RACE agrBTGCGAATGGTATTAGCAACGBR2CGAAATCAACACTACCGCCATB345'RACE agrATTGATGTGTAGGCATTCGTGA44ACCATTCATGTCCGGCTGCCA46CCTTCACTATCATAGTATTTCGTTAGCAACGC35FRACE agrATTGATGTGTAGGCATTCGTGA46Primer extension agrBGACAAAGGGACTTTTGCTAGCGCA46CTTCATCATCATCATCATCATCTA50Primer extension agrBGACAAAGGGACTTTTGCCAGCGB18CTTCATCATCATCATCATCATCCACCGCB18CTTCATCATCATCATCATCACCGCCB26	RTT CR ug/DD	TTGCATTTTCACAAATGGACTT	Timatti	DR
RT-PCR agrCGGCACTTACAAAACAATCAATACC35RT-PCR agrC and Northern blot agrCGGAATGTTGGCGAATTTGTTC11CTTCAAAACCGGCATATCATC10RT-PCR agrCACAAAAGGAGAGGTCGTGGAC23ACCATTCATGTCCGGCTGCCA46Northern blot agrBACCATGGGTAAAATTCGTTGTAAAAATANcoIRT-PCR agrCAAGCTAGCAACAGGAGAGTCCGTGTGAAAATANcoINorthern blot agrBACCATGGGTAAAATTCGTTGTGAAAATANcoIS'RACE agrBTGCGAATGGTATTAGCAACGBR2CGAAATCAACACATCCGCCATB345'RACE agrCGGCACTTACAAAAACAATCAATACC35S'RACE agrATTGATGTGTAGGCATTCGTGA44ACCATTCATGTATGTGTAGGCATTCGTGA44ACCATTCATGTTATGCTACACACCC365'RACE agrATTGATGTGTAGGCATTCGTGA44ACCATTCATGTTATCTTCAA46CGCGCGCATTCGTTGTAACCTA50Primer extension agrBGACAAAGGGACTTTTGCAGGB18CTTCTCATCATCATCTTCCAGCGB26CTCATCATCATCTTCCCAGCGB26CTCATCATCATCTTCCAGCGB26	RT-PCR agrDC	CCATGAAAGTTGCGGATTCT		DF
RT-PCR agrC and Northern blot agrCGGAATGTTGGCGAATTTGTTC11RT-PCR agrCACAAAAGGAGAAGGTCGTGGAC23ACCATTCATGTCCGGCTGCCA46Northern blot agrBACCATGGGTAAATTCGTTGTAAAATANcoIRT-PCR agrCACGTGCAATTCAATGTTTTGGBRNorthern blot agrAAGCTAGCAACAGGAGAGCCGTGTGAGNteIS'RACE agrBTGCGAATGCAACAGGAGATCCGCATE25'RACE agrCCGAAATGCAACACACACACGCCATB345'RACE agrATTGATGTGTAGGCATTCGTGA44ACCATTCATGTCGTGGTAGGCATCGTGGA44ACCATTCATGTCGTGGTAGGCATTCGTGA44ACCATTCATGTCGGGCTGCCA46CGTAAGTATAAGGGAAGGCTAC365'RACE agrATTGATGTGTAGGCATTCGTGA44ACCATTCATTGTCTGGGCTGCCA46CGCTGCATTCTGTTATCTTCAA48CTTCAATATATTTCGTTATCTTCAA50Primer extension agrBGACAAAGGGACTTTTGCAGTB18CTTCTCATCATCATCATCTTCCAGCGB26	111101103020	GGCACTTACAAAAACAATCAATAC		C35
Introduction outrigitCTTCAAACCCGGCATATCATC10RT-PCR agrCACAAAAGGAGAAGGTCGTGGAC23Northern blot agrBACCATTCATGTCCGGCTGCCA46Northern blot agrAACCATGGGTAAATTCATTGTTTGGBRNorthern blot agrACGTGCAATCAATGTTTTGGBRS'RACE agrBTGCGAATGGTATTAGCAACGBR25'RACE agrCGGCACTTACAAAAAACAATCCAATACC355'RACE agrATTGATGTGTGAGGAAGGCTAC365'RACE agrATTGATGTGTGAGCACCGTGGGA44ACCATTCATGTCCGGCTGCCA46CGAAATCAACACATCCGCCATB345'RACE agrATTGATGTGTGAGCACTCGTGA44ACCATTCATGTCCGGCTGCCA46CGCACTTACAAAAACAATCAATACC365'RACE agrATTGATGTGTCGGCGCCA46CGCCTGCATTCGTTATCTCAA48CTTCCAATATATTTCGTTAACCTA50Primer extension agrBGACAAAGGGACTTTTGCAGTB18CTTCTTCATCATCTTTCCACCGCGCGCB26	RT-PCR <i>agrC</i> and Northern blot <i>agrC</i>	GGAATGTTGGCGAATTTGTT		C11
RT-PCR agrCACAAAAGGAGAAGGTCGTGGAC23 AddNorthern blot agrBACCATTCATGTCCGGCTGCCA46Northern blot agrAACCATGGGTAAATTCGTTGTAAAATANcoIS'RACE agrBCTTACCATAAAGGAAGATCCGTTTGAGNheIS'RACE agrCGGCAATCGAATGGTATTAGCAACGBR2S'RACE agrCGGCAATCAACACATCCGCCATB34S'RACE agrATIGCGAATGGTATAAAAATCAATACC35F'RACE agrATIGATGTGTAGGCATTCGTGGA44ACCATTCATGTCCGGCTGCCA44ACCATTCATGTCCGGCTGCCA46CGCTGCATTCTGTTATCTTCAA48CTTCATCATATATTTCGTTAACCTA50Primer extension agrBGACAAAGGGACTTTTGCAGGCB18CTTCATCATCATCTTTCCATCATCTTCCAGCGB18CTTCATCATCTTTCCATCATCTTCCAGCGB26		CTTCAAACCCGGCATATCAT		C10
ACCATTCATGTCCGGCTGCCA46Northern blot agrBACCATTGATGTCCGGCTGCCA46Northern blot agrAAGCTAGCAACAGGAGATCCGTTTGAAAATANcoIB17CGTGCAATTCAATGTTTTGGBRNorthern blot agrAAGCTAGCAACAGGAGATCCGTTTGAGNheIA15CTTACCATAAAATTCCACTTE25'RACE agrBTGCGAATGGTATTAGCAACGBR2CGAAATCAACACATCCGCCATB345'RACE agrCGGCACTTACAAAAACAATCAATACC355'RACE agrATTGATGTGTAGGCATTCGTGA44ACCATTCATGTCCGGCTGCCA44ACCATTCATGTCCGGCTGCCA46CGCTGCATTCTGTTATCTTCAA48CTTCAATATATTTCGTTAACCTA50Primer extension agrBGACAAAGGGACTTTTGCAGTB18CTTCATCATCTTTCCATCATCTTCCAGCGB26	RT-PCR agrCA	CAAAAGGAGAAGGTCGTGGA		C23
