Deciphering regulatory mechanisms involved in phenotypic heterogeneity of the autoinducer synthase genes *traI* and *ngrI* in *Sinorhizobium fredii* NGR234 using single cell technologies and RNA-seq

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This is a corrected version.

Declaration on oath

I hereby declare, on oath, that I have written the present dissertation by my own and have not used other than the acknowledged resources and aids.

Hamburg,

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Jessica Grote

Contributions to the quoted articles

Grote J, Krysciak D, Streit WR. 2015. Phenotypic heterogeneity, a phenomenon that may explain why quorum sensing does not always result in truly homogeneous cell behavior. *Appl Environ Microbiol.* **81**(16):5280-5289

- Literatur inquiry
- Summary of literature results
- Writing parts of the text

Krysciak D, Grote J, Rodriguez Orbegoso M, Utpatel C, Förstner KU, Li L, Schmeisser C, Krishnan HB, Streit WR. 2014. RNA sequencing analysis of the broad-host-range strain *Sinorhizobium fredii* NGR234 identifies a large set of genes linked to quorum sensing-dependent regulation in the background of a *traI* and *ngrI* deletion mutant. *Appl Envrion Microbiol*. **80**(18):5655-5671

- Sample preparation for the RNA sequencing analyses
- Assistance with the evaluation and interpretation of the RNA sequencing results
- RNA isolation for the qRT-PCR control reactions
- Realization of the sedimentation assays

Grote J, Krysciak D, Schorn A, Dahlke RI, Soonvald L, Müller J, Hense BA, Schwarzfischer M, Sauter M, Schmeisser C, Streit WR. 2014. Evidence of autoinducer-dependent and -independent heterogeneous gene expression in *Sinorhizobium fredii* NGR234. *Appl Environ Microbiol*. **80**(18): 5572-5582

- Planning, realization, data analysis and interpretation of promoter fusion experiments
- Plant root control experiments with *S. fredii* NGR234 carrying the promoter fusions

Grote J & Krysciak D, Petersen K, Schmeisser C, Förstner KU, Krishnan HB, Streit WR. The absence of *N*-acyl homoserine lactones results in flavonoid-independent transcription of the symbiotic genes in *Sinorhizobium fredii* NGR234. Unpublished

- Planning and realization of the qPCR copy number verification
- RNA isolation and qRT-PCR
- Assistance with root hair curling experiments and RNA sequencing data evaluation and interpretation

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1 Abstract

The Gram-negative, symbiotic and nitrogen-fixing bacterium *Sinorhizobium fredii* NGR234 (NGR234) is able to nodulate over 120 different legume plant genera (Pueppke, Broughton 1999a). No other bacterium is currently known with such a broad host range. In recent years, notable achievements in deciphering the mechanisms responsible for this exceptional host range were accomplished. However there are still significant gaps in understanding the involved inter- and intraspecific communication mechanisms and their role in host range. To further identify possible molecular mechanisms linked to the biological phenomenon of broad host range two lines of work were followed:

First, population-wide and global transcriptome studies indicated that NGR234 has two major quorum sensing regulons each controlling several hundred genes. The *traI/R* regulon exclusively controls 130 genes, whereby the *ngrI/R* system regulates 280 genes. Additionally, 186 genes are co-regulated by both quorum sensing systems. To obtain these data a NGR234-△*traI* and a NGR234-△*ngrI* deletion mutant were constructed, and their transcriptomes compared with the corresponding transcriptome of the wild type strain. In general, flagellar and Type 4 pilus genes were regulated as well as genes for the biosynthesis of exopolysaccharides. Astonishingly, the second T3SS seemed to be regulated in both mutants, although no function could be identified for this secretion system so far. Further, we analyzed the transcriptome of a newly constructed NGR234-△*traI*-△*ngrI* double deletion mutant to better understand the processes in the absence of any autoinducer. The surprising finding in the background of this mutant was that 98 % of the genes encoded on the symbiotic plasmid pNGR234*a* were upregulated. Among the highest upregulated genes were the *repABC* genes and this probably resulted in a general increase of the copy number of the pNGR234*a* replicon. The increase in copy number and *repABC* transcription was linked to the high level expression of two hitherto not identified small open reading frames (ORFs), located in the intergenic region between *repA* and *traI*. These novel ORFs were designated *repX*, probably coding for a 58 aa protein and *repA0*, probably encoding a 143 aa protein. Both ORFs were highly transcribed in the absence of autoinducer in NGR234-△*traI*-△*ngrI* but not in the presence of autoinducers or in wild type. In line with this finding, it was observed that symbiotic proteins such as the nodulation outer membrane proteins NopABCLPX were produced in the NGR234-△*traI*-△*ngrI* but not in the wild type and in the

absence of plant-released flavonoids. Similar, the NGR234-△*traI*-△*ngrI* produced sufficient levels of nodulation factor to induce root hair curling in the absence of plant-released flavonoids. In addition to NGR234-△*traI*-△*ngrI*, NGR234-△*traI* and NGR234-△*ngrI*, the deletion mutants NGR234-△*traM* and NGR234-△*traR* were constructed and tested for the copy number of their symbiotic plasmid pNGR234*a.*

Second, and in addition to the population-wide studies, single cell analyses were performed. Therefore, seven promoter fusions were constructed and mobilized into NGR234 wild type cells and some of those additionally into a NGR234-△*traI* and a NGR234-△*ngrI* mutant, using the DsRed2 protein as marker. Our tests implied that under laboratory conditions high levels of phenotypic heterogeneous gene expression exist. Tests indicated that heterogeneity was highest after 24 h growth for the P*traI* and P*ngrI* and after 48 h growth in case of the P*dlhR* and P*qsdR1* promoter fusions. The percentage of fluorescing cells in case of P*traI* and P*ngrI* were nearly 31 % for P*traI* and nearly 40 % for P*ngrI* after 24 h and nearly 40 % for P*dlhR* and nearly 48 % for P*qsdR1* after 48 h growth.

Thereby, we provided strong evidence that under laboratory conditions surprisingly many genes in NGR234 are heterogeneously regulated especially the two autoinducer synthase genes *traI* and *ngrI*. However, the addition of external autoinducer and the addition of plant exudates led to a homogenization of the culture. These results imply that there are mechanisms that can overwrite NGR234's heterogeneous gene regulation and that heterogeneity may perhaps be not of high relevance in rhizospheres.

Most results of this work were published in Krysciak *et al.* 2014, Appl Environ Microbiol, Grote *et al.* 2014, Appl Environ Microbiol, and Grote & Krysciak *et al.* 2016, unpublished manuscript.

2 Introduction

2.1 *Sinorhizobium fredii* NGR234 and its symbiosis with plants

The α-proteobacterium *Sinorhizobium fredii* NGR234 (subsequently referred to as NGR234) was the fastest growing isolate from root nodules of the Fabacea *Lablab purpureus* (Trinick 1980). Since its discovery, NGR234 attracted the interest of ongoing research, because it is able to nodulate more than 110 different plant genera, which is unique in the 'rhizobial world' (Pueppke, Broughton 1999a).

NGR234 carries a tripartite genome with a chromosome (*c*NGR234) and two mega plasmids pNGR234*a* and pNGR234*b*. Already in the mid-1990s, the first sequencing of the pNGR234*a* replicon was initiated to better understand the phenomenon of broad host range. The symbiotic plasmid (pNGR234*a*; 0.5 Mbp) was the first genomic part to be sequenced and analyzed according to the symbiosis-related genes. It comprises the nitrogen fixation genes (*nif/fix* genes) and the nodulation genes (*nod*) that are all required for a successful symbiosis. Additionally, many genes of the symbiotic plasmid show a high identity to genes of the *Agrobacterium* tumor inducing (Ti) plasmid leading to the assumption that both genera have a common ancestor (Freiberg *et al.* 1997). In 2004, two fragments of the megaplasmid were sequenced. This led to the hypothesis that many genes of the megaplasmid are not strain specific, because they share high similarities to genes in other organisms (Streit *et al.* 2004). Finally, in 2009, the whole genome sequence of the strain was published. The main essential genes required for growth are encoded on the chromosome (*c*NGR234; 6.9 Mbp). The majority of all genes that are encoded on the megaplasmid (pNGR234*b*; 2.4 Mbp) are nonessential. An additional feature of the symbiotic plasmid is the encoded quorum sensing (QS) system, similar to that of *A. tumefaciens*. A second QS system is encoded on the chromosome (Schmeisser *et al.* 2009a).

Besides the sequencing projects researchers focused on unravelling specific proteins, e.g. NopP/NopM, or substances, e.g. exo-oligosaccharides, required for a NGR234-legume symbiosis (e.g. Broughton *et al.* 2006; Skorpil *et al.* 2005; Staehelin *et al.* 2006; Xin *et al.* 2012).

In general, rhizobia start colonizing the root by exchanging signal molecules with the plant root. Plant cells continuously release flavonoids, secondary plant metabolites that are recognized by the *Rhizobia*. As an answer to these substances, bacteria release nod factors,

lipochitooligosaccharides with various functional groups that are themselves recognized by the plants. After this molecule-exchange the root hair starts curling and an infection thread is formed, through which the bacteria can enter the plant root cells. The bacteria inside the root differentiate into another phenotypic state called bacteroids and start fixing nitrogen. The synthesized ammonium can afterwards be metabolized by the plant, whereby the plant delivers carbon sources needed for survival to the bacterial cells (Figure 1).

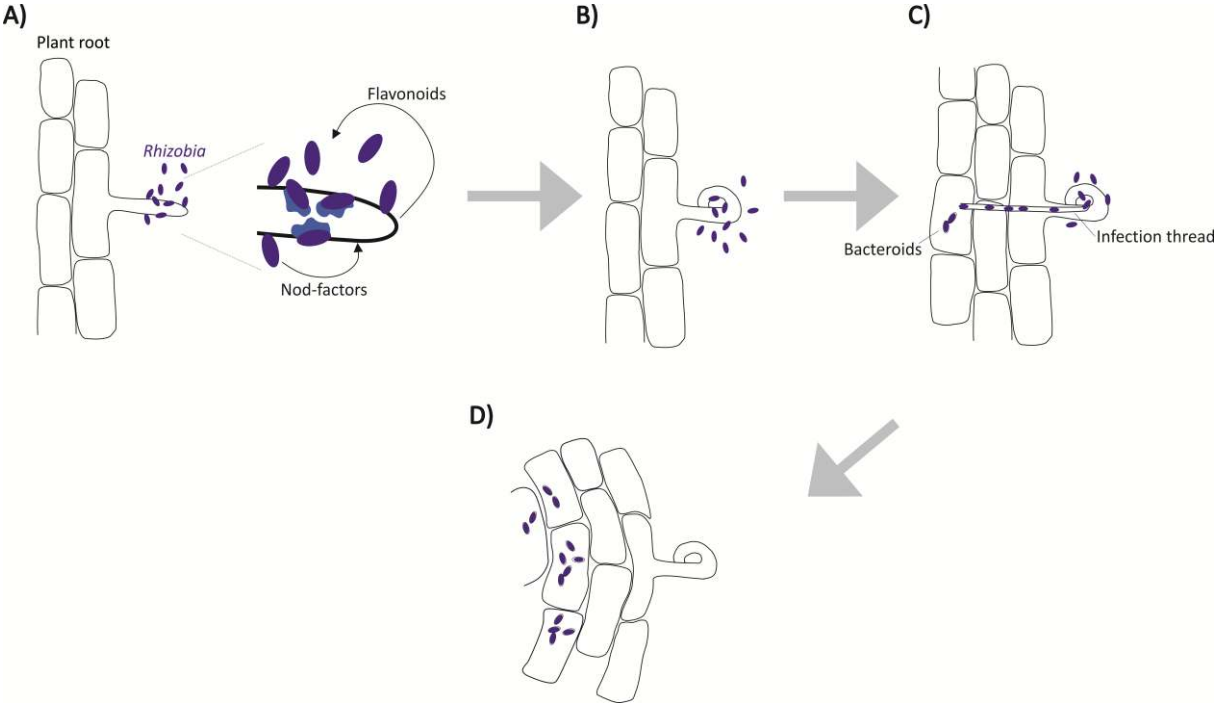

Figure 1: General events during early stages of a symbiosis event between a plant and *Rhizobia*. A) Bacteria colonize a plant root hair and the signal exchange between the plant and the attached bacteria takes place. B) The root hair curls as an answer to the bacterial nod factor signals. C) An infection thread is formed, through which the rhizobia can enter the plant root. Bacteria develop into bacteroids inside the root cells. D) The bacteroids in the nodules start fixing the gaseous nitrogen to turn it into ammonium, which can then be utilized by the plant.

To get a better understanding of the changes inside the root of a plant as an answer to bacterial invasion, Boukli *et al.* took a closer look into the plant response to NGR234's released nod factors via 2D-PAGE and expression analyses. Thereby, they discovered a change in the H^+-translocating ATPase level inside the roots. This pump generates the proton electrochemical gradient and can be located in the vacuole membrane or any other secondary membrane within a plant cell. The up-regulation of this protein suggests an involvement in the symbiosis process between plants and NGR234 (Boukli *et al.* 2007).

An additional study that uncovered details on the transcriptomic behavior of NGR234 bacteroids was published in 2013 by Li *et al*. (Li *et al.* 2013). The researchers analyzed two different nodule types, spherical and elongated, from two different legumes and compared

the transcriptomes with each other and with exponentially free living bacteria. Most of the up-regulated genes in bacteroids compared to the free living cells were found on the symbiotic plasmid pNGR234*a* that was already described as necessary for symbiosis (Freiberg *et al.* 1997; Schmeisser *et al.* 2009). General features for both non-growing bacteroid samples are the down-regulation of for example ribosomes, flagellar assembly, aminoacyl-tRNA biosynthesis, DNA replication and cell division genes. Mainly up-regulated genes were the ABC transporters, especially for phosphonate and sulfonate, and Type III secretion system (T3SS) related genes. In nodules from *Leuconostoc lecocephala* the *exo* genes, responsible for the synthesis of exopolysaccharides, genes necessary for the synthesis of rhamnan O-antigen, important for the nodulation process and the *pqq*-gene cluster, responsible for the synthesis of the cofactor pyrroloquinoline that is needed for the activity of many oxidoreductases, were up-regulated while in *Vigna unguiculata* only parts of these clusters were regulated. This leads to the assumption that there are regulatory differences between bacteroids from different legume hosts (Li *et al.* 2013). The QS systems of NGR234, described in chapter 2.2, were not found to be differently regulated in the nodules in comparison to free living cells (Li *et al.* 2013). This shows the subordinated role of the QS systems inside the root nodules.

2.2 Quorum sensing (QS) and quorum quenching (QQ)

Bacterial QS systems are cell density-dependent networks, through which bacteria coordinate the cell behavior from a single cell level at low cell densities to multicellular levels at high cell densities (Waters, Bassler 2005). For this, bacteria synthesize, release and detect small diffusible signaling molecules, named autoinducers (AIs). Five main groups of AI molecules are described in detail: acyl-homoserine lactones (AHLs) synthesized by a large number of Gram-negative bacteria, oligopeptides mainly used by Gram-positive bacteria, γ-butyrolactones produced by *Streptomyces*, and signaling molecules belonging to the AI-2 family that are probably used for interspecies communication (Garg *et al.* 2014; Waters, Bassler 2005). The class of AI-3 molecules is so far only described in the *E. coli* serotype O157:H7 as second AI system of this strain (Sperandio *et al.* 2003). In addition to the previously mentioned classes, more systems and signal classes were described in the past years. Schaefer *et al.* describe a new class of homoserine lactones that consist of a *p*-coumaroyl rather than of a long carbon chain added to the homoserine lactone moiety. The molecule was first described for *Rhodopseudomonas palustris* but was afterwards also found

in other bacteria, e.g. *Bradyrhizobium* sp. or *Silicibacter pomeroyi* (Schaefer *et al.* 2008). Another system linked to QS is the quinolone signaling pathway, which was first described in *Pseudomonas aeruginosa* and directly interferes with the AHL QS systems of *P. aeruginosa* (Diggle *et al.* 2007; Dubern, Diggle 2008; Pesci *et al*. 1999).

The first described AHL-dependent QS system is the one from *Vibrio fischeri*, a symbiont of the squid *Euprymna scolopes* (Nealson *et al.* 1970). In this bacterium, the QS system regulates, besides others, the transcription of genes responsible for the bioluminescence (Nealson, Hastings 1979). The AI synthase produces the AHL that diffuses through the membrane into the environment and back again. If a certain threshold of AI concentration in the environment is reached, the AI can bind to the LuxR regulator that afterwards changes its conformation. In this new conformation the regulator can bind to the '*lux box*', consisting of a 20 bp conserved inverted repeat sequence, in the promoter region of the AI synthase as well as the *lux* operon and activate their transcription leading to luminescence (Figure 2; Garg *et al.* 2014; Sitnikov *et al.* 1995; Waters, Bassler 2005). In general, QS networks regulate functions like bacterial biofilm formation, conjugal transfer of Ti plasmids, antibiotic exoenzyme transcription or pectinases and many more (Uroz *et al.* 2009).

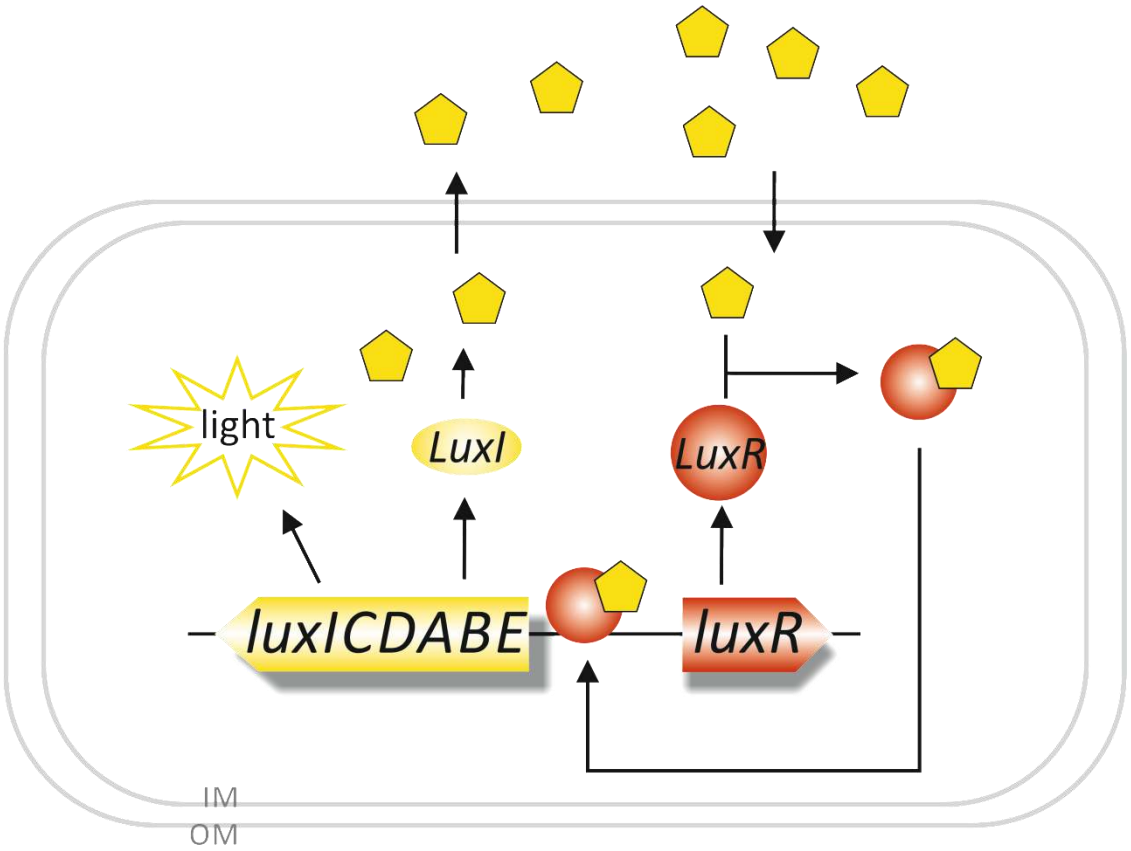

Figure 2: Scheme of the *Vibrio fischeri* quorum sensing system. The autoinducer synthase LuxI synthesizes the acyl-homoserine lactones (AHL, yellow pentagons) that diffuse through the membranes (IM, inner membrane; OM, outer membrane). When a threshold concentration of AHLs is reached, the molecules bind to the LuxR regulator. Afterwards the complex binds to the promoter region of the *luxICDABE* operon and activates their transcription, leading to luminescence (figure according to Garg *et al.* 2014; Sitnikov et al. 1995; Waters, Bassler 2005).

In plant-associated bacteria, most individuals sense their population density by AHLs, like *V. fischeri* (Sanchez-Contreras *et al.* 2007). Thereby, the AIs can, beside other effects, modulate the plant immunity or its development (Hartmann, Schikora 2012). As already mentioned in chapter 2.1, NGR234 encodes for two different AI systems. The genomic organization is shown in Figure 3.

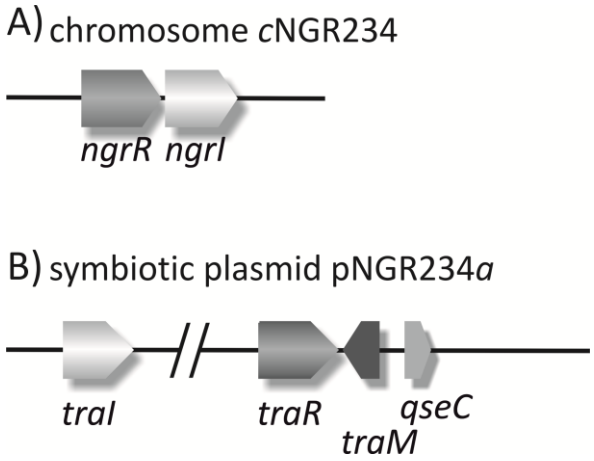

Figure 3: Genomic organization of the two autoinducer systems of NGR234. A) The chromosomally encoded *ngrI/R* system and B) the *traI/R* system encoded on the symbiotic plasmid.

The regulators, NgrR and TraR, as well as the AI synthases, NgrI and TraI, function in a similar way as described for the corresponding enzymes in *V. fischeri* (Gonzalez, Marketon 2003). Additionally to the standard components of *V. fischeri*, NGR234's genome codes for an antiactivator TraM (Schmeisser *et al.* 2009). The function of TraM was first described in the plant pathogen *Agrobacterium tumefaciens* (Fuqua *et al.* 1995; Hwang *et al.* 1995). TraM is a direct antagonist of TraR and inhibits the transcription of *traI*. Therefore, TraM directly binds to TraR, leading to a conformational change that prevents TraR from binding to its target promoter region (Chen *et al.* 2007; Luo *et al.* 2000; Swiderska *et al.* 2001). A second feature of the *traI/R* system is the modulator of the *traM* expression QseC. QseC was first described in *Mesorhizobium loti* where it represses *traM* transcription by binding to operator sites within the promoter region of *traM*. By this mechanism, QseC is proposed to restrict quorum sensing and ICE*M*/Sym^{R7A}, an integrative and conjugative element, transfer to a small proportion of the population. NGR234 encodes a *qseC* upstream of *traM*, which leads to the hypothesis that the regulatory mechanism of QseC in NGR234 as a modulator might be equal to that of *M. loti* (Ramsay *et al.* 2013).

The antagonist of QS is quorum quenching (QQ). This term describes mechanisms that interfere with bacterial cell-cell communication. The system is of importance when, for example, a plant defends itself, by disrupting the QS network of an invading pathogen (Waters, Bassler 2005). QQ can be achieved by: I) inhibiting the recognizing enzymes by inhibiting the synthase; II) synthesizing a molecule of an analogues structure to the AI molecule that binds to the sensor recognition site of the target molecule and thereby disrupting the sensing process; III) changing surrounding abiotic factors like pH or

temperature; IV) degrading the AI molecule by enzymatic activity. So far, four different enzyme classes are known to be able to degrade AIs, namely lactonases, aminohydrolases, oxido-reductases, oxidases and a recently identified enzyme cleaving the PQS signal from *Pseudomonas* (Müller *et al.* 2014; Uroz *et al.* 2009).

NGR234 codes for at least six genes that are probably involved in AHL degradation, namely *dlhR, qsdR1, qsdR2, aldR* and *hitR-hydR*. All genes were identified during a function-based screen for AI hydrolyzing enzymes. Another screen with heterologous expressed DlhR and QsdR1 confirmed their activity towards AHL degradation. Both enzymes are lactonases, cleaving the lactone ring of the AI molecule. NGR234 probably needs the QQ enzymes for a strict control of the AI concentration especially in the root rhizosphere, where an overexpression of either of the QQ genes leads to a strong inhibited root colonization phenotype (Krysciak *et al.* 2011). Additionally, these findings support the hypothesis that the AI signals are very important for a normal root colonization process.

Since the discovery of the phenomenon called phenotypic heterogeneity, described in chapter 2.3, QS- and QQ-networks, which were always thought to display a group-behavioral mechanism, are now under revision.

2.3 Phenotypic heterogeneity (Grote *et al.* 2015)

In recent years the phenomenon of phenotypic heterogeneity has moved into the focus of many research projects. It describes non-genetic variations in an otherwise isogenic population. This phenomenon is of advantage as it assists a small subpopulation within a larger population surviving rapidly changing environmental conditions (Ackermann 2015; Davidson, Surette 2008; de Jong *et al.* 2011; Dhar, McKinney 2007; Dubnau, Losick 2006; Grote *et al.* 2015; Smits *et al.* 2006; Veening *et al.* 2008a).

One of the best-studied and longest known examples for phenotypic heterogeneity are persister cells (Grote *et al.* 2015). These cells enter a different physiological state, by which they can survive antibiotic treatments. The phenomenon was first observed by Brigger, who treated exponentially growing isogenic and antibiotic sensitive *Streptococcus pyogenes* with penicillin and found surviving cells (Bigger 1944). The main regulatory switch leading to persistence is the toxin-antitoxin level within a cell, whereby a certain threshold concentration of the toxin finally leads to the molecular switching in cell morphology (Bertram, Schuster 2014; Maisonneuve, Gerdes 2014; Schuster, Bertram 2013).

Other examples for phenotypic heterogeneity are the spontaneous prophage induction, movement, bacterial competence or metabolic activities (Grote *et al.* 2015). A list with further examples is given in Table 1.

Table 1: Recent examples of phenotypic heterogeneity in bacterial isogenic populations (Grote *et al.* 2015).

Phenotypic heterogeneity traits	**Microorganisms**	**References**
Persister cells, resistance to antibiotics and heavy metals	*Staphylococcus* sp,. *E. coli, S. typhimurium, P. aeruginosa* and others	(Conlon 2014; Helaine, Kugelberg 2014; Lewis 2010; Maisonneuve, Gerdes 2014) and references herein
SOS response[a]	*E. coli, C. glutamicum*	(Kamensek *et al.* 2010; Nanda *et al.* 2014)
Response to peptide antibiotics	*B. subtilis*	(Kesel *et al.* 2013)
Prophage induction	*C. glutamicum, S. oneidensis, S. pneumoniae*	(Carrolo *et al.* 2010; Godeke *et al.* 2011; Nanda *et al.* 2014)
Quorum sensing, autoinducer synthesis genes	*V. campbellii, V. fischeri, L. monocytogenes, D. shibae, P. syringae, P. putida, S. fredii, S. meliloti*	(Anetzberger *et al.* 2009; Anetzberger *et al.* 2012a; Anetzberger *et al.* 2012b; Carcamo-Oyarce *et al.* 2015; Garmyn *et al.* 2011; Grote *et al.* 2014; Hense *et al.* 2012; Patzelt *et al.* 2013; Perez, Hagen 2010; Pradhan, Chatterjee 2014; Schluter *et al.* 2015)
Arabinose utilization	*E. coli*	(Fritz *et al.* 2014; Megerle *et al.* 2008)
Poly-hydroxy-butyrate utilization	*S. meliloti*	(Ratcliff, Denison 2011)
Secretion related genes	*S. fredii*	(Grote *et al.* 2014)
Quorum quenching genes	*S. fredii*	(Grote *et al.* 2014)
Motility, secondary flagella formation	*S. putrefaciens, S. typhimurium*	(Bubendorfer *et al.* 2014; Stewart *et al.* 2011)

Table 1 (continued)

Biofilm escape, motility after putrisolvin production	*P. putida*	(Carcamo-Oyarce *et al.* 2015)
Genomic island excision/transfer	*M. loti, P. knackmussii*	(Ramsay *et al.* 2013) (Minoia *et al.* 2008; Miyazaki *et al.* 2012)
Bacterial competence, DNA uptake	*B. subtilis, S. mutans*	(Hamoen *et al.* 2003; Lemme *et al.* 2011; Smits *et al.* 2005; Son *et al.* 2012)
Sporulation	*B. subtilis*	(Chung *et al.* 1994; Siebring *et al.* 2014; Veening *et al.* 2008b)
Colony heterogeneity	*S. aureus*	(Koch *et al.* 2014)
Increased lag phase	*E. coli*	(Fridman *et al.* 2014)
Surface pilus	*S. pneumoniae*	(De Angelis *et al.* 2011)
Myo-inositol utilization	*S. enterica*	(Kroger *et al.* 2011)
Biofilm formation	*S. enterica, B. subtilis*	(Chai *et al.* 2008; Grantcharova *et al.* 2010)
Antibiotic production	*S. coelicor*	(Mehra *et al.* 2008)

[a]SOS response is in part linked to the formation of persister cells, see reference (Maisonneuve, Gerdes 2014) and references herein

2.3.1 Phenotypic heterogeneity in QS systems

As already mentioned in chapter 2.2, quorum sensing is thought to be a mechanism, through which cells coordinate a specific cell behavior as a function of the population's cell density. Newest research results gave evidence that QS does not always lead to an equal cell behavior in all isogenic cells of a population but rather ends up in a heterogeneous gene regulation (Grote *et al.* 2015). One of the first descriptions of a heterogeneous response to QS signals was described for *V. fischeri*. In this bacterium the bioluminescence that depends on the AI concentration (see chapter 2.2) is turned on individually by every single cell with regard to response time and brightness (Perez, Hagen 2010). A similar observation was described for *V. harveyi*, recently reclassified as *Vibrio campbellii*, where the bioluminescence is controlled by QS and heterogeneous as well. Despite the general belief that at high cell densities, which means high AI concentrations, all cells of a population luminesce, only 69 % of the cells started to glow. And equal to *V. fischeri*, the strength of the luminescence varied throughout the glowing cells. Beside the luminescence genes, other AI dependent genes were heterogeneously expressed in *V. campbellii* as well (Anetzberger *et al.* 2009; Anetzberger *et al.* 2012b).

An example for AI-linked heterogeneity apart from bioluminescence was described for *Dinoroseobacter shibae*. In this organism the cell morphology is regulated by the three synthesized AIs and is highly variable. Only if one of the three AI synthases is deleted, all bacteria of this culture have the same morphology (Patzelt *et al.* 2013).

The AI systems of *Pseudomonas syringae* and *Xanthomonas campestris* are heterogeneously expressed in these two strains in laboratory culture. The heterogeneity cannot be influenced by the addition of external AI, which leads to the conclusion that the regulation of the AI systems is not a direct response to the synthesized AIs (Pradhan, Chatterjee 2014).

Despite these examples for Gram-negative bacteria, it is noteworthy that phenotypic heterogeneity with respect to QS exists in Gram-positive strains, too. The *arg* AI system in *Listeria monocytogenes*, for example, is expressed in a highly heterogeneous manner (Garmyn *et al.* 2011).

2.4 Aims of this study

This study followed several goals.

1) Get an idea on the general regulatory networks of both AI-systems in NGR234.
2) Find some basic principles that are responsible for the spatial and temporal distributed phenotypic heterogeneity in NGR234.
3) Demonstrate the biological significance of phenotypic heterogeneity/ the decrease of existing phenotypic heterogeneity.

The goal of this study was to investigate the two QS systems of *S. fredii* NGR234 on a population wide and on a single cell level. It was intended to get an idea on the regulatory networks modulated by the two autoinducer systems of NGR234 and to find out whether these data fit into recently published work of close relatives of NGR234. On the other hand, the expression levels of the autoinducer levels as well as autoinducer-dependent and -indepentent genes should be investigated on a single cell level. Thereby, we would like to get an idea about the homogeneity of the expression of certain genes among individual cells. This should give a validation of the transcriptional analyses of the autoinducer regulatory networks. After the description of the heterogeneous transcription behavior of genes in NGR234 we would like to find first hints on the regulatory mechanisms responsible for the heterogenous expression on a single cell level.

3 RNA sequencing analysis of the broad-host-range strain *Shinorhizobium fredii* NGR234 identifies a large set of genes linked to quorum sensing-dependent regulation in the background of a *traI* and *ngrI* deletion mutant

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The alphaproteobacterium *Sinorhizobium fredii* NGR234 has an exceptionally wide host range, as it forms nitrogen-fixing nodules with more legumes than any other known microsymbiont. Within its 6.9-Mbp genome, it encodes two *N*-acyl-homoserine-lactone synthase genes (i.e., *traI* and *ngrI*) involved in the biosynthesis of two distinct autoinducer I-type molecules. Here, we report on the construction of an NGR234-Δ*traI* and an NGR234-Δ*ngrI* mutant and their genome-wide transcriptome analysis. A high-resolution RNA sequencing (RNA-seq) analysis of early-stationary-phase cultures in the NGR234-Δ*traI* background suggested that up to 316 genes were differentially expressed in the NGR234-Δ*traI* mutant versus the parent strain. Similarly, in the background of NGR234-Δ*ngrI* 466 differentially regulated genes were identified. Accordingly, a common set of 186 genes was regulated by the TraI/R and NgrI/R regulon. Coregulated genes included 42 flagellar biosynthesis genes and 22 genes linked to exopolysaccharide (EPS) biosynthesis. Among the genes and open reading frames (ORFs) that were differentially regulated in NGR234-Δ*traI* were those linked to replication of the pNGR234*a* symbiotic plasmid and cytochrome *c* oxidases. Biotin and pyrroloquinoline quinone biosynthesis genes were differentially expressed in the NGR234-Δ*ngrI* mutant as well as the entire cluster of 21 genes linked to assembly of the NGR234 type III secretion system (T3SS-II). Further, we also discovered that genes responsible for rhizopine catabolism in NGR234 were strongly repressed in the presence of high levels of *N*-acyl-homoserine-lactones. Together with nodulation assays, the RNA-seq-based findings suggested that quorum sensing (QS)-dependent gene regulation appears to be of higher relevance during nonsymbiotic growth rather than for life within root nodules.

The ability of bacteria to sense a certain population density has become known as quorum sensing (QS). Quorum sensing is a cell density-dependent system of gene regulation in prokaryotes (1, 2). Through the accumulation of bacterially produced signaling molecules (autoinducers [AIs]), the bacterial population is able to sense increases in cell density and alter gene expression accordingly. Many examples of QS-dependent gene regulation processes have been described in a wide variety of Gram-negative and Gram-positive species. Thereby, it was found that pathogenicity, biofilm formation, production of extracellular proteins, secondary metabolite production, and other processes are often subject to QS-dependent regulation. *N*-Acyl-homoserine-lactones (AHLs) are the key signaling molecules in the cell density-dependent system of gene regulation in many Gram-negative bacteria (3, 4). The AHLs are synthesized through a LuxI-like protein (EC 2.3.1.184), using *S*-adenosylmethionine (SAM) and an acyl-acyl carrier protein (acyl-ACP) from the fatty acid biosynthesis pathway (5). Within the cells, the signals are recognized by the LuxR-type receptor/regulator proteins (6), building up complexes that subsequently stimulate subordinated processes.

Many soil bacteria interact with plants in ways that range from symbiotic and beneficial to pathogenic associations. Some symbiotic alpha- and betaproteobacteria (commonly called rhizobia) are able to form nitrogen-fixing root nodules together with the legume plant. The symbiosis is initiated by a signal exchange between the legume plant and the microbe (7–9). Within this framework, it is noteworthy that some rhizobia have evolved mechanisms that allow them to nodulate a larger variety of legume plants than others. These strains have been designated "broad-host-range" strains, and they are promiscuous with respect to the selection of their host plants (10). *Sinorhizobium fredii* NGR234 (here called NGR234) nodulates more than 120 genera of legumes and the nonlegume *Parasponia andersonii* (10, 11). No other strain with such a wide host range is currently known, and mainly because of its host range, NGR234 is a well-studied model organism. NGR234 was isolated in 1965 from the lablab bean (*Lablab purpureus*) in Papua New Guinea (12). Its 6.9-Mbp genome encodes a remarkable number of secretion systems and other interesting features that give some clues to the molecular keys to broad host range (13 and references therein).

The NGR234 genome encodes two distinct QS systems where

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TABLE 1 Primers used in this study to construct and verify NGR234-Δ*traI*, NGR234-Δ*ngrI*, and NGR234-Δ*ngrI*/Δ*traI* deletion and NGR234-cΔ*traI* and NGR234-cΔ*ngrI* complementation mutant strains

Oligonucleotide	Sequence 5′–3′[a]	Size (bp)	Target region/description
traI_-500_for	GAATTCTCGAGTTCTGAGTTGCTGCGG	27	5′ region of *traI*
traI_-500_rev	TCTAGAGAATTTCTCCGTCGTTGTTG	26	
traI_ + 500_rev	CTGCAGCTGCATTTGCGAGCGTCGTT	26	3′ region of *traI*
traI_ + 500_for	TCTAGAGGAGAAATCAGTGGAACAGC	26	
GmR_F_XbaI	GACATCTAGAGACGCACACCGTGGAAAC	28	Gm^r cassette
GmR_R_XbaI	TAATCTAGACCGCGATCATCAAGGCCGTG	29	
ngrI_A_for	GCGAATTCTGCTGGCGATCAGTGCCAAC	28	5′ region of *ngrI*
ngrI_A_rev	GCTCTAGACGGTCTTGACGTCCCATTTC	28	
ngrI_B_for	GCTCTAGAATCGCCTGGGAAGTATCGAG	28	3′ region of *ngrI*
ngrI_B_rev	GCCTGCAGGAAACCGCGCCGGTGAAATC	28	
traI_del_for	CAACGTCACCGCGAAATAG	19	External control primers
traI_del_rev	TCGCTGGTACGAAGAAGAAC	20	
traI_fragm_for	GACGATCTTCAACCGACCTAC	21	Internal control primers
traI_fragm_rev	GGAGCCTCATGAATGTGTCTG	21	

[a] Inserted restriction sites are underlined.

one of the AI synthases is designated *traI* and the second gene is designated *ngrI*. TraI synthesizes an AHL that is *N*-(3-oxooctanoyl)-L-homoserine lactone (here called 3-oxo-C_8-HSL) (14), and *ngrI* encodes an enzyme that probably synthesizes a not-yet-characterized derivative of an autoinducer I-type molecule (13). TraI is encoded on the 0.54-Mbp symbiotic 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* (13, 14). This conserved cluster contains, beside the *traI* gene, the *traR* and *traM* regulatory genes as well as other genes required for conjugative DNA transfer and replication of the plasmid. With respect to the high synteny with *A. tumefaciens*, it can be postulated that TraR associates with 3-oxo-C_8-HSL and that it is involved in transcriptional activation of QS-dependent promoters (14). TraM is a homolog of the corresponding antiactivator protein in *A. tumefaciens*, preventing TraR from activating the target genes in the presence of low levels of AIs. Furthermore, NgrI is encoded on the NGR234 chromosome together with its cognate receptor/regulator protein NgrR. NgrI is a functional homolog of *Sinorhizobium meliloti* SinI. However, the *sinI* gene is involved in the synthesis of several long-chain AHLs ranging from 12 to 18 carbons in length (15). The SinI/SinR- and ExpR-dependent gene regulation has been intensively characterized in this model organism (16–21); thereby, the different researchers have outlined a very complex and partially strain-specific regulatory network in this narrow-host-range strain.

Recently, high-resolution transcriptome studies using RNA sequencing (RNA-seq) technologies have given us a very detailed and reliable insight into expression profiles of many model organisms (22–25). However, only a few studies so far have used this technology for the genome-wide analysis of QS-dependent expression profiles. The focus of these studies was on the opportunistic pathogenic microorganism *Burkholderia cenocepacia* (26) and on *Pseudomonas aeruginosa* (27, 28). Interestingly, no study has yet focused on the QS-dependent gene regulation in sinorhizobia or closely related species using RNA-seq. Only very recently, an RNA-seq analysis was published on NGR234 with respect to a genome-wide change of the expression profile of the symbiotic lifestyle in bacteroids of two legume plants (29). This study has already given us a very detailed insight into the complexity of many processes linked to the bacterial infection process.

In the current study, high-resolution RNA-seq was used to analyze the expression profile of the NGR234 wild-type strain compared to newly constructed NGR234-Δ*traI* and NGR234-Δ*ngrI* deletion mutants. The focus of the study was thus on the identification of QS-regulated genes. Our data suggested that a common set of 186 genes is QS regulated. Further RNA-seq data generated in early exponential phase and by challenging NGR234 with moderate and high levels of AI suggested that QS plays only a minor role during the onset of growth but that it has profound effects on motility, vitamin biosynthesis, secretion, and other key features of the organism during the stationary phase.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Sinorhizobium fredii* NGR234 was grown at 30°C in liquid TY medium (0.5% tryptone, 0.25% yeast extract, 10 mM $CaCl_2$, pH 7.0) at 200 rpm and supplemented with rifampin (25 μg/ml). NGR234 QS deletion mutants were cultivated under the same conditions as those for the NGR234 wild-type strain in TY medium which was additionally supplemented with gentamicin (10 μg/ml), and complemented NGR234 QS mutant strains were cultivated in TY medium supplemented with kanamycin (25 μg/ml). *Escherichia coli* was grown at 37°C on LB medium supplemented with the appropriate antibiotics. *Agrobacterium tumefaciens* NTL4 (30), carrying a *traI*::*lacZ* promoter fusion, was grown at 28°C in AT medium (31) containing 0.5% glucose per liter and supplemented with spectinomycin (50 μg/ml) and tetracycline (4.5 μg/ml). For sedimentation assays, the NGR234 parent strain as well as the constructed mutant strains was grown at 30°C and 200 rpm in 5 ml TY medium supplemented with appropriate antibiotics for 48 h. Then, cells were allowed to sediment at room temperature for up to 24 h without shaking.

