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Evidence of Autoinduction Heterogeneity via Expression of the Agr System of *Listeria monocytogenes* at the Single-Cell Level[∇]

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To investigate if the primary function of the Agr system of *Listeria monocytogenes* is to monitor cell density, we followed Agr expression in batch cultures, in which the autoinducer concentration was uniform, and in biofilms. Expression was heterogeneous, suggesting that the primary function of Agr is not to monitor population density.

Quorum sensing (QS) is the mechanism by which bacteria secrete signaling molecules called autoinducers that are sensed by neighboring cells in a population (30). The binding of these autoinducers to cognate receptors results in transcriptional regulation of gene expression. So far, for the species *Listeria monocytogenes*, one QS system, mediated by the *agrBDCA* operon, has been described (2, 7). Deletion of *agrD* or *agrA* results in impairment of major adaptive strategies, such as biofilm development (22, 23) and virulence (2, 21).

Historically, the term QS was coined to illustrate that accumulation of autoinducers enables a coordinated control of gene expression resulting in a population-wide phenotype switch when the population reaches a threshold or quorum (6, 8, 18). However, recent reports indicate that adaptive functions of QS can be diverse and are not limited to population density sensing (20).

For example, phenotypic heterogeneity of QS-regulated traits was reported in biofilms. Several subpopulations with distinct phenotypes organize *Bacillus subtilis* biofilms (13, 14). Extracellular DNA release during the sessile growth of *Enterococcus faecalis* is directed by a fratricidal mechanism triggered by a quorum-responsive subpopulation (26). Heterogeneity was also observed in QS-regulated bioluminescence of *Vibrio harveyi* (1).

Recent reports showed that confocal laser scanning microscopy (CLSM) associated with fluorescent reporter fusions may be used to trace the spatiotemporal expression of specific genes at a single-cell level within the overall biofilm structure (9, 12). When we traced Agr expression in biofilms, we detected green fluorescent protein (GFP) mainly in a network of elongated chains reminiscent of scaffoldings that surrounded densely populated microcolonies (22). This heterogeneous expression was surprising; indeed, maximum expression was expected within microcolonies, where the autoinducer concentration is maximum (19).

Thus, the question of whether the function of this QS system was primarily to monitor population density arose. In order to test this hypothesis, a P_{agr}-*gfp* fusion was integrated upstream of the *agr* locus of the *L. monocytogenes* EGD-e genetic background. This construct was designed to develop Agr expression reporters without affecting expression of the downstream *agrBDCA* operon (22).

We followed GFP fluorescence by flow cytometry and microscopy during growth in batch homogenized liquid cultures, which represents environmental conditions prone to facilitate responses to cell density (confined cultures and no diffusion). Cells were collected by centrifugation (10 min at 8,000 × *g*), washed, and diluted in 150 mM filtered NaCl solution before flow cytometry analysis. For each sample, at least 100,000 cells were analyzed on a Cyflow flow cytometer (Partec, Germany) operating a 20-mW solid-state laser (488 nm). Data containing the green fluorescent signals were collected by a fluorescein isothiocyanate (FITC) filter, and the photomultiplier voltage was set at 380 V.

Agr expression in confined liquid environments is heterogeneous. *L. monocytogenes* ARD009 (EGD-e background) was cultivated in tryptic soy broth (TSB) in a rotary incubator (150 rpm) at 25°C. Agr expression was low and represented approximately 15% of the total early-exponential-phase population (Fig. 1A). It increased until late exponential phase (28%), and the subpopulation of cells expressing Agr (Agr-ON) stabilized to 35%. The percentage of viability was close to 100% during the length of the experiment (data not shown). Less than 4% of Agr-ON cells were detected when *agrD* and *agrA* mutants were used. The percentage of fluorescent cells of the positive control *L. monocytogenes* EGD-e(pNF8-GFP) was significantly higher throughout growth, and over 95% of fluorescent cells were detected by flow cytometry (Fig. 1A) as well as epifluorescence microscopy (Fig. 1C).