Northern blot agrBACCATGGGTAAATTCGTTGTAAAATANcoIB17 BRNorthern blot agrAAGCTAGCAACAGGAGATCCGTTTGAGNheIA15 CTTACCATAAAATTCCACTTE25'RACE agrBTGCGAATGGTATTAGCAACGBR2 CGAAATCAACACTCGCCATB345'RACE agrCGGCACTTACAAAAACAATCAATACC35 CTAAAGTAAATAAAGGGAAGGCTAC365'RACE agrATTGATGTGTAGGCATTCGTGGA44 ACCATTCATGTCCGGCTGCCA44 A48 CTTCAATATATTTCGTTAACCATA50Primer extension agrBGACAAAGGGACTTTTGCAGTB18 CTTCATCATCTTTCCAGCGB18 B26 D26		ACCATTCATGTCCGGCTGCC		A46
CGTGCAATTCAATGTTTTGGBRNorthern blot agrAAGCTAGCAACAGGAGATCCGTTTGAGNheIA15CTTACCATAAAATTCCACTTE25'RACE agrBTGCGAATGGTATTAGCAACGBR2CGAAATCAACACATCCGCCATB345'RACE agrCGGCACTTACAAAAACAATCAATACC355'RACE agrATTGATGTGTAGGCATTCGTGA44ACCATTCATGTCAGGCCCA44ACCATTCATGTCGGCCCA46CGCTGCATTCTGTTATCTTCAA48CTTCAATATATTTCGTTAACCTA50Primer extension agrBGACAAAGGGACTTTTGCAGTB18CTTCATCATCTTTCCATCATCTTCCAGCGB26	Northern blot <i>agrB</i>	ACCATGGGTAAATTCGTTGTAAAATA	NcoI	B17
Northern blot agrAAGCTAGCAACAGGAGATCCGTTTGAGNheIA15CTTACCATAAAATTCCACTTE25'RACE agrBTGCGAATGGTATTAGCAACGBR2CGAAATCAACACATCCGCCATB345'RACE agrCGGCACTTACAAAAACAATCAATACC35CTAAAGTAAATAAAGGGAAGGCTAC365'RACE agrATTGATGTGTAGCAACGGCTAA44ACCATTCATGTCCGGCTGCCA46CGCTGCATTCTGTTAACCTA50Primer extension agrBGACAAAGGGACTTTTGCAGTB18CTTCATCATCATCTTTCCATCATCTTCCAGCGB26		CGTGCAATTCAATGTTTTGG		BR
CTTACCATAAAATTCCACTTE25'RACE agrBTGCGAATGGTATTAGCAACGBR2CGAAATCAACACATCCGCCATB345'RACE agrCGGCACTTACAAAAACAATCAATACC35CTAAAGTAATAAAGGGAAGGCTAC365'RACE agrATTGATGTGTAGGCATTCGTGA44ACCATTCATGTCCGGCCCA46CGCTGCATTCIGTTAACCTA48CTTCAATATATTTCGTTAACCTA50Primer extension agrBGACAAAGGGACTTTTGCAGTB18CTTCATCATCTTCATCATCATCTTCCAGCGB26	Northern blot <i>agrA</i>	AGCTAGCAACAGGAGATCCGTTTGAG	NheI	A15
5'RACE agrB TGCGAATGGTATTAGCAACG BR2 CGAAATCAACACATCCGCCAT B34 5'RACE agrC GGCACTTACAAAAACAATCAATAC C35 CTAAAGTAAATAAAGGGAAGGCTA C36 5'RACE agrA TTGATGTAGGCATTCGTG A44 ACCATTCATGTCGGCC A46 CGCTGCATTCIGTTATCTTCA A48 CTTCAATATATTTCGTTAACCT A50 Primer extension agrB GACAAAGGGACTTTTGCAGT B18 CTTCATCATCTTCCACGCG B26	8	CTTACCATAAAATTCCACTT		E2
CGAAATCAACACATCCGCCATB345'RACE agrCGGCACTTACAAAAACAATCAATACC355'RACE agrATTGATGTGTAGGCATTCGTGA44ACCATTCATGTCCGGCTGCCA46CGCTGCATTCTGTTATCTTCAA48CTTCAATATATTTCGTTAACCTA50Primer extension agrBGACAAAGGGACTTTTGCAGTB18CTTCATCATCATCGTCCAGCGB26CTTCATCATCATCTTTCCAGCGB26	5'RACE agrB	TGCGAATGGTATTAGCAACG		BR2
5'RACE agrC GGCACTTACAAAAACAATCAATAC C35 5'RACE agrA TTGATGTGTAGGCATTCGTG A44 ACCATTCATGTCCGGCTGCC A46 CGCTGCATTCTGTTATCTTCA A48 CTTCAATATATTTCGTTAACCT A50 Primer extension agrB GACAAAGGGACTTTTGCAGT B18 CTTCATCATCTTTCCAGCG B26	0	CGAAATCAACACATCCGCCAT		B34
CTAAAGTAAATAAAGGGAAGGCTA C36 5'RACE agrA TTGATGTGTAGGCATTCGTG A44 ACCATTCATGTCCGGCTGCC A46 CGCTGCATTCTGTTATCTTCA A48 CTTCAATATATTTCGTTAACCT A50 Primer extension agrB GACAAAGGGACTTTTGCAGT B18 CTTCCATCATCATCTTTCCAGCG B26	5'RACE agrC	GGCACTTACAAAAACAATCAATAC		C35
5'RACE agrA TTGATGTGTAGGCATTCGTG A44 ACCATTCATGTCCGGCTGCC A46 CGCTGCATTCTGTTATCTTCA A48 CTTCAATATATTTCGTTAACCT A50 Primer extension agrB GACAAAGGGACTTTTGCAGT B18 CTTCATCATCTTTCCATCATCTTCCAGCG B26		CTAAAGTAAATAAAGGGAAGGCTA		C36
ACCATTCATGTCCGGCTGCC A46 CGCTGCATTCTGTTATCTTCA A48 CTTCAATATATTTCGTTAACCT A50 Primer extension <i>agrB</i> GACAAAGGGACTTTTGCAGT B18 CTTCTTCATCATCATCTTTCCAGCG B26	5'RACE agrA	TTGATGTGTAGGCATTCGTG		A44
CGCTGCATTCTGTTATCTTCAA48CTTCAATATATTTCGTTAACCTA50Primer extension agrBGACAAAGGGACTTTTGCAGTB18CTTCATCATCCATCCATCCAGCGCTTCTTCCAGCGB26CTTCTTCGCAGCTD16	0	ACCATTCATGTCCGGCTGCC		A46
Primer extension agrBCTTCAATATATTTCGTTAACCTA50GACAAAGGGACTTTTGCAGTB18CTTCTTCATCATCTTTCCAGCGB26CTTCTTCATCATCTTTCCAGCGB26		CGCTGCATTCTGTTATCTTCA		A48
Primer extension agrBGACAAAGGGACTTTTGCAGTB18CTTCTTCATCATCTTTCCAGCGB26D21D21		CTTCAATATATTTCGTTAACCT		A50
CTTCTTCATCATCTTTCCAGCG B26	Primer extension agrB	GACAAAGGGACTTTTGCAGT		B18
	0	CTTCTTCATCATCTTTCCAGCG		B26
CGAAATCAACACATCCGCCAT B34		CGAAATCAACACATCCGCCAT		B34