Construction of NGR234 AI synthase mutants. Molecular cloning steps were in general done as outlined in reference 32, and mutant strains NGR234-Δ*traI* and NGR234-Δ*ngrI* were constructed as previously described (33). For the construction of a deletion mutant in the *traI* gene, an ~2.0-kb PCR fragment containing the 486-bp upstream region of *traI*, a 986-bp gentamicin resistance gene, and a 500-bp downstream fragment flanking the *traI* gene was cloned in the suicide vector pNPTS138-R6KT (34). For this purpose, the different PCR fragments were amplified from genomic DNA of NGR234 using primers as indicated in Table 1. The gentamicin gene was derived from the broad-host-range cloning vector pBBR1MCS-5 (35). The resulting construct (pNPTS138-*traI*::*gm*) was transformed into NGR234 by conjugation. Single recombinant clones carrying this construct were selected on TY medium containing gentamicin and rifampin. To obtain double recombinant mutants, bacteria were

TABLE 2 Overall transcriptome statistics for the 12 analyzed NGR234 samples[a]

Sample no.	Treatment	NGR234 genotype	Growth phase/OD_{600}	3-Oxo-C_8-HSL added	No. of reads generated (10^6)	No. of uniquely mapped reads (10^6)
1	A	wt	Stationary/3.26	None	7.15	3.55
2	A	wt	Stationary/3.37	None	6.79	3.34
3	B	Δ*traI*	Stationary/3.08	None	6.39	3.24
4	B	Δ*traI*	Stationary/3.10	None	8.07	4.06
5	C	Δ*ngrI*	Stationary/3.30	None	6.79	3.62
6	C	Δ*ngrI*	Stationary/3.24	None	7.82	4.37
7	D	wt	Exponential/0.22	None	4.35	1.51
8	D	wt	Exponential/0.22	None	8.74	2.83
9	E	wt	Exponential/0.26	0.05 μM	8.04	1.58
10	E	wt	Exponential/0.27	0.05 μM	6.68	1.41
11	F	wt	Exponential/0.22	50 μM	3.86	1.77
12	F	wt	Exponential/0.21	50 μM	9.16	2.73

[a] "Stationary" indicates cultures grown to early stationary phase; "Exponential" indicates cultures grown to early exponential phase (see Fig. S1 in the supplemental material). Cultures 1 to 6 were harvested after 34 to 40 h of growth. Cultures 7 to 12 were harvested after 3 h upon addition of 3-oxo-C_8-HSL. Controls were supplemented with an equal amount of ethyl acetate. Two cultures represent one treatment (experiment). wt, wild type, NGR234 parent strain; Δ*traI*, NGR234-Δ*traI* mutant strain; Δ*ngrI*, NGR234-Δ*ngrI* mutant strain.

streaked on the same medium (lacking the antibiotics) in the presence of 10% sucrose. In our deletion mutant, the entire *traI* gene was deleted starting from the ATG to the last codon of the gene. The *ngrI* mutant strain was generated as described above using an ~2.1-kb EcoRI-PstI fragment containing the flanking regions of *ngrI* and the same gentamicin resistance gene. Thereby, the majority of the *ngrI* gene (645 bp) ranging from bp 116 to bp 534 was deleted. The obtained mutations were verified by PCR using different primer pairs flanking the *traI* and *ngrI* genes (Table 1) and by sequencing.

For complementation of the above-constructed QS mutants, the wild-type *traI* and *ngrI* genes, including promoter regions, were amplified using individual flanking primer pairs (Table 1), inserted into pBBR1MCS-2 (35), and reintroduced by conjugation into the respective mutant strain NGR234-Δ*traI* or NGR234-Δ*ngrI*. Recombinant NGR234 QS mutant clones carrying the construct were selected on TY medium containing kanamycin and rifampin and designated NGR234-cΔ*traI* and NGR234-cΔ*ngrI*, respectively. The correctness of the complemented mutant strains was verified by PCR.

Furthermore, to study the importance of the AIs for rhizosphere colonization of NGR234, a double QS mutant designated NGR234-Δ*ngrI*/Δ*traI* having a deletion in both the *traI* and *ngrI* loci was generated. The construction of the double mutant was based on the NGR234-Δ*ngrI* deletion mutant in which the *traI* locus was deleted as described above. The double QS mutant was verified by PCR and sequencing.

TLC coupled with *A. tumefaciens* soft agar overlay assay. Separation of AI molecules produced by the NGR234 parent and the above-constructed mutant strains was carried out using thin-layer chromatography (TLC) and by using the *A. tumefaciens* reporter strain NTL4 (30). Therefore, AIs were extracted from stationary-phase cultures with an equal volume of ethyl acetate. The extraction was carried out twice. Pooled extracts were concentrated *in vacuo* and resuspended in 1 ml ethyl acetate. Sample volumes of 1 to 10 μl were applied to cellulose TLC plates (Polygram Cel MN300 AC-30; Macherey-Nagel, Düren, Germany), developed with methanol-water (60:40, vol/vol), and air dried. Detection of AIs was carried out according to the method of Zhu et al. (36) by overlaying the TLC plates with *A. tumefaciens* soft agar containing *A. tumefaciens* NTL4. A 10^{-8} M solution of 3-oxo-C_8-HSL and a 10^{-6} M solution of *N*-(3-oxododecanoyl)-L-homoserine lactone (Sigma-Aldrich, Heidelberg, Germany) were prepared in ethyl acetate and used as standards. Overlaid TLC plates were incubated overnight at 30°C.

Preparation of transcriptome samples. The different NGR234 cultures used in this study for a transcriptome analysis are summarized in Table 2. Prior to cultivation of large 200-ml cultures, precultures were established from cryocultures in 5 ml TY medium and cultivated at 30°C and 200 rpm. For the transcriptome analyses of early-stationary-growth-phase cultures, 200 ml TY medium was inoculated with freshly grown precultures of NGR234 wild type as well as the mutant strains and cultivated for approximately 34 to 40 h at 30°C and 200 rpm as batch cultures (Table 2, samples 1 to 6, treatments A to C). After reaching an optical density at 600 nm (OD_{600}) of 3.1 to 3.3, cultures were separated into fractions of 50 ml, which were then transferred into Falcon tubes containing 10 ml ethanol-phenol (95:5). Samples were mixed well, directly frozen in liquid nitrogen, and stored at −70°C until further use. For transcriptome analyses of early-exponential-growth-phase cultures, 200 ml TY medium was inoculated with a freshly grown preculture of NGR234 wild type and cultivated at 30°C and 200 rpm till it reached an OD_{600} of 0.1 (Table 2, samples 7 to 12, treatments D to F). Cultures were then divided into two separate fractions of 100 ml of which one was supplemented with either 0.05 μM or 50 μM 3-oxo-C_8-HSL (stock solution of 3-oxo-C_8-HSL was prepared in ethyl acetate) and the other culture fraction (control) was treated with an equal aliquot of ethyl acetate. Cultures were allowed to grow for 3 additional hours and then harvested into Falcon tubes and treated as outlined above.

RNA extraction, library construction, sequencing, and bioinformatic analysis of transcriptome samples. For all NGR234 wild-type and mutant strains, RNA-seq libraries were constructed from independent biological duplicates of RNA samples. Six samples were harvested at the early exponential phase, and six samples were obtained from stationary-growth-phase cultures (Table 2; see also Fig. S1 in the supplemental material). Total RNA was extracted using the hot-phenol method described previously (37). The residual genomic DNA was removed from the total isolated RNA by DNase I treatment. The cDNA libraries for sequencing were constructed by Vertis Biotechnology AG, Germany, as described by Sharma et al. (24). The transcripts were not fragmented in order to get mainly sequencing reads of the 5′ end of the transcripts. The obtained cDNA libraries were sequenced using a HiSeq 2500 machine (Illumina) in single-read mode and running 100 cycles. For the 12 analyzed samples, we sequenced between 3.86 and 9.16 million cDNA reads. The bioinformatic analysis was done as described in the work of Dugar et al. (38). To ensure a high sequence quality, the Illumina reads in FASTQ format were trimmed with a cutoff phred score of 20 by the program fastq_quality_trimmer from FASTX-Toolkit version 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/). The alignment of reads, coverage calculation, gene-wise read quantification, and differential gene expression were performed with READemption (K. U. Förstner, J. Vogel, and C. M. Sharma, posted on bioRxiv under doi:10.1101/003723) which was relying on 'segemehl' version X (39) and DESeq version V. Visual inspection of the coverages was done using the Integrated Genome Browser (IGB) (40). The reference

TABLE 3 Gene-specific primers used for qRT-PCR

Oligonucleotide	Sequence, 5′–3′	Size (bp)	Target gene/ORF
RT_rpoD_for	ACATCACCAATGTCGGCGGTGAAG	162	*rpoD*
RT_rpoD_rev	TGCAGCTTGCGGAGCTTCTTGTAG		
RT_flgB_for	AGAATGTCGTGGCCGGCAACATC	238	*flgB*
RT_flgB_rev	GCCCGTCTTCATCATTTCCTGCTC		
RT_pilA_for	ACCATTTTCGCCCGCCTGATGAAG	172	*pilA*
RT_pilA_rev	ATGTTGGTTTCGGCGGTCGTCATCT		
RT_b22870_for	CCTCTGACGCTCGATGTCCTGAA	110	NGR_b22870
RT_b22870_rev	GTCGCTGAGCATCAGGTCGCAAT		
RT_exoI_for	CGATGGCGACACCATCGAAATTGC	257	*exoI*
RT_exoI_rev	GATTCAACCATCCAGCGGTTGACG		
RT_c14830_for	CGGGTTGCTGCATATGTATCGACCT	220	NGR_c14830
RT_c14830_rev	GGCCTGATACTCTTCGATCAGCATC		

sequences and gene annotations for NGR234 were retrieved from the NCBI database (accession numbers: pNGR234*a*, NC_000914.2; pNGR234*b*, NC_012586.1; cNGR234, NC_012587.1) and in part manually reannotated. Genes with a fold change of ≥2.0 and an adjusted *P* value (*P* value was corrected by false discovery rate [FDR] based on the Benjamini-Hochberg procedure) of ≤0.05 were considered differentially expressed.

qRT-PCR. Quantitative RT-PCR (qRT-PCR) experiments were carried out to verify selected QS-regulated genes. Total RNA was extracted from stationary-phase-grown cultures according to the method of Rivas et al. (41), and the residual genomic DNA was removed by DNase I treatment according to the manufacturer's instructions (DNase I, RNase-free; Thermo Scientific, WI, USA). The SuperScript VILO cDNA synthesis kit (Invitrogen, Life Technologies, TX, USA) was used to generate cDNA using 1.9 μg RNA. Gene-specific primers used for qRT-PCR are shown in Table 3. The qRT-PCRs were set up according to the manufacturer's protocol using the SYBR Select master mix for CFX (Applied Biosystems by Life Technologies, TX, USA) and performed in the MiniOpticon real-time PCR detection system (Bio-Rad Laboratories, Munich, Germany). Standard curves of 10-fold serial dilutions of cDNA were generated for each gene to evaluate the primer efficiency and for data analysis. The efficiency, slope, and correlation coefficient were determined by the CFX Manager software (Bio-Rad Laboratories, Munich, Germany). All qRT-PCRs were run in triplicate and repeated at least three times in separate experiments under the same conditions. To normalize variability in expression levels, *rpoD* was used as the internal control gene. Data were analyzed based on the normalized gene expression (threshold cycle [$2^{-\Delta\Delta CT}$] method) and the above-stated software.

Nodulation assays. Nodulation assays accomplished with NGR234-Δ*traI*, NGR234-Δ*ngrI*, and NGR234-Δ*ngrI*/Δ*traI* and with *Vigna unguiculata*, *Vigna radiata*, and *Tephrosia vogelii* were done as previously described (42). Experiments were repeated two times with five plants per treatment in each experiment. Plants were harvested 30 days after inoculation. Nodules formed on the roots were counted, and the shoot fresh weight was recorded. Plants grown in the absence of rhizobia were used as a control.

Microarray data accession number. The raw, demultiplexed reads as well as coverage files have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (43) under the accession number GSE54381.

RESULTS AND DISCUSSION

Construction and phenotype analysis of *traI* and *ngrI* deletion mutants. To investigate the QS-mediated gene regulation in the broad-host-range strain *Sinorhizobium fredii* NGR234, we followed several strategies. First, we constructed an NGR234-Δ*traI*, an NGR234-Δ*ngrI*, and an NGR234-Δ*ngrI*/Δ*traI* deletion mutant and analyzed their AI profile using TLC separation with subsequent AI detection by *A. tumefaciens* NTL4 (36). While we repeatedly detected two spots in extracts of the NGR234 parent strain, we detected only a single spot in the NGR234-Δ*traI* and the NGR234-Δ*ngrI* mutant strains. No AI was detected in extracts obtained from the double mutant. However, complemented strains NGR234-cΔ*traI* and NGR234-cΔ*ngrI* produced again high levels of the respective AI molecules (see Fig. S2 in the supplemental material). The observation of two AI molecules in the parent strain and one AI in each of the single-knockout mutant strains corresponds well with previous results (13, 14).

Since it is well known that the lack of AI molecules can have profound effects on motility and exopolysaccharide (EPS) production, we analyzed our mutants for possible phenotypes. Interestingly, we did not observe reproducible motility phenotypes on solid media and using swimming and swarming agar (data not shown). Also, growing NGR234 mutant cells on medium containing Congo red or calcofluor white did not produce stable phenotypes. However, analyzing motility in liquid TY medium resulted in the observation of strong sedimentation phenotypes for the mutants (Fig. 1). Thus, parent strain cultures settled within 3 to 24 h, while the mutant strains did not sediment. The observed sedimentation phenotype for NGR234-Δ*ngrI* was completely restored using the NGR234-cΔ*ngrI* strain. The phenotype produced by NGR234-Δ*traI* could be only partially recovered by adding the parental gene back into NGR234-Δ*traI* (Fig. 1). Further tests and chemical complementation using a micromolar concentration of 3-oxo-C_8-HSL produced similar results (data not shown). Therefore, it is likely that the sedimentation phenotype was mainly a

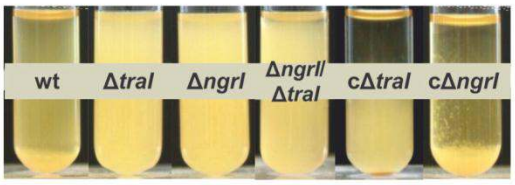


FIG 1 Sedimentation of NGR234 in TY medium. The NGR234 wild type (wt) shows a clear sedimentation phenotype in liquid TY medium after 3 to 24 h of incubation at room temperature without shaking, whereas Δ*traI* (NGR234-Δ*traI*), Δ*ngrI* (NGR234-Δ*ngrI*), and Δ*ngrI*/Δ*traI* (NGR234-Δ*ngrI*/Δ*traI*) strains did not settle even after 24 h of incubation under the same conditions. The NGR234-cΔ*ngrI* (cΔ*ngrI*) strain carries extra copies of the *ngrI* wild-type gene in the background of NGR234-Δ*ngrI* and was completely recovered; NGR234-cΔ*traI* (cΔ*traI*) was partially recovered by adding extra copies of the wild-type gene into the NGR234-Δ*traI* mutant strain.

TABLE 4 Number of genes significantly altered in their expression profile in the NGR234 QS deletion mutants (early stationary phase) and in NGR234 after addition of 3-oxo-C_8-HSL at two different concentrations (early exponential phase)[a]

NGR234 genotype[c]	Growth phase/treatment	Total no. of differentially expressed genes[b]	No. of differentially expressed genes on replicons (cNGR234/pNGR234*b*/pNGR234*a*)
Δ*traI*	Stationary	316	221/82/13
Δ*ngrI*	Stationary	466	303/155/8
wt	Exponential; 0.05 μM 3-oxo-C_8-HSL added	13	8/3/2
wt	Exponential; 50 μM 3-oxo-C_8-HSL added	4	0/4/0

[a] Data are mean values of two independent treatments.
[b] Total number of significantly, differentially expressed genes having a fold change of ≥2.0 and an adjusted *P* value of ≤0.05.
[c] wt, wild type, NGR234 parent strain; Δ*traI*, NGR234-Δ*traI* mutant strain; Δ*ngrI*, NGR234-Δ*ngrI* mutant strain.

result of a strong upregulation of the flagellar gene expression and thus a motility phenotype. Altogether, these findings confirmed the importance of the *traI* and *ngrI* genes for biofilm formation, sedimentation, and motility, and they suggested that the observed phenotypes are mainly caused by the deletion of the respective AI synthase gene.

Global pattern of QS-dependent gene expression in NGR234. In a parallel approach, we examined the global gene expression pattern of the wild type and its two AI synthase mutants (NGR234-Δ*traI* and NGR234-Δ*ngrI*) by comparing their transcriptome profile at the early stationary growth phase to those of the NGR234 wild type and each other. We chose this time point because it can be expected that during the onset of stationary growth phase many QS-dependent processes are turned on. For these experiments, cells were grown for approximately 40 h to a final OD_{600} not greater than 3.4 prior to total RNA extraction. The NGR234 parent strain was cultivated like the control for the same time period to an identical OD_{600} and then treated equally (Table 2).

Further, we analyzed the short-term response of the parent strain to externally added 3-oxo-C_8-HSL in the early exponential growth phase. For this, cells were grown to an OD_{600} of 0.1 and then challenged by the presence of a moderate (0.05 μM) and a high (50 μM) concentration of 3-oxo-C_8-HSL. Total RNA was already extracted after 3 h from the AHL-induced samples and the control cultures. The OD_{600} of these exponentially growing cultures at the time of harvest was 0.24 (±0.03) (Table 2).

For all samples, the cDNA libraries were constructed and sequenced as described in Materials and Methods. For each of the six treatments, two independent biological sample experiments were performed and examined by RNA-seq and protocols for data analysis as previously published (38–40). Therefore, a total of 12 individual samples were analyzed (Table 2). Alignments were established, and for each sample, a minimum of 1.4 to 4.4 million cDNA reads could be uniquely mapped to the NGR234 genome, resulting in 3 to 8 million uniquely mapped reads per treatment (Table 2). We verified that for the early-exponential-phase samples the fraction of mappable reads (on average, ~38%) was 1.6-fold lower than that for the samples obtained in early stationary phase (up to 63%). Other researchers have already observed this variation in mappable reads depending on the selected culture conditions (44).

In the comparative analysis of RNA-seq data, we considered genes with a fold change of ≥2.0 and an adjusted *P* value of ≤0.05 as statistically significant and differentially expressed between two distinct conditions or states. Only values that complied with both requirements were used for subsequent analyses. Unless otherwise specified, Tables 4 to 8 show only those transcriptomic data (data are mean values of two independent treatments) that revealed a fold change of ≥2.0 and an adjusted *P* value of ≤0.05. The final set of differentially regulated genes is given in Table S1 in the supplemental material, and the highlights are discussed below.

A detailed evaluation of sense and antisense (AS) transcripts in all experiments revealed an AS transcript content ranging from 13 to 30%. These data suggest that a small but significant fraction of all genes in NGR234 is regulated through AS transcription. Earlier whole-transcriptome analyses have already emphasized the importance of AS and more recently the *cis*-AS regulation in prokaryotes (24, 45, 46).

TraI/R- and NgrI/R-specific gene regulation. It was determined that 316 genes (4.9% of all predicted genes) are regulated differently in the NGR234-Δ*traI* deletion mutant and the wild-type strain. Moreover, a total of 466 genes (7.3% of all predicted genes) were significantly altered in their expression profile in the NGR234-Δ*ngrI* deletion mutant (Fig. 2). Altogether, a common subset of 186 genes was differentially regulated in both mutant strains in comparison to the wild type (Fig. 2; see also Table S1 in the supplemental material). Surprisingly, almost 98% of these overlapping genes were identically up- or downregulated in the two strains. Thus, 130 genes appeared to be specifically expressed in the NGR234-Δ*traI* strain and 280 genes were uniquely regulated in the NGR234-Δ*ngrI* deletion mutant. All regulated genes were unequally distributed over the three NGR234 replicons (Table 4). The majority of all differentially regulated genes (65 to 70%) was observed on the bacterial chromosome (cNGR234), and only a very few genes were located on the symbiotic plasmid pNGR234*a* (Table 4). In the background of the NGR234-Δ*traI* strain, 13 pNGR234*a*-borne genes were significantly altered in their expression profile, and for the NGR234-Δ*ngrI* mutant, only eight differentially regulated genes were observed on this symbiotic replicon.

Expression analysis by qRT-PCR technology was used to partially confirm the RNA-seq data. Therefore, we analyzed the expression profiles of five different genes in the early stationary phase in the NGR234 wild type compared to both QS deletion mutants. We selected *rpoD* as the internal control gene and the following differentially regulated genes: *flgB* (NGR_c02740) (Table 5), *pilA* (NGR_c34640) (Table 5), NGR_b22870 (hypothetical protein possibly linked to T3SS-II) (Table 8), *exoI* (NGR_b18300) (Table 6), and NGR_c14830 (hypothetical protein; not listed in Tables 5 to 8). Expression data obtained for the five genes by qRT-PCR largely confirmed the data obtained by RNA-seq (see Table S2 in the supplemental material).

To date, only a few studies have performed genome-wide tran-

TABLE 5 T4P, flagellar biosynthesis, and chemotaxis-related genes significantly altered in their expression profile in both NGR234 QS deletion mutants[a]

Gene no.	Locus tag	Predicted function	Fold change	
			Δ*traI*	Δ*ngrI*
1	NGR_c34710	Pilus assembly protein, TadB	+3.2	+3.3
2	NGR_c34700	Pilus protein ATPase, CpaF	+4.3	+3.5
3	NGR_c34690	Pilus assembly protein, CpaE	+4.5	+5.6
4	NGR_c34680	Pilus assembly protein, CpaD	+3.6	+3.7
5	NGR_c34670	Pilus assembly protein, CpaC	+3.3	+2.7
6	NGR_c34660	Pilus assembly protein, CpaB	+3.9	+4.1
7	NGR_c34640	Pilus assembly protein, PilA1	+11.0	+7.3
8	NGR_c34620	Pilus assembly protein, TadG	+5.0	+2.9
9	NGR_c34610	Pilus assembly protein, TadE	+4.6	+2.7
10	NGR_c03010	Flagellar biosynthesis protein, FlhA	+2.2	—
11	NGR_c02990	Flagellar basal body rod modification protein, FlgD	+10.9	+11.8
12	NGR_c02980	Flagellar biosynthesis repressor, FlbT	+4.5	+3.9
13	NGR_c02970	Flagellar biosynthesis regulatory protein, FlaF	+11.3	+8.0
14	NGR_c02960	Flagellar hook-associated protein, FlgL	+22.9	+22.6
15	NGR_c02950	Flagellar hook-associated protein, FlgK	+15.2	+16.4
16	NGR_c02940	Flagellar hook protein, FlgE	+24.9	+23.3
17	NGR_c02930	Two-component transcriptional regulator	+10.5	+6.7
18	NGR_c02920	Lytic transglycosylase-like protein	+5.5	+5.1
19	NGR_c02910	Chemotaxis/motility protein, MotD	+8.9	+15.2
20	NGR_c02900	Chemotaxis/motility protein, MotC	+10.2	+10.8
21	NGR_c02890	Flagellar motor protein, MotB	+7.0	+8.4
22	NGR_c02880	Hypothetical protein	+8.2	+11.3
23	NGR_c02870	Flagellin protein, FlaD	+3.2	+4.7
24	NGR_c02850	Flagellin protein, FlaB	+2.4	—
25	NGR_c02810	Flagellar basal body L-ring protein, FlgH	+9.9	+10.9
26	NGR_c02800	Hypothetical protein	+24.9	+26.0
27	NGR_c02790	Flagellar basal body P-ring protein, FlgI	+25.3	+24.4
28	NGR_c02780	Flagellar P-ring biosynthesis protein, FlgA	+11.6	+12.3
29	NGR_c02770	Flagellar basal body rod protein, FlgG	+20.4	+20.3
30	NGR_c02760	Flagellar hook-basal body protein, FliE	+36.5	+27.2
31	NGR_c02750	Flagellar basal body rod protein, FlgC	+29.0	+25.9
32	NGR_c02740	Flagellar basal body rod protein, FlgB	+69.9	+82.2
33	NGR_c02720	Flagellum-specific ATP synthase, FilL	+12.2	+20.6
34	NGR_c02710	Flagellar basal body rod protein, FlgF	+14.0	(+11.6)
35	NGR_c02700	Hypothetical protein	+7.4	+8.1
36	NGR_c02690	Flagellar motor protein, MotA	+9.8	+8.8
37	NGR_c02680	Flagellar motor switch protein, FliM	+11.8	+11.6
38	NGR_c02670	Flagellar motor switch protein, FliN	+25.0	+28.6
39	NGR_c02660	Flagellar motor switch protein, FliG	+15.9	+14.0
40	NGR_c02630	LuxR family transcriptional regulator	+6.3	+5.5
41	NGR_c02620	LuxR family transcriptional regulator	+10.6	+6.5
42	NGR_c02610	Flagellar MS ring protein, FliF	+4.8	+4.9
43	NGR_c02600	Hypothetical protein	(+2.8)	+4.3
44	NGR_c02590	Chemoreceptor glutamine deamidase, CheD	+4.6	+6.7
45	NGR_c02580	Signal transduction response regulator	(+2.6)	+4.3
46	NGR_c02570	Chemotaxis-specific methylesterase, CheB2	+4.0	+5.6
47	NGR_c02560	Chemotaxis protein methyltransferase, CheR	+4.7	+4.7
48	NGR_c02550	CheW family chemotaxis protein, CheW1	+2.8	+3.2
49	NGR_c02540	Chemotaxis protein, CheA2	+4.8	+6.6
50	NGR_c02530	Two-component response regulator receiver protein	+5.5	+5.3
51	NGR_c02520	Hypothetical protein	+6.9	+5.6
52	NGR_c02510	Chemotaxis methyl-accepting receptor protein	+4.2	+4.3
53	NGR_c00850	Methyl-accepting chemotaxis protein	+3.0	+4.2
54	NGR_c00530	Methyl-accepting chemotaxis protein	+3.9	+3.4
55	NGR_c20850	Methyl-accepting chemotaxis protein	+4.1	+4.2
56	NGR_b22620	Methyl-accepting chemotaxis protein	—	+3.2

[a] Genes 1 to 9 code for the T4P, while genes 10 to 52 code for the flagellum or regulatory components and chemotaxis-associated genes identified within a large conserved cluster on cNGR234. Genes 53 to 56 are located elsewhere in the bacterial genome. Δ*traI*, NGR234-Δ*traI* mutant strain; Δ*ngrI*, NGR234-Δ*ngrI* mutant strain; —, transcriptome data did not match either of the two requirements (fold change of ≥2.0; adjusted *P* value of ≤0.05). Values in parentheses were specified for completeness and indicate an adjusted *P* value of 0.06 for NGR_c02710, 0.18 for NGR_c02600, and 0.09 for NGR_c02580.

TABLE 6 Exopolysaccharide biosynthesis genes differentially regulated in both NGR234 QS deletion mutants

Locus tag	Predicted function	Fold change[b]	
		Δ*traI*	Δ*ngrI*
NGR_b18410	Phosphomethylpyrimidine kinase, ThiD	−2.8	+2.9
NGR_b18400	Succinoglycan biosynthesis transporter, ExoP	−2.2	−3.6
NGR_b18390	UTP-glucose-1-phosphate uridylyltransferase	−2.8	−4.7
NGR_b18380	Succinoglycan biosynthesis protein, ExoO	−2.4	−4.6
NGR_b18370	Succinoglycan biosynthesis protein, ExoM	−2.6	−6.6
NGR_b18360	Succinoglycan biosynthesis protein, ExoA	−2.8	−5.5
NGR_b18350	Succinoglycan biosynthesis protein, ExoL	−2.6	−6.3
NGR_b18340	Endo-beta-glycanase, ExoK	−3.3	−5.7
NGR_b18330	Hypothetical protein	−4.1	−12.4
NGR_b18320[a]	*rpoS* regulatory ncRNA (68 nt[c]), antisense	+36.0	+52.3
NGR_b18320[a]	*rpoS* regulatory ncRNA (68 nt), sense	−2.4	−2.5
NGR_b18310	Hypothetical protein	−13.9	−15.9
NGR_b18300	Succinoglycan biosynthesis protein, ExoI	−29.5	−82.5
NGR_b18270	Exopolysaccharide production protein, ExoY	—	−3.1
NGR_b18260	Exopolysaccharide production protein, ExoF	—	−3.2
NGR_b18250	Succinoglycan biosynthesis protein, ExoQ	—	−3.4
NGR_b18240	Exopolysaccharide production protein, ExoZ	—	−2.5
NGR_b18220	ABC transporter ATP-binding protein, ExsA	−2.0	−2.1
NGR_b18180	Succinoglycan biosynthesis regulator, ExsB	—	−3.0
NGR_b18140	ExsI protein	—	−3.8
NGR_b15690	Endo-beta-glycanase, ExsH	−13.6	−20.1
NGR_a00830	Exopolysaccharide repressor protein	−3.1	−5.0

[a] Data for NGR_b18320 showed it to be significantly differentially regulated at the sense and antisense transcription level.
[b] Δ*traI*, NGR234-Δ*traI* mutant strain; Δ*ngrI*, NGR234-Δ*ngrI* mutant strain; —, transcriptome data did not match either of the two requirements (fold change of ≥2.0; adjusted *P* value of ≤0.05).
[c] nt, nucleotides.

scriptome analyses to identify QS-regulated gene expression patterns in response to exogenous AHLs in bacteria. One of the best-studied model organisms in this research field with respect to QS is the opportunistic pathogenic bacterium *P. aeruginosa*. Several studies have been published analyzing the *P. aeruginosa* QS-dependent transcriptome using microarray technologies and RNA sequencing (27, 44, 47–50). Since the overall setups of these studies were slightly different, the number of genes that were differentially regulated in response to QS processes varied. While in the study by Wagner et al. (47) 11.1% of all genes appeared to be QS dependent, in the study by Schuster et al. (48) 6.3% and in the study by Hentzer and colleagues (49) only 2.9% of all genes were identified that appeared to be differentially regulated through AHLs. Only very recently, Chugani et al. reported on strain-dependent diversity of *Pseudomonas* QS-dependent gene expression. They observed that the QS regulons represent ~0.5 to 6.2% of the coding sequences for a given *P. aeruginosa* genome (27). Similarly, for the Gram-negative pathogenic bacterium *Yersinia pestis*, a total of 335 genes were reported to be QS dependently regulated (51). This equals 8% of the *Y. pestis* genome, which is 4.83 Mbp in size and contains 4,221 open reading frames (ORFs) (52). With respect to these studies, a total of 4.9% differentially regulated genes in the NGR234-Δ*traI* background and a total of 7.3% in NGR234-Δ*ngrI* seem to be reasonable. The overall number of QS-regulated genes in NGR234 appears to be slightly higher than those numbers published for the closely related *S. meliloti*. In this organism, a minimum of 55 to 170 genes were linked to a QS-dependent regulatory circuit (16, 19, 20).

TABLE 7 Upregulated cofactor biosynthesis genes in NGR234-Δ*ngrI*

Locus tag	Predicted function	Fold change
NGR_c25140	Biotin synthase, BioB	+3.0
NGR_c25130	8-Amino-7-oxononanoate synthase, BioF	+3.5
NGR_c25120	Dethiobiotin synthetase, BioD	+3.3
NGR_c25110	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase, BioA	+2.8
NGR_c25100	Biotin synthesis protein, BioZ	+4.7
NGR_c13770	Biotin transport regulator, BioS	+6.3
NGR_b03300	Coenzyme PQQ synthesis protein, PqqE	+7.8
NGR_b03290	Coenzyme PQQ synthesis protein, PqqD	+9.7
NGR_b03280	Coenzyme PQQ synthesis protein, PqqC	+10.0
NGR_b03270	Coenzyme PQQ synthesis protein, PqqB	+10.7
NGR_b03260	Coenzyme PQQ synthesis protein, PqqA	+9.9
NGR_b03250	Alcohol dehydrogenase	+9.5
NGR_b03240	LuxR family transcriptional regulator	+3.0

Function-based interpretation of transcriptome data. The differentially expressed genes in both mutants and the parent strain were classified into seven functional categories based on the KEGG database (http://www.genome.jp/kegg/pathway.html). As indicated in Fig. 3A and B, the genome-wide transcriptome data analysis revealed that upregulated genes were mainly linked to motility, regulators, general metabolism, cell wall and succinoglycan biosynthesis (cell envelope), transporters (mostly ABC), and quite a significant number of hypothetical proteins. Striking changes in gene expression of the most important functional categories are discussed below.

Two hypothetical proteins show the highest expression level in both mutants. Within the set of differentially regulated genes detected in both mutants, a relatively high number of 94 genes in

TABLE 8 T3SS-II-related genes and their fold changes in NGR234-Δ*traI* and NGR234-Δ*ngrI*

Locus tag	Predicted function	Fold change[b]	
		Δ*traI*	Δ*ngrI*
NGR_b23040[a]	Hypothetical protein	—	−3.8
NGR_b23030[a]	Hypothetical protein	—	−2.8
NGR_b23010	Hypothetical protein possibly linked to T3SS-II	—	—
NGR_b23000	Type III secretion component, RhcT	—	−3.3
NGR_b22990	Type III secretion component, RhcS	—	−4.9
NGR_b22980	Translocation protein, RhcR	—	−5.5
NGR_b22970	Translocation protein, RhcQ	—	−5.1
NGR_b22960	Type III secretion component, RhcZ	—	−6.7
NGR_b22950	Type III secretion component, RhcN	—	−5.2
NGR_b22940	Type III secretion component, YOP protein/translocation protein L	—	−4.2
NGR_b22930	Putative type III secretion component	—	−4.6
NGR_b22920	Type III secretion component, RhcJ	−2.1	−4.0
NGR_b22910	Hypothetical protein possibly linked to T3SS-II	—	−4.8
NGR_b22900	Hypothetical protein possibly linked to T3SS-II	—	−4.6
NGR_b22890	Type III secretion component, RhcC1	—	−3.9
NGR_b22880	Hypothetical protein possibly linked to T3SS-II	−2.9	−3.9
NGR_b22870	Hypothetical protein possibly linked to T3SS-II	−3.7	−4.1
NGR_b22860	Hypothetical protein possibly linked to T3SS-II	−3.3	−4.4
NGR_b22850	Hypothetical protein possibly linked to T3SS-II	−3.3	−5.6
NGR_b22840	Type III secretion component, RhcV	−2.6	−4.5
NGR_b22830	Type III secretion component, RhcC2	−2.7	−5.4
NGR_b22820	Hypothetical protein possibly linked to T3SS-II	−2.3	−3.2
NGR_b22810	Hypothetical protein possibly linked to T3SS-II	−2.6	(−3.3)
NGR_b22800	Translocation protein, RhcU	−2.3	−4.3
NGR_b22790[a]	Protein precursor, ErfK/SrfK	—	−3.7

[a] Genes were not directly linked to the T3SS-II cluster but were located upstream/downstream and were obviously simultaneously regulated.
[b] Δ*traI*, NGR234-Δ*traI* mutant strain; Δ*ngrI*, NGR234-Δ*ngrI* mutant strain; —, transcriptome data did not match either of the two requirements (fold change, ≥2.0; adjusted *P* value, ≤0.05). Value in parentheses, adjusted *P* value of 0.08.

NGR234-Δ*ngrI* and 62 genes in the NGR234-Δ*traI* background were classified as hypothetical proteins. It is noteworthy that among these, the two most strongly regulated genes were identified. These were the ORFs NGR_c14820 and NGR_c14830. Both are located on cNGR234 flanking each other but are transcribed in opposite directions. In the NGR234-Δ*traI* mutant strain, NGR_c14820 was 32-fold downregulated by antisense transcripts and NGR_c14830 was 41-fold downregulated by sense transcripts. Further, an even stronger regulation was observed for both genes in the NGR234-Δ*ngrI* background, where NGR_c14820 was 46-fold (antisense) and NGR_c14830 was up to 132-fold (sense) downregulated. Interestingly, no clear function could be assigned to these genes.

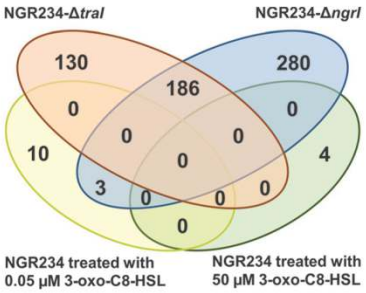


FIG 2 Venn diagram showing the overlap of differentially expressed genes (fold change, ≥2.0; adjusted *P* value, ≤0.05) among NGR234 AI synthase mutants and AHL treatments. The ellipses display the number of uniquely regulated genes in each NGR234 mutant versus the parental strain and for each AHL treatment versus the parental strain. The ellipses also show the number of commonly regulated genes within particular relationships. Only the NGR234 AI synthase mutants share a core set of 186 differentially regulated genes.

Genes linked to general cell and energy metabolism and transport. Previous studies have reported that QS can have profound effects on growth, nodulation efficiency, and nodule development in various rhizobial species (14–16, 19, 20, 53–55). Within this framework, we found that several genes which are related to either energy production, sugar uptake, or respiration were significantly altered in their gene expression levels in either NGR234-Δ*traI*, NGR234-Δ*ngrI*, or both mutants (Fig. 3A and B). Among genes that potentially influence growth were several that encode ATP synthase subunits (NGR_c04460 to -c04500 and NGR_c31110 to -c31140), which were 2- to 4.6-fold downregulated in the background of NGR234-Δ*ngrI*.

In addition, NGR234 contains four loci encoding cytochrome *c* and Cbb3-type cytochrome *c* oxidases, all located on cNGR234. While one of these clusters (NGR_c25510 to -c25550) appeared to be not differentially expressed, almost all genes located in the cluster stretching from NGR_c05230 to NGR_c05300 showed a 2- to 4-fold-reduced expression level in the NGR234-Δ*traI* mutant. In the background of NGR234-Δ*ngrI*, only two out of the six cytochrome *c* oxidase genes were significantly downregulated. The cluster ranging from NGR_c25780 to NGR_c25810 was only partially downregulated in the NGR234-Δ*traI* mutant. A detailed analysis of regulated genes within our transcriptomic data revealed that the Cbb3-type cytochrome *c* oxidase cluster stretching from NGR_c17970 to NGR_c17990 was regulated (fold change,

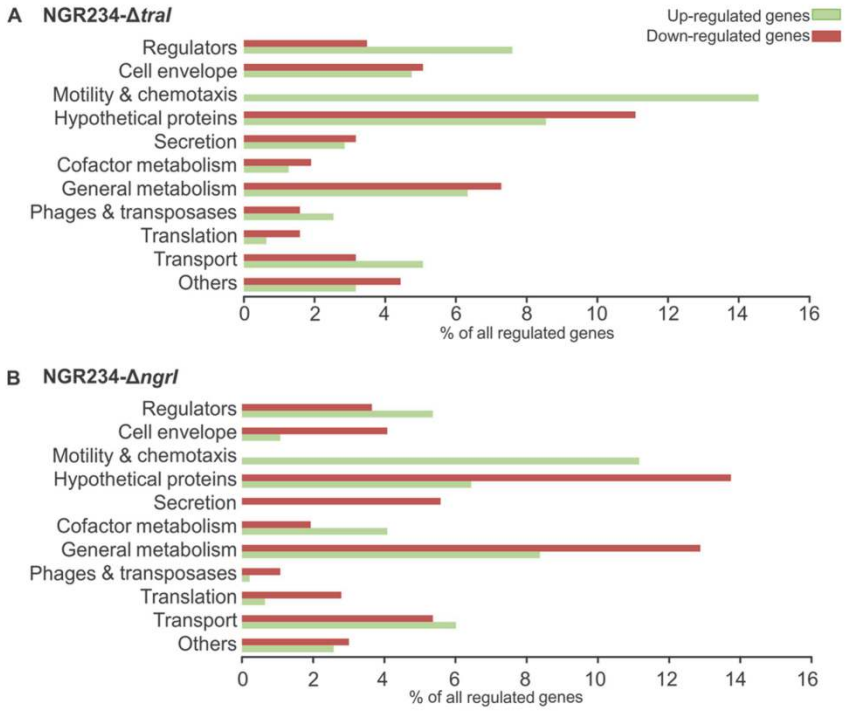


FIG 3 Differentially expressed genes (%) in NGR234 mutant strains. (A) In the background of NGR234-Δ*traI* versus the parent strain. (B) In the background of NGR234-Δ*ngrI* versus the parent strain. The classification was based on the KEGG database (http://www.genome.jp/kegg/pathway.html).

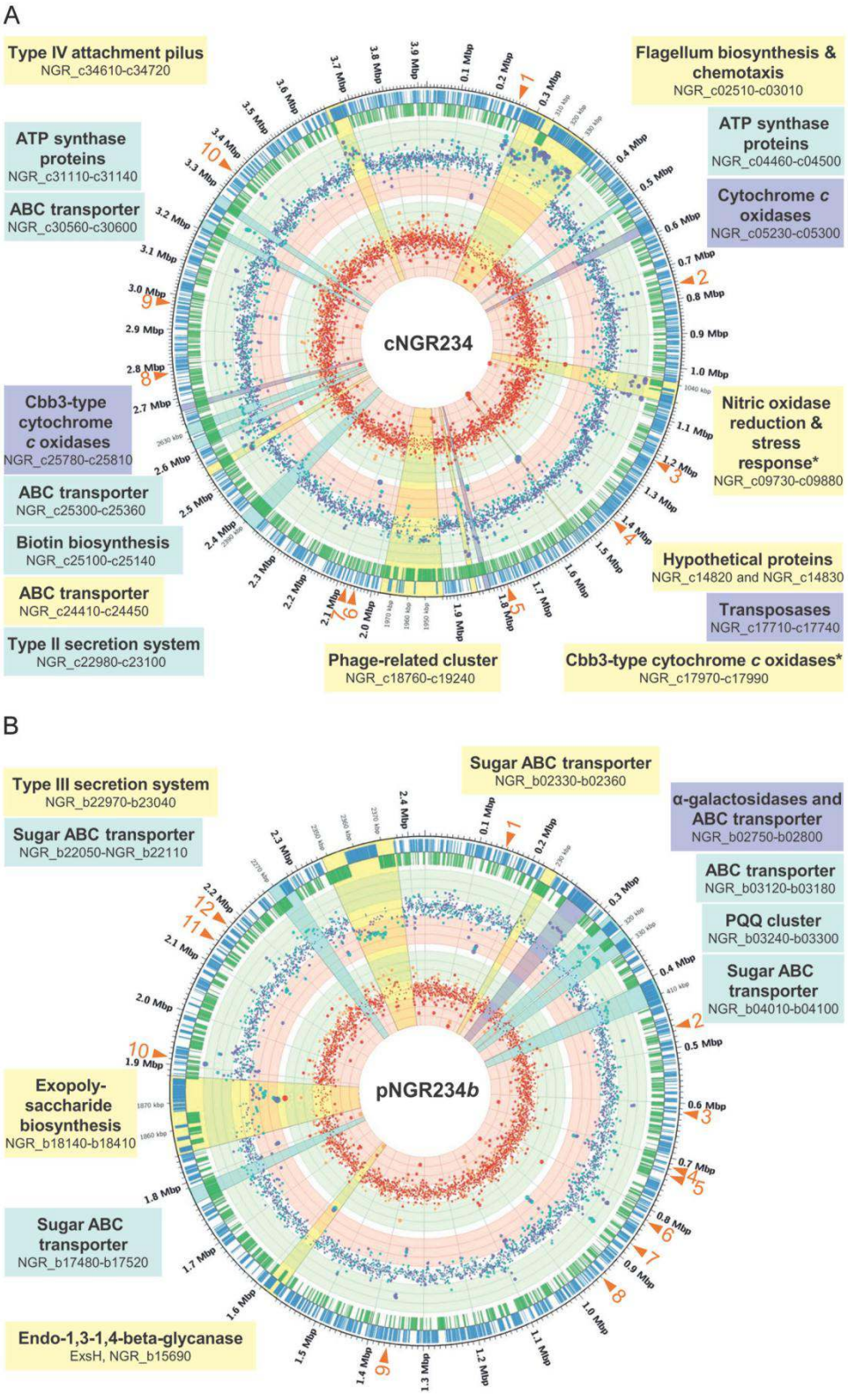
≥2.0; *P* value of ≤0.3; data not shown) in NGR234-Δ*traI* and NGR234-Δ*ngrI*. Although this cluster did not meet our requirements for significantly, differentially regulated genes, data in Fig. 4A clearly indicate a regulation of the Cbb3-type cytochrome *c* oxidase genes. Interestingly, genes within this cluster were upregulated in NGR234-Δ*traI* and downregulated in NGR234-Δ*ngrI*.

