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Evidence of autoinducer-dependent and autoinducer-independent heterogeneous gene expression in *Sinorhizobium fredii* NGR234

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Populations of genetically identical *Sinorhizobium fredii* NGR234 cells differ significantly in their expression profiles of autoinducer (AI)-dependent and AI-independent genes. Promoter fusions of the NGR234 AI synthase genes *traI* and *ngrI* showed high levels of phenotypic heterogeneity during growth in TY medium on a single cell level. However, adding very high concentrations of *N*-(3-oxooctanoyl-)-L-homoserine lactone resulted in a more homogeneous expression profile. Similarly, the lack of internally synthesized AIs in the background of the NGR234-Δ*traI* or the NGR234-Δ*ngrI* mutant resulted in a highly homogenous expression of the corresponding promoter fusions in the population. Expression studies with reporter fusions of the promoter regions of the quorum quenching genes *dlhR*, *qsdR1* and the pNGR234*b* encoded type IV pilus gene cluster suggested that other factors than AI molecules may affect NGR234 phenotypic heterogeneity. Further studies with root exudates and developing *Arabidopsis thaliana* seedlings provide first evidence that plant root exudates have strong impact on the heterogeneity of AI synthase and quorum quenching genes in NGR234. Thereby, plant-released octopine appears to play a key role in modulation of heterogeneous gene expression.

INTRODUCTION

Bacteria are able to monitor their own population density to subsequently synchronize group behavior. This process of cell-to-cell communication is called quorum sensing (QS). By small diffusible chemical molecules, called autoinducer (AI), bacteria convey the status of the single cell to the whole population, allowing bacteria to collectively make decisions with respect to gene expression. The cell density dependent synthesis, release and detection of these AIs was first described in the symbiosis of *Vibrio fischeri* and its marine host the bobtail squid *Euprymna scolopes* (1-4). The production of AIs in *V. fischeri* finally leads to a coordinated expression of the *lux* operon (1). Until today many examples of QS-dependent gene regulations have been described in a wide variety of Gram-negative and Gram-positive bacteria including the regulation of pathogenicity-related genes, genes for biofilm formation and for the formation of extracellular products (5, 6). Many Gram-negative members of the *Proteobacteria* employ acylated homoserine lactones (AHLs) as QS signaling molecules and possess the corresponding LuxR-type receptor/regulator proteins. The LuxR-type regulators are involved in signal binding and transduction (4, 6). It was assumed that QS-dependent signaling would allow well-coordinated and highly homogeneous expression of selected genes or gene clusters at the population level. Recent studies, however, gave a first hint at the heterogeneous gene expression of QS-related genes. Anetzberger and colleagues reported on the heterologous expression of the QS-regulated bioluminescence of *Vibrio harveyi* and the heterogeneous expression of QS responsive genes (7, 8). Other examples for QS-dependent heterogeneity include the Arg QS system of *Listeria monocytogenes* (9), the bioluminescence and AI system of *V. fischeri* (10) and the QS-dependent DNA release of *Enterococcus faecalis* (11). Thus, it appears that QS-dependent gene expression can lead to high levels of phenotypic

heterogeneity in an otherwise isogenic population. Thereby, the term 'phenotypic heterogeneity' describes non-genetic variations that are commonly observed between individual cells within a genetically homogeneous population. It is assumed that heterogeneous gene expression within an isogenic population is essential for the survival and fitness of the population and a prerequisite for bacterial multicellular behavior (12, 13).

Sinorhizobium fredii NGR234 (hereafter NGR234) was first described in 1980 as the only fast-growing isolate from *Lablab purpureus* among 30 other strains (14). Since then it became a model plant-symbiotic organism due to its ability to nodulate over 112 different plant genera (15). The NGR234 genome encodes for two different QS systems, the *tra*-system and the *ngr*-system. The AI synthase TraI produces the AHL *N*-(3-oxooctanoyl-)-L-homoserine lactone (3-oxo-C8-HSL) and NgrI probably synthesizes a derivative of AI-I type molecules (16, 17). The *tra* operon, including the *traI, traR* and *traM* genes, is encoded on the 0.54 Mb replicon pNGR234*a* as part of a conserved cluster of genes that share a high degree of synteny with the Ti-plasmid of *Agrobacterium tumefaciens* (17). With respect to this synteny a highly similar regulation mechanism is likely. The expression of *traI* is kept under control of TraR that initiates a positive feedback loop when bound to 3-oxo-C8-HSL (4, 16, 18). TraR also regulates the expression of the antiactivator *traM*. When TraM is bound to TraR it induces allosteric conformational changes in the regulator, preventing it from DNA-binding and thereby creating a negative feedback loop. Due to a strong affinity of TraM to TraR, an already existing DNA binding can also be disrupted by the TraM-TraR interaction (4, 16, 18-20).

By analyzing the quorum quenching (QQ) potential of NGR234, we could uncover at least five loci that were involved in the hydrolysis of AI-I molecules in NGR234. Our experimental data suggested that out of the five QQ proteins, DlhR,

QsdR1 and QsdR2 resemble lactonases (21). Thereby, the presence of at least five loci involved in AI degradation suggests a complex regulatory circuit in NGR234 that strictly controls the internal AI concentration. Interestingly, RNA-seq data from our laboratory suggested an AI-independent regulation of these QQ genes (22).

Within the current manuscript we have addressed the question to which extent NGR234 shows phenotypic heterogeneity with respect to the expression of selected AI- and non-AI-controlled genes in laboratory cultures and if data obtained in laboratory experiments concur with data in plant rhizospheres. We provide first evidence that the expression of the two NGR234 AI synthase genes is highly heterogeneous in laboratory cultures within isogenic populations. Further, our data suggest that the absence of either one of the AI molecules, the presence of plant-derived octopine or other not yet identified plant-derived compounds affect NGR234's phenotypic heterogeneity of *traI* and *ngrI* promoter fusions. We further provide evidence that NGR234 transcribes other than the *traI* and *ngrI* genes heterogeneously, suggesting additional signaling molecules involved in the regulation of NGR234's phenotypic heterogeneity.

