

N-Acyl-L-Homoserine Lactone Quorum Sensing Controls Butanediol Fermentation in *Serratia plymuthica* RVH1 and *Serratia marcescens* MG1

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Butanediol fermentation in two *Serratia* species is shown to be affected by *N*-acyl-L-homoserine lactone-dependent quorum sensing. Knockout of quorum-sensing signal production caused a shift towards enhanced acid production, resulting in early growth arrest, which was reversible by the addition of synthetic signal molecules.

The *Enterobacteriaceae* are commonly divided into two groups with different fermentation pathways (4). Members of genera such as *Escherichia*, *Salmonella*, and *Shigella* use the mixed-acid pathway, causing strong acidification of their environment due to the production of large amounts of acids, including acetate, lactate, succinate, and formate. In contrast, members of *Klebsiella*, *Enterobacter*, *Serratia*, and a number of other genera ferment glucose predominantly to 2,3-butanediol (18). In the latter case, the production of acidic products is limited because a significant amount of pyruvate from glycolysis is channeled into the butanediol pathway (6, 9). The production of neutral compounds is ecologically relevant since it allows butanediol fermenters to prevent lethal acidification as cells approach stationary phase. In *Klebsiella terrigena*, the LysR-type transcriptional activator BudR appears to regulate at least part of the 2,3-butanediol pathway (10). Recently, microarray analysis showed that the production of 2,3-butanediol in *Vibrio cholerae* is also regulated by the transcriptional activator AphA, in addition to the LysR-type transcriptional activator AlsR (8). The AphA activator is, in turn, regulated by multiple quorum-sensing systems that act in parallel. System 1 uses the CAI-1 autoinducer of unknown structure, while system 2 uses AI-2, a furanosyl borate diester, to trigger a phosphorelay circuit (1, 2, 7, 11, 19). These systems are homologues of the *Vibrio harveyi* systems (11) but differ from the LuxIR paradigm in *Vibrio fischeri*, in which LuxI synthesizes the *N*-acyl-L-homoserine lactone (AHL) signaling molecules and the AHL-activated LuxR protein functions as a transcriptional activator triggering a response (5).

We recently initiated a study of biofilm-forming bacteria from a food-processing environment (16). One isolate, designated RVH1, was identified as *Serratia plymuthica* (17), and we recently characterized its LuxIR-homologous quorum-sensing system, SplIR. The SplIR quorum-sensing system regulates the production of an extracellular chitinase, protease, nuclease,

and antibacterial compound (R. Van Houdt, P. Moons, A. Aertsen, A. Jansen, K. Vanoirbeek, M. Daykin, P. Williams, and C. W. Michiels, submitted for publication). In the current study, we report that 2,3-butanediol production in this strain is quorum sensing regulated via the same AHL-dependent system. Furthermore, we confirmed our observations in *Serratia marcescens* MG1 (previously *Serratia liquefaciens* MG1), the type species of the *Serratia* genus. The demand and manufacture of 2,3-butanediol are still increasing worldwide (annual rate of 4 to 7%) due to a variety of applications, such as its use as a liquid fuel additive, and to the increased demand for polybutylene terephthalate resin, gamma-butyrolactone, spandex, and its precursors (14). Furthermore, rising petroleum prices revived significant interest in producing feedstock chemicals, including 2,3-butanediol, from biomass. These results therefore may also be useful for improving the yield of 2,3-butanediol fermentation via metabolic engineering.

Effect of carbohydrates on growth and acid production of *S. plymuthica* RVH1 and its isogenic quorum-sensing mutants. We observed incidentally that the *splI* mutant of *S. plymuthica* RVH1, which is deficient in the production of *N*-acyl-L-homoserine lactone quorum-sensing signals, grew less well than its parent strain in Luria-Bertani (LB) broth supplemented with glucose. To investigate this effect more systematically, we recorded growth curves for both strains in LB broth at 30°C, with

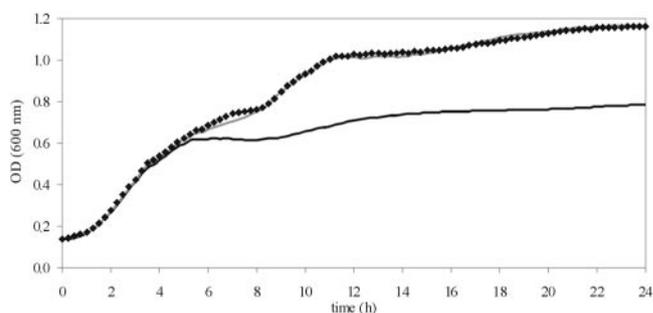


FIG. 1. Growth of *S. plymuthica* RVH1 (gray line) and its isogenic *splI* mutant with (diamonds) or without (black line) the addition of 10 μ M 3-oxo-C₆-HSL in LB medium supplemented with 0.5% galactose.