In this context, we also found genes linked to nitric oxidase reduction and stress response, which were differentially expressed in the mutant strains (fold change, ≥2.0; *P* value of ≤0.5; data not shown) (Fig. 4A). Similar to the above-mentioned regulation of the *cbb3*-type cluster, the nitric oxidase genes were upregulated in NGR234-Δ*traI* and downregulated in NGR234-Δ*ngrI*.

Further, several of the ribosomal subunits (*rplJ*, *rpmH*, *rpmG*, *rpmB*, *rpmJ*, and *rpsT*) were significantly reduced in their expression in both mutants. While *rplJ* was downregulated in NGR234-Δ*traI*, the ribosomal subunits *rpmH*, *rpmG*, *rpmB*, *rpmJ*, and *rpsT* were all downregulated in the NGR234-Δ*ngrI* mutant. Furthermore, several tRNA synthase and tRNA genes were downregulated in both mutants, and in addition, a few ABC-type transporters for different sugars were also subject to a QS-dependent regulation. Finally, more than 30 regulators and two regulatory noncoding RNAs (ncRNAs) (NGR_b18320 and NGR_b21180) out of five were subject to a QS-dependent regulation in the two mutant strains (Fig. 3A and B; see also Table S1 in the supplemental material).

Motility and chemotaxis genes are upregulated in NGR234-Δ*traI* and NGR234-Δ*ngrI*. Among the genes that were most strongly upregulated in both mutant strains, over 50 genes were involved in flagellum biosynthesis, pilus attachment, and chemotaxis (Table 5; Fig. 4A; see also Fig. S3A and B in the supplemental material). The NGR234 chromosome encodes a single flagellum, which is involved in bacterial motility and chemotaxis. The flagellar genes are mainly organized in a single large cluster encompassing more than 40 genes and spanning from NGR_c02610 to NGR_c03010 (Table 5). Most of the flagellum-specific genes were more than 10-fold and some even up to 80-fold upregulated in their transcription in NGR234-Δ*traI* and NGR234-Δ*ngrI* compared to the parent strain (Table 5). Therefore, *flgB* expression was verified by qRT-PCR (see Table S2). Moreover, these findings are consistent with the above-observed motility phenotypes in sedimentation assays where both mutants were not able to settle within the given time period (Fig. 1). Our data are in agreement with earlier findings reported for planktonic *Bradyrhizobium japonicum* cells and for *S. meliloti* suggesting that flagella play a pivotal role during root colonization but that they are most likely not essential during the infection process (56–59). This hypothesis fits well with the recent observation that flagellar genes are downregulated in bacteroids in NGR234 (29) and with the observation that flagellar genes are subject to QS-mediated regulation in *S. meliloti* (60, 61).

Further, the NGR234 genome contains several gene clusters involved in the biosynthesis of type IV attachment pili (T4P). T4P are involved in motility, attachment to surfaces, biofilm formation, twitching motility, and virulence (62), and they are of importance for an initial attachment to the root surface (63). We have previously identified two clusters of T4P-encoding genes on pNGR234*b* along with a third cluster of 12 genes on the chromosome (NGR_c34610 to -c34720) (13). While the two clusters on pNGR234*b* were not affected in their transcription levels, the


A
Type IV attachment pilus
NGR_c34610-c34720
Flagellum biosynthesis & chemotaxis
NGR_c02510-c03010
ATP synthase proteins
NGR_c31110-c31140
ATP synthase proteins
NGR_c04460-c04500
ABC transporter
NGR_c30560-c30600
Cytochrome c oxidases
NGR_c05230-c05300
cNGR234
Cbb3-type cytochrome c oxidases
NGR_c25780-c25810
Nitric oxide reduction & stress response*
NGR_c09730-c09880
ABC transporter
NGR_c25300-c25360
Biotin biosynthesis
NGR_c25100-c25140
Hypothetical proteins
NGR_c14820 and NGR_c14830
ABC transporter
NGR_c24410-c24450
Transposases
NGR_c17710-c17740
Type II secretion system
NGR_c22980-c23100
Phage-related cluster
NGR_c18760-c19240
Cbb3-type cytochrome c oxidases*
NGR_c17970-c17990
B
Type III secretion system
NGR_b22970-b23040
Sugar ABC transporter
NGR_b02330-b02360
α-galactosidases and ABC transporter
NGR_b02750-b02800
Sugar ABC transporter
NGR_b22050-NGR_b22110
ABC transporter
NGR_b03120-b03180
PQQ cluster
NGR_b03240-b03300
Sugar ABC transporter
NGR_b04010-b04100
pNGR234b
Exopoly-saccharide biosynthesis
NGR_b18140-b18410
Sugar ABC transporter
NGR_b17480-b17520
Endo-1,3-1,4-beta-glycanase
ExsH, NGR_b15690

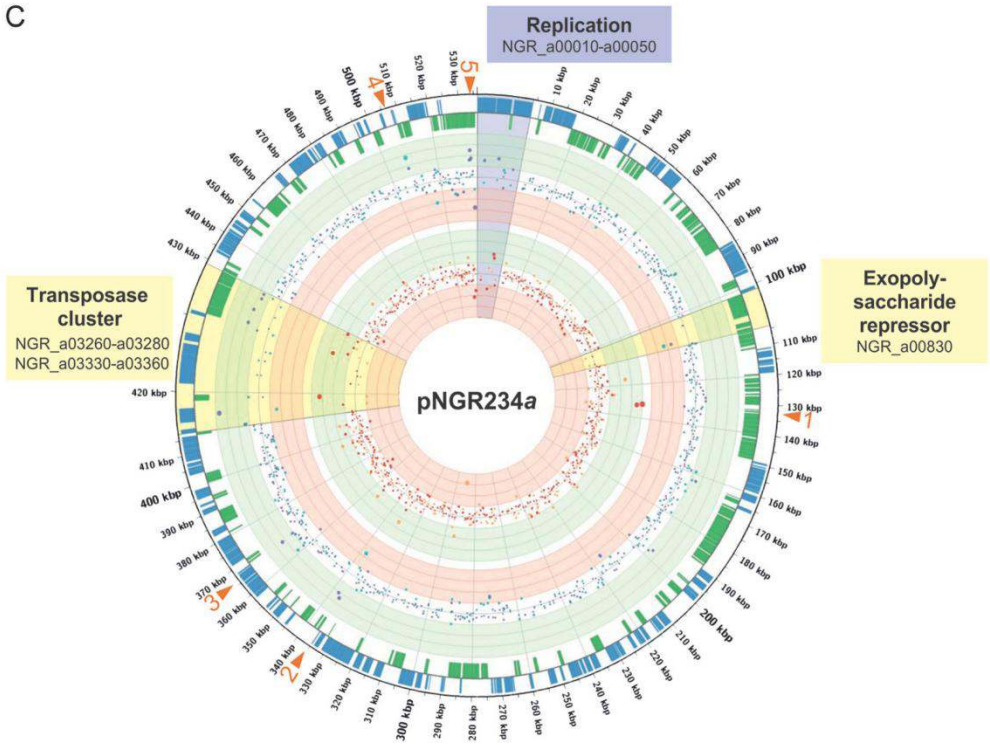


FIG 4 Circular representation of the complete RNA-seq-based transcriptome data set of NGR234-Δ*traI* and NGR234-Δ*ngrI* versus the parent strain for all three replicons (A to C) generated with the Circus 0.64 software (99). The following specifications apply for panels A to C. Fold change cutoff is $\log_2$ 4/−4 (circle size by values). Circles are described from the outside to the innermost circle: the outer circles indicate the coordinates of cNGR234, pNGR234*b*, and pNGR234*a* in megabase pairs and positions of several regulated regions/genes that are not explicitly mentioned in the text. The positions are tagged with orange arrows and corresponding numbers. These genes/ORFs are listed in detail for panels A, B, and C, respectively. Note that several genes within the marked regions have adjusted *P* values of ≥0.05. The second and third outer circles indicate ORFs on the leading (blue) and the lagging (dark green) strands. The next circles are light green areas that indicate $\log_2$ 4, 3, 2, and 1 (from outside to inside); the next circles, represented by purple and cyan dots (scattered over light green/white/light red areas), are sense transcripts for NGR234-Δ*traI* (purple) and NGR234-Δ*ngrI* (cyan); the light red circles/areas are $\log_2$ −1, −2, −3, and −4 (from outside to inside). The next light green and light red areas apply as described above. The following inner circles represented by red and orange dots (scattered over light green/white/light red areas) are antisense transcripts for NGR234-Δ*traI* (red) and NGR234-Δ*ngrI* (orange). Highlighted areas are 5-fold magnified and show regions/genes which are differentially regulated in the mutant strains and are further discussed in the text. Light yellow areas, regions/genes regulated in both mutants (NGR234-Δ*traI* and NGR234-Δ*ngrI*); light purple areas, regions/genes regulated only in NGR234-Δ*traI*; light cyan, regions/genes regulated only in NGR234-Δ*ngrI*. (A) Circular representation of RNA-seq data for cNGR234. Highlighted areas marked with asterisks include also adjusted *P* values of ≥0.05. Tagged positions: 1, hypothetical proteins with ice nucleation domain (NGR_c02320 to -c02360); 2, phosphosulfate reductase/sulfate adenylyltransferases (NGR_c06940 to -c06960); 3, hypothetical proteins (NGR_c11390 to -c11590); 4, nitrogen fixation proteins (NGR_c17900 to -c17930); 5, hypothetical proteins and transposases (NGR_c13830 to -c13890); 6, MerR family transcriptional regulator (NGR_c19960); 7, calcium binding hemolysin-like protein (NGR_c20180); 8, acetyl coenzyme A (acetyl-CoA) synthetase (NGR_c26750); 9, hypothetical protein (NGR_c28550); 10, hypothetical protein (NGR_c31960). (B) Circular representation of RNA-seq data for pNGR234*b*. For specifications of individual circles, see above. Tagged positions: 1, putative secretion protein and putative nuclease inhibitor (NGR_b01620 to -b01640); 2, hypothetical protein (NGR_b04610); 3, hypothetical proteins (NGR_b06210 and NGR_b06130); 4, alanine racemase and D-amino acid dehydrogenase (NGR_b07050 to -b07070); 5, copper-related proteins (NGR_b07130 to -b07180); 6, copper-related proteins (NGR_b08240 to -b08280); 7, C_4-dicarboxylate transport system (NGR_b08880 and NGR_b08920); 8, hypothetical proteins (NGR_b09530 and NGR_b09550); 9, hypothetical proteins (NGR_b13550 and NGR_b13560); 10, hypothetical protein (NGR_b18740); 11, propionyl-CoA carboxylases (NGR_b20820 to -b20920); 12, adenylate cyclase (NGR_b21170). (C) Circular representation of RNA-seq data for pNGR234*a*. For specifications of individual circles, see above. Tagged positions: 1, ferredoxin and hypothetical protein (NGR_a01050 and NGR_a01060); 2, precursor of 26.2 kDa for periplasmic protein and outer membrane protein (NGR_a02560 and NGR_c02570); 3, hypothetical proteins (NGR_a02770 to -a02800); 4, hypothetical protein (NGR_a03910); 5, conjugal transfer cluster (NGR_a03950 to -a04210).

chromosome-carried T4P genes were nearly all 3- to 11-fold upregulated (Table 5). The overall changes in T4P transcription in the NGR234-Δ*ngrI* and the NGR234-Δ*traI* mutant were quite similar (Table 5; Fig. 4A), and for *pilA*, they were verified by qRT-PCR in both mutants (see Table S2 in the supplemental material). This observation is in line with reports on an altered surface motility of a *sinI* mutant in *S. meliloti* (19) and correlates well with observations made for QS-dependent expression of T4P and surface motility in *P. aeruginosa* (64).

Succinoglycan biosynthesis is repressed in NGR234-Δ*traI* and NGR234-Δ*ngrI*. The development of the *Rhizobium*-legume symbiosis requires the timely and spatially regulated bacterial synthesis of four classes of cell envelope-associated polysaccharides: the cyclic β-(1,2)-glucans, the outer membrane lipopolysaccharides (LPSs), the external capsular polysaccharides (KPS), and finally the extracellular polysaccharides (exopolysaccharides [EPSs] composed of succinoglycan [EPS I] and galactoglucan [EPS II]) (65). NGR234 carries several clusters and operons involved in the

synthesis of the bacterial exopolysaccharides, capsular polysaccharides, and lipopolysaccharides (13, 66). The largest of these clusters (encompassing *exo* and *exs* genes) stretches from *thiD* to *exsI* with a total of 31 genes encoding the synthesis of low-molecular-weight exopolysaccharides (67, 68). This large cluster is located on pNGR234*b*, and its overall genetic organization is highly similar to that of the corresponding *exo*/*exs* cluster on the pSymB replicon of *S. meliloti* (67, 69). The most striking differences are mainly found in proximity to the *exoI* gene (NGR_b18300).

Our analysis suggests that most of the genes within the conserved *exo* cluster are downregulated in both the NGR234-Δ*traI* and the NGR234-Δ*ngrI* mutant strains compared to the parent strain (Fig. 4B). The *exoI* gene and a flanking hypothetical protein (NGR_b18310) were strongly repressed in their expression in both mutants on the level of sense transcription (see Fig. S3C in the supplemental material). While *exoI* was 83-fold and NGR_b18310 was 16-fold downregulated in NGR234-Δ*ngrI* (Table 6), the remaining significantly regulated genes within this cluster were not as strongly affected in their expression level.

Within the conserved *exo* cluster, a small 68-nucleotide noncoding RNA was observed that was strongly upregulated on the level of antisense transcription. While the respective ncRNA gene was weakly repressed by sense transcription, by a factor of only 2.4 and 2.5 in both mutant strains, the antisense *rpoS*-like ncRNA of NGR_b18320 was 52-fold upregulated in NGR234-Δ*ngrI* and 36-fold upregulated in NGR234-Δ*traI* (Table 6). Although we do not know the exact target of this ncRNA, it is likely that it interacts with EPS biosynthesis and further suppresses the transcription of the indicated *exo* cluster in the absence of AI molecules. BLAST analyses accomplished with NGR_b18320 revealed that this ncRNA was present only in the genomes of the broad-host-range strains *S. fredii* USDA257 and HH103 but not in *S. meliloti* 1021. We have previously identified the flanking regions of this ncRNA on pNGR234*b* next to *exoI* as part of a region in which gene rearrangement and deletions in the EPS cluster, compared to *S. meliloti* 1021, had been observed (67).

With respect to biosynthesis and regulation of EPS, *S. meliloti* appears to be one of the best-studied model organisms (65). In this strain, the ExpR/Sin QS system controls the expression of the succinoglucan biosynthesis genes (16, 70). Here, the observation that the EPS biosynthesis is also subject to a QS-dependent regulation in NGR234 fits well with the previous observations for *S. meliloti* (65 and references therein). However, in *S. meliloti* 1021 only a single AI synthase has been identified, while NGR234 encodes two AI synthases.

Cofactor biosynthesis genes are upregulated in NGR234-Δ*ngrI*. Beside its role as a cofactor in carboxylation reactions, biotin plays a pivotal role during root colonization in sinorhizobia (71). It is of importance for the synthesis and degradation of polyhydroxybutyrate (72) and various anaplerotic reactions in rhizobia and bradyrhizobia (73, 74). A minimum set of six genes is generally required for biotin biosynthesis in Gram-negative bacteria, and they are usually organized within a single transcriptional unit (75). In NGR234, five essential biotin synthesis genes, *bioADBFZ* (NGR_c25100 to -c25140), are clustered on the chromosome, whereby a *bioA* (NGR_b06270) homolog and a *bioF* (NGR_b10670) ortholog can also be found on pNGR234*b*. In addition, the regulatory locus *bioS* (NGR_c13770) was identified on the chromosome. Furthermore, this gene was detected only in species closely related to *Sinorhizobium fredii*. A copy of the sixth biotin synthesis gene, *bioC* (NGR_c26660), is located further downstream of the *bioADBFZ* cluster. Genes involved in biotin transport (*bioMNY*) were identified elsewhere on the chromosome.

The transcriptomic data set showed that the genes *bioADBFZ* together with the *bioS* regulator were upregulated in the NGR234-Δ*ngrI* mutant. The biotin biosynthesis genes were on average 3-fold upregulated, while the *bioS* gene was almost 6.5-fold upregulated by sense transcripts (Table 7). Since it can be assumed that TY medium contains high levels of this vitamin, the induced regulation of the biotin biosynthesis genes in the absence of the *ngrI*-specific AI suggests a QS-dependent regulation of this *bio* cluster. With respect to the regulation of biotin, this is the first report showing a QS-dependent regulation of bacterial biotin biosynthesis genes.

The *bioS* gene is unique to the sinorhizobia, and it is involved in regulation of biotin-dependent processes and survival under biotin starvation in *S. meliloti* (76, 77). Interestingly, earlier we reported on a possible link between the *bioS* regulation and QS-dependent gene regulation in *S. meliloti* (75). This observation is now supported by the transcriptome data in the background of the NGR234-Δ*ngrI* strain (Table 7; Fig. 3B).

Pyrroloquinoline quinone (PQQ) is required as a cofactor for dehydrogenases involved in the primary oxidation of growth substrates such as alcohols (ethanol, glycerol, and polyvinyl alcohols), amines, and aldose sugars. PQQ-dependent dehydrogenases are mostly secreted into the bacterial periplasm (78). While the presence and function of PQQ enzymes have been reported in many rhizobial genomes, only little is known about their role during root infection and nodule formation. In this respect, the presence of a PQQ-dependent glucose dehydrogenase appears to be essential for nodulation efficiency and competitiveness in *S. meliloti* (79, 80).

In the NGR234 genome, five genes encode the PQQ biosynthesis. The *pqqEDCBA* genes are located on pNGR234*b* in a single cluster (NGR_b03260 to -b03300) together with a possible alcohol dehydrogenase (NGR_b03250) and a potential LuxR regulator (NGR_b03240) (Table 7). In our study, all genes within the PQQ gene cluster were strongly upregulated (up to 10.7-fold) in the NGR234-Δ*ngrI* mutant but not in the NGR234-Δ*traI* mutant (Fig. 4B). This finding suggests that PQQ-associated genes are regulated and controlled by NGR234 QS signaling that is *ngrI* specific. This is a novel finding, since it has previously not been reported that PQQ genes in NGR234 are subject to QS-dependent regulation.

T2SS and T3SS-II are subject to QS-dependent regulation. NGR234 carries a remarkable number of secretion-associated genes (13). Within this framework, we asked the question to what extent the many secretory genes are subject to a QS-dependent gene regulation in NGR234.

Expression of several T2SS-associated genes is diminished. The general secretion-related pathway (T2SS) is widely conserved in Gram-negative bacteria and translocates exoproteins (e.g., cellulases, lipases, etc.) from the bacterial periplasm into the surrounding medium. The T2SS is formed by a set of 12 to 16 proteins (GspA to GspS), and in many ways they resemble type IV pilus assembly systems (81). In NGR234, these genes are required for type II pilus assembly and are all located in a single cluster on cNGR234 encompassing 13 genes (NGR_c22980 to -c23100). With respect to the regulation of secretion pathways, it is notable

that several of the general secretion pathway-related genes were significantly downregulated in the NGR234-Δ*ngrI* mutant (Fig. 4A).

QS-mediated repression of the T3SS-II. Among the many secretion systems that are encoded on the genome of NGR234, we reported on two clusters of genes involved in the buildup of two distinct type III secretion apparatuses (T3SS). Many Gram-negative bacteria utilize T3SS to direct effector proteins into the cytoplasm of their eukaryotic hosts. These specialized protein export machineries are important components of bacterial virulence (82). In NGR234, the first T3SS (T3SS-I) is encoded on the symbiotic plasmid (pNGR234*a*) and a second T3SS (T3SS-II) was identified on pNGR234*b*. The two loci each encompass 22 genes involved in the assembly of the two secretion machineries (13, 83). The T3SS-I locus was found to be one of the key determinants of host range (84–86). Recently, it was also shown that the T3SS-I locus was upregulated in the *Leucaena leucocephala* and *V. unguiculata* bacteroids. Transcription was, however, highest in the *L. leucocephala* nodules in comparison to the *V. unguiculata* nodules (29). Within this framework, our transcriptome study suggests that the T3SS-I is subject to a QS-independent regulation. This observation fits well with the reports on the flavonoid- and NodD1-SyrM1-NodD2-TtsI-dependent regulatory cascade controlling the activity of the T3SS-I locus (86, 87). In contrast to the T3SS-I, not much is known about the function of the second T3SS locus. Only a few of the T3SS-II-associated genes were upregulated in *L. leucocephala* bacteroids (29), and a deletion of NGR_b22890 to NGR_b22950 in the T3SS-II did not result in a symbiotic phenotype, suggesting that the T3SS-II locus is not required for effective nodulation of the few legumes tested (13, 88). In contrast to the QS-independent regulation of T3SS-I, the T3SS-II, however, appears to be regulated in a QS-dependent manner. Our transcriptome analysis of the NGR234-Δ*ngrI* mutant suggests that all genes within the T3SS-II locus (NGR_b22790 to -b23040) were downregulated by a factor of 3 to 7 compared to the parent strain (Table 8; see also Fig. S3D in the supplemental material), and for NGR_b22870, qRT-PCR analyses verified these findings (see Table S2). Interestingly, in the NGR234-Δ*traI* strain only 10 genes (NGR_b22920 and NGR_b22880 to -b22800) were regulated in a QS-dependent manner, suggesting a slightly different expression with respect to the *traI*-dependent regulation.

QS-dependent regulation of transposase activities in NGR234. The NGR234 genome encodes a significant number (4.1%) of phages and transposases which are unequally distributed over the three replicons (13). On pNGR234*a*, 114 transposases and, on pNGR234*b*, 142 transposases were observed, whereas only 79 transposable elements were found on the chromosome. Insertion sequence (IS) elements could be grouped into 36 families, the largest of which includes 10 copies of the NGRIS-4 transposable element, and their sizes range from 623 to 3,316 bp. With respect to our transcriptome study, it was obvious that altogether 14 transposases were regulated in a QS-dependent manner in the NGR234-Δ*traI* mutant. In the NGR234-Δ*ngrI* mutant, five transposases were significantly repressed in their expression levels, while only one was induced.

In NGR234, several phage-related proteins have been identified during the genome analysis. Most strikingly, a large cluster comprising 49 phage-related genes was carried on the bacterial chromosome (Fig. 4A). In the background of the NGR234-Δ*ngrI* mutant, several genes within this cluster were 3- to 4-fold downregulated.

Nodulation is not affected in *traI* or *ngrI* deletion mutant strains. Our transcriptome data revealed that almost none of the symbiotic loci were differentially regulated in the NGR234 mutants (Fig. 4C). To further verify these findings, we performed nodulation assays to monitor the nodulation phenotype of the different AI synthase mutants versus the parent strain. The assays revealed that the QS deletion mutants were able to form nitrogen-fixing nodules on *V. unguiculata*, *V. radiata*, and *T. vogelii* and did not differ significantly in their nodulation efficiencies from the NGR234 wild type (see Table S3 in the supplemental material). We further tested the nodulation phenotype of the NGR234 double mutant with a deletion in both QS loci. Similarly, the NGR234-Δ*ngrI*/Δ*traI* mutant was not affected in its ability to form nitrogen-fixing nodules. Altogether, these observations fit well with the hypothesis that QS is perhaps not of crucial importance for nodulation *per se*.

AHL-dependent gene expression in early exponential phase of NGR234. NGR234 carries a remarkably high number of genes, which are involved in the degradation of various AHL signaling molecules (55). It is worthwhile to speculate whether NGR234 uses this quorum quenching (QQ) arsenal to regulate its own AI concentration inside and outside the cell or whether the QQ-based strategy provides NGR234 a competitive advantage in the rhizosphere. The intertwined regulation of these QQ-associated genes is still unsolved and so far could not be linked to the presence of various levels of AHLs. In our experimental setup, we wanted to investigate the influence of different AHL concentrations on the expression of NGR234's QQ loci. Therefore, we used RNA-seq to analyze overall changes in the expression profiles of NGR234 cells challenged with externally added AHLs.

In order to answer the question whether the QQ-related genes could be induced by the addition of AHLs, we treated exponentially growing cells with 3-oxo-C_8-HSL at two different concentrations (0.05 μM and 50 μM). Interestingly, none of the previously identified QQ genes showed an increased transcription level due to the added AHLs. To our surprise, we identified only a few genes that were significantly altered in their transcription profile (Fig. 2; Table 4) under both conditions. The addition of 0.05 μM 3-oxo-C_8-HSL identified 13 significantly regulated genes, and the addition of 50 μM merely identified four significantly differently regulated genes compared to equally treated NGR234 wild-type cells.

The observation that only a few genes were significantly altered in their expression profiles suggests that QS-dependent gene regulation is less important during exponential growth in NGR234 and that factors other than just an increase in external AHL levels are required for a QS-dependent gene expression. It is noteworthy that other studies have already reported on QS modulators like QscR and antiactivators like QslA that regulate the timing of QS-controlled gene expression and the threshold concentration of the QS signal (89, 90).

Rhizopine catabolism is repressed in the presence of high levels of AHLs. Surprisingly, in the presence of high concentrations of AHLs (50 μM) all four differentially regulated genes could be grouped to the cluster responsible for rhizopine catabolism collectively called the *moc* operon. Rhizopines are secreted plant compounds, which are synthesized by bacteroids inside the plant nodule in *Rhizobium*-legume interactions. Plant-nodulating rhi-

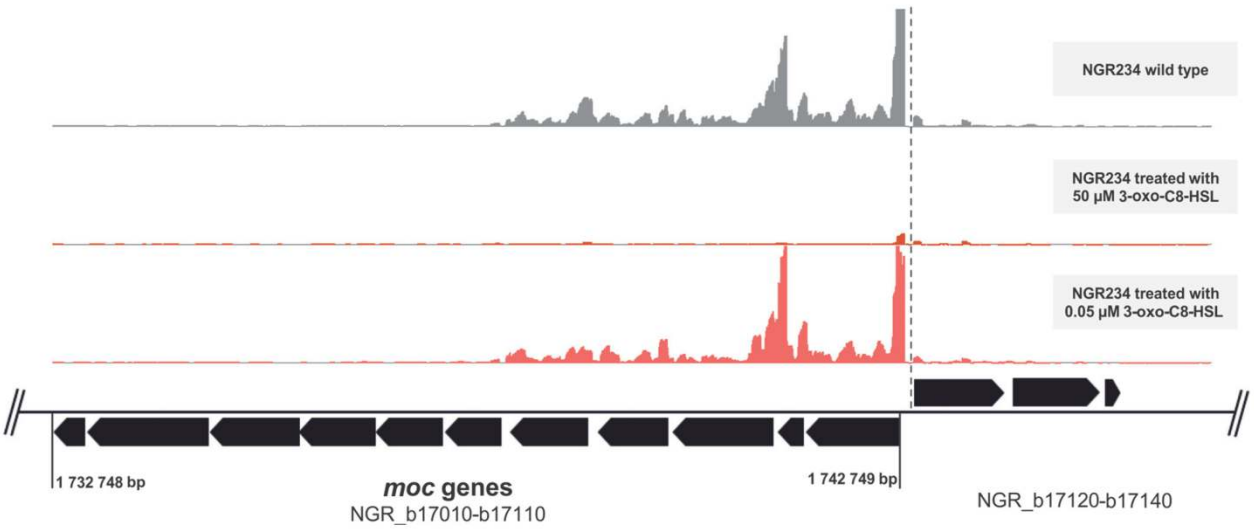


FIG 5 Partial physical map of rhizopine catabolism genes (*moc* genes) located on pNGR234*b* (NGR_b17010 to -b17110) and the relevant transcriptome profiles for NGR234 wild type (control), NGR234 treated with high levels of AHLs (50 μM 3-oxo-C_8-HSL), and NGR234 treated with moderate levels of AHLs (0.05 μM 3-oxo-C_8-HSL). Transcriptome profile images of the leading and lagging strand were generated with IGB (40), merged (indicated with a dashed line), and visualized on the leading strand for a simplified presentation. The *y* axis scale cutoff was set to 800 (leading strand) and to −800 (lagging strand). Black arrows indicate the ORFs identified within and flanking the *moc* region of NGR234.

zobia like NGR234 can obtain a nutritional advantage and a competition success for nodule formation from these symbiotic associations as the plant-associated rhizopines stimulate rhizobial growth (91–93). These compounds function as growth substrates and are catabolized by free-living rhizobia located in nodule infection threads and in the rhizosphere (94). In NGR234, the *moc* gene cluster, which is involved in the degradation of rhizopines, is composed of six genes of which four, *mocABRC*, are essential for the catabolic function. The NGR234 *moc* cluster is located on pNGR234*b*, probably stretching from NGR_b17010 to NGR_b17110. In our transcriptome data, *moc* genes ranging from NGR_b17080 to -b17110 (Fig. 5) were 9- to 12-fold downregulated in NGR234 challenged with 50 μM 3-oxo-C_8-HSL. The *moc* genes have been previously observed to be subject to QS-dependent regulation in *S. meliloti* (19), and in *A. tumefaciens*, opines act as the conjugal signal for octopine-type Ti plasmids. Furthermore, in *A. tumefaciens* the octopine catabolic pathway and QS signal pathways are directly linked and *traR* is activated only when a threshold level of octopine is reached (95–97). The observation that the presence of AI represses rhizopine degradation in NGR234 might suggest, similarly to the *A. tumefaciens* system, a link between rhizopine metabolism and QS. In fact, He and colleagues (14) have previously suggested that rhizopines interfere with the regulation of TraR in NGR234. Thus, our observations are well in line with the hypothesis of He and colleagues.

Conclusions. Within this work, we have identified a set of 186 QS-regulated (*traI* and *ngrI*) genes in NGR234 using RNA-seq. We have provided evidence that the T3SS-II, T4P, biotin biosynthesis, and PQQ genes are QS regulated under the control of the NgrI/R regulon. These genes were so far not identified to be QS regulated in any of the other rhizobial model organisms. Our data further suggested that, similarly to *S. meliloti*, genes for flagellar and EPS (succinoglucan) biosynthesis are subject to QS-dependent regulation. While in general it is assumed that a homogeneous expression of these genes occurred throughout an isogenic population, there is now initial evidence that this may not be the natural situation. Instead, a fraction of the NGR234 genes are most likely heterogeneously expressed on a single-cell level (98). In the light of these novel findings, future work will now have to elucidate the complex pattern of gene expression and regulation on a single-cell level in this fascinating microorganism.

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REFERENCES

1. **Waters CM, Bassler BL.** 2005. Quorum sensing: cell-to-cell communication in bacteria. Annu. Rev. Cell Dev. Biol. **21:**319–346. http://dx.doi.org/10.1146/annurev.cellbio.21.012704.131001.
2. **Ng WL, Bassler BL.** 2009. Bacterial quorum-sensing network architectures. Annu. Rev. Genet. **43:**197–222. http://dx.doi.org/10.1146/annurev-genet-102108-134304.
3. **Shank EA, Kolter R.** 2009. New developments in microbial interspecies signaling. Curr. Opin. Microbiol. **12:**205–214. http://dx.doi.org/10.1016/j.mib.2009.01.003.
4. **de Kievit TR, Iglewski BH.** 2000. Bacterial quorum sensing in pathogenic relationships. Infect. Immun. **68:**4839–4849. http://dx.doi.org/10.1128/IAI.68.9.4839-4849.2000.
5. **Val DL, Cronan JE, Jr.** 1998. In vivo evidence that *S*-adenosylmethionine and fatty acid synthesis intermediates are the substrates for the LuxI family of autoinducer synthases. J. Bacteriol. **180:**2644–2651.
6. **Zeng LR, Xie JP.** 2011. Molecular basis underlying LuxR family transcription factors and function diversity and implications for novel antibiotic drug targets. J. Cell. Biochem. **112:**3079–3084. http://dx.doi.org/10.1002/jcb.23262.
7. **Gage DJ.** 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. Microbiol. Mol. Biol. Rev. **68:**280–300. http://dx.doi.org/10.1128/MMBR.68.2.280-300.2004.

8. **Deakin WJ, Broughton WJ.** 2009. Symbiotic use of pathogenic strategies: rhizobial protein secretion systems. Nat. Rev. Microbiol. **7:**312–320. http://dx.doi.org/10.1038/nrmicro2091.
9. **Jones KM, Kobayashi H, Davies BW, Taga ME, Walker GC.** 2007. How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. Nat. Rev. Microbiol. **5:**619–633. http://dx.doi.org/10.1038/nrmicro1705.
10. **Broughton WJ, Perret X.** 1999. Genealogy of legume-*Rhizobium* symbioses. Curr. Opin. Plant Biol. **2:**305–311. http://dx.doi.org/10.1016/S1369-5266(99)80054-5.
11. **Pueppke SG, Broughton WJ.** 1999. *Rhizobium* sp. strain NGR234 and *R. fredii* USDA257 share exceptionally broad, nested host ranges. Mol. Plant Microbe Interact. **12:**293–318. http://dx.doi.org/10.1094/MPMI.1999.12.4.293.
12. **Trinick MJ.** 1980. Relationships amongst the fast-growing rhizobia of *Lablab purpureus*, *Leucaena leucocephala*, *Mimosa* spp., *Acacia farnesiana* and *Sesbania grandiflora* and their affinities with other rhizobial groups. J. Appl. Microbiol. **49:**39–53.
13. **Schmeisser C, Liesegang H, Krysciak D, Bakkou N, Le Quere A, Wollherr A, Heinemeyer I, Morgenstern B, Pommerening-Röser A, Flores M, Palacios R, Brenner S, Gottschalk G, Schmitz RA, Broughton WJ, Perret X, Strittmatter AW, Streit WR.** 2009. *Rhizobium* sp. strain NGR234 possesses a remarkable number of secretion systems. Appl. Environ. Microbiol. **75:**4035–4045. http://dx.doi.org/10.1128/AEM.00515-09.
14. **He X, Chang W, Pierce DL, Seib LO, Wagner J, Fuqua C.** 2003. Quorum sensing in *Rhizobium* sp. strain NGR234 regulates conjugal transfer (*tra*) gene expression and influences growth rate. J. Bacteriol. **185:**809–822. http://dx.doi.org/10.1128/JB.185.3.809-822.2003.
15. **Marketon MM, González JE.** 2002. Identification of two quorum-sensing systems in *Sinorhizobium meliloti*. J. Bacteriol. **184:**3466–3475. http://dx.doi.org/10.1128/JB.184.13.3466-3475.2002.
16. **Hoang HH, Becker A, González JE.** 2004. The LuxR homolog ExpR, in combination with the Sin quorum sensing system, plays a central role in *Sinorhizobium meliloti* gene expression. J. Bacteriol. **186:**5460–5472. http://dx.doi.org/10.1128/JB.186.16.5460-5472.2004.
17. **Becker A, Bergès H, Krol E, Bruand C, Rüberg S, Capela D, Lauber E, Meilhoc E, Ampe F, de Bruijn FJ, Fourment J, Francez-Charlot A, Kahn D, Küster H, Liebe C, Pühler A, Weidner S, Batut J.** 2004. Global changes in gene expression in *Sinorhizobium meliloti* 1021 under microoxic and symbiotic conditions. Mol. Plant Microbe Interact. **17:**292–303. http://dx.doi.org/10.1094/MPMI.2004.17.3.292.
18. **Gurich N, González JE.** 2009. Role of quorum sensing in *Sinorhizobium meliloti*-alfalfa symbiosis. J. Bacteriol. **191:**4372–4382. http://dx.doi.org/10.1128/JB.00376-09.
19. **Gao M, Chen H, Eberhard A, Gronquist MR, Robinson JB, Rolfe BG, Bauer WD.** 2005. *sinI*- and *expR*-dependent quorum sensing in *Sinorhizobium meliloti*. J. Bacteriol. **187:**7931–7944. http://dx.doi.org/10.1128/JB.187.23.7931-7944.2005.
20. **Chen H, Teplitski M, Robinson JB, Rolfe BG, Bauer WD.** 2003. Proteomic analysis of wild-type *Sinorhizobium meliloti* responses to *N*-acyl homoserine lactone quorum-sensing signals and the transition to stationary phase. J. Bacteriol. **185:**5029–5036. http://dx.doi.org/10.1128/JB.185.17.5029-5036.2003.
21. **Charoenpanich P, Meyer S, Becker A, McIntosh M.** 2013. Temporal expression program of quorum sensing-based transcription regulation in *Sinorhizobium meliloti*. J. Bacteriol. **195:**3224–3236. http://dx.doi.org/10.1128/JB.00234-13.
22. **Croucher NJ, Thomson NR.** 2010. Studying bacterial transcriptomes using RNA-seq. Curr. Opin. Microbiol. **13:**619–624. http://dx.doi.org/10.1016/j.mib.2010.09.009.
23. **Haas BJ, Chin M, Nusbaum C, Birren BW, Livny J.** 2012. How deep is deep enough for RNA-Seq profiling of bacterial transcriptomes? BMC Genomics **13:**734. http://dx.doi.org/10.1186/1471-2164-13-734.
24. **Sharma CM, Hoffmann S, Darfeuille F, Reignier J, Findeiss S, Sittka A, Chabas S, Reiche K, Hackermüller J, Reinhardt R, Stadler PF, Vogel J.** 2010. The primary transcriptome of the major human pathogen *Helicobacter pylori*. Nature **464:**250–255. http://dx.doi.org/10.1038/nature08756.
25. **Westermann AJ, Gorski SA, Vogel J.** 2012. Dual RNA-seq of pathogen and host. Nat. Rev. Microbiol. **10:**618–630. http://dx.doi.org/10.1038/nrmicro2852.
26. **Schmid N, Pessi G, Deng Y, Aguilar C, Carlier AL, Grunau A, Omasits U, Zhang LH, Ahrens CH, Eberl L.** 2012. The AHL- and BDSF-dependent quorum sensing systems control specific and overlapping sets of genes in *Burkholderia cenocepacia* H111. PLoS One **7:**e49966. http://dx.doi.org/10.1371/journal.pone.0049966.
27. **Chugani SA, Kim BS, Phattarasukol S, Brittnacher MJ, Choi SH, Harwood CS, Greenberg EP.** 2012. Strain-dependent diversity in the *Pseudomonas aeruginosa* quorum-sensing regulon. Proc. Natl. Acad. Sci. U. S. A. **109:**E2823–2831. http://dx.doi.org/10.1073/pnas.1214128109.
28. **Wang D, Seeve C, Pierson L, Pierson E.** 2013. Transcriptome profiling reveals links between ParS/ParR, MexEF-OprN, and quorum sensing in the regulation of adaptation and virulence in *Pseudomonas aeruginosa*. BMC Genomics **14:**618. http://dx.doi.org/10.1186/1471-2164-14-618.
29. **Li Y, Tian CF, Chen WF, Wang L, Sui XH, Chen WX.** 2013. High-resolution transcriptomic analyses of *Sinorhizobium* sp. NGR234 bacteroids in determinate nodules of *Vigna unguiculata* and indeterminate nodules of *Leucaena leucocephala*. PLoS One **8:**e70531. http://dx.doi.org/10.1371/journal.pone.0070531.
30. **Fuqua C, Winans SC.** 1996. Conserved *cis*-acting promoter elements are required for density-dependent transcription of *Agrobacterium tumefaciens* conjugal transfer genes. J. Bacteriol. **178:**435–440.
31. **Tempe J, Petit A, Holsters M, Montagu M, Schell J.** 1977. Thermosensitive step associated with transfer of the Ti plasmid during conjugation: possible relation to transformation in crown gall. Proc. Natl. Acad. Sci. U. S. A. **74:**2848–2849. http://dx.doi.org/10.1073/pnas.74.7.2848.
32. **Sambrook J, Russell DW.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
33. **Hornung C, Poehlein A, Haack FS, Schmidt M, Dierking K, Pohlen A, Schulenburg H, Blokesch M, Plener L, Jung K, Bonge A, Krohn-Molt I, Utpatel C, Timmermann G, Spieck E, Pommerening-Röser A, Bode E, Bode HB, Daniel R, Schmeisser C, Streit WR.** 2013. The *Janthinobacterium* sp. HH01 genome encodes a homologue of the *V. cholerae* CqsA and *L. pneumophila* LqsA autoinducer synthases. PLoS One **8:**e55045. http://dx.doi.org/10.1371/journal.pone.0055045.
34. **Lassak J, Henche AL, Binnenkade L, Thormann KM.** 2010. ArcS, the cognate sensor kinase in an atypical Arc system of *Shewanella oneidensis* MR-1. Appl. Environ. Microbiol. **76:**3263–3274. http://dx.doi.org/10.1128/AEM.00512-10.
35. **Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM, II, Peterson KM.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175–176. http://dx.doi.org/10.1016/0378-1119(95)00584-1.
36. **Zhu J, Beaber JW, More MI, Fuqua WC, Eberhard A, Winans SC.** 1998. Analogs of the autoinducer 3-oxooctanoyl-homoserine lactone strongly inhibit activity of the TraR protein of *Agrobacterium tumefaciens*. J. Bacteriol. **180:**5398–5405.
37. **Blomberg P, Wagner EG, Nordström K.** 1990. Control of replication of plasmid R1: the duplex between the antisense RNA, CopA, and its target, CopT, is processed specifically *in vivo* and *in vitro* by RNase III. EMBO J. **9:**2331–2340.
38. **Dugar G, Herbig A, Förstner KU, Heidrich N, Reinhardt R, Nieselt K, Sharma CM.** 2013. High-resolution transcriptome maps reveal strain-specific regulatory features of multiple *Campylobacter jejuni* isolates. PLoS Genet. **9:**e1003495. http://dx.doi.org/10.1371/journal.pgen.1003495.
39. **Hoffmann S, Otto C, Kurtz S, Sharma CM, Khaitovich P, Vogel J, Stadler PF, Hackermüller J.** 2009. Fast mapping of short sequences with mismatches, insertions and deletions using index structures. PLoS Comput. Biol. **5:**e1000502. http://dx.doi.org/10.1371/journal.pcbi.1000502.
40. **Nicol JW, Helt GA, Blanchard SG, Jr, Raja A, Loraine AE.** 2009. The Integrated Genome Browser: free software for distribution and exploration of genome-scale datasets. Bioinformatics **25:**2730–2731. http://dx.doi.org/10.1093/bioinformatics/btp472.
41. **Rivas R, Vizcaíno N, Buey RM, Mateos PF, Martínez-Molina E, Velázquez E.** 2001. An effective, rapid and simple method for total RNA extraction from bacteria and yeast. J. Microbiol. Methods **47:**59–63. http://dx.doi.org/10.1016/S0167-7012(01)00292-5.
42. **Krishnan HB, Pueppke SG.** 1991. Sequence and analysis of the *nodABC* region of *Rhizobium fredii* USDA257, a nitrogen-fixing symbiont of soybean and other legumes. Mol. Plant Microbe Interact. **4:**512–520. http://dx.doi.org/10.1094/MPMI-4-512.
43. **Edgar R, Domrachev M, Lash AE.** 2002. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. **30:**207–210. http://dx.doi.org/10.1093/nar/30.1.207.
44. **Dötsch A, Eckweiler D, Schniederjans M, Zimmermann A, Jensen V, Scharfe M, Geffers R, Häussler S.** 2012. The *Pseudomonas aeruginosa*

transcriptome in planktonic cultures and static biofilms using RNA sequencing. PLoS One **7:**e31092. http://dx.doi.org/10.1371/journal.pone.0031092.