MATERIAL & METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. NGR234, if not otherwise stated, was cultivated at 30°C in TY medium (0.5% tryptone, 0.3% yeast extract and 5 mM $CaCl_2$) supplemented with rifampicin (25 µg/ml). *Escherichia coli* strains were cultured at 37°C in LB medium (1% NaCl, 1% tryptone, 0.5% yeast extract) with the appropriate antibiotics. NGR234 carrying pBBR1MCS-5 was cultivated in the presence of gentamycin (30 µg/ml); NGR234 carrying pBBR1MCS-2 was cultivated

130 in the presence of kanamycin (25 µg/ml). *E. coli* cultures were supplemented with
131 ampicillin (75-100 µg/ml), gentamycin (10 µg/ml) or kanamycin (25 µg/ml) depending
132 on the different vectors.
133
134 **Molecular methods.** Cloning of the various promoter fusions was performed
135 according to standard techniques (23). Primers used are listed in Table 2. Promoter
136 fusions and genes that were synthesized are listed in Table S1 and purchased from
137 Eurofins MWG GmbH (Ebersberg, Germany). Plasmid transformation in *E. coli* was
138 done following standard heat shock protocols (23). NGR234 was manipulated using
139 di-parental conjugation following standard protocols (16, 23). For the live/death
140 staining, we incubated the cells for 10 min with 5 µM propidium iodide at room
141 temperature and verified the number of red fluorescing cells.
142
143 **Single cell fluorescence microscopic analyses in laboratory cultures.** For
144 the analyses of phenotypic heterogeneity on a single cell level, cultures were
145 generally grown in 20 ml TY medium supplemented with gentamycin (30 µg/ml) in
146 100 ml flasks under aerobic conditions at 30°C on a rotary shaker (200 rpm).
147 Cultures were inoculated to a starting OD_{600} of 0.1. 1 ml sample were drawn at the
148 indicated time points and prepared for microscopic analysis. Throughout the
149 experiments, the optical densities of the different cultures were recorded. For the
150 chemical complementation, 50 µM 3-oxo-C8-HSL (Sigma-Aldrich, Heidelberg,
151 Germany) were added to the growing cultures after 24 h and cells were analyzed
152 after 48 h. The phase-contrast and fluorescence images were recorded using a Zeiss
153 AxioCam microscope with a MRm camera mounted on the fluorescence microscope
154 (Zeiss Axio Imager.M2; Carl Zeiss AG, Oberkochen, Germany) equipped with a 25x,
155 40x, 63x and 100x lens. For fluorescence imaging the microscope was equipped with

filter BP546/12 (red), the emission filter 605/75 (red) and a Zeiss Illuminator HXP 120 C. Phase-contrast and fluorescence images were obtained from the same area and matched using AxioVision (release 4.8). The fluorescence phenotypes of single cells were recorded by evaluating in general a minimum of 500-700 cells per biological sample. For each time point at least three independent samples were analyzed by using the image analysis program ImageJ (version 1.48k; National Institutes of Health, USA [http://imagej.nih.gov/ij]) and by manually inspecting the obtained images.

To further verify the obtained fluorescence data and to get full quantitative fluorescence intensities, phase-contrast images of selected P*ngrI* experiments have been segmented. P*ngrI* cells were analysed by using the MSER software (24). Absolute intensities of individual cells were obtained by integrating the fluorescence signals within the cell segmentation boundaries. Images of control cells were used to determine the level of autofluorescence allowing a classification of cells into positive or negative fluorescence signals.

Mathematic modelling. To test the obtained data for homogeneity/heterogeneity, we independently fitted at selected time points one gamma distribution using maximum likelihood estimation and a finite mixed model with two gamma distributions, describing the mixture of two homogeneous subpopulations. Thereby, our basic modeling assumption was that gene expression in a homogeneous population would be well described by a gamma distribution but not in a heterogeneous population (25, 26). For the mixture model, an adapted version of the EM-algorithm published by Benaglia *et al.* (27) has been used.

Rhizosphere colonization experiments. Rhizosphere colonization tests were accomplished with *Arabidopsis thaliana* (ecotype Columbia-0). *A. thaliana* is not a host for NGR234. For root colonization experiments, *A. thaliana* seeds were surface-sterilized for 20 min in 2% NaClO (w/v) and finally washed 5 times with sterile H_2O_{bidist}. Water was completely removed and the bacterial suspension (OD_{600} 0.7) was added to cover the seed. After 5 min seeds were placed under sterile conditions on a square plate. After stratification for 2 days at 8°C in the dark, seedlings were grown at long day conditions (16 h light with 80 µE) at 22°C for 4 days on plates with half strength Murashige-Skoog media (28) and 1.5% sucrose (w/v) solidified with 0.4% (w/v) gelrite. Co-localizations of *A. thaliana* and NGR234 bacterial strains were visualized with a Leica SP5 CLSM (Leica, Wetzlar, Germany) or using a Zeiss Axio Imager 2 fluorescence microscope (Zeiss, Jena, Germany). RFP fluorescence was detected with an excitation wavelength of 543 nm and an emission wavelength of 570 nm to 600 nm.

Plant root exudates. For experiments in which we used *Vigna unguiculata*-exudates, beans were surface-sterilized for 20 min in 0.12% NaClO (w/v) and washed 5 times in sterile H_2O_{bidist}. To detect contaminated seeds, infected with surviving bacteria, sterilized beans were placed on 0.5x TY agar (lacking $CaCl_2$) and germinated for 2 days at 30°C in the dark. Subsequently, beans showing no contaminations were transferred into 190 ml sterile plastic pots (Greiner bio-one, Frickenhausen, Germany) filled with 30 ml sterile glass beads (Ø 2.85 - 3.45 mm, Carl Roth GmbH, Karlsruhe, Germany) and 5 ml sterile 0.25x Hoagland solution (29). After 8 days of growth, the Hoagland solution was sterile filtered and used as aqueous extract. Additionally, the roots of up to 8 *V. unguiculata* plants were cut off,

transferred into 15 ml methanol and incubated on a rotary shaker for 4 days for the extraction of methanolic root extracts.

RESULTS

Promoter gene construction, evaluation of phenotypic heterogeneity and image analyses. To test if NGR234 shows a heterogeneous behavior with respect to the expression of QS-, QQ- and secretion-dependent genes on a single cell level, we constructed a series of promoter fusions employing the red fluorescent protein (DsRed2, in the following abbreviated with *rfp*) (30). Since this protein had already been successfully used in laboratory and rhizosphere studies with *Rhizobium* (31) and in other prokaryotic systems (32), we chose DsRed2 as a marker protein. The generated plasmids were verified by sequencing and are listed together with the bacterial strains in Table 1. All constructs were inserted into the broad host range vector pBBR1MCS-2 for experiments with the NGR234 deletion mutants or pBBR1MCS-5 for experiments with the NGR234 wild type strain (33) and mobilized via conjugation into NGR234. NGR234 grew in TY medium at 30°C with doubling times of 3-4 h and reached its stationary growth phase after 28 to 32 h. Aliquots of the cultures were transferred on glass slides and phase-contrast as well as fluorescence images taken.