TABLE 2. Oligonucleotides used in this study

^{*a*} qRT-PCR, quantitative RT-PCR.

^b Specific restriction sites are underlined, and extra nucleotides added to include restriction sites to the PCR product are indicated in boldface.

strain DG125A ($\Delta agrA$) (Fig. 1B) and the deletion of 49 bp in strain DG119D ($\Delta agrD$) (Fig. 1C) resulted in a frameshift causing the premature appearance of a stop codon and thus the premature stop of translation.

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. A portion (5 μ l) of the culture to be tested was then deposited on a glass slide, and the glass slide was incubated for 2 h 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 1 min and washed with distilled water. Adhering cells were observed by using a Zeiss Axioplan 2 imaging microscope. For each experiment, 12 replicates resulting from three independent inocula were analyzed.

Biofilm formation on polystyrene microplate. An overnight culture of the bacterium in TSB at 25°C was used to inoculate (1/100, vol/vol) fresh TSB. A total of 100 μ l per well was dispensed on a 96-well microtiter plate (Nunc, Dominique Dutscher S.A., Brumath, France), which was then 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 by using a microtiter plate washer (Cellwash; Thermolabsystems, Cergy Pontoise, France) with 100 μ l

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 μ l of 96% ethanol (vol/vol) were added to each well, and the optical density at 595 nm (OD₅₉₅) was determined. For each experiment, 15 replicates resulting from three independent inocula were analyzed. Each microtiter plate included eight wells with sterile TSB as control.