45. **Georg J, Hess WR.** 2011. *cis*-antisense RNA, another level of gene regulation in bacteria. Microbiol. Mol. Biol. Rev. **75:**286–300. http://dx.doi.org/10.1128/MMBR.00032-10.
46. **Lasa I, Toledo-Arana A, Gingeras TR.** 2012. An effort to make sense of antisense transcription in bacteria. RNA Biol. **9:**1039–1044. http://dx.doi.org/10.4161/rna.21167.
47. **Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH.** 2003. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. J. Bacteriol. **185:**2080–2095. http://dx.doi.org/10.1128/JB.185.7.2080-2095.2003.
48. **Schuster M, Lostroh CP, Ogi T, Greenberg EP.** 2003. Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. J. Bacteriol. **185:**2066–2079. http://dx.doi.org/10.1128/JB.185.7.2066-2079.2003.
49. **Hentzer M, Wu H, Andersen JB, Riedel K, Rasmussen TB, Bagge N, Kumar N, Schembri MA, Song Z, Kristoffersen P, Manefield M, Costerton JW, Molin S, Eberl L, Steinberg P, Kjelleberg S, Høiby N, Givskov M.** 2003. Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. EMBO J. **22:**3803–3815. http://dx.doi.org/10.1093/emboj/cdg366.
50. **Bijtenhoorn P, Mayerhofer H, Müller-Dieckmann J, Utpatel C, Schipper C, Hornung C, Szesny M, Grond S, Thürmer A, Brzuszkiewicz E, Daniel R, Dierking K, Schulenburg H, Streit WR.** 2011. A novel metagenomic short-chain dehydrogenase/reductase attenuates *Pseudomonas aeruginosa* biofilm formation and virulence on *Caenorhabditis elegans*. PLoS One **6:**e26278. http://dx.doi.org/10.1371/journal.pone.0026278.
51. **LaRock CN, Yu J, Horswill AR, Parsek MR, Minion FC.** 2013. Transcriptome analysis of acetyl-homoserine lactone-based quorum sensing regulation in *Yersinia pestis*. PLoS One **8:**e62337. http://dx.doi.org/10.1371/journal.pone.0062337.
52. **Parkhill J, Wren BW, Thomson NR, Titball RW, Holden MT, Prentice MB, Sebaihia M, James KD, Churcher C, Mungall KL, Baker S, Basham D, Bentley SD, Brooks K, Cerdeño-Tárraga AM, Chillingworth T, Cronin A, Davies RM, Davis P, Dougan G, Feltwell T, Hamlin N, Holroyd S, Jagels K, Karlyshev AV, Leather S, Moule S, Oyston PC, Quail M, Rutherford K, Simmonds M, Skelton J, Stevens K, Whitehead S, Barrell BG.** 2001. Genome sequence of *Yersinia pestis*, the causative agent of plague. Nature **413:**523–527. http://dx.doi.org/10.1038/35097083.
53. **Daniels R, De Vos DE, Desair J, Raedschelders G, Luyten E, Rosemeyer V, Verreth C, Schoeters E, Vanderleyden J, Michiels J.** 2002. The *cin* quorum sensing locus of *Rhizobium etli* CNPAF512 affects growth and symbiotic nitrogen fixation. J. Biol. Chem. **277:**462–468. http://dx.doi.org/10.1074/jbc.M106655200.
54. **Gray KM, Pearson JP, Downie JA, Boboye BE, Greenberg EP.** 1996. Cell-to-cell signaling in the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum*: autoinduction of a stationary phase and rhizosphere-expressed genes. J. Bacteriol. **178:**372–376.
55. **Krysciak D, Schmeisser C, Preuss S, Riethausen J, Quitschau M, Grond S, Streit WR.** 2011. Involvement of multiple loci in quorum quenching of autoinducer I molecules in the nitrogen-fixing symbiont *Rhizobium* (*Sinorhizobium*) sp. strain NGR234. Appl. Environ. Microbiol. **77:**5089–5099. http://dx.doi.org/10.1128/AEM.00112-11.
56. **Fujishige NA, Kapadia NN, De Hoff PL, Hirsch AM.** 2006. Investigations of *Rhizobium* biofilm formation. FEMS Microbiol. Ecol. **56:**195–206. http://dx.doi.org/10.1111/j.1574-6941.2005.00044.x.
57. **Kape R, Parniske M, Werner D.** 1991. Chemotaxis and *nod* gene activity of *Bradyrhizobium japonicum* in response to hydroxycinnamic acids and isoflavonoids. Appl. Environ. Microbiol. **57:**316–319.
58. **Finan TM, Gough C, Truchet G.** 1995. Similarity between the *Rhizobium meliloti fliP* gene and pathogenicity-associated genes from animal and plant pathogens. Gene **152:**65–67. http://dx.doi.org/10.1016/0378-1119(94)00643-7.
59. **Ames P, Bergman K.** 1981. Competitive advantage provided by bacterial motility in the formation of nodules by *Rhizobium meliloti*. J. Bacteriol. **148:**728–729.
60. **Gao M, Coggin A, Yagnik K, Teplitski M.** 2012. Role of specific quorum-sensing signals in the regulation of exopolysaccharide II production within *Sinorhizobium meliloti* spreading colonies. PLoS One **7:**e42611. http://dx.doi.org/10.1371/journal.pone.0042611.
61. **Hoang HH, Gurich N, González JE.** 2008. Regulation of motility by the ExpR/Sin quorum-sensing system in *Sinorhizobium meliloti*. J. Bacteriol. **190:**861–871. http://dx.doi.org/10.1128/JB.01310-07.
62. **Shi W, Sun H.** 2002. Type IV pilus-dependent motility and its possible role in bacterial pathogenesis. Infect. Immun. **70:**1–4. http://dx.doi.org/10.1128/IAI.70.1.1-4.2002.
63. **Vesper SJ, Bauer WD.** 1986. Role of pili (fimbriae) in attachment of *Bradyrhizobium japonicum* to soybean roots. Appl. Environ. Microbiol. **52:**134–141.
64. **Barken KB, Pamp SJ, Yang L, Gjermansen M, Bertrand JJ, Klausen M, Givskov M, Whitchurch CB, Engel JN, Tolker-Nielsen T.** 2008. Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in *Pseudomonas aeruginosa* biofilms. Environ. Microbiol. **10:**2331–2343. http://dx.doi.org/10.1111/j.1462-2920.2008.01658.x.
65. **Skorupska A, Janczarek M, Marczak M, Mazur A, Król J.** 2006. Rhizobial exopolysaccharides: genetic control and symbiotic functions. Microb. Cell Fact. **5:**7. http://dx.doi.org/10.1186/1475-2859-5-7.
66. **Le Quere AJ, Deakin WJ, Schmeisser C, Carlson RW, Streit WR, Broughton WJ, Forsberg LS.** 2006. Structural characterization of a K-antigen capsular polysaccharide essential for normal symbiotic infection in *Rhizobium* sp. NGR234: deletion of the *rkpMNO* locus prevents synthesis of 5,7-diacetamido-3,5,7,9-tetradeoxy-non-2-ulosonic acid. J. Biol. Chem. **281:**28981–28992.
67. **Streit WR, Schmitz RA, Perret X, Staehelin C, Deakin WJ, Raasch C, Liesegang H, Broughton WJ.** 2004. An evolutionary hot spot: the pNGR234*b* replicon of *Rhizobium* sp. strain NGR234. J. Bacteriol. **186:**535–542. http://dx.doi.org/10.1128/JB.186.2.535-542.2004.
68. **Staehelin C, Forsberg LS, D'Haeze W, Gao MY, Carlson RW, Xie ZP, Pellock BJ, Jones KM, Walker GC, Streit WR, Broughton WJ.** 2006. Exo-oligosaccharides of *Rhizobium* sp. strain NGR234 are required for symbiosis with various legumes. J. Bacteriol. **188:**6168–6178. http://dx.doi.org/10.1128/JB.00365-06.
69. **Chen H, Gray J, Nayudu M, Djordjevic M, Batley M, Redmond J, Rolfe B.** 1988. Five genetic loci involved in the synthesis of acidic exopolysaccharides are closely linked in the genome of *Rhizobium* sp. strain NGR234. Mol. Gen. Genet. **212:**310–316. http://dx.doi.org/10.1007/BF00334701.
70. **Glenn SA, Gurich N, Feeney MA, González JE.** 2007. The ExpR/Sin quorum-sensing system controls succinoglycan production in *Sinorhizobium meliloti*. J. Bacteriol. **189:**7077–7088. http://dx.doi.org/10.1128/JB.00906-07.
71. **Streit WR, Joseph CM, Phillips DA.** 1996. Biotin and other water-soluble vitamins are key growth factors for alfalfa root colonization by *Rhizobium meliloti* 1021. Mol. Plant Microbe Interact. **9:**330–338. http://dx.doi.org/10.1094/MPMI-9-0330.
72. **Hofmann K, Heinz EB, Charles TC, Hoppert M, Liebl W, Streit WR.** 2000. *Sinorhizobium meliloti* strain 1021 *bioS* and *bdhA* gene transcriptions are both affected by biotin available in defined medium. FEMS Microbiol. Lett. **182:**41–44. http://dx.doi.org/10.1111/j.1574-6968.2000.tb08870.x.
73. **Dunn MF, Encarnación S, Araíza G, Vargas MC, Dávalos A, Peralta H, Mora Y, Mora J.** 1996. Pyruvate carboxylase from *Rhizobium etli*: mutant characterization, nucleotide sequence, and physiological role. J. Bacteriol. **178:**5960–5970.
74. **Dunn MF.** 2011. Anaplerotic function of phosphoenolpyruvate carboxylase in *Bradyrhizobium japonicum* USDA110. Curr. Microbiol. **62:**1782–1788. http://dx.doi.org/10.1007/s00284-011-9928-y.
75. **Streit WR, Entcheva P.** 2003. Biotin in microbes, the genes involved in its biosynthesis, its biochemical role and perspectives for biotechnological production. Appl. Microbiol. Biotechnol. **61:**21–31. http://dx.doi.org/10.1007/s00253-002-1186-2.
76. **Heinz EB, Phillips DA, Streit WR.** 1999. BioS, a biotin-induced, stationary-phase, and possible LysR-type regulator in *Sinorhizobium meliloti*. Mol. Plant Microbe Interact. **12:**803–812. http://dx.doi.org/10.1094/MPMI.1999.12.9.803.
77. **Streit WR, Phillips DA.** 1997. A biotin-regulated locus, *bioS*, in a possible survival operon of *Rhizobium meliloti*. Mol. Plant Microbe Interact. **10:**933–937. http://dx.doi.org/10.1094/MPMI.1997.10.7.933.
78. **Goodwin PM, Anthony C.** 1998. The biochemistry, physiology and genetics of PQQ and PQQ-containing enzymes. Adv. Microb. Physiol. **40:**1–80. http://dx.doi.org/10.1016/S0065-2911(08)60129-0.
79. **Bernardelli CE, Luna MF, Galar ML, Boiardi JL.** 2008. Symbiotic phenotype of a membrane-bound glucose dehydrogenase mutant of *Sinorhi-*

zobium meliloti. Plant Soil **313:**217–225. http://dx.doi.org/10.1007/s11104-008-9694-1.

80. **Bernardelli CE, Luna MF, Galar ML, Boiardi JL.** 2001. Periplasmic PQQ-dependent glucose oxidation in free-living and symbiotic rhizobia. Curr. Microbiol. **42:**310–315. http://dx.doi.org/10.1007/s002840010222.
81. **Sandkvist M.** 2001. Biology of type II secretion. Mol. Microbiol. **40:**271–283. http://dx.doi.org/10.1046/j.1365-2958.2001.02403.x.
82. **Coburn B, Sekirov I, Finlay BB.** 2007. Type III secretion systems and disease. Clin. Microbiol. Rev. **20:**535–549. http://dx.doi.org/10.1128/CMR.00013-07.
83. **Freiberg C, Fellay R, Bairoch A, Broughton WJ, Rosenthal A, Perret X.** 1997. Molecular basis of symbiosis between *Rhizobium* and legumes. Nature **387:**394–401. http://dx.doi.org/10.1038/387394a0.
84. **Ausmees N, Kobayashi H, Deakin WJ, Marie C, Krishnan HB, Broughton WJ, Perret X.** 2004. Characterization of NopP, a type III secreted effector of *Rhizobium* sp. strain NGR234. J. Bacteriol. **186:**4774–4780. http://dx.doi.org/10.1128/JB.186.14.4774-4780.2004.
85. **Viprey V, Del Greco A, Golinowski W, Broughton WJ, Perret X.** 1998. Symbiotic implications of type III protein secretion machinery in *Rhizobium*. Mol. Microbiol. **28:**1381–1389. http://dx.doi.org/10.1046/j.1365-2958.1998.00920.x.
86. **Marie C, Deakin WJ, Ojanen-Reuhs T, Diallo E, Reuhs B, Broughton WJ, Perret X.** 2004. TtsI, a key regulator of *Rhizobium* species NGR234 is required for type III-dependent protein secretion and synthesis of rhamnose-rich polysaccharides. Mol. Plant Microbe Interact. **17:**958–966. http://dx.doi.org/10.1094/MPMI.2004.17.9.958.
87. **Kobayashi H, Naciri-Graven Y, Broughton WJ, Perret X.** 2004. Flavonoids induce temporal shifts in gene-expression of *nod*-box controlled loci in *Rhizobium* sp. NGR234. Mol. Microbiol. **51:**335–347. http://dx.doi.org/10.1046/j.1365-2958.2003.03841.x.
88. **Bakkou N, Perret X.** 2008. Functional analysis of a second type III secretion system in *Rhizobium* sp. NGR234, p 209. *In* Holsters M (ed), Abstr. 8th Eur. Nitrogen Fixation Conf., Ghent, Belgium.
89. **Chugani SA, Whiteley M, Lee KM, D'Argenio D, Manoil C, Greenberg EP.** 2001. QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U. S. A. **98:**2752–2757. http://dx.doi.org/10.1073/pnas.051624298.
90. **Seet Q, Zhang LH.** 2011. Anti-activator QslA defines the quorum sensing threshold and response in *Pseudomonas aeruginosa*. Mol. Microbiol. **80:**951–965. http://dx.doi.org/10.1111/j.1365-2958.2011.07622.x.
91. **Murphy PJ, Heycke N, Banfalvi Z, Tate ME, de Bruijn FJ, Kondorosi A, Tempe J, Schell J.** 1987. Genes for the catabolism and synthesis of an opine-like compound in *Rhizobium meliloti* are closely linked and on the Sym plasmid. Proc. Natl. Acad. Sci. U. S. A. **84:**493–497. http://dx.doi.org/10.1073/pnas.84.2.493.
92. **Saint CP, Wexler M, Murphy PJ, Tempe J, Tate ME, Murphy PJ.** 1993. Characterization of genes for synthesis and catabolism of a new rhizopine induced in nodules by *Rhizobium meliloti* Rm220-3: extension of the rhizopine concept. J. Bacteriol. **175:**5205–5215.
93. **Rossbach S, Rasul G, Schneider M, Eardly B, de Bruijn FJ.** 1995. Structural and functional conservation of the rhizopine catabolism (*moc*) locus is limited to selected *Rhizobium meliloti* strains and unrelated to their geographical origin. Mol. Plant Microbe Interact. **8:**549–559. http://dx.doi.org/10.1094/MPMI-8-0549.
94. **Kohler PR, Zheng JY, Schoffers E, Rossbach S.** 2010. Inositol catabolism, a key pathway in *Sinorhizobium meliloti* for competitive host nodulation. Appl. Environ. Microbiol. **76:**7972–7980. http://dx.doi.org/10.1128/AEM.01972-10.
95. **Oger P, Kim K-S, Sackett RL, Piper KR, Farrand SK.** 1998. Octopine-type Ti plasmids code for a mannopine-inducible dominant-negative allele of *traR*, the quorum-sensing activator that regulates Ti plasmid conjugal transfer. Mol. Microbiol. **27:**277–288. http://dx.doi.org/10.1046/j.1365-2958.1998.00671.x.
96. **Fuqua C, Burbea M, Winans SC.** 1995. Activity of the *Agrobacterium* Ti plasmid conjugal transfer regulator TraR is inhibited by the product of the *traM* gene. J. Bacteriol. **177:**1367–1373.
97. **Fuqua C, Winans SC.** 1996. Localization of OccR-activated and TraR-activated promoters that express two ABC-type permeases and the *traR* gene of Ti plasmid pTiR10. Mol. Microbiol. **20:**1199–1210. http://dx.doi.org/10.1111/j.1365-2958.1996.tb02640.x.
98. **Grote J, Krysciak D, Schorn A, Dahlke RI, Soonvald L, Müller J, Hense BA, Schwarzfischer M, Sauter M, Schmeisser C, Streit WR.** 2014. Evidence of autoinducer-dependent and -independent heterogeneous gene expression in *Sinorhizobium fredii* NGR234. Appl. Environ. Microbiol. **80:**5572–5582. http://dx.doi.org/10.1128/AEM.01689-14.
99. **Krzywinski MI, Schein JE, Birol I, Connors J, Gascoyne R, Horsman D, Jones SJ, Marra MA.** 2009. Circos: an information aesthetic for comparative genomics. Genome Res. **19:**1639–1645. http://dx.doi.org/10.1101/gr.092759.109.

4 Evidence of autoinducer-dependent and –independent heterogeneous gene expression in *Shinorhizobium fredii* NGR234

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Evidence of Autoinducer-Dependent and -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* and *qsdR1* and the type IV pilus gene cluster located on pNGR234*b* suggested that factors other than AI molecules affect NGR234 phenotypic heterogeneity. Further studies with root exudates and developing *Arabidopsis thaliana* seedlings provide the first evidence that plant root exudates have strong effects on the heterogeneity of AI synthase and quorum-quenching genes in NGR234. Therefore, plant-released octopine appears to play a key role in modulation of heterogeneous gene expression.

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 autoinducers (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 regulation 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 genes 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. Therefore, the term "phenotypic heterogeneity" describes nongenetic 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 was first described in 1980 as the only fast-growing isolate from *Lablab purpureus* among 30 other strains (14). Since then it has become a model plant-symbiotic organism due to its ability to nodulate over 112 different plant genera (15). The NGR234 genome encodes 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-C_8-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 located 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 the control of TraR, which initiates a positive feedback loop when bound to 3-oxo-C_8-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

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid[a]	Relevant trait(s)[b]	Source or reference
Strains		
S. fredii NGR234	Wild-type strain, Rf^r	14
S. fredii NGR234-Δ*traI*	Rf^r, Gm^r, Δ*traI*	44
S. fredii NGR234-Δ*ngrI*	Rf^r, Gm^r, Δ*ngrI*	44
E. coli DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*argF-lacZYA*)*U169 endA1 hsdR17*(r_K^-, m_K^-) *supE44 thi-1 recA1 gyrA96 relA1*	22
E. coli XL1-Blue	*endA1 gyrA96*(nal^r) *thi-1 recA1 relA1 lac glnV44* [F′ Tn*10 proAB*$^+$ *lacI*^{q}*Z*ΔM15] *hsdR17*(r_K^- m_K^+)	Stratagene, LA Jolla, CA
E. coli S17-1	*thi-1 proA hsdR17* (r_K^- m_K^+) *recA1 tra*; genes of plasmid RP4 integrated into the genome	23
Plasmids		
pBBR1MCS-2	Broad-host-range vector, low copy no., Km^r	24
pBBR1MCS-5	Broad-host-range vector, low copy no., Gm^r	24
pBBR1MCS-5::*rfp*	pBBR1MCS-5 carrying the DsRed2 gene (GenBank accession 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

[a] Asterisks indicate constructs that were made in pBBR1MCS-2 and pBBR1MCS-5.
[b] MCS, multiple cloning site.

from binding DNA and thereby creating a negative feedback loop. Due to a strong affinity of TraM for TraR, already-existing DNA binding events also can be disrupted by the TraM-TraR interaction (4, 16, 18–20).

By analyzing the quorum-quenching (QQ) potential of NGR234, we uncovered 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). Therefore, 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 sequencing (RNA-seq) data from our laboratory suggested an AI-independent regulation of these QQ genes (44).

In the current report, we have addressed to what 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 from plant rhizospheres. We provide the 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 genes other than the *traI* and *ngrI* genes heterogeneously, suggesting that additional signaling molecules are involved in the regulation of NGR234's phenotypic heterogeneity.

MATERIALS AND 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 rifampin (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 gentamicin (30 μg/ml); NGR234 carrying pBBR1MCS-2 was cultivated in the presence of kanamycin (25 μg/ml). *E. coli* cultures were supplemented with ampicillin (75 to 100 μg/ml), gentamicin (10 μg/ml), or kanamycin (25 μg/ml), depending on the different vectors.

Molecular methods. Cloning of the various promoter fusions was performed according to standard techniques (25). Primers used are listed in Table 2. Promoter fusions and genes that were synthesized are listed in

TABLE 2 Primers synthesized in this study

Oligonucleotide	Sequence (5′–3′)[a]	Size (bp)	Promoter region used for PCR
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	

[a] Lowercase and underlined letters indicate restriction sites.

Table S1 in the supplemental material and were purchased from Eurofins MWG GmbH (Ebersberg, Germany). Plasmid transformation in *E. coli* was done by following standard heat shock protocols (25). NGR234 was manipulated using diparental conjugation by following standard protocols (16, 25). For the live/dead staining, we incubated the cells for 10 min with 5 μM propidium iodide at room temperature and verified the number of red fluorescing cells.

Single-cell fluorescence microscopic analyses in laboratory cultures. For the analyses of phenotypic heterogeneity on the single-cell level, cultures generally were grown in 20 ml TY medium supplemented with gentamicin (30 μg/ml) in 100-ml flasks under aerobic conditions at 30°C on a rotary shaker (200 rpm). Cultures were inoculated to a starting optical density at 600 nm (OD_{600}) of 0.1. One-ml samples were drawn at the indicated time points and prepared for microscopic analysis. Throughout the experiments, the optical densities of the different cultures were recorded. For the chemical complementation, 50 μM 3-oxo-C_8-HSL (Sigma-Aldrich, Heidelberg, Germany) was added to the growing cultures after 24 h, and cells were analyzed after 48 h. The phase-contrast and fluorescence images were recorded using a Zeiss AxioCam microscope with an MRm camera mounted on the fluorescence microscope (Zeiss Axio Imager.M2; Carl Zeiss AG, Oberkochen, Germany) equipped with 25×, 40×, 63×, and 100× lenses. 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 to 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 analyzed by using the MSER software (26). 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 modeling. To test the obtained data for homogeneity/heterogeneity, we independently fitted one gamma distribution at selected time points using maximum-likelihood estimation and a finite mixed model with two gamma distributions, describing the mixture of two homogeneous subpopulations. 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 (27, 28). For the mixture model, an adapted version of the expectation-maximization (EM) algorithm published by Benaglia et al. (29) 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% (wt/vol) NaClO 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 under long-day conditions (16 h of light at 80 μE) at 22°C for 4 days on plates with half-strength Murashige-Skoog media (30) and 1.5% (wt/vol) sucrose solidified with 0.4% (wt/vol) gelrite. Colocalizations of *A. thaliana* and NGR234 bacterial strains were visualized with a Leica SP5 confocal laser scanning microscope (Leica, Wetzlar, Germany) or a Zeiss Axio Imager 2 fluorescence microscope (Zeiss, Jena, Germany). Red fluorescent protein (RFP) fluorescence was detected at an excitation wavelength of 543 nm and an emission wavelength of 570 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% (wt/vol) NaClO and washed 5 times in sterile H_2O_{bidist}. To detect contaminated seeds, infected with surviving bacteria, sterilized beans were placed on 0.5× 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 (diameter, 2.85 to 3.45 mm; Carl Roth GmbH, Karlsruhe, Germany) and 5 ml sterile 0.25× Hoagland solution (31). After 8 days of growth, the Hoagland solution was sterile filtered and used as the 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; designated *rfp* in fusion constructs) (32). Since this protein had already been used successfully in laboratory and rhizosphere studies with *Rhizobium* (33) and in other prokaryotic systems (34), 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 (24) and mobilized via conjugation into NGR234. NGR234 grew in TY medium at 30°C with doubling times of 3 to 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 were taken.

For the initial setup of our system, we used the promoter of the constitutively expressed NGR234 *rpoD* gene, encoding 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 (Fig. 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 (32). RFP is known to require significant maturation times (32). Although virtually all cells expressed the RFP protein, a more detailed image analysis suggested that approximately 5.7% ± 2.3% of the cells showed a less intense fluorescence (Fig. 1B). Further, we verified that the cell counts were not affected by large numbers of dead cells. We estimated the number of living versus dead cells by employing propidium iodide staining of an NGR234 wild-type culture. It appeared that in exponential- and stationary-growth-phase cultures with an age of up to 72 h, less than 2% of dead cells were observed (data not shown).

***traI* and *ngrI* gene expression in cultures is heterogeneous on a single-cell level.** Since recent publications reported on the heterogeneous expression of AI synthase genes in different organisms (7, 8, 10, 11), we were interested in learning if NGR234 shows phenotypic heterogeneity with respect to its own AI synthase

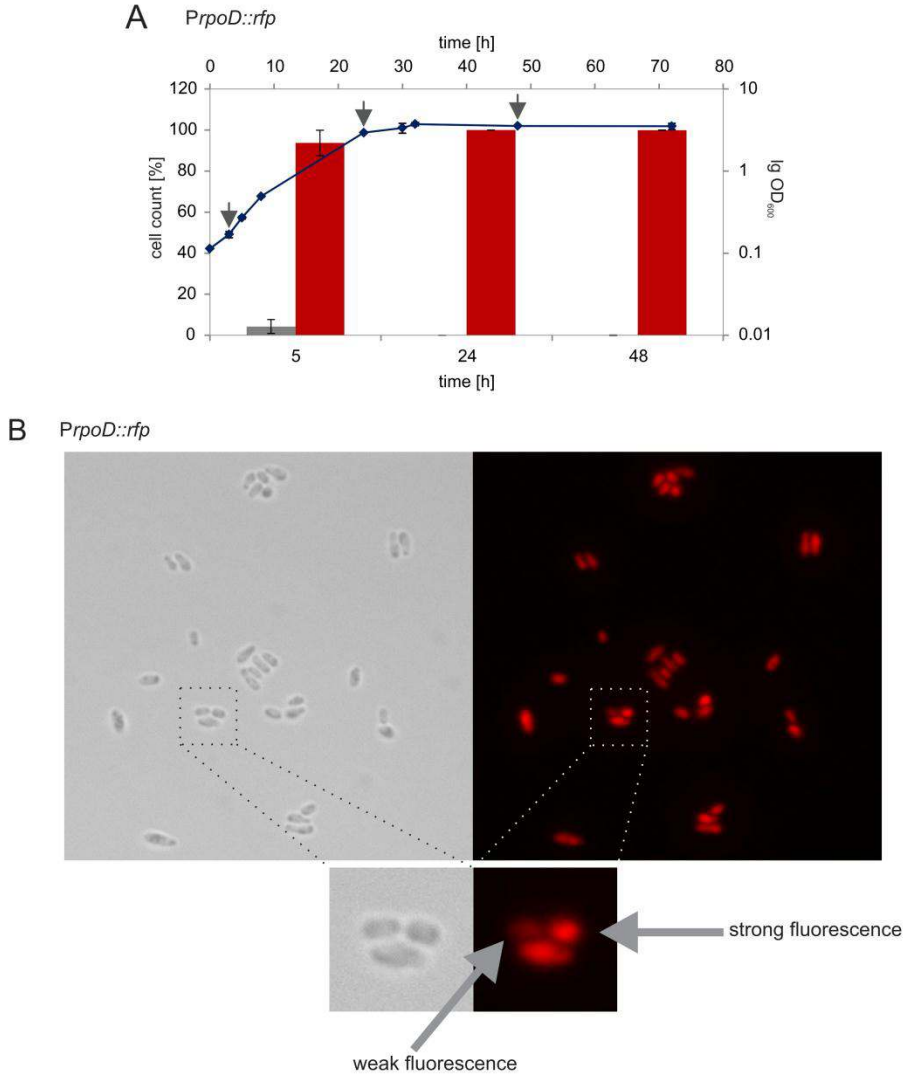

FIG 1 Analysis of gene expression of individual NGR234 cells carrying a P*rpoD*::*rfp* promoter fusion in the vector pBBR1MCS-5. (A) Percentage of cells expressing the marker genes versus nonexpressing cells. Bacteria were grown in 20-ml batch cultures in TY medium. Gray bars, percentage of cells for which no fluorescence was observed; red bars, percentage of cells showing red fluorescence. Samples were taken after 5, 24, and 48 h (gray arrows). Data are mean values from at least 500 cells for each time point and from three independent biological samples. Bars indicate the simple standard deviations. The upper line represents the corresponding growth curve. (B) Microscopic images of NGR234 cells carrying the promoter fusions as described for panel A. Visualization was performed using a Zeiss Axio Imager 2 fluorescence microscope (Zeiss, Jena, Germany). Images were obtained with a Zeiss LD Achroplan 100×/1.3-numeric-aperture oil objective. Phase-contrast and fluorescence images were obtained from the same area and matched using AxioVision (release 4.8).

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 versus those cells that showed no red fluorescence. *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 weak or strong fluorescence (*traI*-ON). As expected during stationary growth phase, almost 74.1% ± 9.1% of all cells expressed the *traI* promoter fusion (Fig. 2A). The relatively high expression of *traI* at 5 h after inoculation could be explained by 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 an *ngrI*-ON mode, while during exponential growth phase the majority of cells (60.5% ± 12.6%) was in an *ngrI*-OFF mode (Fig. 2B).

To verify the data described above and the presence of two stable subpopulations, we used mathematic modeling for the P*ngrI* reporter fusion as outlined in Materials and Methods. When we compared a homogeneous population to the mixture of two homogeneous populations described by a finite mixture model

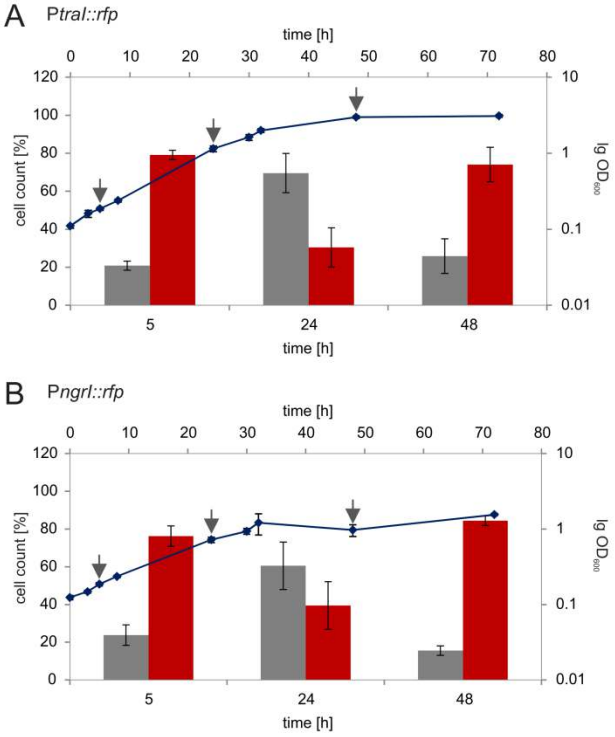

FIG 2 Phenotypic heterogeneity of NGR234 cells carrying the P*traI*::*rfp* fusion (A) and the P*ngrI*::*rfp* fusion (B). The percentage of cells expressing the marker genes versus nonexpressing cells is shown. Bacteria were grown in 20-ml batch cultures in TY medium. Gray bars, percentage of cells for which no fluorescence was observed; red bars, percentage of cells showing red fluorescence. Samples were taken after 5, 24, and 48 h (gray arrows). Data are mean values from at least 500 cells for each time point and from three independent biological samples. Bars indicate the simple standard deviations. The upper line represents the corresponding growth curve.

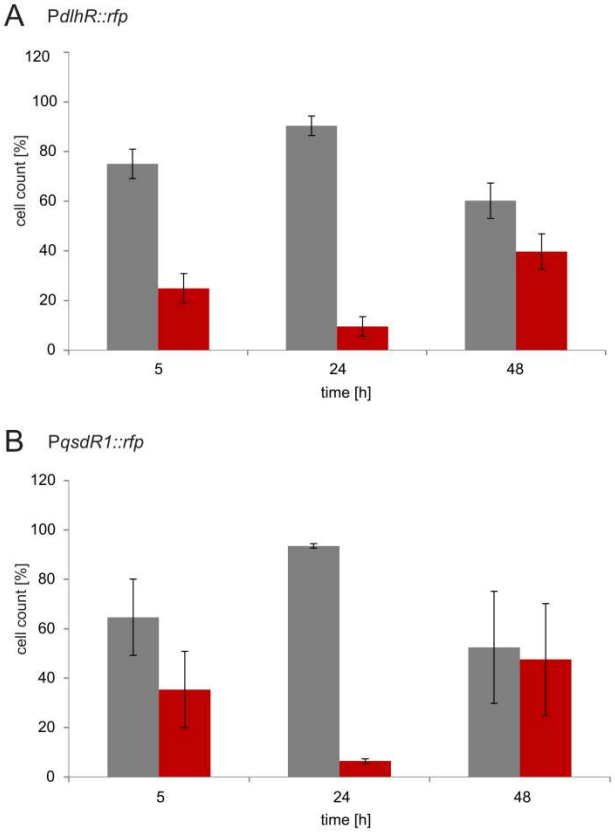

FIG 3 Fluorescence and heterogeneity analyses of the two QQ genes *dllhR* and *qsdR1* during growth in laboratory cultures. NGR234 cells carrying the P*dllhR*::*rfp* (A) and P*qsdR1*::*rfp* (B) reporter constructs. Data indicate the percentage of cells expressing the marker genes versus nonexpressing cells. Bacteria were grown in 20-ml batch cultures in TY medium. Samples were taken after 5, 24, and 48 h. Gray bars, percentage of cells for which no fluorescence was observed; red bars, percentage of cells showing red fluorescence. Data are mean values from at least 500 cells for each time point and from three independent biological samples. Bars indicate the simple standard deviations.

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 (Fig. 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, likewise were heterogeneous in their expression profiles. For this purpose we first chose two QQ genes, *dllhR* and *qsdR1*. Both genes encode 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 of the presence of AIs (21, 44). 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 (Fig. 3). Under these conditions, less than 10% of the cells were in an ON mode. This was similar for both *dllhR* and *qsdR1* of the reporter strain.

Second, 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 also is 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 (44). For the *gspD* promoter fusion, the 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 *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* gene at high levels.

Further tests using a *virB* (P*virB*::*rfp*) promoter fusion produced a similar result. The genome of NGR234 encodes two possible conjugative pili (type 4 secretion system [T4SS]); one is located on the symbiotic plasmid pNGR234*a* and is regulated within the *traI/R* regulon. The second one is 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

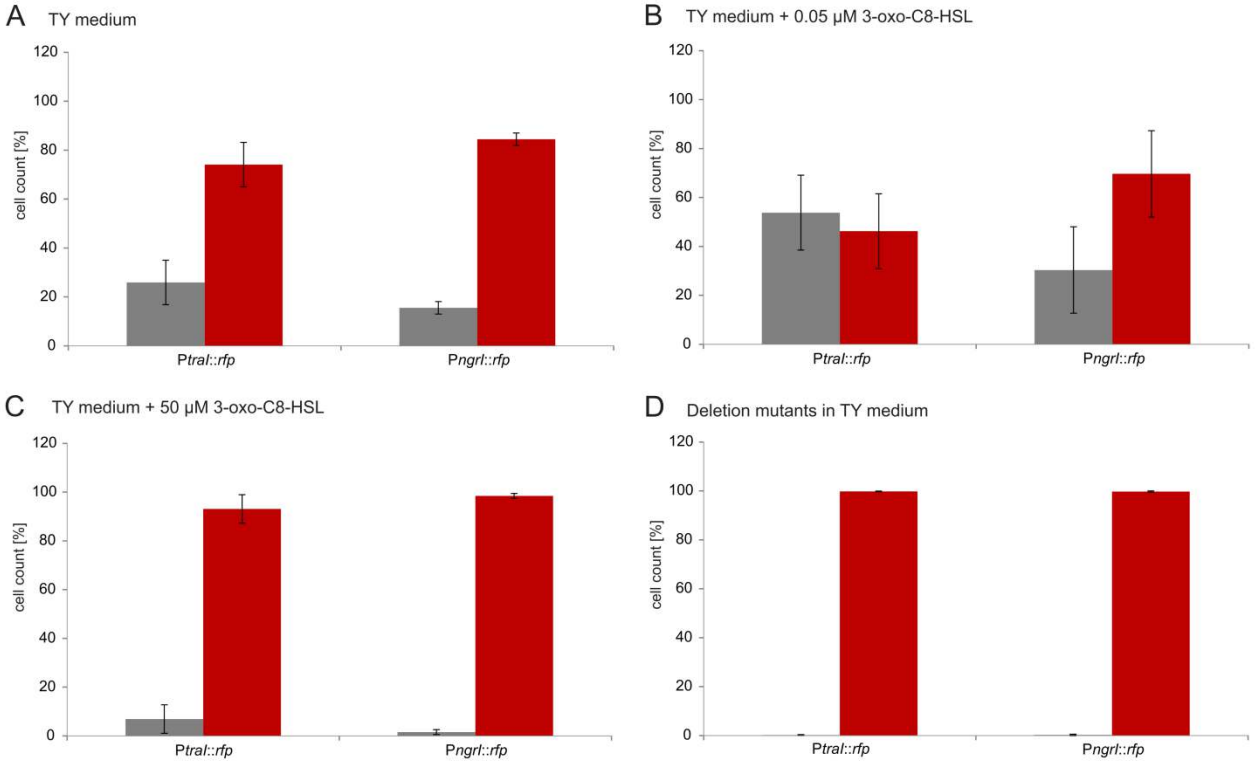

FIG 4 Relative fluorescence of NGR234 carrying P*traI*::*rfp* and the P*ngrI*::*rfp* constructs during growth in TY medium in batch cultures. (A) NGR234 after 48 h of incubation without supplements and in the wild-type background; (B) wild-type NGR234 cells carrying the same constructs but treated with 0.05 μM 3-oxo-C_8-HSL; (C) NGR234 wild type treated with 50 μM 3-oxo-C_8-HSL; and (D) NGR234-Δ*traI* and NGR234-Δ*ngrI* mutants carrying the P*traI*::*rfp* and the P*ngrI*::*rfp* constructs. Gray bars, percentage of cells for which no fluorescence was observed; red bars, percentage of cells showing red fluorescence. Data are mean values from at least 500 cells for each time point and from three independent biological samples. Bars indicate the simple standard deviations.

chose the promoter region in front of the first gene (NGR_b10240, hypothetical protein) of the T4SS operon. NGR_b10240 and the T4SS cluster most likely are regulated in an AI-independent manner and are transcribed from a single promoter located in the 5′ direction of NGR_b10240 (44). During growth in TY medium, almost equal amounts of cells were in a *virB*-ON and a *virB*-OFF mode in the exponential growth phase. However, in stationary growth phase, almost all cells were in a *virB*-ON mode.