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TABLE 1. Plate count determinations and pH measurements for cultures grown for 24 h

Strain and supplement	Data for cultures grown in indicated medium ^a			
	LB + 0.5% galactose		MR-VP broth	
	Log CFU/ml	pH	Log CFU/ml	pH
RVH1	8.89 ± 0.05 ^b	6.03 ± 0.05 ^b	8.95 ± 0.04 ^b	6.50 ± 0.01 ^b
RVH1 <i>splI</i>	8.51 ± 0.07 ^c	4.94 ± 0.01 ^c	8.58 ± 0.04 ^c	5.04 ± 0.05 ^c
RVH1 <i>splI</i> + 3-oxo-C ₆ -HSL	9.02 ± 0.02 ^b	5.96 ± 0.02 ^b	9.01 ± 0.31 ^b	6.45 ± 0.05 ^b

^a Data are means ± standard deviations. Different superscripts indicate significant differences ($P < 0.05$) between values in the same column.

or without the addition of several sugars, using a Bioscreen C growth analyzer (Thermo Life Sciences, Brussels, Belgium). This equipment incubates up to 200 cultures in a microplate format and automatically measures their optical densities by using a wide-band filter (405 to 600 nm). Measurements were taken every 15 min after shaking of cultures for 60 s. No growth differences between the strains were observed in unamended LB broth (data not shown). However, in the presence of 0.5% of glucose, galactose, arabinose, fructose, or mannose, the *splI* mutant entered more rapidly into stationary phase than the parent strain. In LB broth supplemented with 0.5% galactose (Fig. 1), the onset of stationary phase occurred at an optical density of about 1.0 for the parent strain, compared to 0.6 for the *splI* mutant, and the corresponding cell densities at the end of the experiment (24 h) were 9.5 and 8.7 log CFU/ml, respectively. Furthermore, there was an important difference in the pHs of the spent culture media after 24 h, with values of 7.48 and 4.87, respectively, for the parent and the mutant. Interestingly, the apparent growth defect of the *splI* mutant could be nullified by the addition of 10 μM synthetic *N*-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C₆-HSL) (Sigma, Bornem, Belgium), the major AHL produced by *S. plymuthica* RVH1 (Van

Houdt et al., submitted for publication). Growth curves for the mutant in LB broth with galactose and 3-oxo-C₆-HSL were indistinguishable from those of the parent strain, and the cell density (9.5 log CFU/ml) and medium pH (7.37) after 24 h of growth were also restored to those of the parent strain. Growth of the *splR* and *splI splR* mutants in LB broth, with and without added sugar, was indistinguishable from that of the parent strain (data not shown). The same experiment was repeated with *S. marcescens* MG1, in which AHL-dependent quorum sensing has been studied in much detail. In this strain, quorum sensing affects phenotypes such as swarming, biofilm formation, and production of exoenzymes (3, 12, 13). Like the case in *S. plymuthica*, we observed an early onset of stationary phase accompanied by acidification in LB broth supplemented with sugars for the *S. marcescens swrI* mutant, which is deficient in the production of the signals *N*-butanoyl-L-homoserine lactone (C₄-HSL) and *N*-hexanoyl-L-homoserine lactone (C₆-HSL).

We next investigated whether the observed acidification and early growth arrest in *S. plymuthica* would also take place in methyl red-Voges-Proskauer (MR-VP) medium, which is a glucose-containing broth used in the classical test to distinguish between the mixed-acid and butanediol fermentation types in the *Enterobacteriaceae* family. The same effect was indeed noticeable after 24 h of growth in glass tubes (4 ml) but was somewhat less pronounced than that in LB-based media, probably because MR-VP medium is buffered. The effect was also reversible by the addition of 10 μM synthetic 3-oxo-C₆-HSL (Table 1). Similar results were obtained with the methyl red test for *S. marcescens* MG1 and its *swrI* mutant, either complemented or not with C₄-HSL (data not shown). Together, these results suggested that activation of the butanediol pathway at the end of the exponential growth phase is quorum sensing regulated, a hypothesis that was further investigated.