We then modified the growth medium in order to simulate a range of environmental conditions, including low nutrient levels (half-strength TSB), osmolarity (TSB plus 3% NaCl), and high glucose concentration (TSB plus 1% glucose or TSB

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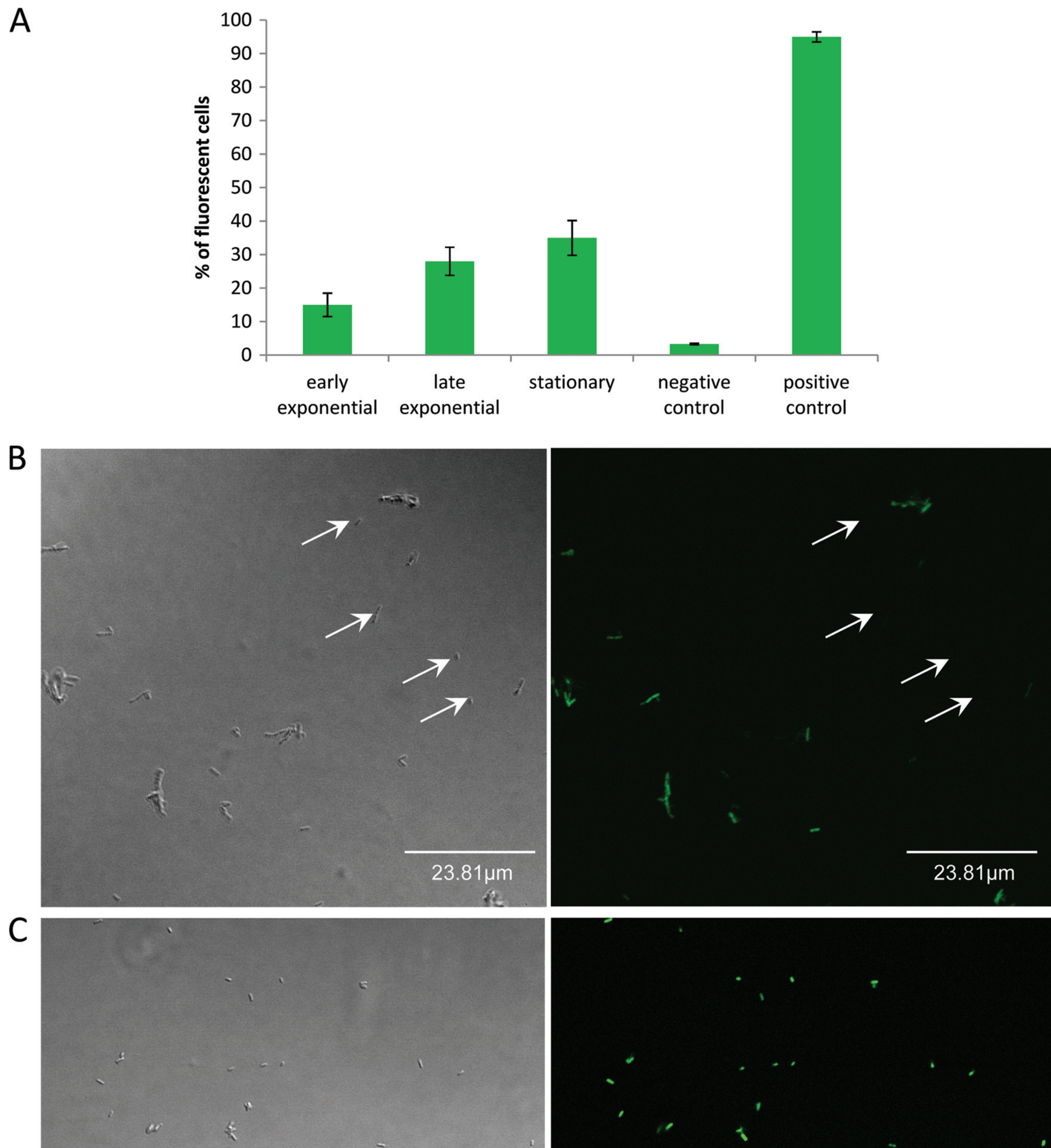

FIG. 1. Individual cell measurement of Agr expression during growth of *L. monocytogenes* ARD009 in homogenized liquid cultures incubated at 25°C. (A) Percentages of GFP fluorescent cells detected by flow cytometry. A positive GFP signal is detected in Agr-ON cells (cells expressing P_{agr}-*gfp*). (B) Phase-contrast microscopy and fluorescence microscopy of *L. monocytogenes* ARD009 cells expressing P_{agr}-*gfp* after 16 h of incubation at 25°C. (C) Phase-contrast microscopy and fluorescence microscopy of *L. monocytogenes* EGD-e(pNF8-GFP). White arrows show examples of Agr-OFF cells.