Together, these results indicated that heterogeneous gene expression on the single-cell level not only is restricted to the *traI* and *ngrI* genes but also is 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 extent on the presence of different AHL concentrations, we added 3-oxo-C_8-HSL to liquid laboratory cultures. 3-Oxo-C_8-HSL was chosen because it is synthesized by NGR234 TraI (16). In cultures that were supplemented with 0.05 μM 3-oxo-C_8-HSL, a 2-fold increase in nonfluorescing cells was observed for the P*traI*::*rfp* and P*ngrI*::*rfp* fusions. Since the standard deviations in both tests were rather high, the increase was not significant on a single-cell level ($P < 0.05$) (Fig. 4A and B). However, the addition of 50 μM 3-oxo-C_8-HSL clearly resulted in a significantly decreased heterogeneity within the rhizobial population (Fig. 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, showed the same levels of phenotypic heterogeneity as those 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 of growth with 3-oxo-C_8-HSL 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 the NGR234-Δ*traI* mutant did not show significant levels of heterogeneity. In fact, 99.8% ± 0.2% of the counted cells were in a *traI*-ON mode,

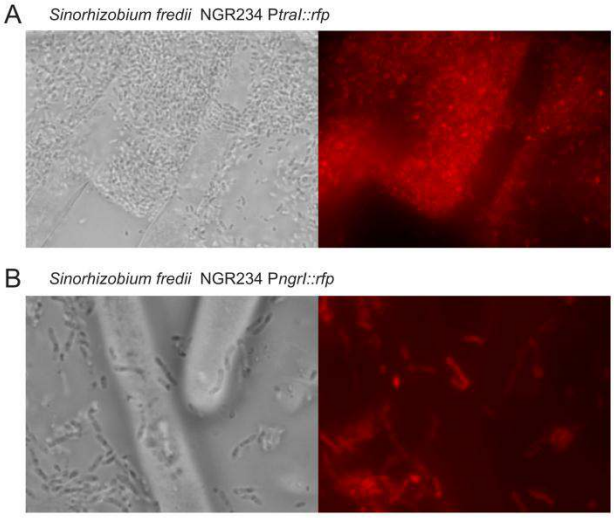

FIG 5 P*traI*::*rfp* and P*ngrI*::*rfp* gene expression of NGR234 cells in proximity of *A. thaliana* root hairs. (A) NGR234 carrying the P*traI*::*rfp* fusion and (B) NGR234 carrying the P*ngrI*::*rfp* fusion. The images show representative 3-day-old sections of root hairs. The developing seedling was treated with approximately 2×10^8 cells/ml 24 h prior to the analysis. The images on the left show light microscopic pictures, and images on the right give an overlay of the fluorescence and the light microscopy image. The phase-contrast and fluorescence images were recorded using a Zeiss AxioCam microscope with an MRm camera mounted on the fluorescence microscope (Zeiss Axio Imager M2). Images were recorded at ×100 magnification. Cells that were not growing in proximity of the *A. thaliana* roots showed normal levels of phenotypic heterogeneity (see Fig. S2B in the supplemental material).

and only 0.2% ± 0.2% did not express the P*traI*::*rfp* fusion. Similar data were obtained for the P*ngrI*::*rfp* fusion in the NGR234-Δ*ngrI* mutant background (Fig. 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* mutants by adding 50 μM 3-oxo-C_8-HSL. In these tests, we observed for the NGR234-Δ*traI* mutant 90.3% ± 4.0% of cells in the *traI*-ON mode and 9.7% ± 4.0% in the *traI*-OFF mode. In the case of the NGR234-Δ*ngrI* mutant strain, 92.0% ± 1.4% of cells were in an *ngrI*-ON mode and 8.0% ± 1.4% in an *ngrI*-OFF mode. As a further control, we used the P*dllhR*::*rfp* reporter fusion. It was heterogeneously expressed, and this phenotypic heterogeneity was not affected by the addition of 50 μM 3-oxo-C_8-HSL (see Fig. S1 in the supplemental material). Together, data obtained for AI synthase promoter fusions expressed in the 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 plant signal molecules modulate NGR234's phenotypic heterogeneity. To test this hypothesis, we incubated NGR234 carrying the P*traI* and P*ngrI* reporter fusions together with *A. thaliana*, which is a nonlegume; thus, it is not a 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 (Fig. 5). Almost all cells (>90%) attached to the *A. thaliana* roots, or cells within a distance of 1 to 2 μm were in an ON mode (Fig. 5), suggesting that unidentified plant-derived molecules influence *traI* and *ngrI* expression levels. Therefore, *A. thaliana* was an ideal model, because we observed almost no red autofluorescence that interfered with our promoter fusions (Fig. 5; also see Fig. S2A in the supplemental material).

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 the subject of 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 (Fig. 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 (Fig. 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 (Fig. 6B).

The plant-derived molecule octopine affects phenotypic heterogeneity of AI synthase and QQ genes in NGR234. Unpublished RNA-seq data from our laboratory 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 already has been linked to QS signal pathways in *A. tumefaciens* (35). To test our hypothesis, we added 50 μM octopine (Chemos GmbH, Regenstauf, Germany) to growing cultures of NGR234 carrying the P*dllhR*::*rfp* and P*qsdR1*::*rfp* fusions. While in the absence of octopine almost equal cell numbers were in an ON versus OFF mode, the addition of 50 μM octopine resulted in a strong increase of the fraction of *dllhR*-OFF cells (81.2% ± 8.2%), while 18.8% ± 8.2% of all cells remained in a *dllhR*-ON mode (Fig. 7). Further, the addition of 0.05 μM octopine already resulted in an altered ratio of cells in the ON versus OFF mode, albeit not as obvious (data not shown). The effects observed for the P*qsdR1* promoter fusion were similar but not as pronounced (Fig. 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% *ngrI*-ON cells. In summary, these data support 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 survival of bacterial populations in nature (12, 13). This phenomenon has been studied widely in a number of model organisms, such as *Bacillus subtilis* (36, 37), *E. coli* (38–40), *L. monocytogenes* (9), and *V. harveyi* (7, 8, 10, 41). In 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.

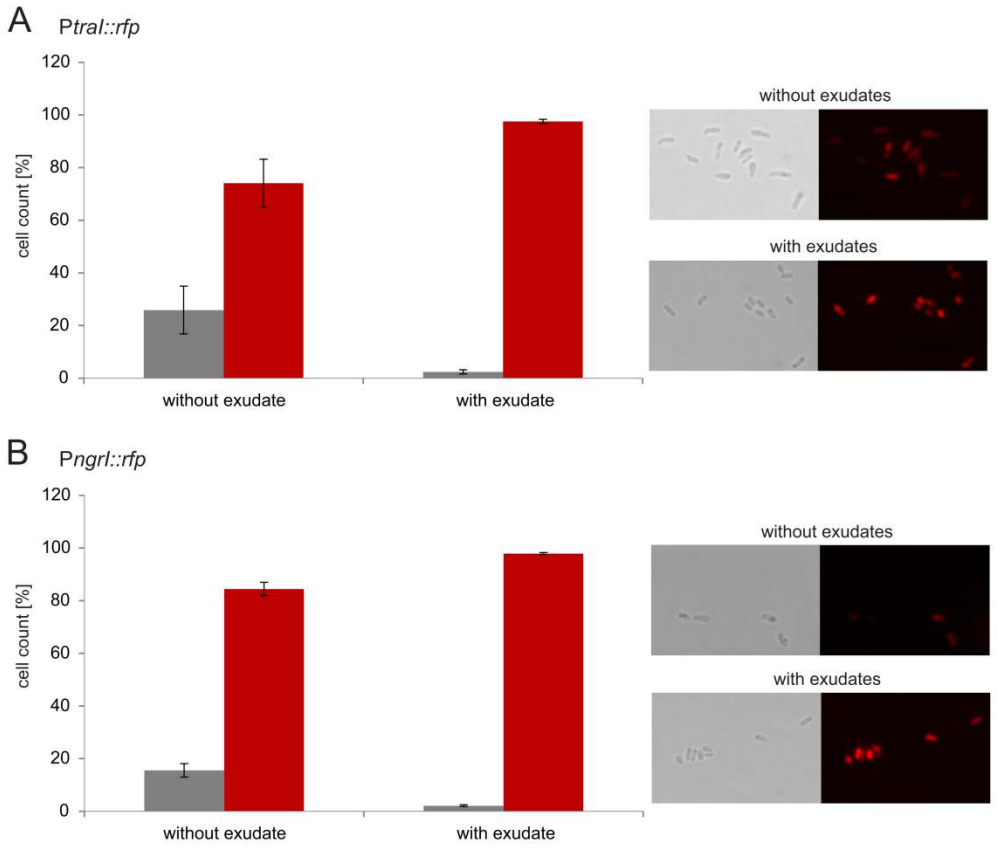

FIG 6 Fluorescence and heterogeneity analyses of NGR234 cells carrying P*traI*::*rfp* and P*ngrI*::*rfp* treated with water-soluble root exudate. Bacteria were grown in 20-ml batch cultures in TY medium. Samples were analyzed after 48 h, and root exudate of *V. unguiculata* seedlings, dissolved in Hoagland solution, was added after 24 h. Gray bars, percentage of cells for which no fluorescence was observed; red bars, percentage of cells showing red fluorescence. Data are mean values from at least 500 cells for each time point and three independent biological samples. Bars indicate the simple standard deviations.

We have chosen NGR234, because this microbe is unique in the sense that its genome carries not only two distinct AI synthase genes (17) but also a remarkable number of genes involved in the quenching of the produced AI signaling molecules (21).

In the current study, we observed that NGR234 revealed different levels of phenotypic heterogeneity in the presence of various AI concentrations and with respect to the expression of its AI synthase genes *traI* and *ngrI* during growth in laboratory cultures (Fig. 2 and 3). The heterogeneity of *traI* and *ngrI* was significantly decreased when we added high concentrations of synthesized AI (50 μM 3-oxo-C_8-HSL) or in the background of the two AI deletion mutants (Fig. 4). Heterogeneity of both AI synthases was high within the cell population in the background of the nontreated NGR234 cells or if only small amounts (0.05 μM) of AI were added. These findings support the notion that NGR234 can distinguish between very high and very low concentrations of specific AI molecules, and it suggests that phenotypic heterogeneity is a well-controlled process that depends on a more or less defined concentration range of the AI molecules. In this respect, our data are in line with earlier reports on the QS-dependent expression of phenotypic heterogeneity in other microbes, such as *V. harveyi* (7, 8, 41) and *L. monocytogenes* (9). Possible candidate sensor proteins or regulators in NGR234 that are involved in intracellular AI measurements are TraR, NgrR, and any of the other four identified individual LuxR proteins encoded by the bacterial genome.

Further data from this study indicated that not only the *traI* and *ngrI* genes but also genes linked to the degradation of QS signals (i.e., the QQ genes *dlhR* and *qsdR1*) and genes linked to the buildup of secretion apparatuses (*gspD* and *virB*) are subject to heterogeneous expression. Our recent RNA-seq data indicate that the two QQ genes and the *virB* cluster encoded on pNGR234*b* are transcribed in an AI-independent manner and the *gspD* gene in an AI-dependent manner (44). This observation is interesting, since it implies that the heterogeneous regulation of genes is a more common phenomenon in NGR234 and does not depend only on the presence of AI. This hypothesis is in part supported by observations made for AI-dependent gene expression in *V. harveyi* (8). However, the observation that NGR_b10240, *dlhR*, and *qsdR1* expression is heterogeneous suggests a major difference from the *V. harveyi* gene expression patterns of AI-independent gene regulation. In *V. harveyi*, it is postulated that AI-independent genes basically are expressed in a homogeneous manner (8). Thus, our findings suggest that factors other than AI-I control heterogeneity in NGR234. In light of this hypothesis, we observed that the heterogeneous expression of the *traI* and *ngrI* genes was affected by the presence of plant root exudates (Fig. 5 and 6). The addition of

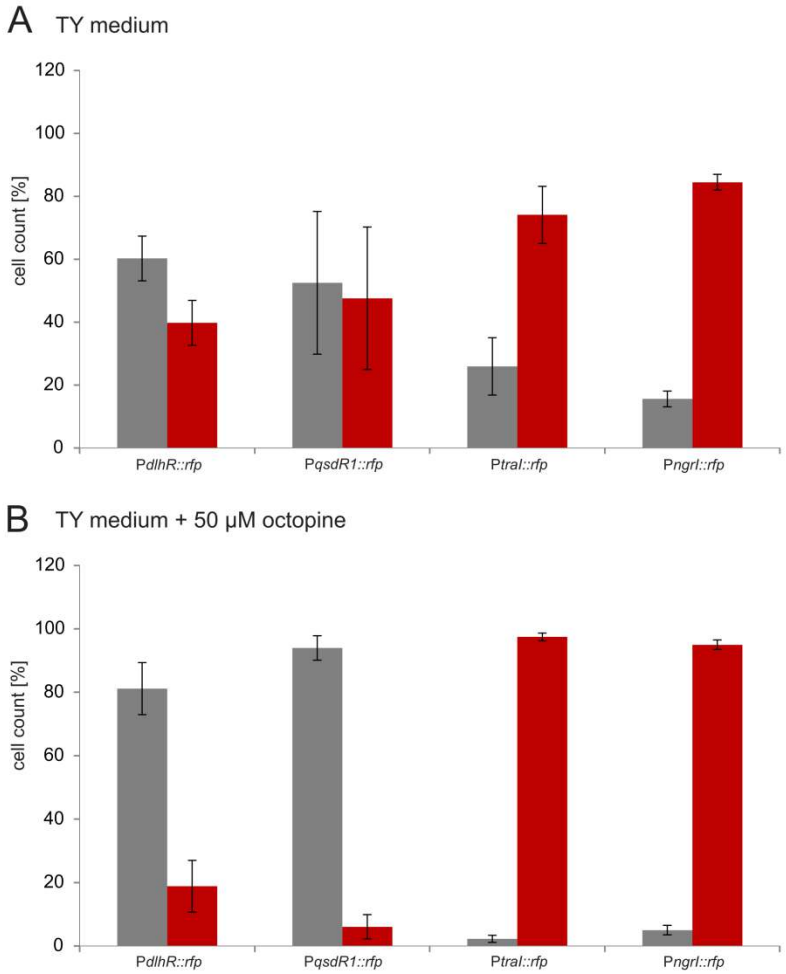

FIG 7 Fluorescence and heterogeneity analyses of the two QQ genes *dlhR* and *qsdR1* and the AI synthase genes *traI* and *ngrI* in response to added octopine. NGR234 cells carrying the different reporter constructs were treated with water (A) and with 50 μM octopine (B). Data indicate percentages of cells expressing the marker genes versus nonexpressing cells. Bacteria were grown in 20-ml batch cultures in TY medium. Samples were analyzed after 48 h. Gray bars, percentage of cells for which no fluorescence was observed; red bars, percentage of cells showing red fluorescence. Data are mean values from at least 500 cells for each time point and from three independent biological samples. Bars indicate the simple standard deviations.

small amounts of either water-soluble or methanol-extracted root exudates (data not shown) strongly altered the expression profile of both the *traI* and the *ngrI* genes and induced highly homogeneous expression (Fig. 6). Virtually the same effect was observed when NGR234 was grown in direct contact with developing seedlings of *A. thaliana* (Fig. 5). This finding implies that the plant is able to control phenotypic heterogeneity by the release of natural compounds that affect gene expression in a way that does not allow heterogeneity. Since *A. thaliana* is not a host of NGR234, we further speculate that this is a general effect independent from the host plant and that it is a result of general compound releases of developing roots.

Within this framework, we further provide evidence that octopine plays a role as a modulator of phenotypic heterogeneity in NGR234. Although legumes are not reported to produce opines *per se* within the root nodules, several rhizobial isolates are known to synthesize rhizopines, and it is generally accepted that these rhizopines are released to feed the free-living rhizobia in the rhizosphere (42). Surprisingly, adding 50 μM 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 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 (35). Furthermore, it was recently demonstrated that opines provide cooperative *A. tumefaciens* cells within groups a fitness advantage over saprophytic agrobacteria (43).

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 is 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 sig-

naling molecules. Second, the observation that plant-released compounds reduce phenotypic heterogeneity indicates that plants 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 suggests a broader role of this molecule as a common good.

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REFERENCES

1. **Engebrecht J, Nealson K, Silverman M.** 1983. Bacterial bioluminescence-isolation and genetic-analysis of functions from *Vibrio fischeri*. Cell **32:** 773–781. http://dx.doi.org/10.1016/0092-8674(83)90063-6.
2. **Engebrecht J, Silverman M.** 1984. Identification of genes and gene-products necessary for bacterial bioluminescence. Proc. Natl. Acad. Sci. U. S. A. **81:**4154–4158. http://dx.doi.org/10.1073/pnas.81.13.4154.
3. **Kaplan HB, Greenberg E.** 1985. Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. J. Bacteriol. **163:** 1210–1214.
4. **Waters CM, Bassler BL.** 2005. Quorum sensing: cell-to-cell communication in bacteria. Annu. Rev. Cell Dev. Biol. **21:**319–346. http://dx.doi.org/10.1146/annurev.cellbio.21.012704.131001.
5. **Fuqua C, Parsek MR, Greenberg EP.** 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. Annu. Rev. Genet. **35:**439–468. http://dx.doi.org/10.1146/annurev.genet.35.102401.090913.
6. **Frederix M, Downie AJ.** 2011. Quorum sensing: regulating the regulators. Adv. Microb. Physiol. **58:**23–80. http://dx.doi.org/10.1016/B978-0-12-381043-4.00002-7.
7. **Anetzberger C, Pirch T, Jung K.** 2009. Heterogeneity in quorum sensing-regulated bioluminescence of *Vibrio harveyi*. Mol. Microbiol. **73:**267–277. http://dx.doi.org/10.1111/j.1365-2958.2009.06768.x.
8. **Anetzberger C, Schell U, Jung K.** 2012. Single cell analysis of *Vibrio harveyi* uncovers functional heterogeneity in response to quorum sensing signals. BMC Microbiol. **12:**209. http://dx.doi.org/10.1186/1471-2180-12-209.
9. **Garmyn D, Gal L, Briandet R, Guilbaud M, Lemaître JP, Hartmann A, Piveteau P.** 2011. Evidence of autoinduction heterogeneity via expression of the Agr system of *Listeria monocytogenes* at the single-cell level. Appl. Environ. Microbiol. **77:**6286–6289. http://dx.doi.org/10.1128/AEM.02891-10.
10. **Pérez PD, Hagen SJ.** 2010. Heterogeneous response to a quorum-sensing signal in the luminescence of individual *Vibrio fischeri*. PLoS One **5:**e001547. http://dx.doi.org/10.1371/journal.pone.0015473.
11. **Thomas VC, Hiromasa Y, Harms N, Thurlow L, Tomich J, Hancock LE.** 2009. A fratricidal mechanism is responsible for eDNA release and contributes to biofilm development of *Enterococcus faecalis*. Mol. Microbiol. **72:**1022–1036. http://dx.doi.org/10.1111/j.1365-2958.2009.06703.x.
12. **Veening JW, Smits WK, Kuipers OP.** 2008. Bistability, epigenetics, and bet-hedging in bacteria. Annu. Rev. Microbiol. **62:**193–210. http://dx.doi.org/10.1146/annurev.micro.62.081307.163002.
13. **Smits WK, Kuipers OP, Veening JW.** 2006. Phenotypic variation in bacteria: the role of feedback regulation. Nat. Rev. Microbiol. **4:**259–271. http://dx.doi.org/10.1038/nrmicro1381.
14. **Trinick MJ.** 1980. Relationships amongst the fast-growing *Rhizobia* of *Lablab purpureus*, *Leucaena leucocephala*, *Mimosa* spp, *Acacia farnesiana* and *Sesbania grandiflora* and their affinities with other rhizobial groups. J. Appl. Microbiol. **49:**39–53.
15. **Pueppke SG, Broughton WJ.** 1999. *Rhizobium* sp. strain NGR234 and *R. fredii* USDA257 share exceptionally broad, nested host ranges. Mol. Plant Microbe Interact. **12:**293–318. http://dx.doi.org/10.1094/MPMI.1999.12.4.293.
16. **He XS, Chang W, Pierce DL, Seib LO, Wagner J, Fuqua C.** 2003. Quorum sensing in *Rhizobium* sp strain NGR234 regulates conjugal transfer (*tra*) gene expression and influences growth rate. J. Bacteriol. **185:**809–822. http://dx.doi.org/10.1128/JB.185.3.809-822.2003.
17. **Schmeisser C, Liesegang H, Krysciak D, Bakkou N, Le Quéré A, Wollherr A, Heinemeyer I, Morgenstern B, Pommerening-Röser A, Flores M, Palacios R, Brenner S, Gottschalk G, Schmitz RA, Broughton WJ, Perret X, Strittmatter AW, Streit WR.** 2009. *Rhizobium* sp. strain NGR234 possesses a remarkable number of secretion systems. Appl. Environ. Microbiol. **75:** 4035–4045. http://dx.doi.org/10.1128/AEM.00515-09.
18. **González JE, Marketon MM.** 2003. Quorum sensing in nitrogen-fixing rhizobia. Microbiol. Mol. Biol. Rev. **67:**574–592. http://dx.doi.org/10.1128/MMBR.67.4.574-592.2003.
19. **Chen GZ, Jeffrey PD, Fuqua C, Shi YG, Chen LL.** 2007. Structural basis for antiactivation in bacterial quorum sensing. Proc. Natl. Acad. Sci. U. S. A. **104:**16474–16479. http://dx.doi.org/10.1073/pnas.0704843104.
20. **Churchill MEA, Chen LL.** 2011. Structural basis of acyl-homoserine lactone-dependent signaling. Chem. Rev. **111:**68–85. http://dx.doi.org/10.1021/cr1000817.
21. **Krysciak D, Schmeisser C, Preuss S, Riethausen J, Quitschau M, Grond S, Streit WR.** 2011. Involvement of multiple loci in quorum quenching of autoinducer I molecules in the nitrogen-fixing symbiont *Rhizobium* (*Sinorhizobium*) sp. strain NGR234. Appl. Environ. Microbiol. **77:**5089–5099. http://dx.doi.org/10.1128/AEM.00112-11.
22. **Hanahan D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580. http://dx.doi.org/10.1016/S0022-2836(83)80284-8.
23. **Simon R, Priefer U, Pühler A.** 1983. A broad host range mobilization system for *in vivo* genetic-engineering transposon mutagenesis in Gram-negative bacteria. Nat. Biotechnol. **1:**784–791. http://dx.doi.org/10.1038/nbt1183-784.
24. **Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM, Peterson KM.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175–176. http://dx.doi.org/10.1016/0378-1119(95)00584-1.
25. **Sambrook J, Russell DW.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
26. **Matas J, Chum O, Urban M, Pajdla T.** 2004. Robust wide-baseline stereo from maximally stable extremal regions. Image Vision Comput. **22:**761–767. http://dx.doi.org/10.1016/j.imavis.2004.02.006.
27. **Müller J, Kuttler C, Hense BA, Zeiser S, Liebscher V.** 2008. Transcription, intercellular variability and correlated random walk. Math. Biosci. **216:**30–39. http://dx.doi.org/10.1016/j.mbs.2008.08.003.
28. **Lipniacki T, Paszek P, Marciniak-Czochra A, Brasier AR, Kimmel M.** 2006. Transcriptional stochasticity in gene expression. J. Theor. Biol. **238:** 348–367. http://dx.doi.org/10.1016/j.jtbi.2005.05.032.
29. **Benaglia T, Chauveau D, Hunter DR, Young DSY.** 2009. An R package for analyzing finite mixture models. J. Stat. Softw. **32:**1–29.
30. **Murashige T, Skoog F.** 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plantarum **15:**473–497. http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x.
31. **Hoagland DR, Arnon DI.** 1950. The water-culture method for growing plants without soil. Calif. Agric. Exp. Station C **347:**1–32.
32. **Baird GS, Zacharias DA, Tsien RY.** 2000. Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. Proc. Natl. Acad. Sci. U. S. A. **97:**11984–11989. http://dx.doi.org/10.1073/pnas.97.22.11984.
33. **Gage DJ.** 2002. Analysis of infection thread development using Gfp- and DsRed-expressing *Sinorhizobium meliloti*. J. Bacteriol. **184:**7042–7046. http://dx.doi.org/10.1128/JB.184.24.7042-7046.2002.
34. **Uliczka F, Pisano F, Kochut A, Opitz W, Herbst K, Stolz T, Dersch P.** 2011. Monitoring of gene expression in bacteria during infections using an adaptable set of bioluminescent, fluorescent and colorigenic fusion vectors. PLoS One **6:**e20425. http://dx.doi.org/10.1371/journal.pone.0020425.
35. **Oger P, Kim K-S, Sackett RL, Piper KR, Farrand SK.** 1998. Octopine-type Ti plasmids code for a mannopine-inducible dominant-negative allele of *traR*, the quorum-sensing activator that regulates Ti plasmid conjugal transfer. Mol. Microbiol. **27:**277–288. http://dx.doi.org/10.1046/j.1365-2958.1998.00671.x.
36. **Ozbudak EM, Thattai M, Kurtser I, Grossman AD, van Oudenaarden A.** 2002. Regulation of noise in the expression of a single gene. Nat. Genet. **31:**69–73. http://dx.doi.org/10.1038/ng869.
37. **Lopez D, Vlamakis H, Kolter R.** 2009. Generation of multiple cell types in *Bacillus subtilis*. FEMS Microbiol. Rev. **33:**152–163. http://dx.doi.org/10.1111/j.1574-6976.2008.00148.x.
38. **Rosenfeld N, Young JW, Alon U, Swain PS, Elowitz MB.** 2005. Gene regulation at the single-cell level. Science **307:**1962–1965. http://dx.doi.org/10.1126/science.1106914.

39. **Elowitz MB, Levine AJ, Siggia ED, Swain PS.** 2002. Stochastic gene expression in a single cell. Science **297:**1183–1186. http://dx.doi.org/10.1126/science.1070919.
40. **Kamenšek S, Podlesek Z, Gillor O, Zgur-Bertok D.** 2010. Genes regulated by the *Escherichia coli* SOS repressor LexA exhibit heterogeneous expression. BMC Microbiol. **10:**283. http://dx.doi.org/10.1186/1471-2180-10-283.
41. **Long T, Tu KC, Wang Y, Mehta P, Ong NP, Bassler BL, Wingreen NS.** 2009. Quantifying the integration of quorum-sensing signals with single-cell resolution. PLoS Biol. **7:**e1000068. http://dx.doi.org/10.1371/journal.pbio.1000068.
42. **Murphy PJ, Heycke N, Trenz SP, Ratet P, de Bruijn FJ, Schell J.** 1988. Synthesis of an opine-like compound, a rhizopine, in alfalfa nodules is symbiotically regulated. Proc. Natl. Acad. Sci. U. S. A. **85:**9133–9137. http://dx.doi.org/10.1073/pnas.85.23.9133.
43. **Platt TG, Fuqua C, Bever JD.** 2012. Resource and competitive dynamics shape the benefits of public goods cooperation in plant pathogen. Evolution **66:**1953–1965. http://dx.doi.org/10.1111/j.1558-5646.2011.01571.x.
44. **Krysciak D, Grote J, Rodriguez Orbegoso M, Utpatel C, Förstner KU, Li L, Schmeisser C, Krishnan HB, Streit WR.** 2014. RNA sequencing analysis of the broad-host-range strain *Sinorhizobium fredii* NGR234 identifies a large set of genes linked to quorum sensing-dependent regulation in the background of a *traI* and *ngrI* deletion mutant. **80:**5655–5671. http://dx.doi.org/10.1128/AEM.01835-14.

5 The absence of *N*-acyl homoserine lactones results in a flavonoid-independent transcription of the symbiotic genes in *Sinorhizobium fredii* NGR234

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Unpublished

ABSTRACT

Almost 30 years ago it was shown that plant-released flavonoids induce nodulation (*nod*) genes in the alphaproteobacteria commonly called rhizobia. The corresponding Nod proteins produce Nod factors also referred to as chito-lipo-oligosaccharides. Nod factors are responsible for root hair curling as one of the primary responses during the onset of root nodule development. This signal exchange is believed to be essential for the initiation of the nitrogen fixation symbiosis in rhizobia affiliated with the alphaproteobacterial phylum. Here we provide evidence that in the broad host range strain *Sinorhizobium fredii* NGR234 the complete lack of autoinducer molecules results in an elevated copy number of its symbiotic plasmid pNGR234*a* and an up to 40-fold increased transcription of all symbiotic genes on this replicon. Thereby *nod* gene transcription is at levels high enough to allow Nod factor production and root hair curling in the absence of plant-released flavonoids. From an evolutionary point of view this feature appears to be common to broad host range strains as it is linked to a highly conserved intergenic region of 803 bp between the *traI* and *repA* genes of *repABC* type plasmids and the occurrence of two novel, previously not identified, open reading frames, designated *repX* and *repA0.* This observation implies that quorum sensing regulation attenuates the response to plant-released flavonoids and it suggests that on a single cell level, when quorum sensing signals are not yet available, infection can be successful even in the absence of plant-released flavonoids.

INTRODUCTION

The rhizobium–legume symbiosis is considered to be one of the best-studied model systems of mutualistic interactions between eukaryotic hosts and the alpha- and betaproteobacteria that are commonly called 'rhizobia'. The symbiosis is initiated by a signal exchange between the legume plant and the microbe that ideally results in the production of nitrogen-fixing plant root nodules[1-3]. The infection depends in part on the release of plant-produced specific *nod* gene-inducing flavonoids[2,4-6]. These polyphenolic compounds are perceived by bacterial sensors, i.e. NodD proteins that themselves induce expression of genes responsible for the synthesis of lipo-chito-oligo-saccharidic Nod factors. The Nod factors trigger the nodulation pathway in susceptible legumes. Then, the rhizobia enter the root tissue through infection threads in the root hairs, find their way into the cortex, multiply and colonize the intracellular spaces in root nodules[2,7]. While most rhizobia and closely related species are known to establish a symbiosis with a rather small number of plant legume genera some rhizobia have evolved mechanisms that allow them to nodulate a larger variety of legume plants. These strains have been designated 'broad host range' strains and they are promiscuous with respect to the selection of their host plants[8]. *Sinorhizobium fredii* NGR234 (hereafter NGR234) is well known for its exceptional wide host range. It nodulates more than 120 different genera of legumes and the non-legume *Parasponia andersonii*[8,9].

The NGR234 tripartite 6.9 Mbp genome encodes for two distinct quorum sensing (QS) systems that regulate the expression of genes involved in the cell envelope biosynthesis, motility, cofactor metabolism and secretion related genes in this bacterium[10]. One of the autoinducer (AI) synthases is designated *traI* and the second gene is designated *ngrI*. TraI synthesizes an *N*-(3-oxooctanoyl-)L-homoserine lactone (3-oxo-C8-HSL) and *ngrI* encodes for a synthase most likely producing an *N*-(3-oxododecanoly-)L-homoserine lactone. NgrI is encoded on the bacterial chromosome together with its cognate receptor NgrR and TraI is encoded on the 0.54 Mbp symbiotic replicon pNGR234*a*[10-12]. pNGR234*a* is a *repABC* type plasmid similar to the *Agrobacterium tumefaciens* Ti plasmid and many of the known rhizobial symbiotic plasmids[13,14]. These plasmids are exclusively found within the alphaproteobacteria-clade. The NGR234 *traI* gene is part of a conserved cluster that shares a high degree of synteny with the well-studied Ti plasmid of *A. tumefaciens*[11,12,14]. This

conserved cluster includes the *repABC* genes, the *traI* gene as well as other genes involved in conjugative DNA transfer and plasmid replication.

Due to the obvious synteny between the *repABC* plasmids harbored in both microorganisms and the known AI-dependent increase in copy number of the *A. tumefaciens* Ti plasmid, we asked if the copy number control of pNGR234*a* in NGR234 is regulated in a similar way as it has been published for *A. tumefaciens*[15,16].

RESULTS AND DISCUSSION

Analysis of the traI-repA region indicates significant differences between A. tumefaciens and known S. fredii broad host range strains.

The pNGR234*a* replicon belongs to the *repABC*-type plasmids. There is a high degree of synteny with respect to the overall organization of the region encoding the *traI* and the *repABC* genes with *A. tumefaciens* and many other *repABC*-type plasmids. Since it is well-known that in *A. tumefaciens* the *repABC* copy number is increased in the presence of 3-oxo-C8-HSL, we wondered, if in NGR234 a similar effect on the copy number could be observed and if any of the above identified proteins would influence this regulatory circuit. To address these questions, we analysed the copy number of the pNGR234*a* symbiotic plasmid using qPCRs in the background of the parent strain and in the background of various recently constructed AI synthase mutants[10]. In addition, we constructed a *traR* and a *traM* mutant as outlined in the material and methods section. The deletion mutants were all verified by PCR analyses and sequencing of the complete genome using Miseq technology generating 4-5.5 million reads for each mutant.

Analogous to the observations made for *A. tumefaciens*, the copy number of pNGR234*a* increased in response to the addition of micro molar concentrations of exogenous AI. While the addition of 0.05 and 5 µM AI resulted in a 2.7-3.9 fold higher copy number of pNGR234*a*, the addition of 50 µM AI had no significant impact on the copy number of pNGR234*a* in the parent strain (Table 1). The supplementation with octopine, apigenin or DMSO resulted in a 1.7-3.8-fold increase in copy number. Octopine was used because it is known to increase copy number of the Ti plasmid in *A. tumefaciens*[17]. Apigenin was used as a *nod* gene inducer[18]. DMSO was included as a further control because it was used as a solvent for the AI molecules.

The copy number of pNGR234*a* appeared to be slightly increased in the background of a mutant carrying a single deletion in *traI*. However, no significant increase in copy number was observed for mutations in *ngrI*, *traR* and *traM* (Table 1)

Surprisingly, the complete absence of any AI resulted in an elevated copy number of pNGR234*a*. In the background of a double deletion mutant NGR234-Δ*traI*-Δ*ngrI* the copy number of pNGR234*a* was increased by a factor of 2.3-2.8 when no AI or apigenin were added (Table 1). Further, adding AI molecules at either 0.05 µM, 5 µM or 50 µM

concentration to cultures of NGR234-Δ*traI*-Δ*ngrI* resulted in a dramatic increase in copy number of up to 11.9-fold (Table 1).

Within this framework it is notably, that In *A. tumefaciens* the copy number of the Ti plasmid is upregulated by the introduction of an additional regulator copy of TraR and by the addition of external AI molecules[15]. These findings are in line with the results of this study, where the addition of external AI leads to an increased copy number of about 3-fold. In addition to the findings in *A. tumefaciens*, NGR234 shows an increased copy number in total absence of any AHL-type autoinducer. This finding was unexpected and indicated that possibly different or additional regulatory elements were involved in copy number control in pNGR234*a* compared to the well-characterized *A. tumefaciens* systems. In *A. tumefaciens* the TraR- and QS-dependent expression of the *repABC* genes has been studied extensively over the last decade[14,15,19]. The *repABC* genes together with a small non-coding RNA (ncRNA) (*repE*/inc-alpha) are responsible for the copy number control of the corresponding symplasmid. Thereby, increased levels of AI or plant released Nopaline induce an elevated transcription of the *repABC* genes resulting in a 5-8-fold increased copy number. Furthermore, the increased copy number corresponds with a three to fourfold-increase in tumorigenesis[15,16,20]. In addition to the QS-dependent copy number control, phenolic compounds released by the plants have influence on the copy number via the two component regulatory system VirA/VirG[19,21]. It is notably, that in the *A. tumefaciens* 363 bp region between *traI* and *repA* four distinct promoter sites, two *tra* boxes for binding of the regulator TraR and binding sites for VirG and CtrA have been identified[14,15].

Two novel, previously not detected, ORFs within the traI-repA intergenic region.

In *repABC* type plasmids the copy number is controlled by *repA*[22]. Because of the above-observed variations in copy number we asked, if in NGR234 the *repA* or flanking genes such as *traI* would be different from those of other known *repABC* plasmids (Figure 1A). A more detailed analysis of pNGR234*a traI-repA* intergenic region indicated that the region is twice as large as observed for *A. tumefaciens* and most other rhizobial isolates (Figure 1A and Table 2). We observed that in NGR234 and the two other broad host range strains HH103 and USDA257 an 803 bp long intergenic region separates *traI* and *repA*. In *A. tumefaciens*, *S. meliloti*, and *R. etli* this intergenic region is usually not larger than 376 bp and only in

R. rhizogenes NBRC13257 a 441 bp intergenic region was observed (Table 2). In NGR234, USDA257 and HH103 this intergenic region codes for two hitherto not identified genes. We have designated these genes *repX* and *repA0* (Figure 1A). *RepX* probably codes for a 51 aa protein and *repA0* for a 143 aa protein. The analysis of both sequences with PROMALS3D (http://prodata.swmed.edu/promals3d/promals3d.php) revealed for the longer variant of *repX* two β-sheet-regions and one α-helix-region and in case of *repA0* two β-sheets and four α-helices secondary structure regions. No known conserved domains could be detected in both peptide sequences with the conserved domain search provided by NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Although both genes have not been reported in other rhizobial species a detailed analysis of the intergenic region of *A. tumefaciens* and others indicated that *repX* is present in some of the closely related species but often in a shortened version. *RepA0* could not be identified in other strains (Figure 1B). Notably, the expression of *repA0* on a self replicable plasmid resulted in a 3.3.-4.1-fold increased copy number of pNGR234*a.* Overexpression of *repX* had no influence on the pNGR234*a* copy number.

RNA-seq data identify possible regulatory elements involved in copy number control of pNGR234a.

To further verify that the lack of AI and the increase in copy number of pNGR234*a* would have a global effect on the transcription of pNGR234*a* we used RNA-seq in the background of the NGR234-Δ*traI*-Δ*ngrI* double deletion mutant and compared the data to the parent strain. Transcriptomes of cells that were grown to early stationary growth phase were analysed. We choose this time point, since our previous data indicated that during the onset of stationary growth phase many QS-dependent processes were turned on. For the experiments cells were grown for 48 h to a final OD_{600} not greater than 9.1 prior to total RNA extraction (Table S1). For all samples cDNA libraries were constructed and sequenced as described in material and methods. For each of the treatments three independent biological experiments were performed and examined by RNA-seq. Protocols for data analyses were employed as previously published[10]. Alignments were established and for each sample a min. of 0.5-2.2 million cDNA reads could be uniquely mapped to the NGR234 genome resulting in 3.9-5.2 million uniquely mapped reads per treatment (Table S1).

In the further analysis of RNA-seq data we considered genes with a fold-change of ≥ 2.0 and an adjusted p-value of ≤ 0.05 as statistically significant and differentially expressed between two distinct. Only those values that complied with both requirements were used for subsequent analyses.

The data obtained using RNA-seq surprisingly indicated, that in the double deletion mutant NGR234-△*traI*-△*ngrI* many more genes are differentially regulated than in any of the previously reported single AI synthase deletion mutants compared to the parent strain. Altogether more than 1,261 genes were differentially regulated in the double mutant. This equals almost 20 % of all predicted genes. Of these, the majority was upregulated (75 %) and regulated through sense transcripts (68 % of all regulated genes). Given the large differences in size of the three replicons, we observed a rather unequal distribution of the regulated genes over the three replicons (pNGR243*a* – 32.43 %; pNGR243*b* – 21.33 %; cNGR234 – 46.24 %). Additionally, our data indicated that of the 418 previously predicted genes on pNGR234*a* 408 (98 %) were upregulated. For 357 of the 408 genes the sense and anti-sense transcripts were increased. However, sense transcripts dominated. In contrast, on pNGR234*b* 269 genes were regulated (11.5 %) and only two of these genes were simultaneously regulated via sense and antisense transcripts. Similarly, on the chromosome 583 genes (16.05 %) were regulated and 15 genes were simultaneously regulated via sense and antisense transcripts. Within this framework, the most surprising observation was that virtually all the pNGR234a encoded genes were transcribed at significantly higher levels. Thereby, the average of the pNGR234*a* genes was increased by a factor of 7. However, the *repABC* showed a 23-43-fold increased level of transcription (Figure 2 and Table 3). It is further noteworthy that the transcription of the two predicted ncRNAs (NGR_a00030, an antisense RNA regulator of the RepB translation and NGR_a00040, a negative antisense RNA regulator of RepC) flanking *repB* and *repC* was also strongly upregulated in the double deletion mutant. NGR_a00030 is most likely a homologue of the *repE/incA* gene[23].

Reporter gene studies, qRT-PCR and immune blotting confirm the elevated transcription of nod, nop, repA and *NGR_a00860 gene expression.*

Further tests with reporter strains using the *Escherichia coli lacZ* gene fused to the *nodABC* promoter and the *nopB* promoter confirmed the elevated expression of these genes

(Table 4). The expression was most pronounced after 120 hours of growth. For the P*nodABC* reporter fusion, we observed an eight-fold increased transcription in the double deletion mutant in the absence of apigenin vs. the parent strain (Table 4). In the presence of apigenin, the transcription in the mutant was 3.7-fold higher compared to the induced wild type. Similar expression levels were observed for the P*nopB* reporter fusion where we observed a 20-fold higher level of *lacZ* expression in NGR234-△*traI*-△*ngrI* compared to the untreated parent strain; and in the presence of apigenin the mutant produced 333-fold more ß-galactosidase compared to the wild type (Table 4). Altogether these data implied that in the absence of any AI an increased level of transcription of the pNGR234*a* genes can be observed and that this increased transcription is in part be linked to an increased copy number. Further qRT-PCR test results confirmed largely the above made findings (Table S3 and S4). Thereby, we assayed the transcription of genes NGR_a00860 and NGR_a00010.

Additionally, we analysed the extracellular protein profile of the NGR234 wild type and NGR234-Δ*traI*-Δ*ngrI* with respect to the production of selected Nop proteins. Nop proteins are secreted through the T3SS-I, which is encoded on pNGR234*a*. Their expression is under the control of a nod-dependent promoter[1]. Nop proteins in the supernatants were detected using a cocktail of highly specific antibodies as previously described[24]. These immunoblot analyses further indicated that Nops are produced and secreted in the mutant strain at higher levels compared to the parent strain (Figure 3). Consistent with the ß-galactosidase data the highest expression was detected in the mutant strain after 120 hours and in the presence of apigenin. No Nops were detected in the cultures that were not treated with apigenin. Altogether these tests support the above made findings with respect to the increased transcription of all the pNGR234*a*-harboured genes in NGR234-△*traI*-△*ngrI* in the presence and in the absence of added apigenin or AI.

NGR234-△traI-△ngrI induces root hair curling in the absence of plant-released flavonoids.

While it is well-known that in *A. tumefaciens an elevated* copy number of the Ti plasmid is associated with an increased level of tumorgenesis[15] we asked if in NGR234 an increased copy number of the symplasmid would have a plant phenotype, as well. An early response to plant released flavonoids is the activation of the *nod* gene transcription, which leads to the

production of Nod factors. The release of these factors induces, through a Nod factor specific signal cascade, the curling of the root hairs, which initiates the symbiosis process[25]. Up to know it was thought that the symbiosis process can only be initiated by the activation of *nod* gene transcription induced through flavonoids or related plant-released molecules[26,27]. In the light of the observations made above, we asked if the elevated transcription of all genes on pNGR234*a* in NGR234-△*traI*-△*ngrI* would be sufficient to allow production of Nod factors.

Therefore, we extracted Nod factors from culture supernatants of the wt and the double deletion mutant grown with and without apigenin. These tests indicated that Nod factors produced from non-induced NGR234-△*traI*-△*ngrI* cells were sufficient to induce root hair curling on germinating *Vigna unguculata* roots (Figure 4A). In contrast, when we tested Nod factor extracts of non-induced wt cells, no root hair curling was observed after a 24 h time period (Figure 4B). A further test implied that mutant cells treated with 1 µM apigenin induced root hair curling already after 4 hours, like the induced wild type (Figure 4C and 4D).

Altogether these data imply that in NGR234 the absence of AI molecules causes an increased copy number of pNGR234*a* and an elevated transcription of virtually all symbiotic genes. Whether the copy number alone is responsible for the observed elevated expression needs to be exploited in future work. The findings made in this work suggest that NGR234 and probably other broad host range strains are capable to induce root hair curing in the absence of plant flavonoids. Since it was assumed that plant flavonoids are specific signals, the observation here that NGR234 induces root hair curling in the absence of such specific signal suggests that this may be part of its broad host range strategy and that it does not necessarily need specific-flavonoids. From a microbiological point of view it is likely that single cells infect the root tissue and not high density populations. Thus under these condition AI molecules will only be present at very low levels if at all. Therefore it might be advantageous for NGR234 to activate its nod gene transcription in the absence of AI molecules to be able to infect plant roots even though the matching flavonoid signal is absent.

MATERIAL AND METHODS

Bacterial strains and growth conditions.