For the initial setup of our system, we used the promoter of the constitutively expressed NGR234 *rpoD* gene, encoding for the sigma70 transcription factor, to verify the level of expression by image analysis. Since *rpoD* belongs to the genes that are most strongly expressed in NGR234, we chose this gene as a positive control. As expected cells carrying the P*rpoD::rfp* fusion did not show significant levels of phenotypic heterogeneity during exponential and stationary growth (Figure

1A). Only cultures that were freshly inoculated (5 h) showed low levels of heterogeneity (<5%), which we interpreted as cells that contained high levels of not fully matured RFP (30). RFP is known to require significant maturation times (30). Although virtually all cells were expressing the RFP protein, a more detailed image analysis suggested that approximately 5.7 +/- 2.3% of the cells showed a less intense fluorescence (Figure 1B). Further, we verified that the cell counts were not affected by high numbers of dead cells. We estimated the number of living vs. dead cells employing a propidium iodide staining of a NGR234 wild type culture. It appeared that in exponentially and stationary growth phase cultures with an age of up to 72 h, less than 2% of dead cells were observed (data not shown).

The *traI* and *ngrI* gene expression in cultures is heterogeneous on a single cell level. Since recent publications reported on a heterogeneous expression of AI synthase genes in different organisms (7, 8, 10, 11) we were interested, if NGR234 shows phenotypic heterogeneity with respect to its own AI synthase genes (*traI* and *ngrI*) during growth in complex medium under laboratory conditions. Using P*traI::rfp* and P*ngrI::rfp* promoter fusions (Table 1), we assayed the number of cells expressing the reporter gene vs. those cells that showed no red fluorescence. The *traI* gene expression in the NGR234 parent strain was lowest during the mid to late exponential growth phase. Here, 69.6 +/- 10.3% of all cells showed no fluorescence (*traI*-OFF), while 30.4 +/- 10.3% of the cells showed a weak or strong fluorescence (*traI*-ON). As expected during stationary growth phase almost 74.1 +/- 9.1% of all cells were expressing the *traI* promoter fusion (Figure 2A). The relative high expression of *traI* at 5 h after inoculation could be explained with cells being transferred from the preculture that had already entered stationary phase. Tests in which we inoculated cultures with cells from the exponential growth phase confirmed this hypothesis (data

not shown). The levels of heterogeneity were similar for the *ngrI* promoter fusion. In stationary growth phase the majority (84.4 +/- 2.5%) of cells was in a *ngrI*-ON mode while during exponential growth phase the majority of cells (60.5 +/- 12.6%) was in a *ngrI*-OFF mode (Figure 2B).

To verify the above obtained data and the presence of two stable subpopulations, we used mathematic modeling exemplarily for the P*ngrI* reporter fusion as outlined in material and methods. When we compared a homogeneous and the mixture of two homogeneous populations described by a finite mixture model with two gamma distributions, we were able to verify the presence of two distinct subpopulations for the P*ngrI* reporter strain at the 24 h value but not at the 48 h value. This finding fits well with the observation made above (Figure 2) and clearly suggests that distinct subpopulations have been formed.

Phenotypic heterogeneity is not restricted to AI synthase genes in NGR234. Since the AI synthase genes *traI* and *ngrI* were expressed heterogeneously in laboratory cultures on a single cell level, we wanted to know if other genes such as QQ and secretion-related genes were likewise heterogeneous in their expression profiles. For this purpose we first chose two QQ genes, *dlhR* and *qsdR1*. Both genes encode for lactonases in NGR234 and we have previously shown that they are involved in AI degradation (21). RNA-seq data and previous studies in our laboratory suggested that these genes are expressed independently from the presence of AIs (21, 22). Interestingly, the expression levels of both QQ genes were highly heterogeneous in fresh and 48 h old cultures on a single cell level. Phenotypic heterogeneity was lowest in exponentially growing cultures (Figure 3). Under these conditions less than 10% of the cells were in an ON mode. This was similar for both, the *dlhR* and *qsdR1* reporter strain.

Secondly, we conducted tests employing secretion-related promoter fusions. Experiments with NGR234 cells harboring pBBR1MCS-5 with the inserted P*gspD*::*rfp* fusion suggested that this gene is also heterogeneously expressed during growth in laboratory cultures. *GspD* is the first gene of the *gsp* (general secretion pathway, type II) gene cluster in NGR234 and previous studies indicated that this gene is regulated in a QS-dependent manner (22). For the *gspD* promoter fusion highest levels of phenotypic heterogeneity were observed during the exponential growth phase. After 24 h of growth in TY medium 26.6 +/- 2.4% of cells showed a *rfp* expression and 73.4 +/- 2.4% of all cells were in a *gspD*-OFF mode. However, during stationary growth phase (48 h) the majority of cells (97.6 +/- 0.6%) expressed the *rfp* at high levels.

Further tests using a *virB* (P*virB*::*rfp*) promoter fusion produced a similar result. The genome of NGR234 encodes for two possible conjugative pili (T4SS) whereby one is located on the symbiotic plasmid, pNGR234*a*, and is regulated within the *TraI/R*-regulon. The second one is encoded on the megaplasmid, pNGR234*b*. To test whether the pNGR234*b*-borne T4SS pilus is hetero- or homogeneously expressed on a single cell level, we chose the promoter region in front of the first gene (NGR_b10240, hypothetical protein) of the T4SS operon. NGR_b10240 and the T4SS cluster are most likely regulated in an AI-independent manner and transcribed from a single promoter located in the 5'-direction of NGR_b10240 (22). During the growth in TY medium, almost equal amounts of cells were in a *virB*-ON and in a *virB*-OFF mode in the exponential growth phase. However, in stationary growth phase almost all cells were in a *virB*-ON mode.

Altogether, these results indicated that heterogeneous gene expression on a single cell level is not only restricted to the *traI* and *ngrI* genes but is also of relevance for genes linked to other regulatory circuits in NGR234. The data further

suggest that not yet known growth phase dependent factors have a strong impact on heterogeneous expression patterns in NGR234 populations in laboratory cultures.