plus 2% glucose). We also tested the rich medium brain heart infusion (BHI). The composition of the growth medium affected Agr expression. The stationary-phase Agr-ON subpopulation was significantly smaller in the rich medium BHI (23.1% ± 1.37%) and in TSB supplemented with glucose (11.1% ± 0.22%). On the other hand, nutrient limitation (half-strength TSB) increased Agr expression (37.3% ± 0.56%), while the percentage of Agr-ON cells did not significantly change upon the addition of 3% NaCl. As temperature is an important cue for the environmental adaptation of *L. monocytogenes* (10), we followed cultures incubated at 37°C. Agr expression was significantly higher at this temperature (48.5% ± 8.03%) than at 25°C. This effect of environmental cues on Agr expression suggests that Agr is involved in a complex regulatory network integrating various environmental cues, including temperature and the energy status of the cell. Indeed, interconnection of several regulons in a network was reported recently (3).

Agr expression is heterogeneous in other genetic backgrounds. Considering the biodiversity of the species *L. monocytogenes* (4), we decided to investigate Agr expression during growth of other isolates from lineage I and lineage II (Table 1).

TABLE 1. Percentages of Agr-ON cells detected after 24 h of growth in homogenized TSB cultures incubated at 25°C[a]

Strain	Genotype	Lineage	Origin of parental strain	Reference	% of Agr-ON cells
ARD009	EGD-e::pGID128 P*agr-gfp*	I	Reference strain/rabbit listeriosis outbreak (17)	22	35 ± 5.2
ARD010	DG119D::pGID128 P*agr-gfp*	I	EGD-e Δ*agrD* (23)	This study	3.5 ± 0.25
ARD011	DG125A::pGID128 P*agr-gfp*	I	EGD-e Δ*agrA* (23)	22	3.3 ± 0.22
DG303	LO28::pGID128 P*agr-gfp*	I	Healthy pregnant women (16)	This study	15.1 ± 0.10
DG305	NV4::pGID128 P*agr-gfp*	I	Minced beef (24)	This study	68.4 ± 0.12
DG304	12749::pGID128 P*agr-gfp*	II	South Nation River sample (15)	This study	67.2 ± 0.57
DG306	ScottA::pGID128 P*agr-gfp*	II	Human listeriosis outbreak (31)	This study	72.0 ± 0.13
DG307	3E::pGID128 P*agr-gfp*	II	Sink at a cheese-making plant (23)	This study	73.5 ± 0.03
EGD-e(pNF8-GFP)	EGD-e(pAT18Ω(P*dlt*Ω*gfp-mut1*))		Reference strain/rabbit listeriosis outbreak (17)	5	95 ± 1.50

[a] Percentages were determined after cytometry analysis.

P_{agr}-*gfp* fusions within five other genetic backgrounds were constructed. Agr expression was heterogeneous, indicating that this phenotype was not specific to the domesticated laboratory strain *L. monocytogenes* EGD-e. The percentages of Agr-ON cells significantly differed among genetic backgrounds and ranged from 15% to 73% and were different from the positive-control value (Table 1). Regardless of the genetic background, Agr expression was heterogeneous. This suggests that it may be a generic phenomenon in this species.

In this experimental setup, environmental cues, including autoinducer concentration, are uniform. Still, we did not observe a population-wide switch to the Agr-ON phenotype, but two subpopulations were detected; this heterogeneity suggests that the Agr system of *L. monocytogenes* may not be primarily dedicated to population density sensing but could mediate other adaptive functions (11, 20).