Biofilm formation 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), followed by incubation 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 for 24 and 72 h at 25°C. After incubation, the medium was removed, and 10 ml of saline solution (150 mM NaCl) was softly poured twice onto chips, followed by agitation for 1 min on an orbital shaker at 240 rpm (IKAKS 130 basic) to remove loosely adhering bacteria. Sessile cells were detached from chips in 10 ml of 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 $(10,000 \times g, 5 \text{ min})$, resuspended in 1 ml of Tri-reagent (Sigma), and disrupted with glass beads $(106 \ \mu\text{m})$ in a FastPrep FP120 instrument (Thermo Savant-Bio



FIG. 2. (A) *L. monocytogenes* EGD-e (\blacksquare), DG125A ($\Delta agrA$) (\blacksquare), and DG119D ($\Delta agrD$) (\square) cell adhesion after 2 h of 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 ($\Delta agrA$), and DG119D ($\Delta agrD$) cells adhering to glass slides after 2 h of incubation at 25°C. Magnification, ×63.

101) at 4°C for eight rounds of 30 s at $6,000 \times g$. 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 the absorbance at 260 nm using a SmartSpec Plus spectrophotometer (Bio-Rad, Marnes La Coquette, France).

Before reverse transcription (RT), $2 \mu g$ 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 by 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 and 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 genespecific 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 ($\Delta agrA$) and DG119D ($\Delta agrD$). This method was used to analyze their mRNA level during planktonic growth at early exponential phase (6.5×10^7 CFU ml⁻¹; OD₆₀₀ of 0.1), mid-exponential phase (2.5×10^8 CFU ml⁻¹; OD₆₀₀ of 0.4), and stationary phase (4×10^8 CFU ml-1; OD₆₀₀ of 0.6) and after 2, 24, and 72 h of biofilm formation on AISI 304 stainless steel chips. The specificity of each primer pair was controlled by melting curves, and the mean of efficiencies for the five primer pairs was $98\% \pm 9\%$. The results were analyzed by using the comparative critical threshold method $(\Delta\Delta C_T)$ in which the amount of targeted mRNA was first of all normalized using a specific mRNA standard and then compared to a calibrator condition (15). drm, a gene coding for a phosphopentomutase, was selected as an internal standard since drm transcript levels were stable in the conditions tested. For our work, the calibrator condition corresponded to the agrA mRNA level at an OD₆₀₀ of 0.1. The relative level of agrA mRNA at an OD₆₀₀ 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 to RT.

Northern blotting. Northern blot analysis were performed by fractionation of RNA samples on 1% (wt/vol) agarose gel containing 20% (wt/vol) formaldehyde. Then, 30 µg of total RNA was loaded into 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. Portions (10 ng) of the PCR products were labeled with $[\alpha^{-32}P]$ dATP (Amersham) by the random priming method according to the manufacturer's instructions (Invitrogen) to produce specific RNA probes. Sizes were determined with a RNA ladder (Invitrogen) as the molecular weight standard.

mRNA chemical half-life determination. The chemical half-lives of mRNA of the four genes of the *agr* operon were determined from mid-exponential phase cultures (OD_{600} of 0.4). Total RNA was isolated as described above from samples taken 1, 4, 7, and 10 min after rifampin treatment ($250 \ \mu g \ ml^{-1}$; Sigma). The half-life was determined for each gene by real-time PCR using the time at which rifampin was added as a calibrator and the 16S rRNA transcript levels as an 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 $[\alpha^{-32}P]$ dATP (Perkin-Elmer, Courtaboeuf, France), and 100 U of SuperScript II reverse transcriptase (Invitrogen). The oligonucleotides used in this experiment are described in Table 2. The corresponding DNA-sequencing reactions were carried out using the primers and Cycle Reader DNA sequencing kit as recommended by the manufacturer (Fermentas, Euromedex, Souffel Weyersheim, France).

A capping assay was used to distinguish 5' triphosphate (indicating initiation



FIG. 3. Biofilm formation by *L. monocytogenes* EGD-e (\blacksquare), DG125A ($\Delta agrA$) (\blacksquare), and DG119D ($\Delta agrD$) (\Box) in batch conditions in polystyrene 96-well plates after 16, 24, 48, and 72 h of incubation at 25°C. Each histogram indicates the mean of three independent experiments, with five measurements for each point.

points) from 5' monophosphate (indicating processed products) according to the method of Bensing et al. (9), using a 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 nontreated 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 was performed by using 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 the ΔC_T value and (ii) in biofilm quantity. When one-way analysis of variance was significant, the Holm-Sidak test (n = 3, P < 0.05) was used to locate significant differences.

Nucleotide sequence accession numbers. The DNA sequence data of the mutant strains described in the present study have been deposited in GenBank/EMBL/DDBJ under the 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 or planktonic growth (data not shown). In contrast, the glass slide adherence of mutant DG125A ($\Delta agrA$) was affected. 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). Since cell-cell communication affects biofilm formation in many bacterial species, an agrD inframe deletion was designed to generate a mutant unable to produce the putative autoinducer peptide processed from AgrD. As demonstrated in mutant DG125A ($\Delta agrA$), no pleiotropic effect was observed in mutant DG119D ($\Delta agrD$) (data not shown). Moreover, the adhesion phenotype of DG119D ($\Delta agrD$) was similar to that of DG125A ($\Delta agrA$) (Fig. 2A). Micrographs of the microscopic field of adhering cells on glass slides confirmed that the quantity of adhering cells with L. monocytogenes DG119D ($\Delta agrD$) and DG125A ($\Delta agrA$) 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 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 during sessile and planktonic growth.