Dose-dependent influence of AHLs on AI synthases. Earlier reports documented the influence of AIs on the phenotypic heterogeneity of different bacteria (7, 8, 10). To verify that NGR234's phenotypic heterogeneity also depends to some extend on the presence of different AHL concentrations we added 3-oxo-C8-HSL to liquid laboratory cultures. 3-Oxo-C8-HSL was chosen because it is synthesized by the NGR234 TraI (16). In cultures that were supplemented with 0.05 µM 3-oxo-C8-HSL a 2 fold increase in non-fluorescing cells was observed for the P*traI::rfp* and the P*ngrI::rfp* fusions. Since the standard deviations in both tests were rather high, the increase was not significant on a single cell level ($p < 0.05$, Figures 4A and B). However, the addition of 50 µM 3-oxo-C8-HSL clearly resulted in a significantly decreased heterogeneity within the rhizobial population (Figure 4C). Under these conditions 93.1 +/- 5.9% of the cells expressed the P*traI::rfp* promoter fusion and 98.4 +/- 1.0% of the *ngrI*-expressing cells were in the ON mode. Controls treated with equal amounts of ethyl acetate, which had been used as a solvent for the AHLs, did show the same levels of phenotypic heterogeneity as observed for non-treated cells (data not shown). For reasons of control, we repeated this experiment but dissolved the AHLs in dimethyl sulfoxide (DMSO). In these tests only minor differences with respect to the heterogeneity were observed. After 48 h growth with 3-oxo-C8-HSL solved in DMSO 97.9 +/- 1.2% of the cells expressed the P*traI::rfp* promoter fusion and 99.6 +/- 0.2% of the cells were in an *ngrI*-ON mode. Consequently, the data obtained in these control tests indicated that the observed phenotypes were a result of the added AHLs and not caused by the utilized solvent.

Interestingly, tests in which we assayed the phenotypic heterogeneity in the background of NGR234-Δ*traI* and NGR234-Δ*ngrI* mutants suggested that the complete absence of the respective AI signal strongly affected phenotypic heterogeneity. In 48 h cultures the P*traI::rfp* fusion in the background of NGR234-Δ*traI* did not show significant levels of heterogeneity. In fact 99.8 +/- 0.2% of the counted cells were in a *traI*-ON mode and only 0.2 +/- 0.2% were not expressing the P*traI::rfp* fusion. Similar data were obtained for the P*ngrI::rfp* fusion in the NGR234-Δ*ngrI* background (Figure 4D), where the lack of AI led to an almost homogenous expression of the P*ngrI::rfp* fusion. In additional tests we tried to chemically complement the NGR234-Δ*traI* and the NGR234-Δ*ngrI* mutant by adding 50 µM 3-oxo-C8-HSL. In these tests, we observed for the NGR234-Δ*traI* mutant 90.31 +/- 4.0% of cells in the *traI*-ON mode and 9.69 +/- 4.0% in the *traI*-OFF mode. In case of the NGR234-Δ*ngrI* strain 92.03 +/- 1.4% were in a *ngrI*-ON mode and 7.97 +/- 1.4% in a *ngrI*-OFF mode. As a further control we used the P*dlhR::rfp* reporter fusion. It was heterogeneously expressed and this phenotypic heterogeneity was not affected by the addition of 50 µM 3-oxo-C8-HSL (Figure S1). Altogether, data obtained for AI synthase promoter fusions expressed in NGR234 wild type as well as in NGR234 mutant strains suggested that the concentration of the AI molecules has a strong impact on the level of phenotypic heterogeneity on a single cell level.

Plant-released molecules affect phenotypic heterogeneity in NGR234. Since NGR234 lives in the rhizosphere and grows in a close symbiotic association with numerous legume host plants, we hypothesized that potential plant signal molecules might modulate NGR234's phenotypic heterogeneity. To test this hypothesis, we incubated NGR234 carrying the P*traI* and the P*ngrI* reporter fusions together with *A. thaliana*, which is a non-legume and thus no host of NGR234.

Interestingly, image analyses of NGR234 cells that had been grown in the presence of the developing seedlings indicated a very homogenous expression of the AI-synthases (Figure 5). Almost all cells (> 90%) attached to the *A. thaliana* roots, or cells within a distance of 1-2 µm were in an ON mode (Figure 5), suggesting that not yet identified plant-derived molecules may influence *traI* and *ngrI* expression levels. *A. thaliana* was thereby an ideal model because we observed almost no red autofluorescence that interfered with our promoter fusions (Figure 5 and S2 A).

To further support our findings, we examined the influence of plant root exudates on NGR234's heterogeneity in laboratory cultures. Therefore, we treated NGR234 cells with water-soluble root exudates derived from *V. unguiculata* seedlings and analyzed the expression of the P*traI::rfp* and P*ngrI::rfp* promoter fusions in cultures that reached the stationary growth phase (48 h). The legume *V. unguiculata* is a natural host of NGR234 and was subject for many symbiotic interaction studies between plants and NGR234 (15). Interestingly, the addition of the exudates resulted in a significantly reduced heterogeneity for both promoter fusions (Figure 6). While in the absence of root exudates about 25.9 +/- 9.1% of the cells carrying the P*traI::rfp* fusion were in an OFF mode (Figure 6A), the addition of root exudates decreased this value to less than 3%. Similarly, NGR234 carrying the P*ngrI::rfp* promoter fusion showed less than 3% of OFF mode cells after incubation in the presence of the root exudates as well (Figure 6B).

The plant-derived molecule octopine affects phenotypic heterogeneity of AI synthase and QQ genes in NGR234. Unpublished RNA-seq data from our lab using NGR234 cells indicated a link between the rhizopine metabolism, which is an octopine derivative synthesized by rhizobia, and QS. Thus, we speculated that octopines could modulate phenotypic heterogeneity. Within this framework, it is

noteworthy that octopine has already been linked to QS signal pathways in *A. tumefaciens* (34). To test our hypothesis, we added 50 µM of octopine (CHEMOS GmbH, Regenstauf, Germany) to growing cultures of NGR234 carrying the P*dlhR::rfp* and the P*qsdR1::rfp* fusions. While in the absence of octopine almost equal cell numbers were in an ON vs. OFF mode, the addition of 50 µM octopine resulted in a strong increase of the fraction of *dlhR*-OFF cells (81.2 +/- 8.2%), while 18.8 +/- 8.2% of all cells remained in a *dlhR*-ON mode (Figure 7). Further, the addition of 0.05 µM octopine already resulted in an altered ratio of cells in the ON vs. the OFF mode albeit not as obvious (data not shown). The effects observed for the P*qsdR1* promoter fusion were similar, but not as pronounced (Figure 7). For the P*traI* and P*ngrI* promoter fusions the added octopine had a contrary effect on the observed phenotypic heterogeneity. The addition of 50 µM octopine resulted in the observation of 97.7 +/- 1.2% *traI*-ON cells and at least 95.0 +/- 1.5% of *ngrI*-ON cells. In summary these data supported the concept that plant-released compounds can modulate the level of phenotypic heterogeneity in NGR234.