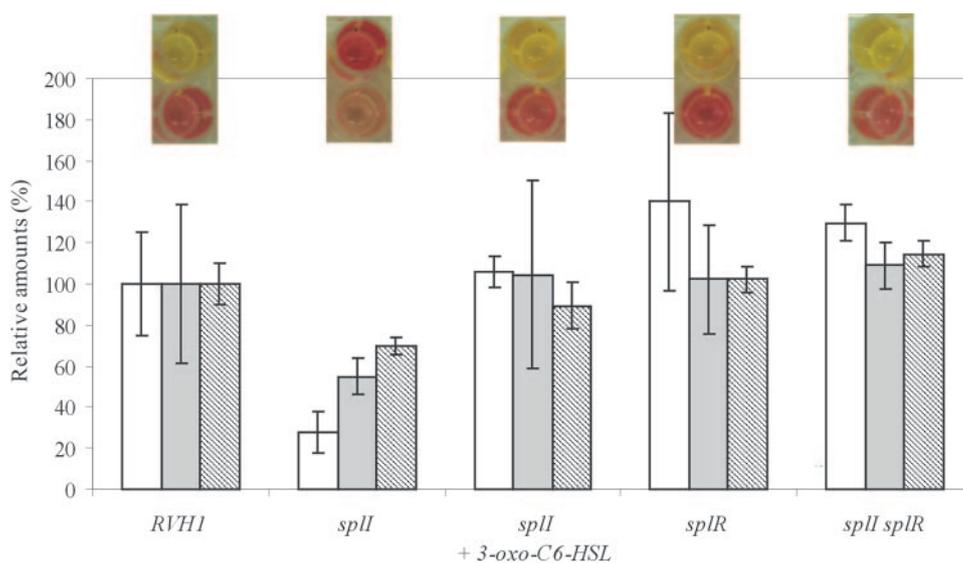


FIG. 2. Voges-Proskauer (top images, bottom row) and methyl red (top images, top row) assays and semiquantitative determination of amounts of acetoin (white bars), 2,3-butanediol (gray bars), and diacetyl (diagonally striped bars) for the *S. plymuthica* RVH1 wild type (left) and its isogenic *splI* mutant, isogenic *splI* mutant complemented with 10 μM 3-oxo-C₆-HSL, isogenic *splR* mutant, and isogenic *splI splR* double mutant. Amounts are expressed relative to the levels in RVH1 (100%). The experiment was performed in duplicate.

Acetoin production by *S. plymuthica* RVH1 and its isogenic quorum-sensing mutants. The Voges-Proskauer test was used as a qualitative method to demonstrate 2,3-butanediol fermentation by the detection of acetoin, a precursor of 2,3-butanediol. To 100 μ l of bacterial culture grown for 24 h in MR-VP medium, 30 μ l freshly prepared 5% α -naphthol in absolute ethanol and 10 μ l of 40% KOH were added, and the mixture was stirred vigorously. The formation of a red color is indicative of the presence of acetoin. As shown in Fig. 2, an intensive red color was obtained for *S. plymuthica* RVH1 and the *splI* mutant grown in the presence of 10 μ M 3-oxo- C_6 -HSL, while only a pale pink color developed for the *splI* mutant in the absence of the quorum signal. Furthermore, an intensive red color was also observed for the *splR* and *splI splR* mutants. Similarly, the test indicated acetoin production by *S. marcescens* MG1 and also by its *swrI* mutant in the presence of 10 μ M C_4 -HSL, but not in its absence (data not shown). A more specific analysis of 2,3-butanediol and its precursors diacetyl and acetoin by gas chromatography coupled to mass spectrometry was performed as previously described (15). Briefly, gas chromatography was performed by using a Fisons GC 8000 gas chromatograph (Fisons, Mainz, Germany) equipped with a Chrompack CP-WAX-52-CB column (Varian, Palo Alto, CA), and total ion mass chromatograms were obtained with a Fisons MD 800 quadrupole mass spectrometer (Fisons, Mainz, Germany) and analyzed using the Masslab software program (ThermoQuest, Manchester, United Kingdom) for identification and quantification of volatiles. This analysis showed decreased levels of all three compounds in the *splI* mutant, which were restored to parental levels in the presence of 10 μ M 3-oxo- C_6 -HSL, and comparable or slightly increased levels for the *splR* and *splI splR* mutants. The results for the *splR* and *splI splR* mutants are in accordance with our previous observation that SplR acts as a repressor, since the knockout of *splR* results in comparable levels of butanediol fermentation products to those in the parent strain and since the reduced amounts of these products in the absence of 3-oxo- C_6 -HSL (*splI* mutant) could be fully restored by inactivation of SplR (*splI splR* mutant).

In conclusion, butanediol fermentation is dependent on AHL-dependent quorum sensing in *S. plymuthica* RVH1 and *S. marcescens* MG1, and although butanediol fermentation has been well studied, it was not known to be regulated by AHL-dependent quorum sensing in any bacterium until now. Inactivation of the quorum-sensing system leads to continued production of acidic end products at the end of the exponential phase and throughout the stationary growth phase, which in turn leads to early growth arrest in the presence of fermentable sugars. Although the precise mechanism of regulation remains to be investigated, this finding may have important implications for the ecology of these bacteria. For example, Rice et al. (12) observed that in minimal medium supplemented with glucose and Casamino Acids, an *swrI* mutant formed thin, undifferentiated biofilms, in contrast to the well-developed biofilms formed by its parent strain, MG1, whereas in 0.1 \times LB

medium, both strains formed biofilms that resembled the wild-type architecture. Their proposed model for quorum-sensing-controlled biofilm development should take into account these new findings, which demonstrate additional metabolic regulation that could lead to differential acidification of the environment in the presence of fermentable sugars.

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