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, b [BF2-BR2], d [DF2-DR2], c [CF2-CR2], and a [AF2-AR2], and Table 2). 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 and 72 h of sessile growth (Fig. 4A). For each gene, the differences



FIG. 4. Semilogarithmic representations. (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, 24, and 72 h [the first three columns in each gene, respectively] and planktonic growth (early exponential phase OD₆₀₀ of 0.1, mid-exponential phase OD₆₀₀ of 0.4, and stationary phase OD₆₀₀ of 0.6 [the second three columns for each gene, respectively]). (B) Relative expression levels of the *agrB*, *agrD*, *agrC*, and *agrA* genes of *L*. *monocytogenes* determined during mid-exponential phase OD₆₀₀ of 0.4: EGD-e strain (\blacksquare), DG125A (*AagrA*) strain (\blacksquare), and DG119D (*AagrD*) strain (\square). For both graphs, gene expression was quantified by using real-time PCR and the comparative critical threshold ($\Delta\Delta C_T$) method. The *drm* gene was used as the internal standard, and expression of the *agrA* gene in the early exponential phase (OD₆₀₀ 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, lane numbers 1 to 4, were separated by electrophoresis on 1% agarose gel and correspond, respectively, to the expected sizes of 567, 388, 435, and 353 bp. The sizes of the DNA marker fragments are indicated in base pairs.

in the relative expression observed between 24 and 72 h of biofilm growth were not significant. The levels of *agrA* transcripts were similar at 2, 24, 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 (OD₆₀₀ of 0.4) the *agrC* and *agrA* transcript levels were, respectively, 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 (OD₆₀₀ of 0.4) in the parental and mutant ($\Delta agrA$ and $\Delta agrD$) backgrounds. In mutant DG125A ($\Delta agrA$), 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 ($\Delta agrA$) (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 transcript strain of the relative suggest an autoregulation of the transcript strain strains.



FIG. 6. Chemical half-life of the transcripts of the *agr* operon. Semilogarithmic plots of mRNA decay corresponding to the *agrB* (–), *agrD* (\blacklozenge), *agrC* (\blacksquare), and *agrA* (\blacktriangle) genes. Total RNA was prepared 0, 1, 4, 7, and 10 min after treatment with rifampin (250 µg ml⁻¹). The 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.

scription 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 posttranscriptional 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 posttranscriptional 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 RT 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; only small size products were detected (data not shown). The mRNA chemical half-life was determined by using real-time PCR experiments with the primer sets described above (Fig. 1A and Table 2). The results indicated that the chemical half-lives of agrB and agrD transcripts were 7.4 and 6.8 min, respectively, while they were lower for agrC and agrA (4.3 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 posttranscriptional cleavage and degradation.

5'-RACE was also used to characterize the 5' end of the

agrB	ta	cat	ttt	tgc	<u>itta</u>	tta	tgg	igta	iaat	tcg	rt <u>tq</u>	Itaa	<u>aat</u>	att	agt	<u>qqa</u>	<u>qq t</u>	gaa	itta	dtt	gag	rtaa	ttt	tac	tgc	aaa	agt	ccc	ttt	gtca
0																				М	S	Ν	F	Т	А	Κ	V	Ρ	L	S
agrC	ag	aac	gca	aac	tta	cat	att	taa	iaca	itag	ata	ttt	tag	aca	gtg	igaq	gat	taa	tat	gtt	tag	rtat	ttt	gat	ggc	aat	tat	aca	gat	aacg
																	_		М	F	S	I	L	М	A	Ι	Ι	Q	I	Т
	qc	rtat	ttt	tat	tqc	aat	.cca	gat	ttt	aåc	aaa	caa	aqt	ttt	ttc	aåt	taa	aqa	adc	att	qqt	tac	tat	aqc	aat	tqc	tat	qct	.aqc	cttc
	G	Ι	F	Ι	Ā	Ι	0	Ī	L	т	Ν	к	v	F	S	I	К	Ē	G	L	v	т	Ι	Ā	Ι	Ā	М	L	Ā	F
							~																							
aorA	gg	ŗatt	agg	rcc t	tgc	tag	ttt	geg	igg a	aat	tat	gaa	gaa	ata	ttc	gca	cgt	tgc	ctt	aga	tac	gaa	agt	gac	cga	tag	aga	agt	tat	tcaa
	G	L	G	L	Ā	s	L	R	E	I	М	ĸ	ĸ	Y	S	Н	v	Ā	L	D	Т	ĸ	v	Т	D	R	Ē	v	I	Q
	αa	at.t	aαa	aat	tat	αt.a	αaa	aa a	itaa	iaaa	t.αå	att	tlat.	act.	acc	aat.	t.t.t.	tat	ttc	rt.αa	aαa	taa	cad	aat	aca	aco	aαa	aad	at.t.	aacq
	Ē	L	E	I	М	**	*	· <u>)</u>					M	L	P	V	F	I	C	E	D	N	R	М	0	R	E	R	L	T
																									~					
	aa	ata	tat	taa	ana	cta	tat	tat	aat	taa	aca	+++	taa	tat	aa a	at t	ana	act	tt c	aac	ano	ana	tee	at t	t da	at t	* aut	ato	aca	aato
	ĸ	v	т	r F	n	v	т	M	.990 W	r F	ucu u	ਸ ਸ	n n	M	yuu v	т	ugu F	т	c	-uuuc π	C	n	D	900 F	r F	т	w	e	D	M
	17	1	1	2	D	Ŧ	Τ.	141	v	-	п	£	J	14	17	ш	-11	ц	3	. 1	9	D	r	r	c		v	5	1	11
olucic of	tho	51	and	of	ant) a	nel'	012	daa	ne / .	tror	cor	nto	11011	$n\alpha t$	otol	DN		1 100	lote	d fi	rom	- 67	210	0.00	lla .	aoll	anto	d d	uring