DISCUSSION

Bacterial phenotypic heterogeneity is an essential parameter for the persistence and the survival of bacterial populations in nature (12, 13). This phenomenon has widely been studied in a number of model organisms such as *B. subtilis* (35, 36), *E. coli* (37-39), *L. monocytogenes* (9) and *V. harveyi* (7, 8, 10, 40). In the light of these reports, we wanted to know, if Gram-negative rhizobacteria also show heterogeneity during the expression of AI-dependent and AI-independent genes.

Therefore, we have investigated the phenotypic heterogeneity of the broad host range and nitrogen-fixing symbiont NGR234. We have chosen NGR234, because

413 this microbe is unique in the sense that its genome not only encodes for two distinct
414 AI synthase genes (17), but it also encodes for a remarkable number of genes
415 involved in quenching of the produced AI signaling molecules (21).

416 In the current study we observed that NGR234 revealed different levels of
417 phenotypic heterogeneity in the presence of varying AI concentrations and with
418 respect to the expression of its AI synthase genes *traI* and *ngrI* during growth in
419 laboratory cultures (Figures 2 and 3). Heterogeneity of *traI* and *ngrI* was significantly
420 decreased when we added high concentrations of synthesized AI (50 µM 3-oxo-C8-
421 HSL), or in the background of the two AI deletion mutants (Figure 4). Heterogeneity
422 of both AI synthases was high within the cell population in the background of the non-
423 treated NGR234 cells or if only low amounts (0.05 µM) of AI were added. These
424 findings support the notion that NGR234 can distinguish between very high and very
425 low concentrations of specific AI molecules and it suggests that phenotypic
426 heterogeneity is a well-controlled process that depends on a more or less defined
427 concentration range of the AI molecules. In this respect our data are in line with
428 earlier reports on the QS-dependent expression of phenotypic heterogeneity in other
429 microbes such as *V. harveyi* (7, 8, 40) and *L. monocytogenes* (9). Possible candidate
430 sensor proteins or regulators in NGR234 that are involved in intracellular AI
431 measurements are TraR, NgrR and any of the other four identified LuxR solos
432 encoded within the bacterial genome.

433 Further data from this study indicated that not only the *traI* and *ngrI* genes are
434 subject to heterogeneous expression but also genes linked to the degradation of QS
435 signals (i.e. the QQ genes *dlhR* and *qsdR1*) and genes linked to the build up of
436 secretion apparatuses (*gspD* and *virB*). Our recent RNA-seq data indicate that the
437 two QQ genes and the *virB* cluster encoded on pNGR234*b* are transcribed in an AI-
438 independent manner and the *gspD* gene in an AI-dependent manner (22). This

439 observation is interesting, since it implies that heterogeneous regulation of genes is a
440 more common phenomenon in NGR234 and not only depending on the presence of
441 AI. This hypothesis is in part supported by observations made for AI-dependent gene
442 expression in *V. harveyi* (8). However, the observation that NGR_b10240, *dlhR* and
443 *qsdR1* expression is heterogeneous suggests a major difference to the *V. harveyi*
444 gene expression patterns of AI-independent gene regulation. In *V. harveyi* it is
445 postulated that AI-independent genes are basically expressed in a homogeneous
446 manner (8). Thus, our findings may suggest that other factors than AI-I control
447 heterogeneity in NGR234. In the light of this hypothesis we observed that the
448 heterogeneous expression of the *traI* and *ngrI* genes was affected by the presence of
449 plant root exudates (Figures 5 and 6). The addition of small amounts of either water-
450 soluble or methanol extracted root exudates (data not shown) strongly altered the
451 expression profile of both the *traI* and the *ngrI* genes and induced a highly
452 homogeneous expression (Figure 6). Virtually the same effect was observed, when
453 NGR234 was grown in direct contact with developing seedlings of *A. thaliana* (Figure
454 5). This finding implies that the plant is able to control phenotypic heterogeneity by
455 the release of natural compounds that affect gene expression in a way that does not
456 allow heterogeneity. Since *A. thaliana* is not a host of NGR234, we further speculate
457 that this is a general effect independent from the host plant and that it is a result of
458 general compound-releases of developing roots.

459 Within this framework, we further provide evidence, that octopine might play a
460 role as a modulator of phenotypic heterogeneity in NGR234. Although legumes are
461 not reported to produce opines per se within the root nodules, several rhizobial
462 isolates are known to synthesize rhizopines and it is generally accepted that these
463 rhizopines are released to feed the free-living rhizobia in the rhizosphere (41).
464 Surprisingly, adding 50 µM of plant-released octopine resulted in homogenous *traI*,

ngrI and *dlhR* expression. This is a novel finding that further supports the notion that plant-released compounds may have influence on phenotypic heterogeneity in NGR234. Within this framework it is noteworthy that octopine is well known for its regulatory role on conjugation genes in octopine-mannityl opine-type Ti plasmids (34). Furthermore, it was recently demonstrated that opines provide cooperative *A. tumefaciens* cells within groups a fitness advantage over saprophytic agrobacteria (42).

In summary, results from this study have different implications. First, the observation, that NGR234 expresses several of its AI-independent genes heterogeneously, suggests that phenotypic heterogeneity might be a more general mechanism to control gene expression in this microbe. It also implies a more complex regulatory network and the involvement of other not yet identified signaling molecules. Second, the observation that plant-released compounds reduce phenotypic heterogeneity indicates that plants may have evolved mechanisms to control bacterial gene expression on a population level and override the AI signal. This mechanism may be a key to successful rhizosphere colonization and with respect to the observed role of octopine it may suggest a broader role of this molecule as a common good.