Sinorhizobium fredii NGR234 was routinely grown at 30°C in liquid TY medium (0.5 % tryptone, 0.25 % yeast extract, 10 mM $CaCl_2$, pH 7.0) or YEM medium (55 mM mannitol, 0.1 % yeast extract, 3 mM K_2HPO_4, 0.8 mM $MgSO_4$, 1.7 mM NaCl, 0.013 mM $CaCl_2$, 0.14 mM $FeCl_3$) at 160 rpm and supplemented with rifampicin (25 µg/ml).

Construction of NGR234 deletion mutants.

Molecular cloning steps were in general done as outlined in the reference[28] and mutant strains NGR234-Δ*traR* and NGR234-Δ*traM* were constructed as previously described[29]. For the construction of a deletion mutant in the *traR* gene a 650 bp PCR fragment containing the 249 bp upstream region of *traR* and the 399 bp downstream fragment flanking the *traR* gene was cloned in the suicide vector pNPTS138-R6KT[30]. For the deletion mutant in the *traM* gene, a flanking region of 485 bp upstream and 744 bp downstream were amplified, ligated and cloned into pNPTS138-R6KT. For this purpose the different PCR fragments were amplified from genomic DNA of NGR234 using primers as indicated in Table S3. The resulting constructs (pNPTS138-R6KT::△*traR* and pNPTS138-R6KT::△*traM*) were transformed into NGR234 by conjugation. Single recombinant clones carrying this construct were selected on TY medium containing kanamycin and rifampicin. To obtain double recombinant mutants, bacteria were cultured overnight in liquid TY medium lacking the antibiotics and plated on TY medium in the presence of 10 % sucrose the next day. In our deletion mutants the entire genes were deleted starting from the ATG to the last codon of the gene. The correctness of the mutants was verified by PCR and sequencing.

Preparation of transcriptome samples.

The different NGR234 cultures used in this study are summarized in Table 1. Prior to cultivation of large 200 ml cultures, precultures were established from cryocultures in 5 ml TY medium and cultivated at 30°C and 200 rpm. For the transcriptome analyses of early stationary growth phase cultures 200 ml TY medium were inoculated with freshly grown precultures of NGR234 wild type as well as the mutant strains and cultivated for approximately 48 h at 30°C and 200 rpm as batch cultures (Table 1; samples No. 1-6; treatment A-C). After reaching an OD_{600} of 3.1-3.3 cultures were separated into fractions of

50 ml, which were then transferred into falcon tubes containing 10 ml ethanol/phenol (95:5). Samples were mixed well, directly frozen in liquid nitrogen and stored at -70°C until further use. For transcriptome analyses of early exponential growth phase cultures 200 ml TY medium were inoculated with a freshly grown preculture of NGR234 wild type and cultivated at 30°C, 200 rpm till they reached an OD_{600} of 0.1 (Table 1; samples No. 7-12; treatment D-F). Cultures were then divided into two separate fractions of 100 ml of which one was supplemented with either 0.05 µM or 50 µM of 3-oxo-C8-HSL (stock solution of 3-oxo-C8-HSL was prepared in ethyl acetate) and the other culture fraction (control) was treated with an equal aliquot of ethyl acetate. Cultures were allowed to grow for three additional hours, then harvested into falcon tubes and treated as outlined above.

RNA extraction, library construction, sequencing and bioinformatical analysis of transcriptome samples.

For all NGR234 wild type and mutant strains RNA-seq libraries were constructed from independent biological duplicates of RNA samples. Six samples were harvested at the early exponential phase and six samples were obtained from stationary growth phase cultures (Table 1, Figure S1). Total RNA was extracted using the hot-phenol method described previously[31]. The residual genomic DNA was removed from the total isolated RNA by DNase I treatment. The cDNA libraries for sequencing were constructed by vertis Biotechnology AG, Germany (http://www.vertis-biotech.com/) as described by[32]. The transcripts were not fragmented in order to get mainly sequencing reads of the 5'-end of the transcripts. The obtained cDNA libraries were sequenced using a HiSeq 2500 machine (Illumina) in single-read mode and running 100 cycles. For the twelve analyzed samples we sequenced between 3.86 and 9.16 mio. cDNA reads. The bioinformatical analysis was done as described in[33]. To assure a high sequence quality, the Illumina reads in FASTQ format were trimmed with a cut-off phred score of 20 by the program fastq_quality_trimmer from FASTX toolkit version 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/). The alignment of reads, coverage calculation, gene wise read quantification and differential gene expression was performed with READemption[34] which was relying on 'segemehl' version X[35] and DESeq version V. Visual inspection of the coverages was done using the Integrated Genome Browser (IGB). The reference sequences and gene annotations for NGR234 were retrieved from the NCBI database (accession no: pNGR234*a,* NC_000914.2; pNGR234*b,* NC_012586.1; cNGR234,

NC_012587.1) and in part manually re-annotated. Genes with a fold-change of ≥ 2.0 and an adjusted p-value (p-value was corrected by FDR (false discovery rate) based on Benjamini-Hochberg procedure) ≤ 0.05 were considered as differentially expressed. The raw, de-multiplexed reads as well as coverage files have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus under the accession number GSE54381 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=gse54381).

Quantitative real time PCR (qRT-PCR) and quantitative PCR (qPCR)

QRT-PCR experiments were carried out to verify selected QS-regulated genes. Total RNA was extracted from stationary grown cultures according to Rivas *et al.*[36] and the residual genomic DNA was removed by DNase I treatment according to the manufactures instructions (DNase I, RNase-free, Thermo Scientific, WI, USA). The SuperScript® VILO™ cDNA synthesis kit (Invitrogen™, life technologies, TX, USA) was used to generate cDNA using 1.9 µg RNA. Gene-specific primers used for qRT-PCR are shown in TableS3. The qRT-PCR reactions were set up according to manufactures protocol using the SYBR® Select Mater Mix for CFX (Applied biosystems® by life technologies, TX, USA) and performed by the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Munich, Germany). Standard curves of 10-fold serial dilutions of cDNA were generated for each gene to evaluate the primer efficiency and for data analysis. The efficiency, slope and correlation coefficient were determined by the CFX Manager™ software (Release 3.1, Bio-Rad Laboratories, Munich, Germany). All qRT-PCR reactions were run in triplicate and repeated at least three times in separate experiments under the same conditions. To normalize variability in expression levels, *rpoD* and *recA* were used as the internal control genes. Data were analyzed based on the normalized gene expression ($2^{-\Delta\Delta C(t)}$ method) and the above stated software. To verify the copy number of the symplasmid, gDNA was extracted after 48 h growth of the corresponding *S. fredii* NGR234 culture with the peqGOLD Bacterial DNA Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany). The qPCR reactions were set up according to manufactures protocol using the SYBR® Select Mater Mix for CFX (Applied biosystems® by life technologies, TX, USA) and performed by the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Munich, Germany). Standard curves of 10-fold serial dilutions of DNA were generated for each gene to evaluate the primer efficiency and for data analysis. The efficiency, slope and correlation coefficient were determined by the

CFX Manager™ software (Release 3.1, Bio-Rad Laboratories, Munich, Germany). All qPCR reactions were run in triplicate and repeated at least three times in separate experiments under the same conditions. To normalize variability in expression levels, NGR_c03800 and *recA* were used as the internal control genes.

SDS-Page and western blot analysis.

Extracellular proteins from rhizobia were obtained following the procedure described earlier[37]. NGR234 and NGR234-ΔtraI-ΔngrI double deletion mutant were grown in 5 ml YEM broth for 3 days at 30°C. Five hundred µL of 3-day old cultures were transferred into 250 ml flasks containing 50 mL liquid YEM medium in either the presence or absence of 1 µM apigenin. Cultures were harvested at 24 h, 72 h, and 120 h of growth and subjected to centrifugation at 12,000 g for 15 min. To the resulting supernatant, three volumes of ice-cold acetone was added and placed at -20° C for 16 h. Precipitated proteins were recovered by centrifugation as before, briefly air-dried and resuspended in SDS sample buffer (125 mM Tris-HCl buffer pH 6.8, 4% sodium dodecyl sulfate (w/v), 20% glycerol (v/v), 0.03 mM bromophenol blue and 5% (v/v) 2-mercaptoethanol). Protein aliquots were resolved by 15% SDS-PAGE using the Hoefer SE260 minigel electrophoresis apparatus (GE Healthcare, Piscataway, NJ) and resolved proteins were visualized by silver stain.

Immunoblot blot analysis was performed using a cocktail of antibodies raised against the individual Nop protein at a final dilution of 1:10,000. Immunoreactive proteins were detected with an enhanced chemiluminescent substrate (Super Signal West Pico kit; Pierce Biotechnology, Rockford, IL) according to the manufacturer's recommendations.

Nodulation assays.

Nodulation assays accomplished with NGR234-Δ*traI*, NGR234-Δ*ngrI* and NGR234-Δ*ngrI*/Δ*traI* and with *Vigna unguiculata, Vigna radiata and Tephrosia vogelii* were done as previously described[24]. Experiments were repeated two times with five plants per treatment in each experiment. Plants were harvested 30 days after inoculation. Nodules formed on the roots were counted and the shoot fresh weight was recorded. Plants grown in the absence of rhizobia were used as a control.

Root hair curling assay.

For the root hair curling assay, supernatants of 48 h old 500 ml cultures grown in TY medium of either uninduced or induced (1 µM apigenin) populations of NGR234 or NGR234-△*traI*-△*ngrI* were collected. The unpurified *Nod factor*s were extracted with 0.4 volumes of *n*-butanol according to Lopez *et al.*[38]. The butanol layer was collected, the butanol removed by rotary evaporation and the residue was resolved in H_2O_{bidist} to a final volume of 1/100 of the starting culture.

Vigna unguiculata seedlings were sterilized in 0.12 % NaClO for 15 min. Afterwards, they were washed three times in sterile H_2O_{bidist} and air dried, before they were transferred to 0.5-fold TY agar plate (0.25 % tryptone, 0.125 % yeast extract, pH 7.0) and incubated at 30 °C for 24 h in the dark for germination. Sterile germinated seedlings were transferred to glass containers filled supplemented with glass beads and Hoagland medium[39]. The seedlings were grown for additional 48 h under the following conditions: day/night; 24/19 °C; 16/8 h; 60 % relative humidity. The small plants were transferred into a small plastic container and coated with 500 µl Hoagland medium and 500 µl supernatant extract of the NGR234 cultures. After 24 h incubation in the dark, the root hairs were analysed using a Zeiss AxioCam microsope with an MRm camera mounted on the microscope (Zeiss Axio Imager.M2). Images were recorded with a 40x magnification.

FIGURES

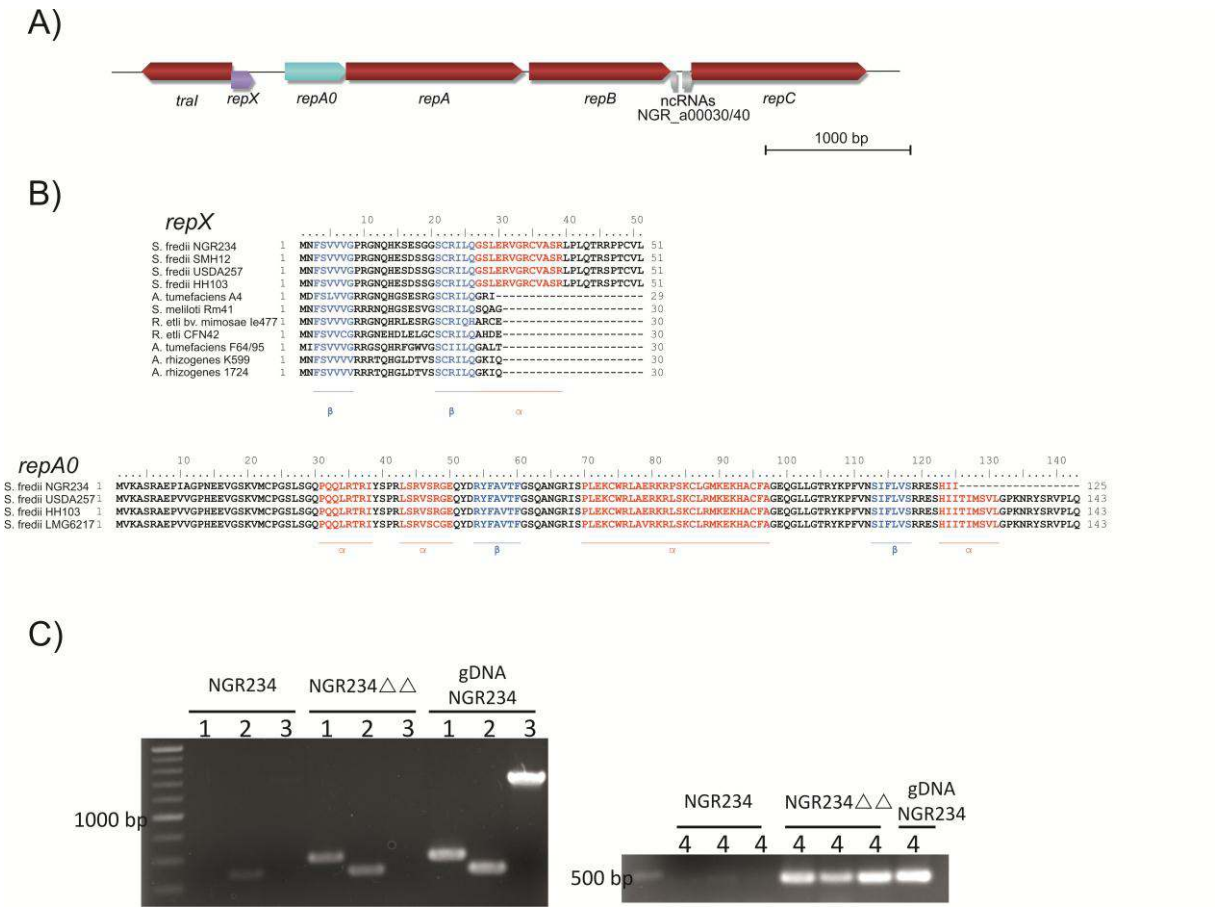

Figure 1. Genomic organization and analyses of *repX* and *repA0*. A) Genomic organization of the *traI* and *repABC* genes including the noncoding RNAs (NGR_a00030/40) and the two ORFs *repX* and *repA0* in the intergenic region between *traI* and *repA*. B) Amino Acid ClustalW Alginments of *repX* and *repA0* of *S. fredii* NGR234 and related strains. C) End point RT-PCR of *repA0* and *repX* on cDNA generated of RNA from the wild type and NGR234-△*traI*-△*ngrI*. Samples are shown on a 0.8 % agarose gel with a 1 kb λ marker (Thermo Scientific, Braunschweig, Germany). Primer pairs used for the amplification: 1, parA_int_fw and parA_int_rev; 2, RT_repA_fw and RT_repA_ rev; 3, parA_int_fw and RT_repA_ rev; 4, repX_int_fw and repX_int_rev.

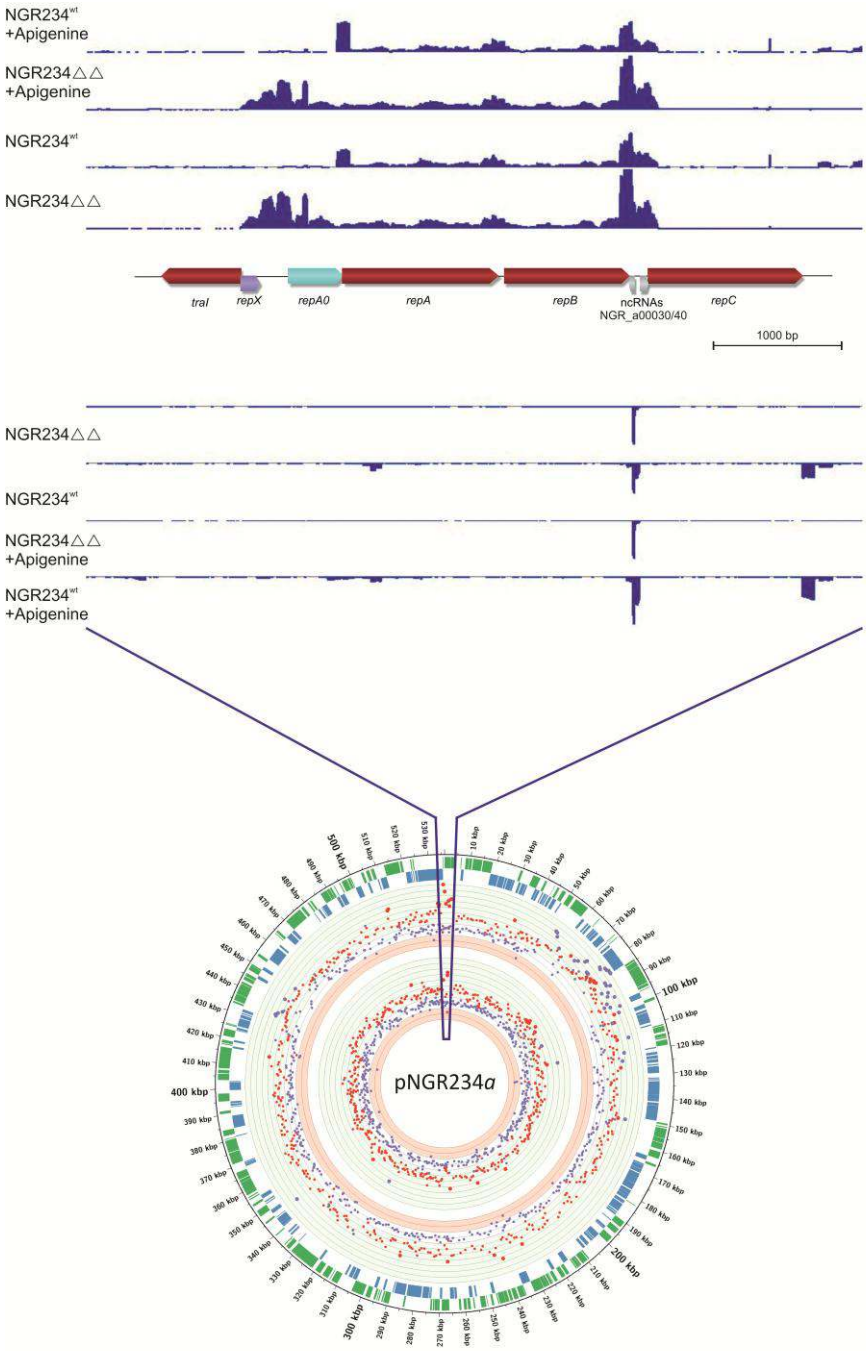

Figure 2. Circular representation of the RNA-seq-based transcriptome symplasmid data set of NGR234-△*traI*-△*ngrI* versus the wild type with a zoom into the replication related zone around the *ori*. The upper part of this picture represents the mapped transcripts on the genomic section around the origin of replication in induced (1 µM apigenin) and in uninduced NGR234-△*traI*-△*ngrI* and wild type cells. The image was composed using the integrated genome browser software version 8.4.4. The circular diagram was calculated with the circus software 0.64[40]. Fold change cut-off: $\log_2$ 8/-3 (circle size by values). The circle described from the outside to the innermost circle: Outer circle indicates coordinates of the pNGR234*a* in kbp. The second and third circles indicate the ORFs on the leading (green) and

the lagging (blue) strand. The circles in light green indicate $\log_2$ 8; 7; 6; 5; 4; 3; 2, the white circles in-between the coloured once represent $\log_2$ 1/-1 and the light red circles indicate $\log_2$ -2; -3 for the sense transcripts. The next circles represent the same $\log_2$ values for the antisense transcripts. The dots scattered over the light green, light red and white circles represent the transcripts of NGR234-Δ*traI*-△*ngrI* in comparison to the wild type strain (red) and the wild type strain induced with 1 µM apigenin in comparison to the wild type strain (purple).

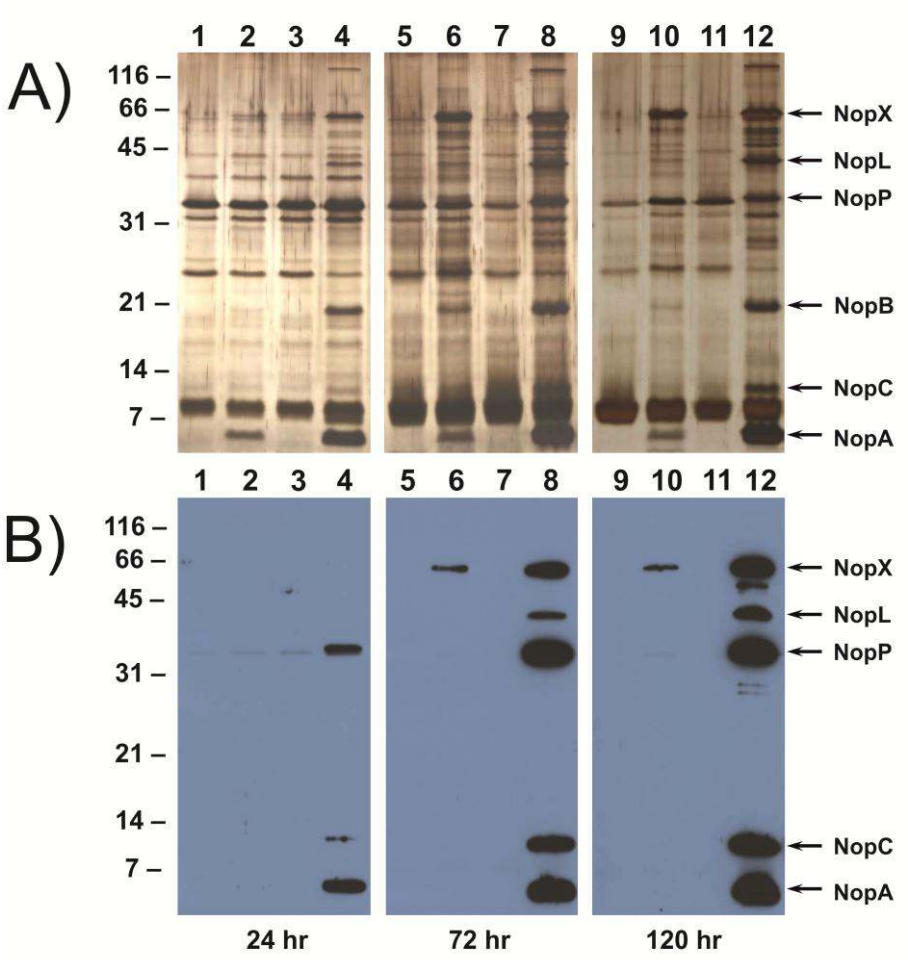

Figure 3. Extracellular protein profile and immunoblot analysis of *Sinorhizobium fredii* NGR234 and NGR234-ΔtraI-ΔngrI double deletion mutant. Extracellular proteins isolated from cells grown in the absence (odd number lanes) or presence (even numbered lanes) of 1 µM apigenin were resolved by 15% SDS-PAGE and silver stained (A) or transferred to nitrocellulose membrane (B) for immunological analysis with a cocktail of antibodies raised against the individual Nop proteins. Lanes 1-4 contains protein samples harvested from 24 h, lanes 5-8 from 72 h, and lanes 9-12 from120 h of growth. The size of the molecular weight markers in kDa and the identity of the immunoreactive proteins are also shown.

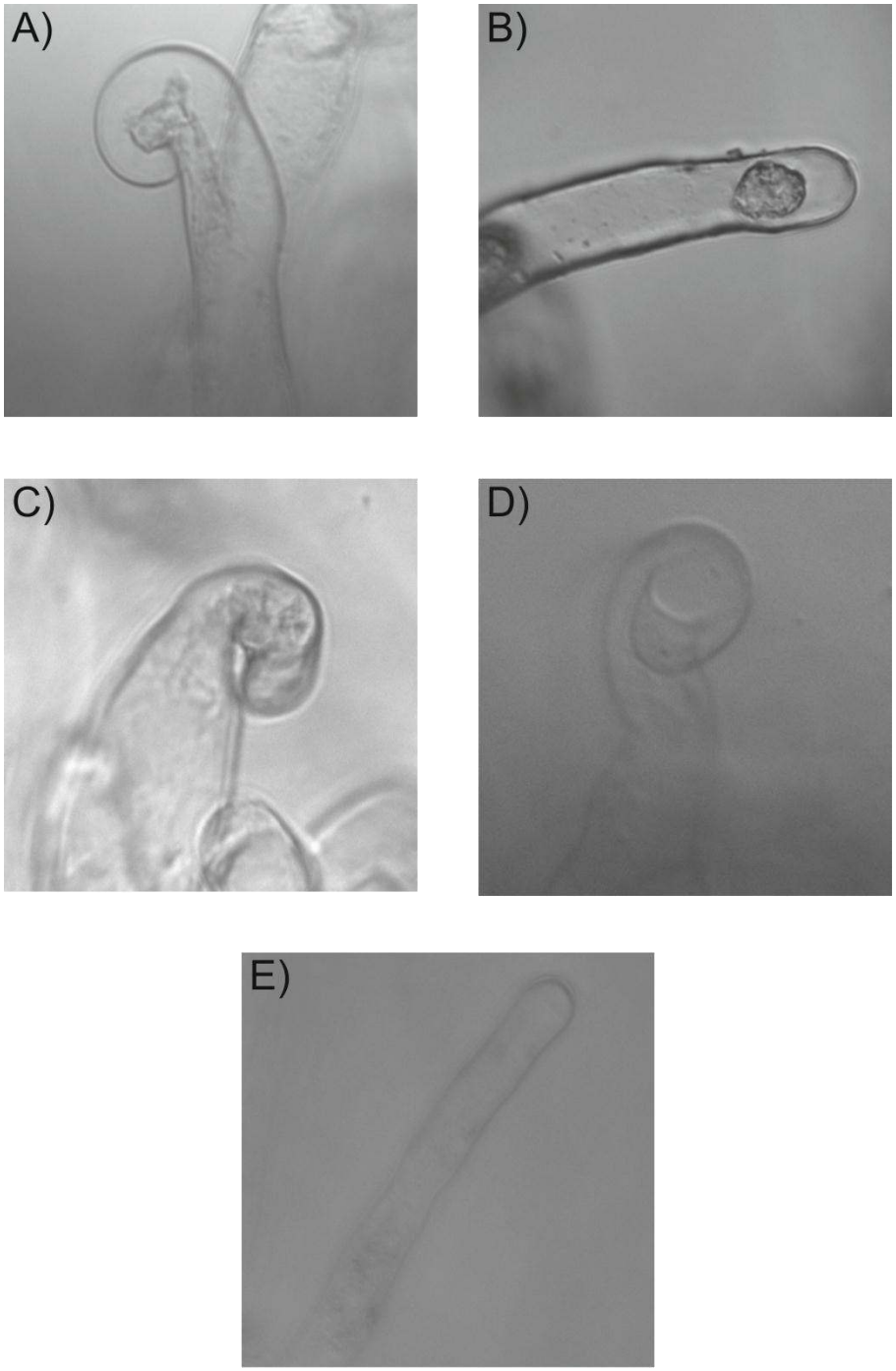

Figure 4. Root hair curling assay with apigenin induced NGR234-△*traI*-△*ngrI* and uninduced NGR234 and NGR234-△*traI*-△*ngrI*. A) Root hair after 24 h induction with extracts from an uninduced culture of NGR234-△*traI*-△*ngrI.* B) Root hair after 24 h induction with extracts from an uninduced culture of NGR234. C) Positive control, root hair curling with extracts of an NGR234-△*traI*-△*ngrI* culture induced with 1 µM apigenin. D) Positive control, root hair curling with extracts of an NGR234 culture induced with 1 µM apigenin. E) Control sample before the induction with NGR234-extracts.

TABLES

Table 1. Copy number of pNGR234*a* estimated using qPCR and using primers for the ORFs NGR234_a00010 and NGR234_a01270. Results are mean values of three technical replicates and of three independent biological samples. The primer efficiencies were 90.1 % for the NGR234_a00010 primers and 90.5 % for the NGR234_a01270 primers. Copy number was calculated based on control qPCRs for chromosomal genes (NGR234_c03800 and NGR234_c16470) and in the background of the wildtype strain.

Strain/ Treatment[a]	Target gene	Copy number	Strain/ Treatment[a]	Target gene	Copy number
NGR234	NGR_a01270	1.0 ± 0.3	NGR234-△△	NGR_a01270	2.8 ± 0.2
	NGR_a00010	1.0 ± 0.3		NGR_a00010	2.3 ± 0.2
NGR234 0.05 µM AI	NGR_a01270	3.1 ± 0.4	NGR234-△△ 0.05 µM AI	NGR_a01270	4.6 ± 0.8
	NGR_a00010	2.7 ± 0.0		NGR_a00010	3.0 ± 0.5
NGR234 0.5 ‰ DMSO	NGR_a01270	3.8 ± 0.4	NGR234-△△ 0.5 ‰ DMSO	NGR_a01270	6.8 ± 1.6
	NGR_a00010	2.8 ± 0.3		NGR_a00010	5.4 ± 1.3
NGR234 5 µM AI	NGR_a01270	3.9 ± 0.3	NGR234-△△ 5 µM AI	NGR_a01270	2.9 ± 0.2
	NGR_a00010	3.3 ± 0.3		NGR_a00010	2.3 ± 0.2
NGR234 0.05 % DMSO	NGR_a01270	3.5 ± 0.2	NGR234-△△ 0.05 % DMSO	NGR_a01270	2.6 ± 0.5
	NGR_a00010	2.9 ± 0.2		NGR_a00010	1.9 ± 0.4
NGR234 50 µM AI	NGR_a01270	1.5 ± 0.3	NGR234-△△ 50 µM AI	NGR_a01270	11.9 ± 1.0
	NGR_a00010	1.3 ± 0.3		NGR_a00010	9.3 ± 0.9
NGR234 0.5 % DMSO	NGR_a01270	3.0 ± 0.5	NGR234-△△ 0.5 % DMSO	NGR_a01270	5.4 ± 1.3
	NGR_a00010	2.4 ± 0.3		NGR_a00010	4.6 ± 1.1
NGR234 1 µM apigenin	NGR_a01270	2.0 ± 0.1	NGR234-△△ 1 µM apigenin	NGR_a01270	3.4 ± 0.3
	NGR_a00010	1.7 ± 0.1		NGR_a00010	2.7 ± 0.2
NGR234 50 µM octopine	NGR_a01270	2.2 ± 0.1	NGR234 pBBR1MCS-2::*repX*	NGR_a01270	1.1 ± 0.1
	NGR_a00010	2.3 ± 0.2		NGR_a00010	0.9 ± 0.1
NGR234 pBBR1MCS-2	NGR_a01270	0.9 ± 0.0	NGR234 pBBR1MCS-2::*repA0*	NGR_a01270	4.1 ± 0.3
	NGR_a00010	0.8 ± 0.0		NGR_a00010	3.3 ± 0.3
NGR234-△*ngrI*	NGR_a01270	1.6 ± 0.2	NGR234-△*traI*	NGR_a01270	2.0 ± 0.2
	NGR_a00010	1.4 ± 0.1		NGR_a00010	1.6 ± 0.2
NGR234-△*ngrI* c*ngrI*	NGR_a01270	3.2 ± 0.2	NGR234-△*traI* c*traI*	NGR_a01270	3.7 ± 0.3
	NGR_a00010	2.0 ± 0.1		NGR_a00010	2.7 ± 0.3
NGR234-△*traM*	NGR_a01270	1.6 ± 0.1	NGR234-△*traR*	NGR_a01270	1.2 ± 0.1
	NGR_a00010	1.2 ± 0.1		NGR_a00010	1.2 ± 0.1

a) NGR234-△△, NGR234-△*traI*-△*ngrI*; AI, autoinducer *N*-(3-oxooctanoyl-)L-homoserine lactone; c*ngrI*, complementation construct of △*ngrI*; c*traI*, complementation construct of △*traI*

Table 2. DNA sequence analysis of the intergenic region separating *traI* and *repA* in various rhizobial and agrobacterial species carrying *repABC*-like plasmids and with a conserved organization of the *traI-repABC* DNA region as previously described.

Strain	*traI-repA* (bp)	Strain	*traI-repA* (bp)
Sinorhizobium		*A. tumefaciens* pTiBo542	365
S. fredii NGR234	803	*A. tumefaciens* LBA4213 (Ach5)	342
S. fredii USDA257	803	*A. tumefaciens* str. C58	363/723
S. fredii HH103	803	*R. rhizogenes* A4	315
S. fredii GR4 (in ncbi meliloti)	383	*R. rhizogenes* NBRC13257	441
S. fredii GR64	442/382	*R. rhizogenes* YR147	393
S. terangae WSM1721	354	*A. rhizogenes* ATCC 15384	388
S. arboris LMG1419	354	*A. rhizogenes* plasmid pRi1724	316
S. americanum CCGM7	382	*A. rhizogenes* plasmid pRi2659	316
S. melilloti GVPV12	442	*Agrobacterium* sp. ATCC 31749	377
S. meliloti Rm41	291	*Agrobacterium* sp. Cherry 2E 2-2	358
S. meliloti 4H41	382	*Agrobacterium vitis* S4 pTiS4	361
		A. albertimagni AOL15	316
Rhizobium sp. and others			
Rhizobium sp. OK665	366	*Rhizobium etli*	
Rhizobium sp. OR191	688	*R. etli* bv. *mimosae* Mim1	162
Rhizobium sp. YR295	376	*R. etli* bv. *mimosae* IE4771	337
Rhizobium sp. YR519	365	*R. etli* CFN 42 (p42d)	374
Rhizobium sp. CF258	376	*R. etli* CFN 42 (p42a)	373
Rhizobium sp. CF080	351		
Rhizobium sp. BR816	383	*Ensifer*	
Rhizobium sp. CCGE 502	372	*Ensifer sp.* USDA 6670	295
Rhizobium sp. STM6155	373	*Ensifer adhaerens* OV14	2683
N. galegae HAMBI 1141	337		
A. doebereinrae UFLA1-100	577	*Rhizobium tropici*	
M. tianhanense CGMCC 1.2546	531	*R. tropici* CIAT 899	376
B. japonicum USDA 135	531	*R. tropici* USDA 9039	376
B. japonicum USDA 123	494	*R. tropici* USDA 9039	393
R. leucaenae USDA 9039	376	*R. tropici* PRF 81	389
R. lusitanum P1-7	388	*R. tropici* CF286	376
R. giardinii bv. *giardinii* H152T	210	*R. tropici* YR635	393
R. selenitireducens BAA-1503	388		
R. loessense CGMCC 1.3401	356	*Rhizobium leguminosarum*	
R. mesoamericanum STM 3625	388	*R. leguminosarum* bv. *trifolii* CC287f	360
R. mongolense USDA1844	304/374	*R. leguminosarum* bv. *trifolii* CC283b	1652
R. hainannese CCBAU 57015	379	*R. leguminosarum* bv. *trifolii* CB782	375
R. sullae WSM1592	372	*R. leguminosarum* bv. *trifolii* TA1	364
R. larrymoorei ATCC 51759	367	*R. leguminosarum* bv. *trifolii* WSM1325	364
R. rubi NBRC13261	366	*R. leguminosarum* bv. *viciae* 128C53	376
R. giardinii bv. *giardinii* H152T	210	*R. leguminosarum* bv. *viciae* Vc2	376
		R. leguminosarum bv. *viciae* UPM1131	376
Agrobacterium		*R. leguminosarum* bv. *viciae* UPM1137	376
A. tumefaciens GW 4	377	*R. leguminosarum* bv. *viciae* 3841 (pRL7)	387
A. tumefaciens A4	388	*R. leguminosarum* bv. *viciae* 248	376
A. tumefaciens 5A	490	*R. leguminosarum* bv. *viciae* WSM1455	364
A. tumefaciens CCNWGS0286	367	*R. leguminosarum* bv. *viciae* GB30	364
A. tumefaciens F2	239	*R. leguminosarum* bv. *viciae* Ps8	364
A. tumefaciens LBA4404	365	*R. leguminosarum* bv. *phaseoli* 4292	434

Table 3. RNA-seq results for the replication machinery of NGR234-△*traI*-△*ngrI* compared to the wild type strain.

Start	End	Strand	Orientation	Locus_tag	Gene	Protein function	Fold Change
101	1324	+	sense	NGR_a00010	*repA*	replication protein RepA	**23.97**
101	1324	+	anti-sense	NGR_a00010	*repA*	replication protein RepA	**2.07**
1381	2361	+	sense	NGR_a00020	*repB*	replication protein RepB	**33.57**
1381	2361	+	anti-sense	NGR_a00020	*repB*	replication protein RepB	**2.90**
2363	2409	-	anti-sense	NGR_a00030	ncRNA	antisense regulator of RepB translation	**29.47**
2363	2409	-	sense	NGR_a00030	ncRNA	antisense regulator of RepB translation	**18.19**
2450	2518	-	anti-sense	NGR_a00040	ncRNA	negative antisense regulator of RepC translation	**42.37**
2516	3730	+	sense	NGR_a00050	*repC*	replication initiation protein RepC	**43.85**
2516	3730	+	anti-sense	NGR_a00050	*repC*	replication initiation protein RepC	**3.15**

Table 4. β-galactosidase activities of NGR234 and NGR234-△*traI*-△*ngrI* carrying either a nodABC or a nopB promoter fusion. Data are mean values of 3 measurements. Promoter genes are given in brackets.

Rhizobium (construct)	24 hours	48 hours	72 hours	96 hours	120 hours
NGR234 (*nodABC* promoter)	79.32 ± 9.9	55.45 ± 2.41	11.75 ± 1.87	13.08 ± 0.59	5.97 ± 0.64
NGR234 (*nodABC* promoter) + apigenin	1597.05 ± 170.32	1518.06 ± 101.32	907.16 ± 212.13	785.80 ± 25.78	528.24 ± 232.87
NGR234 (*nopB* promoter)	146.12 ± 0.77	54.98 ± 8.49	11.46 ± 0.75	9.87 ± 0.04	2.50 ± 0.20
NGR234 (*nodB* promoter) + apigenin	1238.19 ± 60.35	1086.54 ± 51.51	86.98 ± 55.71	84.75 ± 2.21	5.23 ± 0.0
*ngr*IΔ/*tra*IΔ (nodABC promoter)	147.07 ± 2.00	92.18 ± 2.02	53.21 ± 05.00	71.02 ± 0.67	47.35 ± 0.45
*ngr*IΔ/*tra*IΔ (nodABC promoter) + apigenin	2430.34 ± 253.73	3090.85 ± 35.53	2299.20 ± 1.74	2026.67 ± 16.51	1952.91 ± 0.41
*ngr*IΔ/*tra*IΔ (nopB promoter)	125.97 ± 16.48	119.69 ± 0.41	65.22 ± 5.52	62.2 ± 3.06	52.12 ± 0.91
*ngr*IΔ/*tra*IΔ (nodB promoter) + apigenin	670.58 ± 199.42	2684.47 ± 6.83	2023.53 ± 29.54	1835.47 ± 8.95	1733.38 ± 19.18

SUPPLEMENTAL MATERIAL

Table S1. Overall transcriptome statistics for the analyzed NGR234 and NGR234-Δ*traI*-Δ*ngrI*.

Sample #	NGR234 genotype	OD_{600} after 48 h growth	Treatment[a]	No. of reads generated X10^6	No. of uniquely mapped reads X10^6
1	wt	9.10	none	1.36	0.55
2	wt	8.96	none	2.48	1.57
3	wt	8.46	none	2.95	1.75
4	wt	7.76	apigenine (1 µM)	3.45	2.21
5	wt	8.10	apigenine (1 µM)	2.36	1.47
6	wt	6.84	apigenine (1 µM)	2.35	1.42
7	Δ*traI*-Δ*ngrI*	4.12	none	3.57	1.72
8	Δ*traI*-Δ*ngrI*	4.69	none	3.44	1.92
9	Δ*traI*-Δ*ngrI*	4.21	none	2.66	1.39
10	Δ*traI*-Δ*ngrI*	3.47	apigenine (1 µM)	2.96	1.20
11	Δ*traI*-Δ*ngrI*	3.24	apigenine (1 µM)	3.39	1.62
12	Δ*traI*-Δ*ngrI*	3.12	apigenine (1 µM)	3.31	1.72
13	wt	5.92	none	2.35	1.32
14	wt	7.92	none	3.77	2.15
15	wt	6.72	none	2.55	1.56

a) Controls for apigenin samples were supplemented with an equal amount of KOH

Table S2. Primers used in this study.

Oligonucelotide	Sequence 5'-3'[a]	Size (bp)	Target region/description
parA_int_fw	CAACCGCAGCAACTCAGAAC	20	amplification of parts of *parA*
parA_int_rev	ATTCTCGCCGACTGACAAGG	20	
RT_a00010_fw	GCAGCAGTTCCCACCGAATG	20	amplification of parts of *repA* and qRT-PCR for RNA-seq verification
RT_a00010_rev	GCACGTAGTTCCTGGCTTCC	20	
repX_int_fw	ATGAATTTCTCCGTCGTTGTTG	22	amplification of *repX*
repX_int_rev	CTACAAGACGCAAGGGGGCCTTC	23	
RT_recA_fw	CGGCTCGTAGAGGACAAATCG	21	reference gene 1 qRT-PCR
RT_recA_rev	CAATGATGCGCCCTTTCGG	19	
RT_rpoD_fw	ACATCACCAATGTCGGCGGTGAAG	24	reference gene 2 qRT-PCR
RT_rpoD_rev	TGCAGCTTGCGGAGCTTCTTGTAG	24	
RT_c24920_fw	GCCCGCACAAGAGGCAATTCTAGA	24	qRT-PCR for RNA-seq verification
RT_c24920_rev	GCGATCCAGGAACATGGCGTTCA	23	
RT_a00860_fw	CCCGGTTTACTCGAGCTGTC	20	qRT-PCR for RNA-seq verification
RT_a00860_rev	GCTGCTCCTCCGTAACTGTG	20	
RT_c09750_fw	CAATTCGAAAGTGATCTTCGGGCAGCA	27	qRT-PCR for RNA-seq verification
RT_c09750_rev	GAGCCACATCGACTCGGCGTAT	22	
RT_a00550_fw	AGGTACTCTCACGAGTGCAGTTGG	24	qRT-PCR for RNA-seq verification
RT_a00550_rev	CTGTACGCGTTCATCCGCCG	20	
RT_a00440_fw	ACCGCCTTGCTGCACTTCAGCAAT	24	qRT-PCR for RNA-seq verification
RT_a00440_rev	CGTCCATTTCCATGCCGTCGACAA	24	
RT_c17900_fw	ATGAACACGCTCGCCTATCTCATCC	25	qRT-PCR for RNA-seq verification
RT_c17900_rev	TCAATGCCTGTCCGGCTTGCCAT	23	
RT_c09120_fw	ACCAAACTGTCGCTCGCGCACAA	23	qRT-PCR for RNA-seq verification
RT_c09120_rev	CAGGTAAAGGGCGATGTCGAAGCT	24	
TraR_up_fw	aaatctagaCTCTCCTATCTTCTTGACCG	29	amplification of *traR* upstream flank
TraR_up_rev	aaaggatccTGTGGCGCTACAATCACTG	28	
TraR_do_fw	aaaggatccTGTAGCGGCTAGACCGATACAATG	33	amplification of *traR* downstream flank
TraR_do_rev	aaatctagaGTTCTAGTTGTCATCGTCGAAAGGC	34	
TraM_up_fw	aaaggatccCGCTCGATTTTACCCTGCAGTAG	32	amplification of *traM* upstream flank
TraM_up_rev	aaatctagaCCGCTTCTCCCTGATTTGATTTCC	33	
TraM_do_fw	aaatctagaGACAACTAGAACTCAGACCAGG	31	amplification of *traM* downstream flank
TraM_do_rev	aaaggatccGCGGTCAAGAAGATAGGAG	28	

a) Restriction sites are underlined

Table S3: qPCR verification of transcriptome results of comparison between NGR234-△*traI*-△*ngrI* and NGR234. Green number, upregulation; red number, downregulation

Target	RNA-seq results	qPCR results
NGR_c24920	9.0 ↓	1.8 ↓
NGR_a00860	24.4 ↑	1.1 ↑
NGR_c09750	7.1 ↓	1.1 ↓
NGR_a00010	24 ↑	3.9 ↑

Table S4: qPCR verification of transcriptome results of comparison between NGR234-△*traI*-△*ngrI* treated with 50 µM apigenin and untreated NGR234-△*traI*-△*ngrI*. Green number: upregulation; red number: downregulation

Target	RNA-seq results	qPCR results
NGR_a00550	28.0 ↑	68.9 ↑
NGR_a00440	35.3 ↑	8.1 ↑
NGR_c17900	14.0 ↓	11.4 ↓
NGR_c09120	21.6 ↑	39.6 ↑

REFERENCES

1 Deakin, W. J. & Broughton, W. J. Symbiotic use of pathogenic strategies: rhizobial protein secretion systems. *Nat Rev. Microbiol.* **7**, 312-320, doi:10.1038/nrmicro2091 (2009).