The relative ratio of Agr-ON to non-Agr-expressing (Agr-OFF) subpopulations was strain dependent. We wondered what these ratios would be during sessile growth. We cultivated and monitored biofilms in BST FC 81 flow cells (Biosurface Technologies Corporation, Bozeman, MT) for 24 h at 25°C as described previously (22). Briefly, flow chambers were inoculated with cultures grown overnight in TSB medium and left to rest for 1 h to allow bacterial adhesion prior to TSB circulation at 10 ml/h. Biofilms were observed on a Leica TCS SP2 AOBS CLSM (at the MIMA2 INRA microscopic platform) fitted with a 63×/1.2-numerical-aperture water immersion objective lens (Fig. 2). Strains DG303, DG304, DG305, and DG307 developed structures similar to those we reported previously regarding biofilms of *L. monocytogenes* EGD-e. Ball-shaped microcolonies were linked together by chains of elongated cells organized as a network at the apex of the biofilm (22). The biofilms of strain DG306 were less structured. Biovolumes (μm^3) are indicators of the ability of the strains to grow on surfaces. Strains DG306 and ARD009 produced large amounts of biofilm; the biovolume of biofilms produced by DG304 was intermediate, while DG303, DG305, and DG307 were poor colonizers (Fig. 2). Whatever the strain tested, Agr expression

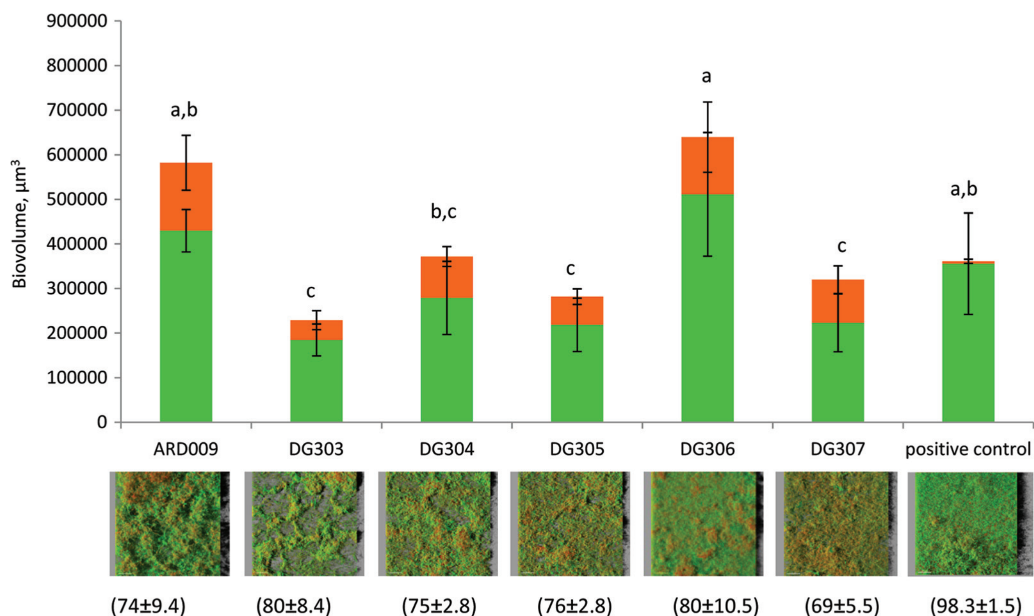

FIG. 2. Biovolumes of Agr-ON (green bars) and Agr-OFF (orange bars) cells in biofilms of six P*agr-gfp* reporter strains grown for 24 h at 25°C under flowing conditions. The percentages of Agr-ON cells are presented in parentheses. Letters indicate groups of biovolumes identified after analysis of variance ($P < 0.05$).

was heterogeneous. The percentages of Agr-ON cells ranged from 69% ± 5.5% to 80% ± 10.5%, but these differences were not statistically significant (Fig. 2). These percentages were higher than those in liquid cultures. The percentage of fluorescent cells of the control EGD-e(pNF8-GFP) was always significantly higher and close to 100% whatever the growth condition (biofilms or liquid cultures). Our results differ from those in reports on expression of other QS systems. Population-wide expression of *las* and *rhl* was observed during sessile growth of *Pseudomonas aeruginosa* (25), and Veening et al. proposed that induction of all ComA-regulated genes could be homogenous in populations of *B. subtilis* (27).

In conclusion, our results indicate that in *L. monocytogenes*, population density sensing may not be the sole function of the Agr system. The rationale of generating heterogeneity through the Agr system has to be investigated. The bet-hedging strategy (28, 29) is an appealing theory; the generation of offsprings with different phenotypes could facilitate survival of clonal populations to ever-changing environmental conditions. This phenomenon could indeed drive ubiquity in the species *L. monocytogenes*. It will be of particular interest to decipher the adaptive function of the Agr system considering the various habitats colonized by this species. To this end, further *in situ* investigations are required.

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