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609
610 **FIGURE LEGENDS**
611 **Figure 1. Analysis of gene expression of individual NGR234 cells carrying a**
612 **P*rpoD::rfp* promoter fusion in the vector pBBR1MCS-5**. **(A)** Percent of cells
613 expressing the marker genes vs. non-expressing cells. Bacteria were grown in 20 ml
614 batch cultures in TY medium. Grey bars, percent of cells for which no fluorescence
615 was observed; red bars, percent of cells showing red fluorescence. Samples were
616 taken after 5, 24 and 48 h (grey arrows). Data are mean values of at least 500 cells

617 for each time point and of three independent biological samples. Bars indicate the
618 simple standard deviations. The upper line represents the corresponding growth
619 curve. **(B)** Microscopic images of NGR234 cells carrying the same promoter fusions
620 as described in (A). Visualization was performed using a Zeiss Axio Imager 2
621 fluorescence microscope (Zeiss, Jena, Germany). Images were obtained with a Zeiss
622 LD Achroplan 100x/1.3 oil objective. Phase-contrast and fluorescence images were
623 obtained from the same area and matched using AxioVision (release 4.8).
624
625 **Figure 2. Phenotypic heterogeneity of NGR234 cells carrying (A) the P*tral::rfp***
626 **fusion and (B) the P*ngrI::rfp* fusion.** Percent of cells expressing the marker genes
627 vs. non-expressing cells. Bacteria were grown in 20 ml batch cultures in TY medium.
628 Grey bars, percent of cells for which no fluorescence was observed; red bars,
629 percent of cells showing red fluorescence. Samples were taken after 5, 24 and 48 h
630 (grey arrows). Data are mean values of at least 500 cells for each time point and of
631 three independent biological samples. Bars indicate the simple standard deviations.
632 The upper line represents the corresponding growth curve.
633
634 **Figure 3. Fluorescence and heterogeneity analyses of the two QQ genes *dlhR***
635 **and *qsdR1* during growth in laboratory cultures. (A)** NGR234 cells carrying the
636 P*dlhR::rfp* and **(B)** the P*qsdR1::rfp* reporter constructs. Data indicate the percentage
637 of cells expressing the marker genes vs. non-expressing cells. Bacteria were grown
638 in 20 ml batch cultures in TY medium. Samples were taken after 5, 24 and 48 h. Grey
639 bars, percent of cells for which no fluorescence was observed; red bars, percent of
640 cells showing red fluorescence. Data are mean values of at least 500 cells for each
641 time point and of three independent biological samples. Bars indicate the simple
642 standard deviations.

643

644 **Figure 4. Relative fluorescence of NGR234 carrying P*tral*::*rfp* and the P*ngrI*::*rfp***
645 **constructs during growth in TY medium in batch cultures. (A)** NGR234 after
646 48 h incubation without supplements and in the wild type background; **(B)** wild type
647 NGR234 cells carrying the same constructs, but treated with 0.05 µM 3-oxo-C8-HSL;
648 **(C)** NGR234 wild type treated with 50 µM 3-oxo-C8-HSL and **(D)** NGR234-Δ*tral* and
649 NGR234-Δ*ngrI* carrying the P*tral::rfp* and the P*ngrI::rfp* constructs. Grey bars,
650 percent of cells for which no fluorescence was observed; red bars, percent of cells
651 showing red fluorescence. Data are mean values of at least 500 cells for each time
652 point and of three independent biological samples. Bars indicate the simple standard
653 deviations.

654

655 **Figure 5. P*tral::rfp* and P*ngrI::rfp* gene expression of NGR234 cells in proximity**
656 **of *A. thaliana* root hairs. (A)** NGR234 carrying the P*tral::rfp* fusion and **(B)** NGR234
657 cells carrying the P*ngrI::rfp* fusion. The images show representative three-day-old
658 section of root hairs. The developing seedling was treated with approximately 2 x 10^8
659 cells x ml^{-1} 24 h prior to the analysis. The left panels in (A) and (B) show an image of
660 the light microscopic picture and right images give an overlay of the fluorescence and
661 the light microscopy image. The phase-contrast and fluorescence images were
662 recorded using a Zeiss AxioCam microsope with a MRm camera mounted on the
663 fluorescence microscope (Zeiss Axio Imager.M2). Images were recorded with a 100x
664 magnification. Cells that were not growing in proximity of the *A. thaliana* roots
665 showed normal levels of phenotypic heterogeneity (Figure S2 B).

666

667

668 **Figure 6. Fluorescence and heterogeneity analyses of NGR234 cells carrying**
669 **the P*tral::rfp* and the P*ngrI::rfp* treated with water-soluble root exudate.**
670 Bacteria were grown in 20 ml batch cultures in TY medium. Samples were analyzed
671 after 48 h and root exudate of *V. unguiculata* seedlings was added after 24 h
672 dissolved in Hoagland solution. Grey bars, percent of cells for which no fluorescence
673 was observed; red bars, percent of cells showing red fluorescence. Data are mean
674 values of at least 500 cells for each time point and of three independent biological
675 samples. Bars indicate the simple standard deviations.
676
677 **Figure 7. Fluorescence and heterogeneity analyses of the two QQ genes *dlhR*,**
678 ***qsdR1* and the AI synthase genes *tral* and *ngrI* in response to added octopine.**
679 NGR234 cells carrying the different reporter constructs were treated **(A)** with water
680 and **(B)** with 50 µM octopine. Data indicate percent of cells expressing the marker
681 genes vs. non-expressing cells. Bacteria were grown in 20 ml batch cultures in TY
682 medium. Samples were analyzed after 48 h. Grey bars, percent of cells for which no
683 fluorescence was observed; red bars, percent of cells showing red fluorescence.
684 Data are mean values of at least 500 cells for each time point and of three
685 independent biological samples. Bars indicate the simple standard deviations.
686
687

688

689 **TABLES**

690 **TABLE 1. Strains and plasmids used in this study.**

691

Strain or plasmid	Relevant Trait	Source or reference
Strains		
S. fredii NGR234	Wild type strain, Rf^r	(14)
S. fredii NGR234-Δ*traI*	Rf^r, Gm^r Δ*traI*	(22)
S. fredii NGR234-Δ*ngrI*	Rf^r, Gm^r Δ*ngrI*	(22)
E. coli DH5α	F^- *endA1 hasdR17 (*rk^-, mk^-) *supE44 thi-1 recA1 gyrA96 relA1*△(*argF-lacZYA*) U169□ 80d*lacZ* △ M15 □	(43)
E. coli XL1blue	*endA1 gyrA96*(nal^r) *thi-1 recA1 relA1 lac gln*V44 F'[::Tn10 *proAB*$^+$ *lacI*^{q} Δ(*lacZ*)M15] *hsdR17*(r_K^- m_K^+)	Stratagene, LA Joalla, CA
E. coli S17-1	*thi*-1 *pro*A *hsd*R17 (rk-mk+) *rec*A1 *tra*-genes of plasmid RP4 integrated into the genome	(44)
Plasmids		
pBBR1MCS-2	broad host range vector, low copy, Km^r	(33)
pBBR1MCS-5	broad host range vector, low	(33)