2 Gage, D. J. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol. Mol. Biol. Rev.* **68**, 280-300, doi:10.1128/mmbr.68.2.280-300.2004 (2004).

3 Jones, K. M., Kobayashi, H., Davies, B. W., Taga, M. E. & Walker, G. C. How rhizobial symbionts invade plants: the *Sinorhizobium medicago* model. *Nat. Rev. Microbiol.* **5**, 619-633, doi:10.1038/nrmicro1705 (2007).

4 Spaink, H. P. Root nodulation and infection factors produced by rhizobial bacteria. *Annu. Rev. Microbiol.* **54**, 257-288, doi:DOI 10.1146/annurev.micro.54.1.257 (2000).

5 Nelson, M. S. & Sadowsky, M. J. Secretion systems and signal exchange between nitrogen-fixing rhizobia and legumes. *Front. Plant Sci.* **6**, doi:Artn 491 10.3389/Fpls.2015.00491 (2015).

6 Mierziak, J., Kostyn, K. & Kulma, A. Flavonoids as important molecules of plant interactions with the environment. *Molecules.* **19**, 16240-16265, doi:10.3390/molecules191016240 (2014).

7 Oldroyd, G. E. D. Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nat. Rev. Microbiol.* **11**, 252-263, doi:10.1038/nrmicro2990 (2013).

8 Broughton, W. J. & Perret, X. Genealogy of legume-*Rhizobium* symbioses. *Curr. Opin. Plant Biol.* **2**, 305-311, doi:10.1016/S1369-5266(99)80054-5 (1999).

9 Pueppke, S. G. & Broughton, W. J. *Rhizobium* sp. strain NGR234 and *R. fredii* USDA257 share exceptionally broad, nested host ranges. *Mol. Plant. Microbe. Interact.* **12**, 293-318, doi:10.1094/MPMI.1999.12.4.293 (1999).

10 Krysciak, D., Grote, J., Rodriguez-Orbegoso, M., Utpatel, C., Förstner, K. U., Lei, L., Schmeisser, C., Krishnan, H. B. & Streit, W. R. RNA sequencing analysis of the broad-host-range strain *Sinorhizobium fredii* NGR234 identifies a large set of genes linked to quorum sensing-dependent regulation in the background of a *traI* and *ngrI* deletion mutant. *Appl. Environ. Microbiol.* **80**, 5655-5671, doi:Doi 10.1128/Aem.01835-14 (2014).

11 He, X., Chang, W., Pierce, D. L., Seib, L. O., Wagner, J. & Fuqua, C. Quorum sensing in *Rhizobium* sp. strain NGR234 regulates conjugal transfer (*tra*) gene expression and influences growth rate. *J. Bacteriol.* **185**, 809-822 (2003).

12 Schmeisser, C., Liesegang, H., Krysciak, D., Bakkou, N., Le Quéré, A., Wollherr, A., Heinemeyer, I., Morgenstern, B., Pommerenin-Röser, A., Flores, M., Palacios, R., Gottschalk, G, Schmitz, R. A., Broughton, W. J., Perret, X., Strittmatter, A. W. & Streit, W. R. *Rhizobium* sp. strain NGR234 possesses a remarkable number of secretion systems. *Appl. Environ. Microbiol.* **75**, 4035-4045, doi:10.1128/AEM.00515-09 (2009).

13 Cevallos, M. A., Cervantes-Rivera, R. & Gutiérrez-Ríos, R. M. The *repABC* plasmid family. *Plasmid* **60**, 19-37, doi:10.1016/j.plasmid.2008.03.001 (2008).

14 Pinto, U. M., Pappas, K. M. & Winans, S. C. The ABCs of plasmid replication and segregation. *Nat. Rev. Microbiol.* **10**, 755-765, doi:10.1038/nrmicro2882 (2012).

15 Pappas, K. M. & Winans, S. C. A LuxR-type regulator from *Agrobacterium tumefaciens* elevates Ti plasmid copy number by activating transcription of plasmid replication genes. *Mol. Microbiol.* **48**, 1059-1073, doi:10.1046/j.1365-2958.2003.03488.x (2003).

16 Pappas, K. M. Cell-cell signaling and the *Agrobacterium tumefaciens* Ti plasmid copy number fluctuations. *Plasmid* **60**, 89-107, doi:10.1016/j.plasmid.2008.05.003 (2008).

17 Veluthambi, K., Krishnan, M., Gould, J. H., Smith, R. H. & Gelvin, S. B. Opines stimulate induction of the *vir* genes of the *Agrobacterium tumefaciens* Ti Plasmid. *J. Bacteriol.* **171**, 3696-3703 (1989).

18 Begum, A. A., Leibovitch, S., Migner, P. & Zhang, F. Specific flavonoids induced *nod* gene expression and pre-activated *nod* genes of *Rhizobium leguminosarum* increased pea (*Pisum sativum* L.) and lentil (*Lens culinaris* L.) nodulation in controlled growth chamber environments. *J. Exp. Bot.* **52**, 1537-1543, doi:DOI 10.1093/jexbot/52.360.1537 (2001).

19 Cho, H. & Winans, S. C. VirA and VirG activate the Ti plasmid *repABC* operon, elevating plasmid copy number in response to wound-released chemical signals. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 14843-14848, doi:10.1073/pnas.0503458102 (2005).

20 Li, P. L. & Farrand, S. K. The replicator of the nopaline-type Ti plasmid pTiC58 is a member of the *repABC* family and is influenced by the TraR-dependent quorum-sensing regulatory system. *J. Bacteriol.* **182**, 179-188 doi:10.1128/JB.182.1.179-188.2000 (2000).

21 Subramoni, S., Nathoo, N., Klimov, E. & Yuan, Z. C. *Agrobacterium tumefaciens* responses to plant-derived signaling molecules. *Front. Plant. Sci.* **5**, doi:10.3389/Fpls.2014.00322 (2014).

22 Mazur, A. & Koper, P. Rhizobial plasmids - replication, structure and biological role. *Cent. Eur. J.Biol.* **7**, 571-586, doi:10.2478/s11535-012-0058-8 (2012).

23 Venkova-Canova, T., Soberón, N. E., Ramírez-Romero, M. A. & Cevallos M.A. Two discrete elements are required for the replication of a repABC plasmid: an antisense RNA and a stem-loop structure. *Mol. Microbiol*. **54,** 1431-1444, doi:10.1111/j.1365-2958.2004.04366.x (2004).

24 Krishnan, H. B., Lorio, J., Kim, W. S., Jiang, G., Kim, K. Y., DeBoer, M. & Pueppke, S. G. Extracellular proteins involved in soybean cultivar-specific nodulation are associated with pilus-like surface appendages and exported by a type III protein secretion system in *Sinorhizobium fredii* USDA257. *Mol. Plant. Microbe Interact.* **16**, 617-625, doi:10.1094/MPMI.2003.16.7.617 (2003).

25 D'Haeze, W. & Holsters, M. Nod factor structures, responses, and perception during initiation of nodule development. *Glycobiology* **12**, 79r-105r, doi:DOI 10.1093/glycob/12.6.79R (2002).

26 Mulligan, J. T. & Long, S. R. Induction of *Rhozobium meliloti nodC* expression by plant exudate requires *nodD*. *Proc. Natl. Acad. Sci. USA.* **82**, 6609-6613, doi:DOI 10.1073/pnas.82.19.6609 (1985).

27 Rossen, L., Shearman, C. A., Johnston, A. W. B. & Downie, J. A. The *nodD* gene of *Rhizobium meguminosarum* is autoregulatory and in the presence of plant exudate induces the *nodA,B,C* genes. *EMBO J.* **4**, 3369-3373 (1985).

28 Sambrook, D.W. *Molecular Cloning: A Laboratory Manual*. 3re ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2001).

29 Hornung, C., Poehlein, A., Haack, F. S., Schmidt, M., Dierking, K., Pohlen, A., Schulenburg, H., Blokesch, M., Plener, L., Jung, K., Bonge, A., Krohn-Molt, I., Utpatel, C., Timmermann, G., Spieck, E., Pommerening-Röser, A., Bode, E., Bode, H. B., Daniel, R., Schmeisser, C. & Streit, W. R. The *Janthinobacterium* sp. HH01 genome encodes a homologue of the *V. cholerae* CqsA and *L. pneumophila* LqsA autoinducer synthases. *PLoS One*. **8**, e55045, doi: 10.1371/journal.pone.0055045(2013).

30 Lassak, J., Henche, A. L., Binnenkade, L. & Thormann, K. M. ArcS, the cognate sensor kinase in an atypical Arc system of *Shewanella oneidensis* MR-1. *Appl. Environ. Microbiol.* **76**, 3263-3274 doi:10.1128/AEM.00512-10 (2010).

31 Dugar, G., Herbig, A., Förstner, K. U., Heidrich, N., Reinhardt, R., Nieselt, K. & Sharma, C. M. High-resolution transcriptome maps reveal strain-specific regulatory features of multiple *Campylobacter jejuni* isolates. *PLoS Genet*. **9**, e1003495 doi: 10.1371/journal.pgen.1003495 (2013).

32 Sharma, C. M., Hoffmann, S., Darfeuille, F., Reignier, J., Findeiss, S., Sittka, A., Chabas, S., Reiche, K., Hackermüller, J., Reinhardt, R., Stadler, P. F. & Vogel, J. The primary transcriptome of the major human pathogen *Helicobacter pylori*. *Nature*. **464**, 250-255 doi:10.1038/nature08756 (2010).

33 Hoffmann, S., Otto, C., Kurtz, S., Sharma, C. M., Khaitovich, P., Vogel, J., Stadler, P. F. & Hackermüller, J. Fast mapping of short sequences with mismatches, insertions and deletions using index structures. *PLoS Comput. Biol*. **5**, e1000502 10.1371/journal.pcbi.1000502 (2009).

34 Förstner, K. U., Vogel, J. O. & Sharma, C. M. READemption - a tool for the computational analysis of deep-sequencing-based transcriptome data. *Bioinformatics.* **30,** 3421-3423 doi:10.1093/bioinformatics/btu533 (2014).

35 Nicol, J. W., Helt, G. A., Blanchard, S. G., Jr., Raja, A. & Loraine, A. E. The Integrated Genome Browser: free software for distribution and exploration of genome-scale datasets. B*ioinformatics*. **25,** 2730-2731 doi:10.1093/bioinformatics/btp472 (2009).

36 Rivas, R., Vizcaíno, N., Buey, R. M., Mateos, P. F., Martínez-Molina, E. & Velázquez, E. An effective, rapid and simple method for total RNA extraction from bacteria and yeast. *J. Microbiol. Meth.* **47,** 59-63 doi:10.1016/S0167-7012(01)00292-5 (2001).

37 Lorio, J. C., Kim, W. S., Krishnan, A. H. & Krishnan, H. B. Disruption of the glycine cleavage system enables *Sinorhizobium fredii* USDA257 to form nitrogen-fixing nodules on agronomically improved North American soybean cultivars. *Appl. Environ. Microbiol.* **76,** 4185-4193 doi:10.1128/AEM.00437-10 (2010).

38 López-Lara, I. M., van den Berg, J. D., Thomas-Oates, J. E., Glushka, J., Lugtenberg, B. J. & Spaink, H. P. Structural identification of the lipo-chitin oligosaccharide nodulation signals of *Rhizobium loti*. *Mol. Microbiol.* **15,** 627-638 doi:10.1111/j.1365-2958.1995.tb02372.x (1995).

39 Hoagland, D. R. & Arnon, D. I. *The water-culture method for growing plants without soil*. The College of Agriculture, (1950).

40 Krzywinski, M. I., Schein, J. E., Birol, I., Connors, J., Gascoyne, R., Horsman, D., Jones, S. J., Marra, M. A. Circos: An information aesthetic for comparative genomics. *Genome Res.* doi:10.1101/gr.092759.109 (2009).

6 Discussion

Cell-cell communication of bacteria became an interesting research focus since its first description in 1970 in *Vibrio fischeri* (Nealson *et al.* 1970). Since then, many different QS networks and signals have been described in Gram-negative and Gram-positive bacteria. The main QS-dependent regulated features are virulence, biofilm formation and the production of antibiotics (Bassler 1999). In plant-associated bacteria, QS additionally controls the plasmid transfer (e.g. in *Agrobacterium tumefaciens*), the symbiosome development/root nodulation process (e.g. *Rhizobium etli* and *Rhizobium leguminosarum*), EPSII production (e.g. *Sinorhizobium meliloti* and *Pseudomonas stewartii*) or motility (e.g. *S. meliloti*) (Pierson *et al.* 1998; Sanchez-Contreras *et al.* 2007). To get a deeper understanding of the QS controlled features in NGR234, RNA sequencing analyses from two AI synthase deletion mutants NGR234-△*traI* and NGR234-△*ngrI* were performed and obtained data were compared to the wild type RNA sequencing data. These results represent population wide changes in the transcript levels. Additionally, the AI systems were analyzed on a single cell level with the help of *rfp*-promoter fusions. Single cell analyses showed a heterogeneous transcription behavior of the two AI synthases as well as QQ genes. Further data show that this heterogeneity is influenced by the available AI concentration and added plant exudates, which also has an impact on the copy number of the symbiotic plasmid in NGR234.

6.1 Population wide QS-dependent gene regulation

Due to the above made observations, we wondered if and to which extend QS could affect gene regulation in NGR234. To address this question, we initiated RNA sequencing analyses in the background of the wild type and NGR234-△*traI* and NGR234-△*ngrI*.

In general, 4.9 % of all predicted genes in NGR234-△*traI* and 7.3 % of the predicted genes in NGR234-△*ngrI* were significantly altered in their expression profile compared to the wild type strain (Krysciak *et al.* 2014). These data correspond quite well to QS dependent transcription data of other organisms. One of the best studied organisms with respect to QS is *Pseudomonas aeruginosa* (Smith, Iglewski 2003). A set of microarray RNA analyses of this strain are available, wherein the regulated genes range from 0.5 to 11.1 % of all predicted genes. The diverging percentages are based on slightly different experiment setups chosen in each experiment (Bijtenhoorn *et al.* 2011; Chugani *et al.* 2012; Dotsch *et al.* 2012; Hentzer

et al. 2003; Schuster *et al.* 2003; Wagner *et al.* 2003). In *Yersinia pestis,* 335 genes were QS-dependently regulated, which is 8 % of the genome that consist of 4221 open reading frames (ORFs; LaRock *et al.* 2013; Parkhill *et al.* 2001). In contrast to the previously mentioned transcriptome studies, the amount of regulated genes in NGR234 is slightly higher than the QS-linked regulated genes in *S. meliloti*. In this organism 55 to 170 genes were linked to the QS circuit, which is 0.8 to 2.7 % of the counted ORFs (Chen *et al.* 2003; Gao *et al.* 2005; Hoang *et al.* 2004). Both strains are closely related (Jarvis *et al.* 1992), although *S. meliloti* only codes for a single quorum sensing system, which probably explains their difference in regulated genes (Gao *et al.* 2005).

6.1.1 Challenging NGR234 with high AI concentrations in early exponential growth phase

When induced with high concentrations of AI (50 µM *N*-(3-oxooctanoyl-)-L-homoserine lactone; 3-oxo-C_8-HSL) in the early exponential growth phase, surprisingly only four genes were found to be differently regulated. All four genes belong to the *moc* operon and were down-regulated. This operon encodes genes that are responsible for the degradation of rhizopine (Krysciak *et al.* 2014). Rhizopines are sugar- and amino acid-derivatives and are produced by bacteroids in plant nodules. After their production they are transported to the outside of the plant root to promote growth of non-differentiated bacteria inside and outside the plant. The increase in rhizopine concentration around the plant root leads to a growth advantage of cells of the same species over competitor cells of a different species (Kohler *et al.* 2010; Murphy *et al.* 1987; Rossbach *et al.* 1995; Saint *et al.* 1993). In *S. meliloti* the QS-dependent transcription of the *moc* operon was previously described, as well as the octopine-threshold-dependent activation of *traR* expression in *A. tumefaciens* (Fuqua *et al.* 1995; Fuqua, Winans 1996; Gao *et al.* 2005; Oger *et al.* 1998). In this context, the repression of *moc* expression in the presence of high AI concentrations fits in and represents the link between QS and rhizopine metabolism in NGR234. This connection has already been hypothesized by He and colleagues (He *et al.* 2003b). Differentially regulated genes in the culture that was treated with moderate AI concentration (50 nM 3-oxo-C_8-HSL) were located all over the genome of NGR234 and not specifically located in one operon (Krysciak *et al.* 2014).

6.1.2 Analysis of RNA sequencing data in the background of the deletion mutants NGR234-△*traI* and NGR234-△*ngrI*

A comparison of the data obtained from transcriptome analyses of NGR234-△*traI* and NGR234-△*ngrI* with regard to the wild type identified 130 genes differently regulated in NGR234-△*traI* and 280 genes exclusively regulated in the NGR234-△*ngrI* deletion mutant. Additionally, a common set of 186 genes was differently expressed in both AI deletion mutants. The majority (65 – 70 %) of these genes were located on the chromosome in both deletion mutants. The differently expressed genes could be classified into seven functional categories based on the KEGG database (http://www.genome.jp/kegg/pathway.html). The genes were mainly linked to motility, regulators, general metabolism, cell wall and succinoglycan biosynthesis (cell envelope), transporters (mainly ABC) and hypothetical genes (Figure 4; Krysciak *et al.* 2014).

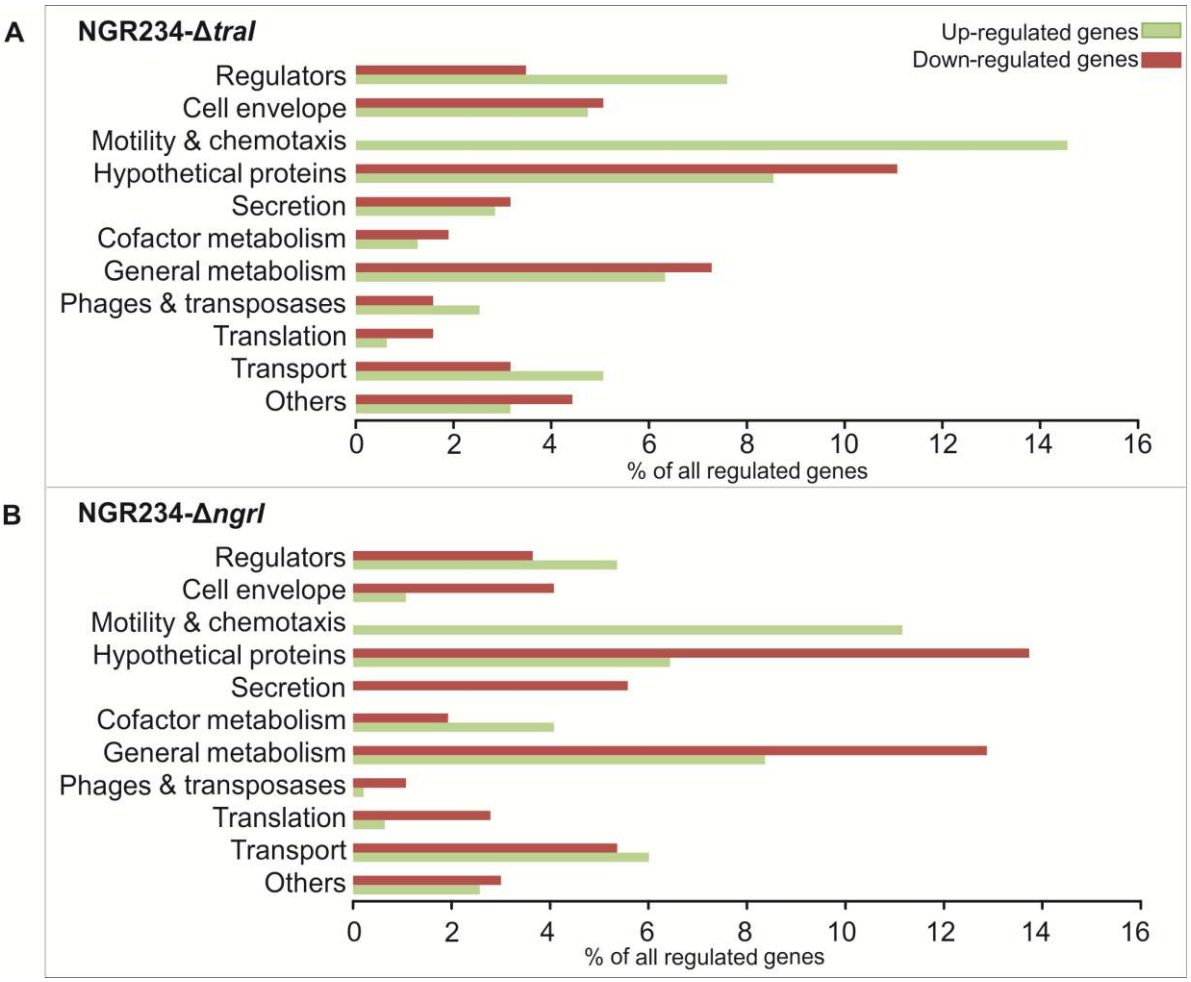

Figure 4: Differently expressed genes (%) in the background of the two autoinducer deletion mutants. A) NGR234-△*traI* compared to the wild type and B) in the background of NGR234-△*ngrI* compared to the wild type. The classification was based on the KEGG database (http://www.genome.jp/kegg/pathway.html; Krysciak *et al.* 2014).

The differently regulated genes of the general metabolism in the two deletion mutants go well together with findings in other rhizobial species. In NGR234 genes linked to energy production, sugar uptake or respiration were significantly regulated (Krysciak *et al.* 2014). In other strains, QS could be linked to growth, nodulation efficiency and nodule development (Chen *et al.* 2003; Daniels *et al.* 2002; Gao *et al.* 2005; Gray *et al.* 1996; He *et al.* 2003; Hoang *et al.* 2004; Marketon, Gonzalez 2002).

Over 50 regulated genes were linked to flagellum biosynthesis as well as genes for a type IV attachment pilus located on the chromosome and chemotaxis related genes. These genes were up-regulated in both investigated deletion mutants (Krysciak *et al.* 2014). The findings correlate with analyses in *S. meliloti*, where the flagellar genes are regulated in a QS-dependent manner (Gao *et al.* 2012; Hoang *et al.* 2008), suggesting a pivotal role of the flagellar systems during root colonization but not during the main infection process. This hypothesis is supported by recently performed RNA sequencing analyses of NGR234 bacteroids, where the flagellar system is found to be down-regulated inside the root nodules (Li *et al.* 2013). The observations for the type IV pilus were in line with the description of an altered surface motility phenotype of an *S. meliloti sinI* mutant and with observations of QS-dependent type IV pilus expression in *P. aeruginosa* (Barken *et al.* 2008; Gao *et al.* 2005).

NGR234 carries several clusters for the biosynthesis of exopolysaccharides (Schmeisser *et al.* 2009a). Four classes of these envelope-associated molecules are especially important for the development of a *Rhizobium*-legume symbiosis, namely cyclic β-(1,2)-glucans, outer membrane lipopolysaccharides (LPSs), external capsular polysaccharides (KPSs) and extracellular polysaccharides (EPSs) (Skorupska *et al.* 2006). The largest exopolysaccharide cluster of NGR234 harbors 31 genes, is located on pNGR234*b* and encodes for the synthesis of low-molecular-weight-exopolysaccharides (Chen *et al.* 1988; Streit *et al.* 2004). The majority of *exo* genes within this cluster are down-regulated in both deletion mutants. Interestingly, an *rpoS*-like noncoding RNA (ncRNA) encoded within the *exo*-cluster was strongly up-regulated on the antisense transcriptional level. The exact target is not described so far, but the strong regulation in both deletion mutants leads to the hypothesis that this ncRNA interacts with EPS biosynthesis and further suppresses the transcription of the indicated *exo* genes in the absence of AI molecules. It is worth mentioning that this particular ncRNA can only be found in other closely related broad host range strains like *S. fredii* USDA257 and HH103, but not in the closely related narrow host range strain *S. meliloti*

1021. In a final conclusion, it could be summarized that the regulatory mechanisms of the EPS synthesis are slightly different in broad host range strains compared to narrow host range strains, being probably one of the reasons for the difference in host ranges (Krysciak *et al.* 2014). In general, *S. meliloti* seems to be the best studies rhizobial strain with respect to the biosynthesis and regulation of EPS. In this strain EPS synthesis is controlled by the QS system, ExpR/SinI, underlining the results of NGR234 (Glenn *et al.* 2007; Hoang *et al.* 2004; Skorupska *et al.* 2006).

The cofactor biosynthesis genes *bioADBFZ* and their regulator *bioS* as well as genes for the PQQ biosynthesis are all up-regulated in NGR234-△*ngrI* but not in NGR234-△*traI* (Krysciak *et al.* 2014). The *bioS* gene is unique to the sinorhizobia and in *S. meliloti* it is involved in the regulation of biotin-dependent processes and the survival under biotin starvation (Heinz *et al.* 1999; Streit, Phillips 1997). In an earlier report, a link between *bioS* regulation and the QS system in *S. meliloti* has already been hypothesized (Streit, Entcheva 2003). Additionally to the *bio* gene regulation, the PQQ synthesis genes were up-regulated in NGR234-△*ngrI*, which is a first finding of a link between PQQ biosynthesis and QS systems in rhizobia (Krysciak *et al.* 2014). So far, a link between quinolone synthesis and QS was only shown for *P. aeruginosa*, whereby in this strain the quinolone signal itself represents a third cell-to-cell communication system. It is linked to the other two AI-systems by regulatory crosstalk (Dubern, Diggle 2008; Lee, Zhang 2015; Pesci *et al.* 1999). These findings could probably give a first hint on the involvement of PQQ in QS of NGR234.

NGR234 encodes a large number of secretion-related genes that were already described in detail (Schmeisser *et al.* 2009a). The T3SS-I of NGR234 is not regulated in the deletion mutant strains, but T3SS-II is partially down-regulated in NGR234-△*ngrI* and NGR234-△*traI* (Krysciak *et al.* 2014). T3SSs are described to be involved in the translocation of effector proteins directly into the cytoplasm of their eukaryotic host and are therefore important for bacterial virulence (Coburn *et al.* 2007). In NGR234 T3SS-I was found to be one of the key determinants of host range and regulated by the NodD1-SyrM1-NodD2-TstI-cascade. This is consistent with its independent regulation and now verified by the RNA sequencing data from the QS systems (Ausmees *et al.* 2004; Freiberg *et al.* 1997; Kobayashi *et al.* 2004; Marie *et al.* 2004; Viprey *et al.* 1998). The function of the second T3SS is not clear since deletion mutants did not lead to a symbiotic phenotype (Schmeisser *et al.* 2009). Finally, the

transcriptomic data did not allow a clear identification of the function of the T3SS-II; but the data indicated that its expression is regulated in a QS-dependent manner.

6.2 Single cell analysis of autoinducer-dependent and -independent gene expression

With respect to the above-made findings on a population wide level, we asked if similar patterns would be seen, when the expression of AI-dependent and -independent regulated genes is analyzed on a single cell level (Grote *et al.* 2014). Thereby, strong phenotypic heterogeneity with regard to the transcription of the selected genes was observed. The phenomenon of phenotypic heterogeneity attracts more and more attention as an essential parameter for the survival of bacterial populations in rapidly changing environments (see chapter 2.3 and Grote *et al.* 2015). First descriptions on phenotypic heterogeneity with reference to AI-systems were reported for *V. fischeri* and *V. harveyi* (Anetzberger *et al.* 2009; Anetzberger *et al.* 2012b; Perez, Hagen 2010). In *V. fischeri*, for example, the individual cells differ widely in the onset and intensity of bioluminescence that is directly linked to quorum sensing as described in chapter 2.3.1 (Perez, Hagen 2010). Like in *V. fischeri* not all cells are bioluminescing in a laboratory culture of *V. harveyi* (chapter 2.3.1 and Anetzberger *et al.* 2009; Anetzberger *et al.* 2012b).

Comparable to these two organisms, both AI synthase genes of NGR234 are subject to a heterogeneous expression in laboratory cultures. The maximum count of 74.1 % ± 9.1 % (P*traI::rfp*) and 84.4 % ± 2.5 % (P*ngrI::rfp*) of the cells in an ON mode were detected during stationary growth phase (see figure 2 in chapter 4). The heterogeneity of both AI synthase genes was significantly decreased either by the addition of high AI concentrations (3-oxo-C_8-HSL; 50 µM) or by the deletion of the corresponding AI synthase in the strain. This led to 93.1 % ± 5.9 % of *traI*-ON cells and 98.4 % ± 1.0 % of *ngrI*-ON cells in case of the AI addition and 99.8 % ± 0.2 % *traI*-ON and 99.7 % ± 0.2 % of *ngrI*-ON cells in case of the corresponding deletion mutant NGR234-△*traI* and NGR234-△*ngrI*. When low amounts of AI (0.05 µM) were added, the heterogeneity between the cells was not significantly influenced (see figure 4 in chapter 4). These findings support the assumption that NGR234 can distinguish between high and low concentrations of AI molecules. Further, it suggests that the phenotypic heterogeneity of the AI synthases is a well-controlled mechanism that depends on a stringent AI-concentration range. Candidate regulators for these processes are TraR, NgrR,

ExpR or any other LuxR solo encoded on the genome of NGR234. All mentioned genes possess an AI binding site within their protein sequence and are therefore able to bind AI molecules. This could lead to an AI dependent regulatory circuit influencing the phenotypic heterogeneity in NGR234 (Grote *et al.* 2014).

Additional experiments with promoter fusions of further AI-dependent and -independent genes gave evidence for a heterogeneous expression of the QQ genes *dlhR* and *qsdR,* and genes linked to secretion apparatuses *gspD* and *virB* (Grote *et al.* 2014). In this context, it is worth knowing that on the basis of the RNA sequencing analyses described in chapter 6.1, *dlhR*, *qsdR1* and *virB* were observed to be transcribed in an AI-independent manner, while *gspD* is transcribed AI-dependently (Krysciak *et al.* 2014). The results for the AI-dependent heterogeneous transcription described here go well together with the already mentioned descriptions made for the AI-dependent heterogeneous transcription of genes in *V. harveyi* (chapter 4 and Anetzberger *et al.* 2012b). In contrast to the AI-independent heterogeneous behavior of some genes in NGR234, no AI-independent genes are transcribed heterogeneously in *V. harveyi*. In this organism the phenotypic heterogeneity is restricted to genes that depend on AI signals (Anetzberger *et al.* 2012b). This leads to the assumption that additional factors beside the AI molecules control phenotypic heterogeneity in NGR234.

Further, the addition of water- or methanol-soluble plant root exudates of *V. unguiculata*, a host of NGR234, and the proximity to roots of vital *A. thaliana*, which is not a host plant of NGR234, decreased the heterogeneity of both AI synthase genes leading to virtually only fluorescing cells in the population in case of both AI synthase promoter fusions (Figures 5 and 6 in chapter 4). These findings support the hypothesis that plants are able to control phenotypic heterogeneity of NGR234. Thereby, it probably doesn't seem to play a role whether the plant is a potential symbiotic host for NGR234 or not. General signals, released by a wide variety of plants could be the key to the homogenization of NGR234's metabolism, at least of the AI synthase genes. This hypothesis should probably be verified by testing more exudates of different plants (Grote *et al.* 2014).

One of these plant-associated molecules could be octoptine. During the RNA sequencing analyses described in chapters 3 and 6.1, the *moc* cluster of NGR234 was differently regulated after induction with 50 µM AI. The *moc* cluster codes for genes involved in the catabolism of rhizopines and are therefore thought to be the corresponding genes to those in *A. tumefaciens* for the catabolism of octopine. Although plants are not able to synthesize

octopine per se in the plant roots, it is generally accepted that bacteriodic rhizobia are able to synthesize rhizopines to feed other free living rhizobia and thereby providing a growth advantage, so that rhizobia can outcompete rivaling bacteria, like the mechanism in *A. tumefaciens* (Murphy *et al.* 1988). Octopine itself was formerly linked to the QS pathway of *A. tumefaciens* (Oger *et al.* 1998) and it was recently demonstrated that opines provide cooperative *A. tumefaciens* cells within a group a fitness advantage over saprophytic cells (Platt *et al.* 2012). Since the symbiotic plasmid as well as the QS pathways of *A. tumefaciens* and NGR234 are closely related, the general influence of octopine on the QS system of NGR234 makes sense.

In summary, results of this study suggest a heterogeneous transcription of AI synthase genes as well as of genes linked to or not linked to AI molecules of nearly all investigated genes. This suggestion implies that phenotypic heterogeneity is a more general mechanism of gene regulation in NGR234, based on very complex regulatory mechanisms and the involvement of not-yet-identified signal molecules. A second observation of plant signals controlling the heterogeneity of NGR234 leads to the conclusion that plants probably evolved a mechanism to control bacterial gene expression and are able to override bacterial QS signals. This mechanism may be a key to successful symbiosis (Grote *et al.* 2014).

6.3 The copy number of the symbiotic plasmid controls the phenotypic heterogeneity in *S. fredii* NGR234

To get a hint for the molecular mechanism of phenotypic heterogeneity in NGR234, RNA sequencing from a double deletion mutant NGR234-△*traI*-△*ngrI* as well as qPCR on genomic DNA from different NGR234 strains under differing culture conditions were performed.

RNA sequencing analyses of NGR234-△*traI*-△*ngrI* were processed in triplicates and transcription results were compared to the wild type. In the analysis of the data, genes with a fold-change of ≥ 2.0 and an adjusted p-value of ≤ 0.5 were regarded as statistically significant and included in further evaluation. Altogether, more than 1,261 genes were differently regulated in NGR234-△*traI*-△*ngrI*, which is nearly 20 % of all predicted genes (Grote *et al*. unpublished). This was astonishing, since in the single deletion mutants less genes were regulated, i.e. 316 genes in NGR234-△*traI* and 466 genes in NGR234-△*ngrI* (Krysciak *et al.* 2014). The majority of the regulated genes (75 %) was upregulated. The

distribution of genes over the three replicons was unusual taken the size of each replicon into account (pNGR234*a* – 32.43 %; pNGR234*b* – 21.33 %; cNGR234 – 46.24 %). This was a striking observation, because these data state that 98 % of all predicted genes on the symbiotic plasmid are upregulated. Since this would mean that nearly every single gene on the symbiotic plasmid is regulated in dependence on quorum sensing, we wondered if there was another mechanism behind this observation.

First, we tested, which transcriptional effects are triggered by the addition of apigenin, as a *nod* inducing flavonoid (Begum *et al.* 2001) and thereby a substance that is thought to take influence on the transcription of genes encoded on the symbiotic plasmid. Therefore, RNA sequencing analyses with cultures supplemented with apigenin were performed. As expected most apigenin-induced genes were located on the symbiotic plasmid, but these genes were less genes than in NGR234-△*traI*-△*ngrI.* In total 213 genes were differently regulated in the induced wild type culture. Further, tests with an apigenin-induced NGR234-△*traI*-△*ngrI* showed additionally 743 differently regulated genes in comparison to the uninduced double mutant (Grote *et al*. unpublished).

Second, the upregulation of parts of the genes encoded on the symbiotic plasmid should be verified. Thereby, promoter fusion studies with promoters of the *nodABC* gene cluster and the *nopB* gene showed an elevated expression level, measured through a *lacZ* fusion protein activity. This confirmed the elevated transcription rate of the symbiotic plasmid at least of these fusion genes. Additional tests for getting the extracellular protein profile of the nodulation outer proteins (Nops) of NGR234 and NGR234-△*traI*-△*ngrI* verified that *nop* gene products are excreted in a higher amount in NGR234-△*traI*-△*ngrI* compared to the wild type after induction with apigenin (Grote *et al*. unpublished and Table 4 and Figure 3 in chapter 5).

Third, a closer look into the transcriptomic data unraveled the *repABC* genes encoded on the symbiotic plasmid among the strongest regulated genes in NGR234-△*traI*-△*ngrI*. These genes showed a 23-43-fold upregulation, while the average increase in transcription was 7-fold. In addition to the *repABC* genes, two noncoding RNAs in the intergenic region between *repB* and *repC* were upregulated. In general the *repABC* operon is responsible for plasmids replication in many rhizobial isolates, whereby RepA/B are necessary for the active segregation/partitioning of the plasmid and RepC is responsible or the replication itself (Mazur, Koper 2012). Furthermore, transcripts were also mapped to the upstream region of

repA. A more detailed analysis of reads mapping to the intergenic region between *traI* and *repA* identified two small open reading frames (ORFs) that weren't described previously. Although the synteny of the region around the origin of replication of pNGR234*a* to the one of *A. tumefaciens'* Ti plasmid is very high, some differences were detected. In an analysis of intergenic regions of many rhizobial isolates, most isolates, like *A. tumefaciens*, only have an intergenic region of around 360 bp between *traI* and *repA*. In contrast to this NGR234, *S. fredii* HH103 and *S. fredii* USDA257 have an intergenic region that is 803 bp in length. These data could explain why the two identified ORFs are not or only in parts present in the other strains summarized in Table 2 in chapter 5 (Grote *et al.* unpublished). The two ORFs were named *repX* and *repA0* and probably code for a 51 aa respectively 143 aa protein, if translated into a protein sequence (Grote *et al*. unpublished). Further investigations have to prove whether the ORFs are transcribed into proteins or whether they are regulatory RNAs.

To test whether the upregulation of the *repABC* genes as well as the newly identified ORFs have an influence on the copy number of the symbiotic plasmid, RT-PCRs on the genomic DNA were performed. These tests showed a 2-3 fold increased copy number of the symbiotic plasmid in NGR234-△*traI*-△*ngrI* compared to the wild type. The addition of moderate concentrations of AI (0.05 µM and 5 µM) to cultures of the double deletion mutant led to a further increase in copy number to 3-4.6-fold. The addition of 50 µM AI to the culture medium finally led to an increase of up to 9-11 copies of symbiotic plasmid on average per cell compared to the uninduced wild type. While the moderate AI additions had equal effects on the wild type, the high concentration had no effect on the copy number of the symbiotic plasmid in NGR234. These results imply that some regulatory mechanisms in NGR234-△*traI*-△*ngrI* are different from those in NGR234 (Grote *et al*. unpublished).

Different reasons for the upregulation of the copy number per cell in NGR234-△*traI*-△*ngrI* exist. In general, the distribution of macromolecules between each daughter cell can vary significantly during cell cycling. In this context, polyploidy of prokaryotic cells is, for example, often triggered by host-bacteria interactions. The nitrogen fixing bacteroids of *S. meliloti* comprise multiple genomic copies to higher the nitrogen fixation functions (Jahn *et al.* 2015). The variable copy number of the chromosome of *B. subtilis* is crucial for the sporulation process and is regulated through QS (Chai *et al.* 2011; Jahn *et al.* 2015). In addition to the chromosomal copy number variation, the number of plasmids per cell is even more variable, which is often determined by the replication system (Jahn *et al.* 2015). It is

already known that in *A. tumefaciens* the copy number of the Ti plasmid was increased after the addition of the corresponding autoinducer molecule and depends on the regulation of the QS related regulator TraR (Pappas, Winans 2003). Additionally, the increased copy number could be linked to an increased tumorigenesis (Li, Farrand 2000b; Pappas 2008; Pappas, Winans 2003).

The findings of *A. tumefaciens* are in part comparable to the results observed for NGR234. In NGR234, the copy number of the symbiotic plasmid is increased by the addition of AI like in *A. tumefaciens.* Additionally, the copy number is increased by the total lack of AI (in NGR234-△*traI*-△*ngrI*). In contrast to *A. tumefaciens*, the nodulation efficiency is not influenced by the increased copy number, since NGR234-△*traI*-△*ngrI* does not show a differing nodulation phenotype (Grote *et al*. unpublished).

In another closely related strain, *R. leguminosarum* bv. *viciae*, the plasmid regulation genes are co-regulated with the plasmid transfer/QS system genes like the regulation network in *A. tumefaciens*. This could give first hints for the regulatory mechanism of plasmid copy number control in NGR234. In *R. leguminosarum*, *repA* was found to be transcribed from two different start sites, from which only one needs the activity of TraR, resulting in a shorter and a longer version of *repA* mRNA (McAnulla *et al.* 2007). With end point RT-PCR on cDNA from NGR234 and NGR234-△*traI*-△*ngrI* we showed that *repX* and *repA0* are only transcribed in NGR234-△*traI*-△*ngrI* but not in the wild type strain. In addition, *repA0* is transcribed independently from *repA*. This opposes the hypothesis, that NGR234 possesses a *repA* with to different lengths according to the situation in *R. leguminosarum* bv. *viciae*. Instead, NGR234 transcribes an additional ORF in dependence to the environmental conditions (Grote *et al*. unpublished). Further tests with an additional copy of the *repA0* in a pBBR1MCS-2 vector inserted into NGR234 proved a direct impact on the copy number of the symbiotic plasmid. Under these circumstances the copy number of the symbiotic plasmid increases up to 3-4 copies per cell (Grote *et al*. unpublished).