##

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 (OD₆₀₀ of 0.4). #, 5' end identified by primer extension; *, processing sites identified by 5'-RACE. The putative -10 with TGn extension and -35 sequences are double underlined, the ribosome binding sites are underlined, the start codons are boxed, and the stop codon of *agrC* is represented by three asterisks.

mRNA agr operon. PCR amplification was observed in TAPtreated 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 an OD₆₀₀ of 0.1, 0.4, and 0.6. Moreover, two hexanucleotides (TGGTTA and TAAAAT) separated by 18 nucleotides were detected upstream. They have 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). Thus far, the role of the agr system has not been clearly described in the pathogenic bacterium L. monocytogenes. In the present 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, which 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 afterward. 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 1.5- and 1.7fold decreases 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 Δagr genotype led to a pronounced attachment to polystyrene, 1.8- and 2.5-fold increases, 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 Δagr biofilm phenotype may have important consequences. For example, the dysfunction of agr is correlated with persistent bacteremia in *S. aureus* (49), and mutation of the *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 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 upregulation (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 after initial attachment. At first glance, this may seem surprising since 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.

ment but not in later stages; in contrast, the Rhl regulon plays a role in the 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 posttranscriptional 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 initiation transcription points, supported the posttranscriptional 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 posttranscriptional 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 the expression of *fsrB*, *fsrD*, and *fsrC* is fsr dependent, whereas the expression of the fsrA is weak, constitutive, and fsr independent (29, 45). It is proposed, on the 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 neighboring cells and prepare the cell for 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 in the adherence of *L. monocytogenes*. Our data show 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 since posttranscriptional processing occurred at the site of the 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*.

ACKNOWLEDGMENTS

This study 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 Sofia Kathariou for providing the pGF-EM vector, Philippe Gaudu for critical reading of the manuscript, and Mary Boulay for reading of the English text.

REFERENCES

- Aguado, V., A. I. Vitas, and I. Garcia-Jalon. 2004. Characterization of *Listeria monocytogenes* and *Listeria innocua* from a vegetable processing plant by RAPD and REA. Int. J. Food Microbiol. 90:341–347.
- Allenby, N. E. E., N. O'Connor, Z. Pragai, N. M. Carter, M. Miethke, S. Engelmann, M. Hecker, A. Wipat, A. C. Ward, and C. R. Harwood. 2004. Post-transcriptional regulation of the *Bacillus subtilis pst* operon encoding a phosphate-specific ABC transporter. Microbiology 150:2619–2628.
- Allison, D. G., B. Ruiz, C. San Jose, A. Jaspe, and P. Gilbert. 1998. Extracellular products as mediators of the formation and detachment of *Pseudo*monas fluorescens biofilms. FEMS Microbiol. Lett. 167:179–184.
- Arvidson, S., and K. Tegmark. 2001. Regulation of virulence determinants in Staphylococcus aureus. Int. J. Med. Microbiol. 291:159–170.
- Auger, S., E. Krin, S. Aymerich, and M. Gohar. 2006. Autoinducer 2 affects biofilm formation by *Bacillus cereus*. Appl. Environ. Microbiol. 72:937–941.
- Autret, N., C. Raynaud, I. Dubail, P. Berche, and A. Charbit. 2003. Identification of the *agr* locus of *Listeria monocytogenes*: role in bacterial virulence. Infect. Immun. 71:4463–4471.
- Balestrino, D., J. A. J. Haagensen, C. Rich, and C. Forestier. 2005. Characterization of type 2 quorum sensing in *Klebsiella pneumoniae* and relationship with biofilm formation. J. Bacteriol. 187:2870–2880.
- Bassler, B. L. 2002. Small talk: cell-to-cell communication in bacteria. Cell 109:421–424.
- Bensing, B. A., B. J. Meyer, and G. M. Dunny. 1996. Sensitive detection of bacterial transcription initiation sites and differentiation from RNA processing sites in the pheromone-induced plasmid transfer system of *Enterococcus faecalis*. Proc. Natl. Acad. Sci. USA 93:7794–7799.
- Bergmann, B., D. Raffelsbauer, M. Kuhn, M. Goetz, S. Hom, and W. Goebel. 2002. InIA- but not InIB-mediated internalization of *Listeria monocytogenes* by non-phagocytic mammalian cells needs the support of other internalins. Mol. Microbiol. 43:557–570.
- Challan Belval, S., L. Gal, S. Margiewes, D. Garmyn, P. Piveteau, and J. Guzzo. 2006. Assessment of the roles of LuxS, S-ribosyl homocysteine, and autoinducer 2 in cell attachment during biofilm formation by *Listeria mono*cytogenes EGD-e. Appl. Environ. Microbiol. **72**:2644–2650.
- Cramton, S. E., C. Gerke, N. F. Schnell, W. W. Nichols, and F. Gotz. 1999. The intercellular adhesion (*ica*) locus is present in *Staphylococcus aureus* and is required for biofilm formation. Infect. Immun. 67:5427–5433.
- Crossman, L., and J. M. Dow. 2004. Biofilm formation and dispersal in Xanthomonas campestris. Microbes Infect. 6:623–629.
- Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of bacterial biofilm. Science 280:295–298.
- Desroche, N., C. Beltramo, and J. Guzzo. 2005. Determination of an internal control to apply reverse transcription quantitative PCR to study stress response in the lactic acid bacterium *Oenococcus oeni*. J. Microbiol. Methods 60:325–333.
- Djordjevic, D., M. Wiedmann, and L. A. McLandsborough. 2002. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. Appl. Environ. Microbiol. 68:2950–2958.
- Donlan, R. M., and J. W. Costerton. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin. Microbiol. Rev. 15:167–193.
- Dunn, A. K., and E. V. Stabb. 2007. Beyond quorum sensing: the complexities of prokaryotic parliamentary procedures. Anal. Bioanal. Chem. 387: 391–398.
- Farber, J. M., and P. I. Peterkin. 1991. Listeria monocytogenes, a food-borne pathogen. Microbiol. Rev. 55:476–511.
- Garmyn, D., T. Ferain, N. Bernard, P. Hols, and J. Delcour. 1995. Cloning, nucleotide sequence, and transcriptional analysis of the *Pediococcus acidilactici* L-(+)-lactate dehydrogenase gene. Appl. Environ. Microbiol. 61:266– 272.
- 21. Guzzo, J., M.-P. Jobin, F. Delmas, L.-C. Fortier, D. Garmyn, R. Tourdot-