	copy, Gm^r	
pBBR1MCS-5::*rfp*	pBBR1MCS-5 carrying the DsRed2 gene (GenBank No. ABS86946.1) in the MCS	This work
pBBR1MCS-5::P*rpoD::rfp*	NGR234 P*rpoD::rfp* promoter fusion in pBBR1MCS-5	This work
*pBBR1MCS-2,5::P*traI::rfp*	NGR234 P*traI::rfp* promoter fusion in pBBR1MCS-2,5	This work
*pBBR1MCS-2,5::P*ngrI::rfp*	NGR234 P*ngrI::rfp* promoter fusion in pBBR1MCS-2,5	This work
pBBR1MCS-5::P*gspD::rfp*	NGR234 P*gspD::rfp* promoter fusion in pBBR1MCS-5	This work
pBBR1MCS-5::P*dlhR::rfp*	NGR234 P*dlhR::rfp* promoter fusion in pBBR1MCS-5	This work
pBBR1MCS-5::P*qsdR1::rfp*	NGR234 P*qsdR1::rfp* promoter fusion in pBBR1MCS-5	This work
pBBR1MCS-5::P*virB::rfp*	NGR234 pNGR234*b vir* gene cluster promoter fusion in pBBR1MCS-5	This work

692 * constructs were made in pBBR1MCS-2 and pBBR1MCS-5

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696 **TABLE 2: Primers synthesized in this study**

697

Oligonucleotide	Sequence 5'-3'	Size [bp]	PCR of Promoter region
P*ngrI*_XbaI_for	GTGGATcTagaATCTGAGCGCGA	23	AI synthase *ngrI*
P*ngrI*_EcoRI_rev	CATCgaattcGTTTTTGCGCGATGC	25	
Prom_b10240_fw	aaaatctagaAACCAGGGGATCAATCGTTT	31	pNGR234*b* *virB* cluster
Prom_b10240_rv	aaaagaattcGGAGAAAGTCCCCGCGAG	28	
P*dlhR*_XbaI_for	GTCCTtctagaGGCGATTACTGCATG	26	QQ gene *dlhR*
P*dlhR*_EcoRI_rev	CATGGCAAGGAgaattcGGGAACCT	25	
P*qsdR1*_XbaI_for	CGCGAAACCtctagaCAGGATCAAC	25	QQ gene *qsdR1*
P*qsdR1*_BamHI_rev	CTGCATggatccTGATGCGCTC	22	

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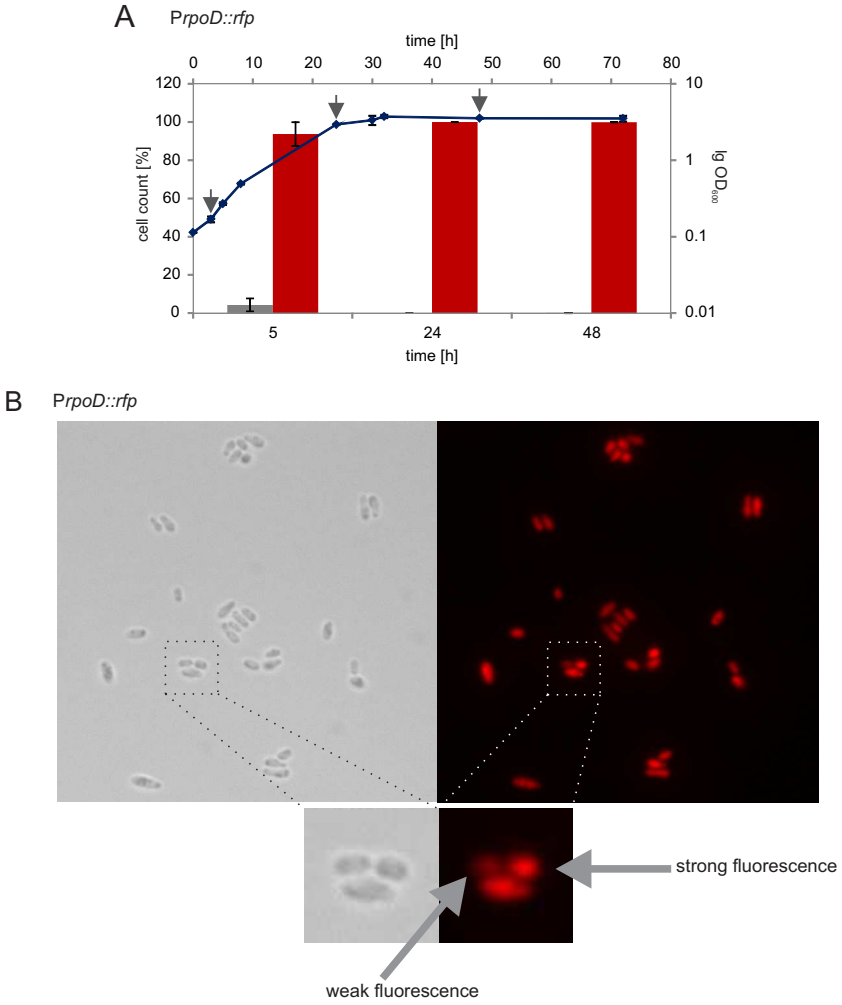
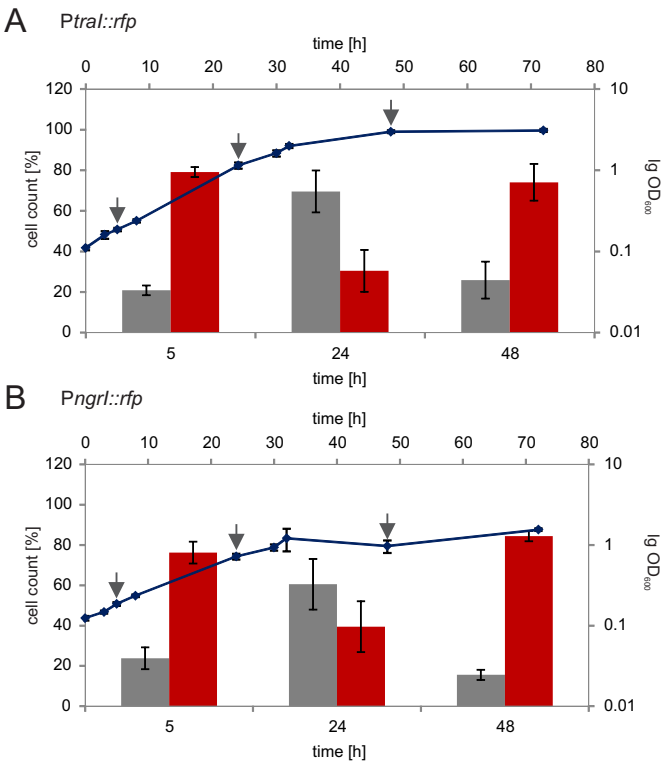
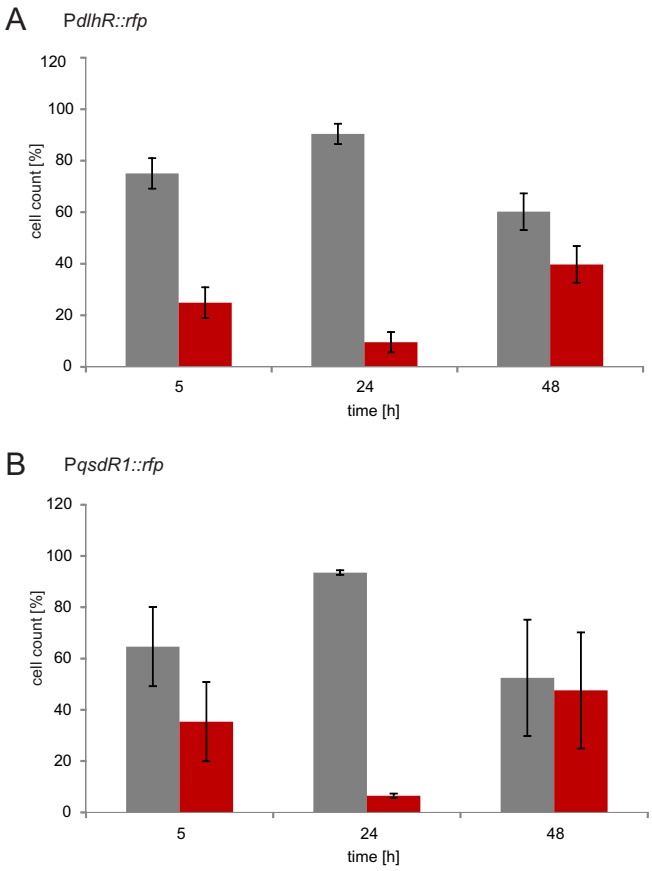
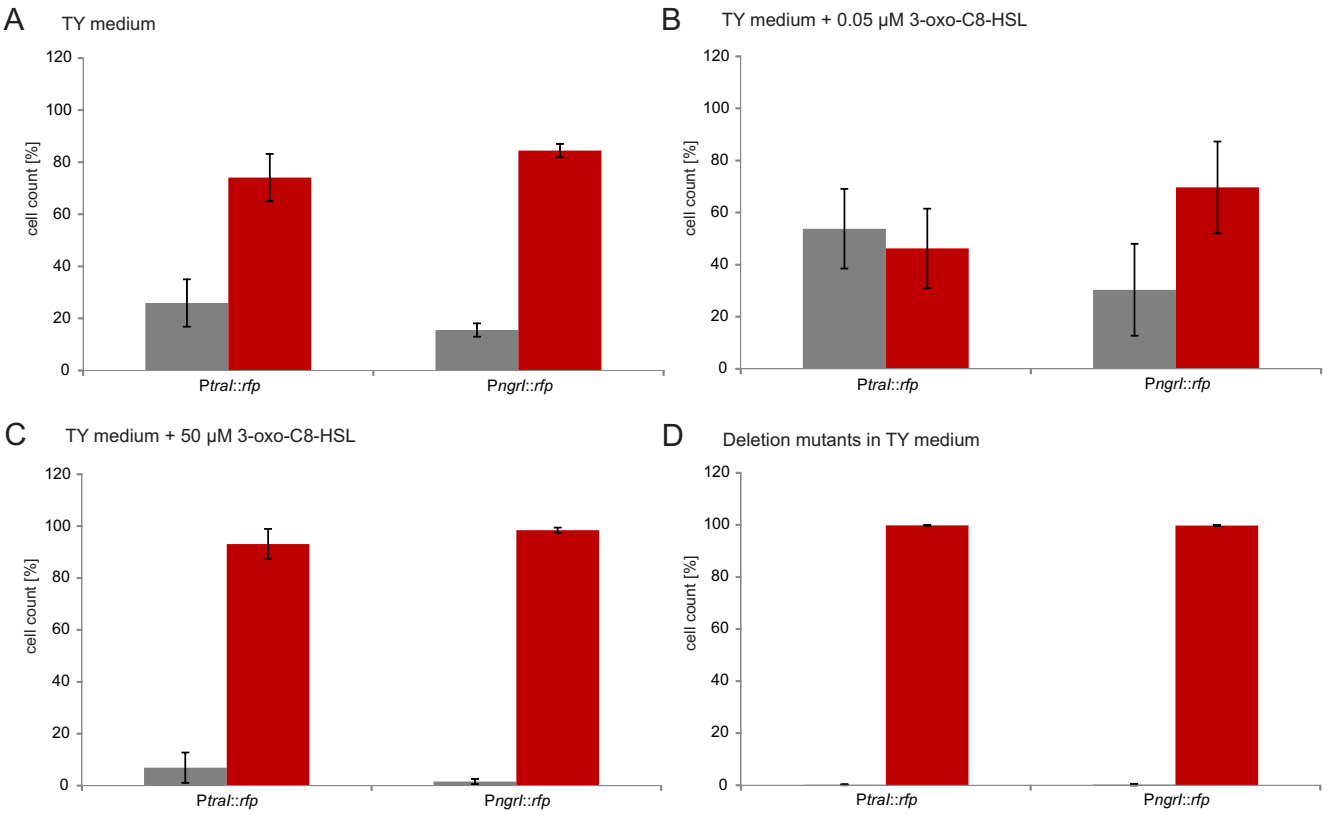
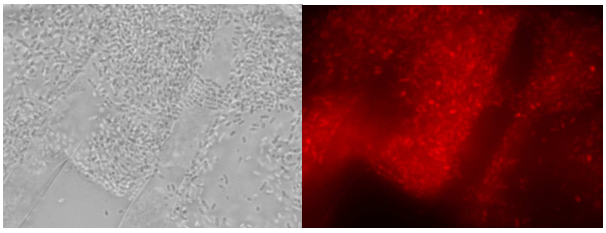








A *Sinorhizobium fredii* NGR234 P*traI::rfp*



B *Sinorhizobium fredii* NGR234 P*ngrI::rfp*