Marechal, B. Lee, and C. Divies. 2000. Regulation of stress response in *Oenococcus oeni* as a function of environmental changes and growth phase. Int. J. Food Microbiol. **55**:27–31.

- Hall-Stoodley, L., and P. Stoodley. 2002. Developmental regulation of microbial biofilms. Curr. Opin. Biotechnol. 13:228–233.
- Harley, C. B., and R. P. Reynolds. 1987. Analysis of *Escherichia coli* promoter sequences. Nucleic Acids Res. 15:2343–2361.
- Hebermehl, M., and G. Klug. 1998. Effect of oxygen on translation and posttranslational steps in expression of photosynthesis genes in *Rhodobacter capsulatus*. J. Bacteriol. 180:3983–3987.
- Homuth, G., A. Mogk, and W. Schumann. 1999. Post-transcriptional regulation of the *Bacillus subtilis dnaK* operon. Mol. Microbiol. 32:1183–1197.
- Horswill, A. R., P. Stoodley, P. S. Stewart, and M. R. Parsek. 2007. The effect of the chemical, biological, and physical environment on quorum sensing in structured microbial communities. Anal. Bioanal. Chem. 387:371–380.
- Huber, B., K. Riedel, M. Hentzer, A. Heydorn, A. Gotschlich, M. Givskov, S. Molin, and L. Eberl. 2001. The *cep* quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. Microbiology 147:2517–2528.
- Jefferson, K. K. 2004. What drives bacteria to produce a biofilm? FEMS Microbiol. Lett. 236:163–173.
- Ji, G., R. C. Beavis, and R. P. Novick. 1995. Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. Proc. Natl. Acad. Sci. USA 92:12055–12059.
- Kjelleberg, S., and S. Molin. 2002. Is there a role for quorum sensing signals in bacterial biofilms? Curr. Opin. Microbiol. 5:254–258.
- Kühn, K., A. Weihe, and T. Börner. 2005. Multiple promoters are a common feature of mitochondrial genes in *Arabidopsis*. Nucleic Acids Res. 33:337–346.
- Lawrence, L., and A. Gilmour. 1995. Characterization of *Listeria monocytogenes* isolated from poultry products and from the poultry-processing environment by random amplification of polymorphic DNA and multilocus enzyme electrophoresis. Appl. Environ. Microbiol. 61:2139–2144.
- Li, G., and S. Kathariou. 2003. An improved cloning vector for construction of gene replacements in *Listeria monocytogenes*. Appl. Environ. Microbiol. 69:3020–3023.
- Mack, D., A. P. Davies, L. G. Harris, H. Rohde, M. A. Horstkotte, and J. K. M. Knobloch. 2007. Microbial interactions in *Staphylococcus epidermidis* biofilms. Anal. Bioanal. Chem. 387:399–408.
- McNab, R., S. K. Ford, A. El-Sabaeny, B. Barbieri, G. S. Cook, and R. J. Lamont. 2003. LuxS-based signaling in *Streptococcus gordonii*: autoinducer 2 controls carbohydrate metabolism and biofilm formation with *Porphyromonas gingivalis*. J. Bacteriol. 185:274–284.
- Merritt, J., F. Qi, S. D. Goodman, M. H. Anderson, and W. Shi. 2003. Mutation of *luxS* affects biofilm formation in *Streptococcus mutans*. Infect. Immun. 71:1972–1979.
- Mongkolsuk, S., W. Panmanee, S. Atichartpongkul, P. Vattanaviboon, W. Whangsuk, M. Fuangthong, W. Eiamphungporn, R. Sukchawalit, and S. Utamapongchai. 2002. The repressor for an organic peroxide-inducible operon is uniquely regulated at multiple levels. Mol. Microbiol. 44:793–802.
- Murakami, S. K., S. Masuda, E. A. Campbell, O. Muzzin, and S. A. Darst. 2002. Structural basis of transcription initiation: an RNA polymerase holoenzyme-DNA complex. Science 296:1285–1290.
- Murray, E. G. D., R. A. Webb, and M. B. R. Swann. 1926. A disease of rabbit characterised by a large mononuclear leucocytosis, caused by a hitherto undescribed bacillus: *Bacterium monocytogenes*. J. Pathol. Bacteriol. 29:407– 439.
- 40. Nilsson, P., S. Naureckiene, and B. Uhlin. 1996. Mutations affecting mRNA

processing and fimbrial biogenesis in the *Escherichia coli pap* operon. J. Bacteriol. **178:**683–690.

- Novick, R. P., S. J. Projan, J. Kornblum, H. F. Ross, G. Ji, B. Kreiswirth, F. Vandenesch, and S. Moghazeh. 1995. The agr P2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. Mol. Gen. Genet. 248:446–458.
- Otto, M. 2004. Quorum-sensing control in Staphylococci-a target for antimicrobial drug therapy? FEMS Microbiol. Lett. 241:135–141.
- Phadtare, S., and K. Severinov. 2005. Extended -10 motif is critical for activity of the *cspA* promoter but does not contribute to low-temperature transcription. J. Bacteriol. 187:6584–6589.
- 44. Qin, X., K. V. Singh, G. M. Weinstock, and B. E. Murray. 2001. Characterization of *fsr*, a regulator controlling expression of gelatinase and serine protease in *Enterococcus faecalis* OG1RF. J. Bacteriol. 183:3372–3382.
- 45. Qin, X., K. V. Singh, G. M. Weinstock, and B. E. Murray. 2000. Effects of *Enterococcus faecalis fsr* genes on production of gelatinase and a serine protease and virulence. Infect. Immun. 68:2579–2586.
- Sauer, K., A. K. Camper, G. D. Ehrlich, J. W. Costerton, and D. G. Davies. 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. J. Bacteriol. 184:1140–1154.
- Silvaggi, J. M., J. B. Perkins, and R. Losick. 2005. Small untranslated RNA antitoxin in *Bacillus subtilis*. J. Bacteriol. 187:6641–6650.
- Sturme, M. H. J., J. Nakayama, D. Molenaar, Y. Murakami, R. Kunugi, T. Fujii, E. E. Vaughan, M. Kleerebezem, and W. M. de Vos. 2005. An *agr*-like two-component regulatory system in *Lactobacillus plantarum* is involved in production of a novel cyclic peptide and regulation of adherence. J. Bacteriol. 187:5224–5235.
- 49. Vance, G., J. Fowler, G. Sakoulas, L. M. McIntyre, V. G. Meka, R. D. Arbeit, C. H. Cabell, M. E. Stryjewski, G. M. Eliopoulos, L. B. Reller, G. R. Corey, T. Jones, N. Lucindo, M. R. Yeaman, and A. S. Bayer. 2004. Persistent bacteremia due to methicillin-resistant *Staphylococcus aureus* infection is associated with *agr* dysfunction and low-level in vitro resistance to thrombininduced platelet microbicidal protein. J. Infect. Dis. 190:1140–1149.
- Vogel, J., I. M. Axmann, H. Herzel, and W. R. Hess. 2003. Experimental and computational analysis of transcriptional start sites in the *Cyanobacterium* prochlorococcus MED4. Nucleic Acids Res. 31:2890–2899.
- 51. Vogel, J., V. Bartels, T. H. Tang, G. Churakov, J. G. Slagter-Jäger, A. Hüttenhofer, E. Gerhart, and H. Wagner. 2003. RNomics in *Escherichia coli* detects new sRNA species and indicates parallel transcriptional output in bacteria. Nucleic Acids Res. 31:6435–6443.
- Vuong, C., H. L. Saenz, F. Gotz, and M. Otto. 2000. Impact of the agr quorum-sensing system on adherence to polystyrene in *Staphylococcus au*reus. J. Infect. Dis. 182:1688–1693.
- Williams, T., S. Bauer, D. Beier, and M. Kuhn. 2005. Construction and characterization of *Listeria monocytogenes* mutants with in-frame deletions in the response regulator genes identified in the genome sequence. Infect. Immun. 73:3152–3159.
- Xu, L., H. Li, C. Vuong, V. Vadyvaloo, J. Wang, Y. Yao, M. Otto, and Q. Gao. 2006. Role of the *luxS* quorum-sensing system in biofilm formation and virulence of *Staphylococcus epidermidis*. Infect. Immun. 74:488–496.
- Yarwood, J. M., D. J. Bartels, E. M. Volper, and E. P. Greenberg. 2004. Quorum sensing in *Staphylococcus aureus* biofilms. J. Bacteriol. 186:1838– 1850.
- Yarwood, J. M., J. K. McCormick, M. L. Paustian, V. Kapur, and P. M. Schlievert. 2002. Repression of the *Staphylococcus aureus* accessory gene regulator in serum and in vivo. J. Bacteriol. 184:1095–1101.
- Yarwood, J. M., and P. M. Schlievert. 2003. Quorum sensing in *Staphylococcus* infections. J. Clin. Investig. 112:1620–1625.