In summary, the described results in this chapter give a first hint on the regulatory mechanisms of the copy number of the symbiotic plasmid in NGR234. Furthermore, they give a first direction to the regulation of phenotypic heterogeneity. The copy number in the single AI deletion mutants increases up to 2-fold. At the same time, the phenotypic heterogeneity that is observed in the wild type strain is decreased and all cells turn into the ON-mode (Grote *et al.* 2014). From these findings it can be hypothesized that NGR234 has

mechanisms for regulating phenotypic heterogeneity of cells in a population. Some of these mechanisms are most likely directly related to the AI level of the surrounding environment. Furthermore, the strict regulation can easily be disturbed by taking influence on the quorum sensing system or the available types of AI molecules, e.g. by deleting AI synthase genes or adding AI molecules to the medium.

Besides the effects on phenotypic heterogeneity, a biological significance could be linked to this phenomenon. In a root hair curling assay, extracted culture supernatant of NGR234-△*traI*-△*ngrI* was able to induce root hair curling of *V. unguiculata* after 24 h induction although the *nod* genes were not specifically induced by a plant excreted flavonoid. Extracted supernatants of the wild type did not lead to a root hair curling phenotype when cultivated under the same uninduced conditions. This induction has no influence on the final nodule number, but could probably help NGR234 to be faster in nodulating host plants than other strains and it would eventually overcome host restriction. This could be an additional explanation for the broad host range of NGR234, being able to start the nodulation process without flavonoid, by elevating the number of symbiotic plasmids per cell (Grote *et al*. unpublished).

7 References

Ackermann M. 2015. A functional perspective on phenotypic heterogeneity in microorganisms. Nat Rev Microbiol. **13**(8): 497-508.

Anetzberger C, Pirch T, Jung K. 2009. Heterogeneity in quorum sensing-regulated bioluminescence of *Vibrio harveyi*. *Mol Microbiol*. **73**(2): 267-277.

Anetzberger C, Reiger M, Fekete A, Schell U, Stambrau N, Plener L, Kopka J, Schmitt-Kopplin P, Hilbi H, Jung K. 2012a. Autoinducers act as biological timers in *Vibrio harveyi*. Plos One. **7**(10): e48310.

Anetzberger C, Schell U, Jung K. 2012b. Single cell analysis of *Vibrio harveyi* uncovers functional heterogeneity in response to quorum sensing signals. BMC Microbiol. **12**: 209.

Ausmees N, Kobayashi H, Deakin WJ, Marie C, Krishnan HB, Broughton WJ, Perret X. 2004. Characterization of NopP, a type III secreted effector of *Rhizobium* sp. strain NGR234. J Bacteriol. **186**(14): 4774-4780.

Barken KB, Pamp SJ, Yang L, Gjermansen M, Bertrand JJ, Klausen M, Givskov M, Whitchurch CB, Engel JN, Tolker-Nielsen T. 2008. Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in *Pseudomonas aeruginosa* biofilms. Environ Microbiol. **10**(9): 2331-2343.

Bassler BL. 1999. How bacteria talk to each other: regulation of gene expression by quorum sensing. Curr Opin Microbiol. **2**(6): 582-587.

Begum AA, Leibovitch S, Migner P, Zhang F. 2001. Specific flavonoids induced *nod* gene expression and pre-activated *nod* genes of *Rhizobium leguminosarum* increased pea (*Pisum sativum* L.) and lentil (*Lens culinaris* L.) nodulation in controlled growth chamber environments. J Exp Bot. **52**(360): 1537-1543.

Bertram R, Schuster CF. 2014. Post-transcriptional regulation of gene expression in bacterial pathogens by toxin-antitoxin systems. Front Cell Infect Microbiol. **4**: 6.

Bigger JW. 1944. Treatment of staphylococcal infections with penicillin by intermittent sterilization. Lancet. **244**: 497-500.

Bijtenhoorn P, Mayerhofer H, Müller-Dieckmann J, Utpatel C, Schipper C, Hornung C, Szesny M, Grond S, Thürmer A, Brzuszkiewicz E, Daniel R, Dierking K, Schulenburg H, Streit WR. 2011. A novel metagenomic short-chain dehydrogenase/reductase attenuates *Pseudomonas aeruginosa* biofilm formation and virulence on *Caenorhabditis elegans*. Plos One. **6**(10): e26278.

Boukli NM, Sunderasan E, Bartsev A, Hochstrasser D, Perret X, Bjourson AJ, Krause A, Broughton WJ. 2007. Early legume responses to inoculation with *Rhizobium* sp NGR234. J Plant Physiol. **164**(6): 794-806.

Broughton WJ, Hanin M, Relić B, Kopciñska J, Golinowski W, Şimşek Ş, Ojanen-Reuhs T, Reuhs B, Marie C, Kobayashi H, Bordogna B, Le Quéré A, Jabbouri S, Fellay R, Perret X, Deakin WJ. 2006. Flavonoid-inducible modifications to rhamnan O antigens are necessary for *Rhizobium* sp strain NGR234-legume symbioses. J Bacteriol. **188**(10): 3654-3663.

Bubendorfer S, Koltai M, Rossmann F, Sourjik V, Thormann KM. 2014. Secondary bacterial flagellar system improves bacterial spreading by increasing the directional persistence of swimming. Proc Natl Acad Sci USA. **111**(31): 11485-11490.

Cárcamo-Oyarce G, Lumjiaktase P, Kümmerli R, Eberl L. 2015. Quorum sensing triggers the stochastic escape of individual cells from *Pseudomonas putida* biofilms. Nat Commun. **6**: 5945.

Carrolo M, Frias MJ, Pinto FR, Melo-Cristino J, Ramirez M. 2010. Prophage spontaneous activation promotes DNA release enhancing biofilm formation in *Streptococcus pneumoniae*. Plos One. **5**(12): e15678.

Chai YR, Chu F, Kolter R, Losick R. 2008. Bistability and biofilm formation in *Bacillus subtilis*. Mol Microbiol. **67**(2): 254-263.

Chai YR, Norman T, Kolter R, Losick R. 2011. Evidence that metabolism and chromosome copy number control mutually exclusive cell fates in *Bacillus subtilis*. EMBO J. **30**(7): 1402-1413.

Chen GZ, Jeffrey PD, Fuqua C, Shi YG, Chen LL. 2007. Structural basis for antiactivation in bacterial quorum sensing. Proc Natl Acad Sci USA. **104**(42): 16474-16479.

Chen H, Gray JX, Nayudu M, Djordjevic MA, Batley M, Redmond JW, Rolfe BG. 1988. Five genetic-loci involved in the synthesis of acidic exopolysaccharides are closely linked in the genome of *Rhizobium* sp strain NGR234. Mol Gen Genet. **212**(2): 310-316.

Chen HC, Teplitski M, Robinson JB, Rolfe BG, Bauer WD. 2003. Proteomic analysis of wild-type *Sinorhizobium meliloti* responses to N-acyl homoserine lactone quorum-sensing signals and the transition to stationary phase. J Bacteriol. **185**(17): 5029-5036.

Chugani S, Kim BS, Phattarasukol S, Brittnacher MJ, Choi SH, Harwood CS, Greenberg EP. 2012. Strain-dependent diversity in the *Pseudomonas aeruginosa* quorum-sensing regulon. Proc Natl Acad Sci USA. **109**(41): E2823-E2831.

Chung JD, Stephanopoulos G, Ireton K, Grossman AD. 1994. Gene-expression in single cells of *Bacillus subtilis*: evidence that a threshold mechanism controls the initiation of sporulation. J Bacteriol. **176**(7): 1977-1984.

Coburn B, Sekirov I, Finlay BB. 2007. Type III secretion systems and disease. Clin Microbiol Rev. **20**(4): 535-549.

Conlon BP. 2014. *Staphylococcus aureus* chronic and relapsing infections: Evidence of a role for persister cells: An investigation of persister cells, their formation and their role in *S. aureus* disease. Bioessays. **36**(10): 991-996.

Daniels R, De Vos DE, Desair J, Raedschelders G, Luyten E, Rosemeyer V, Verreth C, Schoeters E, Vanderleyden J, Michiels J. 2002. The *cin* quorum sensing locus of *Rhizobium etli* CNPAF512 affects growth and symbiotic nitrogen fixation. J Biol Chem. **277**(1): 462-468.

Davidson CJ, Surette MG. 2008. Individuality in Bacteria. Annu Rev Genet. **42**: 253-268.

De Angelis G, Moschioni M, Muzzi A, Pezzicoli A, Censini S, Delany I, Lo Sapio M, Sinisi A, Donati C, Masignani V, Barocchi MA. 2011. The *Streptococcus pneumoniae* pilus-1 displays a biphasic expression pattern. Plos One. **6**(6): e21269.

De Jong IG, Haccou P, Kuipers OP. 2011. Bet hedging or not? A guide to proper classification of microbial survival strategies. Bioessays. **33**(3): 215-223.

Dhar N, McKinney JD. 2007. Microbial phenotypic heterogeneity and antibiotic tolerance. Curr Opin Microbiol. **10**(1): 30-38.

Diggle SP, Matthijs S, Wright VJ, Fletcher MP, Chhabra SR, Lamont IL, Kong XL, Hider RC, Cornelis P, Camara M, Williams P. 2007. The *Pseudomonas aeruginosa* 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. Chem Biol. **14**(1): 87-96.

Dötsch A, Eckweiler D, Schniederjans M, Zimmermann A, Jensen V, Scharfe M, Geffers R, Häussler S. 2012. The *Pseudomonas aeruginosa* transcriptome in planktonic cultures and static biofilms using RNA sequencing. Plos One. **7**(2): e31092.

Dubern JF, Diggle SP. 2008. Quorum sensing by 2-alkyl-4-quinolones in *Pseudomonas aeruginosa* and other bacterial species. Mol Biosyst. **4**(9): 882-888.

Dubnau D, Losick R. 2006. Bistability in bacteria. Mol Microbiol. **61**(3): 564-572.

Freiberg C, Fellay R, Bairoch A, Broughton WJ, Rosenthal A, Perret X. 1997. Molecular basis of symbiosis between *Rhizobium* and legumes. Nature. **387**(6631): 394-401.

Fridman O, Goldberg A, Ronin I, Shoresh N, Balaban NQ. 2014. Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. Nature. **513**(7518): 418-421.

Fritz G, Megerle JA, Westermayer SA, Brick D, Heermann R, Jung K, Rädler JO, Gerland U. 2014. Single cell kinetics of phenotypic switching in the arabinose utilization system of *E. coli*. Plos One. **9**(2): e89532.

Fuqua C, Burbea M, Winans SC. 1995. Activity of the *Agrobacterium* Ti plasmid conjugal transfer regulator TraR is inhibited by the product of the *traM* gene. J Bacteriol. **177**(5): 1367-1373.

Fuqua C, Winans SC. 1996. Localization of OccR-activated and TraR-activated promoters that express two ABC-type permeases and the *traR* gene of Ti plasmid pTiR10. Mol Microbiol. **20**(6): 1199-1210.

Gao MS, Chen HC, Eberhard A, Gronquist MR, Robinson JB, Rolfe BG, Bauer WD. 2005. *sinI*- and *expR*-dependent quorum sensing in *Sinorhizobium meliloti*. J Bacteriol. **187**(23): 7931-7944.

Gao MS, Coggin A, Yagnik K, Teplitski M. 2012. Role of specific quorum-sensing signals in the regulation of exopolysaccharide II production within *Sinorhizobium meliloti* spreading colonies. Plos One. **7**(8): e42611.

Garg N, Manchanda G, Kumar A. 2014. Bacterial quorum sensing: circuits and applications. Antonie Van Leeuwenhoek. **105**(2): 289-305.

Garmyn D, Gal L, Briandet R, Guilbaud M, Lemaître JP, Hartmann A, Piveteau P. 2011. Evidence of autoinduction heterogeneity via expression of the Agr system of *Listeria monocytogenes* at the single-cell level. Appl Environ Microbiol. **77**(17): 6286-6289.

Glenn SA, Gurich N, Feeney MA, González JE. 2007. The ExpR/Sin quorum-sensing system controls succinoglycan production in *Sinorhizobium meliloti*. J Bacteriol. **189**(19): 7077-7088.

Gödeke J, Paul K, Lassak J, Thormann KM. 2011. Phage-induced lysis enhances biofilm formation in *Shewanella oneidensis* MR-1. ISME J. **5**(4): 613-626.

González JE, Marketon MM. 2003. Quorum sensing in nitrogen-fixing rhizobia. Microbiol Mol Biol Rev. **67**(4): 574-592.

Grantcharova N, Peters V, Monteiro C, Zakikhany K, Römling U. 2010. Bistable expression of CsgD in biofilm development of *Salmonella enterica* serovar typhimurium. J Bacteriol. **192**(2): 456-466.

Gray KM, Pearson JP, Downie JA, Boboye BEA, Greenberg EP. 1996. Cell-to-cell signaling in the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum*: Autoinduction of a stationary phase and rhizosphere-expressed genes. J Bacteriol. **178**(2): 372-376.

Grote J, Krysciak D, Schorn A, Dahlke RI, Soonvald L, Müller J, Hense BA, Schwarzfischer M, Sauter M, Schmeisser C, Streit WR. 2014. Evidence of autoinducer-dependent and -independent heterogeneous gene expression in *Sinorhizobium fredii* NGR234. Appl Environ Microbiol. **80**(18): 5572-5582.

Grote J, Krysciak D, Streit WR. 2015. Phenotypic heterogeneity, a phenomenon that may explain why quorum sensing does not always result in truly homogenous cell behavior. Appl Environ Microbiol. **81**(16): 5280-5289.

Hamoen LW, Venema G, Kuipers OP. 2003. Controlling competence in *Bacillus subtilis*: shared use of regulators. Microbiology. **149**(Pt 1): 9-17.

Hartmann A, Schikora A. 2012. Quorum sensing of bacteria and trans-kingdom interactions of N-acyl homoserine lactones with eukaryotes. J Chem Ecol. **38**(6): 704-713.

He XS, Chang W, Pierce DL, Seib LO, Wagner J, Fuqua C. 2003. Quorum sensing in *Rhizobium* sp strain NGR234 regulates conjugal transfer (tra) gene expression and influences growth rate. J Bacteriol. **185**(3): 809-822.

Heinz EB, Phillips DA, Streit WR. 1999. BioS, a biotin-induced, stationary-phase, and possible LysR-type regulator in *Sinorhizobium meliloti*. Mol Plant Microbe In. **12**(9): 803-812.

Helaine S, Kugelberg E. 2014. Bacterial persisters: formation, eradication, and experimental systems. Trends Microbiol. **22**(7): 417-424.

Hense BA, Müller J, Kuttler C, Hartmann A. 2012. Spatial heterogeneity of autoinducer regulation systems. Sensors (Basel). **12**(4): 4156-4171.

Hentzer M, Wu H, Andersen JB, Riedel K, Rasmussen TB, Bagge N, Kumar N, Schembri MA, Song ZJ, Kristoffersen P, Manefield M, Costerton JW, Molin S, Eberl L, Steinberg P, Kjelleberg S, Høiby N, Givskov M. 2003. Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. EMBO J. **22**(15): 3803-3815.

Hoang HH, Becker A, González JE. 2004. The LuxR homolog ExpR, in combination with the Sin quorum sensing system, plays a central role in *Sinorhizobium meliloti* gene expression. J Bacteriol. **186**(16): 5460-5472.

Hoang HH, Gurich N, González JE. 2008. Regulation of motility by the ExpR/Sin quorum-sensing system in *Sinorhizobium meliloti*. J Bacteriol. **190**(3): 861-871.

Hwang IY, Cook DM, Farrand SK. 1995. A new regulatory element modulates homoserine lactone-mediated autoinduction of Ti plasmid conjugal transfer. J Bacteriol. **177**(2): 449-458.

Jahn M, Günther S, Müller S. 2015. Non-random distribution of macromolecules as driving forces for phenotypic variation. Curr Opin Microbiol. **25**: 49-55.

Jarvis BDW, Downer HL, Young JPW. 1992. Phylogeny of fast-growing soybean-nodulating rhizobia supports synonymy of *Sinorhizobium* and *Rhizobium* and assignment to *Rhizobium fredii*. Int J Syst Bacteriol. **42**(1): 93-96.

Kamenšek S, Podlesek Z, Gillor O, Žgur-Bertok D. 2010. Genes regulated by the *Escherichia coli* SOS repressor LexA exhibit heterogenous expression. BMC Microbiol. **10**: 283.

Kesel S, Mader A, Höfler C, Mascher T, Leisner M. 2013. Immediate and heterogeneous response of the LiaFSR two-component system of *Bacillus subtilis* to the peptide antibiotic bacitracin. Plos One. **8**(1): e53457.

Kobayashi H, Naciri-Graven Y, Broughton WJ, Perret X. 2004. Flavonoids induce temporal shifts in gene-expression of nod-box controlled loci in *Rhizobium* sp NGR234. Mol Microbiol. **51**(2): 335-347.

Koch G, Yepes A, Förstner KU, Wermser C, Stengel ST, Modamio J, Ohlsen K, Foster KR, Lopez D. 2014. Evolution of resistance to a last-resort antibiotic in *Staphylococcus aureus* via bacterial bompetition. Cell. **158**(5): 1060-1071.

Kohler PRA, Zheng JY, Schoffers E, Rossbach S. 2010. Inositol catabolism, a key pathway in *Sinorhizobium meliloti* for competitive host nodulation. Appl Environ Microbiol. **76**(24): 7972-7980.

Kröger C, Srikumar S, Ellwart J, Fuchs TM. 2011. Bistability in myo-Inositol utilization by *Salmonella enterica* serovar typhimurium. J Bacteriol. **193**(6): 1427-1435.

Krysciak D, Grote J, Orbegoso MR, Utpatel C, Förstner KU, Li L, Schmeisser C, Krishnan HB, Streit WR. 2014. RNA sequencing analysis of the broad-host-range strain *Sinorhizobium fredii* NGR234 identifies a large set of genes linked to quorum sensing-dependent regulation in the background of a *traI* and *ngrI* deletion mutant. Appl Environ Microbiol. **80**(18): 5655-5671.

Krysciak D, Schmeisser C, Preuss S, Riethausen J, Quitschau M, Grond S, Streit WR. 2011. Involvement of multiple loci in quorum quenching of autoinducer I molecules in the nitrogen-fixing symbiont *Rhizobium* (*Sinorhizobium*) sp strain NGR234. Appl Environ Microbiol. **77**(15): 5089-5099.

LaRock CN, Yu J, Horswill AR, Parsek MR, Minion FC. 2013. Transcriptome analysis of acyl-homoserine lactone-based quorum sensing regulation in *Yersinia pestis*. Plos One. **8**(4): e62337.

Lee J, Zhang LH. 2015. The hierarchy quorum sensing network in *Pseudomonas aeruginosa*. Protein Cell. **6**(1): 26-41.

Lemme A, Gröbe L, Reck M, Tomasch J, Wagner-Döbler I. 2011. Subpopulation-specific transcriptome analysis of competence-stimulating-peptide-induced *Streptococcus mutans*. J Bacteriol. **193**(8): 1863-1877.

Lewis K. 2010. Persister cells. Annu Rev Microbiol. **64**: 357-372.

Li PL, Farrand SK. 2000. The replicator of the nopaline-type Ti plasmid pTiC58 is a member of the *repABC* family and is influenced by the TraR-dependent quorum-sensing: Regulatory system. J Bacteriol. **182**(1): 179-188.

Li Y, Tian CF, Chen WF, Wang L, Sui XH, Chen WX. 2013. High-resolution transcriptomic analyses of *Sinorhizobium* sp NGR234 bacteroids in determinate nodules of *Vigna unguiculata* and indeterminate nodules of *Leucaena leucocephala*. Plos One. **8**(8): e70531.

Luo ZQ, Qin YP, Farrand SK. 2000. The antiactivator TraM interferes with the autoinducer-dependent binding of TraR to DNA by interacting with the C-terminal region of the quorum-sensing activator. J Biol Chem. **275**(11): 7713-7722.

Maisonneuve E, Gerdes K. 2014. Molecular mechanisms underlying bacterial persisters. Cell. **157**(3): 539-548.

Marie C, Deakin WJ, Ojanen-Reuhs T, Diallo E, Reuhs B, Broughton WJ, Perret X. 2004. TtsI, a key regulator of *Rhizobium* species NGR234 is required for type III-dependent protein secretion and synthesis of rhamnose-rich polysaccharides. Mol Plant Microbe In. **17**(9): 958-966.

Marketon MM, González JE. 2002. Identification of two quorum-sensing systems in *Sinorhizobium meliloti*. J Bacteriol **184**(13): 3466-3475.

Mazur A, Koper P. 2012. Rhizobial plasmids – replication, structure and biological role. Cent Eur J Biol. **7**(4): 571-586.

McAnulla C, Edwards A, Sanchez-Contreras M, Sawers RG, Downie JA. 2007. Quorum-sensing-regulated transcriptional initiation of plasmid transfer and replication genes in *Rhizobium leguminosarum* biovar *viciae*. Microbiology. **153**: 2074-2082.

Megerle JA, Fritz G, Gerland U, Jung K, Rädler JO. 2008. Timing and dynamics of single cell gene expression in the arabinose utilization system. Biophys J. **95**(4): 2103-2115.

Mehra S, Charaniya S, Takano E, Hu WS. 2008. A bistable gene switch for antibiotic biosynthesis: The butyrolactone regulon in *Streptomyces coelicolor*. Plos One. **3**(7): e2724.

Minoia M, Gaillard M, Reinhard F, Stojanov M, Sentchilo V, van der Meer JR. 2008. Stochasticity and bistability in horizontal transfer control of a genomic island in *Pseudomonas*. Proc Natl Acad Sci USA. **105**(52): 20792-20797.

Miyazaki R, Minoia M, Pradervand N, Sulser S, Reinhard F, van der Meer JR. 2012. Cellular Variability of RpoS expression underlies subpopulation activation of an integrative and conjugative element. Plos Genet. **8**(7): e1002818.

Müller C, Birmes FS, Niewerth H, Fetzner S. 2014. Conversion of the *Pseudomonas aeruginosa* quinolone signal and related alkylhydroxyquinolines by *Rhodococcus* sp strain BG43. Appl Environ Microbiol. **80**(23): 7266-7274.

Murphy PJ, Heycke N, Banfalvi Z, Tate ME, Debruijn F, Kondorosi A, Tempe J, Schell J. 1987. Genes for the catabolism and synthesis of an opine-like compound in *Rhizobium meliloti* are closely linked and on the Sym plasmid. Proc Natl Acad Sci USA. **84**(2): 493-497.

Murphy PJ, Heycke N, Trenz SP, Ratet P, Debruijn FJ, Schell J. 1988. Synthesis of an opine-like compound, a rhizopine, in alfalfa nodules is symbiotically regulated. Proc Natl Acad Sci USA. **85**(23): 9133-9137.

Nanda AM, Heyer A, Krämer C, Grünberger A, Kohlheyer D, Frunzke J. 2014. Analysis of SOS-induced spontaneous prophage induction in *Corynebacterium glutamicum* at the single-cell level. J Bacteriol. **196**(1): 180-188.

Nealson KH, Hastings JW. 1979. Bacterial bioluminescence: its control and ecological significance. Microbiol Rev. **43**(4): 496-518.

Nealson KH, Platt T, Hastings JW. 1970. Cellular control of synthesis and activity of bacterial luminescent system. J Bacteriol. **104**(1): 313-322.

Oger P, Kim KS, Sackett RL, Piper KR, Farrand SK. 1998. Octopine-type Ti plasmids code for a mannopine-inducible dominant-negative allele of *traR*, the quorum-sensing activator that regulates Ti plasmid conjugal transfer. Mol Microbiol. **27**(2): 277-288.

Pappas KM. 2008. Cell-cell signaling and the *Agrobacterium tumefaciens* Ti plasmid copy number fluctuations. Plasmid. **60**(2): 89-107.

Pappas KM, Winans SC. 2003. The RepA and RepB autorepressors and TraR play opposing roles in the regulation of a Ti plasmid *repABC* operon. Mol Microbiol. **49**(2): 441-455.

Parkhill J, Wren BW, Thomson NR, Titball RW, Holden MTG, Prentice MB, Sebaihia M, James KD, Churcher C, Mungall KL, Baker S, Basham D, Bentley SD, Brooks K, Cerdeñ o-Tárraga AM, Chillingworth T, Cronin A, Davies RM, Davis P, Dougan G, Feltwell T, Hamlin N, Holroyd S, Jagels K, Karlyshev AV, Leather S, Moule S, Oyston PCF, Quail M, Rutherford K, Simmonds M, Skelton J, Stevens K, Whitehead S, Barrell BG. 2001. Genome sequence of *Yersinia pestis*, the causative agent of plague. Nature. **413**(6855): 523-527.

Patzelt D, Wang H, Buchholz I, Rohde M, Gröbe L, Pradella S, Neumann A, Schulz S, Heyber S, Münch K, Münch R, Jahn D, Wagner-Döbler I, Tomasch J. 2013. You are what you talk: quorum sensing induces individual morphologies and cell division modes in *Dinoroseobacter shibae*. ISME J. **7**(12): 2274-2286.

Pérez PD, Hagen SJ. 2010. Heterogeneous response to a quorum-sensing signal in the luminescence of individual *Vibrio fischeri*. Plos One. **5**(11): e15473.

Pesci EC, Milbank JBJ, Pearson JP, McKnight S, Kende AS, Greenberg EP, Iglewski BH. 1999. Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA. **96**(20): 11229-11234.

Pierson LS, Wood DW, Pierson EA. 1998. Homoserine lactone-mediated gene regulation in plant-associated bacteria. Annu Rev Phytopathol. **36**: 207-225.

Platt TG, Fuqua C, Bever JD. 2012. Resource and competitive dynamics shape the benefits of public goods cooperation in a plant pathogen. Evolution. **66**(6): 1953-1965.

Pradhan BB, Chatterjee S. 2014. Reversible non-genetic phenotypic heterogeneity in bacterial quorum sensing. Mol Microbiol. **92**(3): 557-569.

Pueppke SG, Broughton WJ. 1999. *Rhizobium* sp. strain NGR234 and K USDA257 share exceptionally broad, nested host ranges. Mol Plant Microbe Int. **12**(4): 293-318.

Ramsay JP, Major AS, Komarovsky VM, Sullivan JT, Dy RL, Hynes MF, Salmond GPC, Ronson CW. 2013. A widely conserved molecular switch controls quorum sensing and symbiosis island transfer in *Mesorhizobium loti* through expression of a novel antiactivator. Mol Microbiol. **87**(1): 1-13.

Ratcliff WC, Denison RF. 2011. Bacterial persistence and bet hedging in *Sinorhizobium meliloti*. Commun Integr Biol. **4**(1): 98-100.

Rossbach S, Rasul G, Schneider M, Eardly B, Debruijn FJ. 1995. Structural and functional conservation of the rhizopine catabolism (*moc*) locus is limited to selected *Rhizobium meliloti* strains and unrelated to their geographical origin. Mol Plant Microbe Int. **8**(4): 549-559.

Saint CP, Wexler M, Murphy PJ, Tempé J, Tate ME, Murphy PJ. 1993. Characterization of genes for synthesis and catabolism of a new rhizopine induced in nodules by *Rhizobium meliloti* Rm220-3: extension of the rhizopine concept. J Bacteriol. **175**(16): 5205-5215.

Sanchez-Contreras M, Bauer WD, Gao MS, Robinson JB, Downie JA. 2007. Quorum-sensing regulation in rhizobia and its role in symbiotic interactions with legumes. Philos Trans R Soc Lond B Biol Sci. **362**(1483): 1149-1163.

Schaefer AL, Greenberg EP, Oliver CM, Oda Y, Huang JJ, Bittan-Banin G, Peres CM, Schmidt S, Juhaszova K, Sufrin JR, Harwood CS. 2008. A new class of homoserine lactone quorum-sensing signals. Nature. **454**(7204): 595-U596.

Schlüter JP, Czuppon P, Schauer O, Pfaffelhuber P, McIntosh M, Becker A. 2015. Classification of phenotypic subpopulations in isogenic bacterial cultures by triple promoter probing at single cell level. J Biotechnol. **198**: 3-14.

Schmeisser C, Liesegang H, Krysciak D, Bakkou N, Le Quéré A, Wollherr A, Heinemeyer I, Morgenstern B, Pommerening-Röser A, Flores M, Palacios R, Brenner S, Gottschalk G, Schmitz RA, Broughton WJ, Perret X, Strittmatter AW, Streit WR. 2009. *Rhizobium* sp strain NGR234 possesses a remarkable number of secretion systems. Appl Environ Microbiol. **75**(12): 4035-4045.

Schuster CF, Bertram R. 2013. Toxin-antitoxin systems are ubiquitous and versatile modulators of prokaryotic cell fate. FEMS Microbiol Lett. **340**(2): 73-85.

Schuster M, Lostroh CP, Ogi T, Greenberg EP. 2003. Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. J Bacteriol. **185**(7): 2066-2079.

Siebring J, Elema MJH, Vega FD, Kovács AT, Haccou P, Kuipers OP. 2014. Repeated triggering of sporulation in *Bacillus subtilis* selects against a protein that affects the timing of cell division. ISME J. **8**(1): 77-87.

Sitnikov DM, Schineller JB, Baldwin TO. 1995. Transcriptional regulation of bioluminescence genes from *Vibrio fischeri*. Mol Microbiol. **17**(5):801-812.

Skorpil P, Saad MM, Boukli NM, Kobayashi H, Ares-Orpel F, Broughton WJ, Deakin WJ. 2005. NopP, a phosphorylated effector of *Rhizobium* sp strain NGR234, is a major determinant of nodulation of the tropical legumes *Flemingia congesta* and *Tephrosia vogelii*. Mol Microbiol. **57**(5): 1304-1317.

Skorupska A, Janczarek M, Marczak M, Mazur A, Król J. 2006. Rhizobial exopolysaccharides: genetic control and symbiotic functions. Microb Cell Fact. **5**: 7.

Smith RS, Iglewski BH. 2003. *P. aeruginosa* quorum-sensing systems and virulence. Curr Opin Microbiol. **6**(1): 56-60.

Smits WK, Eschevins CC, Susanna KA, Bron S, Kuipers OP, Hamoen LW. 2005. Stripping *Bacillus*: ComK auto-stimulation is responsible for the bistable response in competence development. Mol Microbiol. **56**(3): 604-614.

Smits WK, Kuipers OP, Veening JW. 2006. Phenotypic variation in bacteria: the role of feedback regulation. Nat Rev Microbiol. **4**(4): 259-271.

Son MJ, Ahn SJ, Guo Q, Burne RA, Hagen SJ. 2012. Microfluidic study of competence regulation in *Streptococcus mutans*: environmental inputs modulate bimodal and unimodal expression of *comX*. Mol Microbiol. **86**(2): 258-272.

Sperandio V, Torres AG, Jarvis B, Nataro JP, Kaper JB. 2003. Bacteria-host communication: The language of hormones. Proc Natl Acad Sci USA. **100**(15): 8951-8956.

Staehelin C, Forsberg LS, D'Haeze W, Gao MY, Carlson RW, Xie ZP, Pellock BJ, Jones KM, Walker GC, Streit WR, Broughton WJ. 2006. Exo-oligosaccharides of *Rhizobium* sp strain NGR234 are required for symbiosis with various legumes. J Bacteriol. **188**(17): 6168-6178.

Stewart MK, Cummings LA, Johnson ML, Berezow AB, Cookson BT. 2011. Regulation of phenotypic heterogeneity permits *Salmonella evasion* of the host caspase-1 inflammatory response. Proc Natl Acad Sci USA. **108**(51): 20742-20747.

Streit WR, Entcheva P. 2003. Biotin in microbes, the genes involved in its biosynthesis, its biochemical role and perspectives for biotechnological production. Appl Microbiol Biotechnol. **61**(1): 21-31.

Streit WR, Phillips DA. 1997. A biotin-regulated locus, *bioS*, in a possible survival operon of *Rhizobium meliloti*. Mol Plant Microbe Int. **10**(7): 933-937.

Streit WR, Schmitz RA, Perret X, Staehelin C, Deakin WJ, Raasch C, Liesegang H, Broughton WJ. 2004. An evolutionary hot spot: the pNGR234*b* replicon of *Rhizobium* sp strain NGR234. J Bacteriol. **186**(2): 535-542.

Swiderska A, Berndtson AK, Cha MR, Li L, Beaudoin III GMJ, Zhu J, Fuqua C. 2001. Inhibition of the *Agrobacterium tumefaciens* TraR quorum-sensing regulator - Interactions with the TraM anti-activator. J Biol Chem. **276**(52): 49449-49458.

Trinick MJ. 1980. Relationships amongst the fast-growing Rhizobia of *Lablab purpureus*, *Leucaena leucocephala*, *Mimosa* spp, *Acacia farnesiana* and *Sesbania grandiflora* and their affinities with other rhizobial groups. J Appl Bacteriol. **49**(1): 39-53.

Uroz S, Dessaux Y, Oger P. 2009. Quorum sensing and quorum quenching: The yin and yang of bacterial communication. Chembiochem. **10**(2): 205-216.

Veening JW, Smits WK, Kuipers OP. 2008a. Bistability, epigenetics, and bet-hedging in bacteria. Annu Rev Microbiol. **62**: 193-210.

Veening JW, Stewart EJ, Berngruber TW, Taddei F, Kuipers OP, Hamoen LW. 2008b. Bet-hedging and epigenetic inheritance in bacterial cell development. Proc Natl Acad Sci USA. **105**(11): 4393-4398.

Viprey V, Del Greco A, Golinowski W, Broughton WJ, Perret X. 1998. Symbiotic implications of type III protein secretion machinery in *Rhizobium*. Mol Microbiol. **28**(6): 1381-1389.

Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH. 2003. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: Effects of growth phase and environment. J Bacteriol. **185**(7): 2080-2095.

Waters CM, Bassler BL. 2005. Quorum sensing: Cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol. **21**: 319-346.

Xin DW, Liao S, Xie ZP, Hann DR, Steinle L, Boller T, Staehelin C. 2012. Functional analysis of NopM, a novel E3 ubiquitin ligase (NEL) domain effector of *Rhizobium* sp strain NGR234. Plos Pathog. **8**(5): e1002707.

8 Appendix

Phenotypic heterogeneity, a phenomenon that may explain why quorum sensing does not always result in truly homogeneous cell behaviour

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MINIREVIEW

Phenotypic Heterogeneity, a Phenomenon That May Explain Why Quorum Sensing Does Not Always Result in Truly Homogenous Cell Behavior

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Phenotypic heterogeneity describes the occurrence of "nonconformist" cells within an isogenic population. The nonconformists show an expression profile partially different from that of the remainder of the population. Phenotypic heterogeneity affects many aspects of the different bacterial lifestyles, and it is assumed that it increases bacterial fitness and the chances for survival of the whole population or smaller subpopulations in unfavorable environments. Well-known examples for phenotypic heterogeneity have been associated with antibiotic resistance and frequently occurring persister cells. Other examples include heterogeneous behavior within biofilms, DNA uptake and bacterial competence, motility (i.e., the synthesis of additional flagella), onset of spore formation, lysis of phages within a small subpopulation, and others. Interestingly, phenotypic heterogeneity was recently also observed with respect to quorum-sensing (QS)-dependent processes, and the expression of autoinducer (AI) synthase genes and other QS-dependent genes was found to be highly heterogeneous at a single-cell level. This phenomenon was observed in several Gram-negative bacteria affiliated with the genera *Vibrio*, *Dinoroseobacter*, *Pseudomonas*, *Sinorhizobium*, and *Mesorhizobium*. A similar observation was made for the Gram-positive bacterium *Listeria monocytogenes*. Since AI molecules have historically been thought to be the keys to homogeneous behavior within isogenic populations, the observation of heterogeneous expression is quite intriguing and adds a new level of complexity to the QS-dependent regulatory networks. All together, the many examples of phenotypic heterogeneity imply that we may have to partially revise the concept of homogeneous and coordinated gene expression in isogenic bacterial populations.

Bacteria have evolved multiple strategies to cope with rapid and frequent changes in their environment. These survival strategies may include spore formation, increased production of polysaccharides, biofilm formation or escape from biofilms (i.e., switching from a motile into a sessile form and vice versa), altered motility, change of metabolic capabilities, response to antibiotics, and many more. In general, these switches are initiated by environmental or bacterially produced signals that are perceived by a diverse array of regulators and delegated into the corresponding regulatory networks. This presumably results in altered transcription levels of different genes or operons, which eventually causes a change in the phenotype and a response to the environmental stimulus. Within this context, it is generally assumed that the majority of a population will undergo this switch within a short time period and that the population will show a mostly homogeneous expression profile. During the last decade, it became, however, evident that a certain fraction of cells in an isogenic population behaves differently from the others, even though the environment may not have changed significantly. The term "phenotypic heterogeneity" thereby describes usually "nongenetic" variations that are observed between individual cells in a genetically homogeneous (i.e., isogenic) population (1). Within this context, the term "bistability" describes the situation in which the population has bifurcated into coexisting cell types (2).

It has been postulated that the ecological advantage of producing nonconformists is linked to a spreading-the-risk strategy (3–6). Thereby, switching is a statistical event as a result of noise and can additionally be an evolved event on the basis of a genetically encoded regulatory mechanism or a result of small genetic variations as part of an evolutionary process (7–9). It is further assumed that stochastic switching can be favored over sensing-dependent switching if the environment changes infrequently (10).

In this minireview, we highlight recent findings with respect to phenotypic heterogeneity. We place special emphasis on quorum-sensing (QS)-dependent phenotypic heterogeneity. For a broader overview on the topic of phenotypic heterogeneity, we provide in Table 1 a wide range of published examples observed in Gram-positive and Gram-negative bacteria. Furthermore, Fig. 1 gives a first glance at heterogeneous gene expression observed under a microscope for a plant symbiont, a human pathogen, and an aquatic bacterium in isogenic laboratory cultures. The increasing number of published examples implies that phenotypic heterogeneity is a broadly observed phenomenon within the bacterial world. It should be considered an important component in many regulatory networks.

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TABLE 1 Recent examples of nongenetically determined phenotypic heterogeneity and bistability in bacteria

Phenotypic heterogeneity and/or bistability trait(s)	Microorganism(s)	Reference(s)
Presence of persister cells, resistance to antibiotics and heavy metals	*Staphylococcus* sp., *E. coli*, *S.* Typhimurium, *P. aeruginosa*, and others	37–39, 41 and references herein
SOS response[a]	*E. coli*, *C. glutamicum*	54, 89
Response to peptide antibiotics	*B. subtilis*	90
Prophage induction	*C. glutamicum*, *S. oneidensis*, *S. pneumoniae*	54–56
Quorum sensing, presence of autoinducer synthesis genes	*V. campbellii*, *V. fischeri*, *L. monocytogenes*, *D. shibae*, *P. syringae*, *P. putida*, *S. fredii*, *S. meliloti*	13, 19, 23–25, 29, 30, 32–34, 91
Arabinose utilization	*E. coli*	63, 64
Polyhydroxybutyrate utilization	*S. meliloti*	92
Secretion of related genes	*S. fredii*	30
Quorum quenching genes	*S. fredii*	30
Motility, secondary-flagellum formation	*S. putrefaciens*, *S.* Typhimurium	68, 93
Biofilm escape, motility after putisolvin production	*P. putida*	33
Genomic island excision/transfer	*M. loti*, *Pseudomonas knackmussii*	28, 94, 95
Bacterial competence, DNA uptake	*B. subtilis*, *S. mutans*	71–74
Sporulation	*B. subtilis*	9, 96, 97
Colony heterogeneity	*S. aureus*	7
Increased lag phase	*E. coli*	8
Surface pilus formation	*S. pneumoniae*	98
Myo-inositol utilization	*S. enterica*	99
Biofilm formation	*S. enterica*, *B. subtilis*	100, 101
Antibiotic production	*Streptomyces coelicolor*	102

[a] The SOS response is in part linked to the formation of persister cells (see reference 39 and references therein).

BACTERIAL QS: A SYSTEM THOUGHT TO COORDINATE CELLS INSTEAD OF PRODUCING HETEROGENEOUS CELL BEHAVIOR

The term "quorum sensing" (QS) describes the intra- and interspecific cell-cell communication to sense population densities. For this, cells produce, release, and detect small diffusible molecules named autoinducers (AI). While Gram-negative bacteria release molecules like the homoserine lactones (HSLs), alpha-hydroxy-ketones, quinolone-like compounds, and others, Gram-positive bacteria produce and release autoinducing peptides.

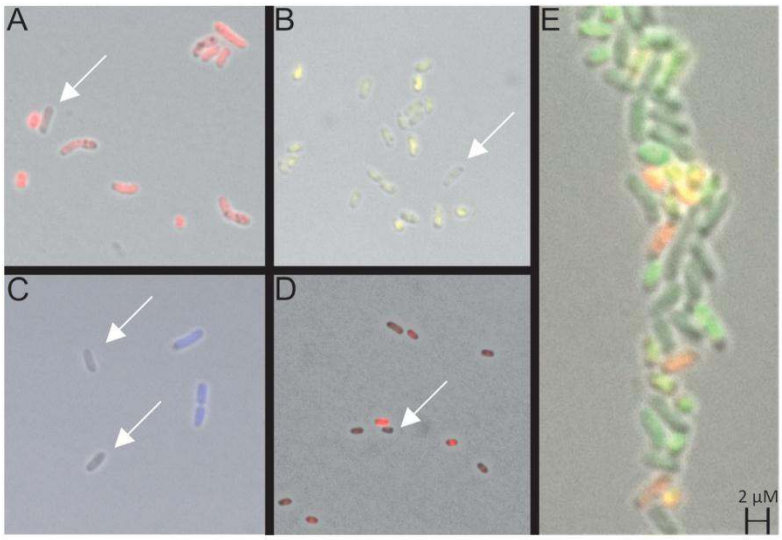

FIG 1 Phenotypic heterogeneous expression of various reporter gene fusions in plant-associated *Sinorhizobium fredii* NGR234, the human pathogen *Stenotrophomonas maltophilia* K279a, and the aquatic *Janthinobacterium* sp. strain HH102. Images are overlays of light and fluorescence microscopic pictures. Arrows indicate nonfluorescing cells. All strains carry low-copy-number plasmids with a promoter fused to a fluorescence protein. (A) *S. fredii* NGR234 carrying P*ngrI*::*dsRed2*, a promoter fusion of the chromosomally encoded AI synthase gene in this organism (for further details, see reference 30); (B) *S. fredii* NGR234 carrying P*traI*::*mVenus*, a promoter fusion of the second AI synthase gene of the organism in panel A; (C) *S. maltophilia* K279a carrying P*bla2*::*mCerulean*, a promoter fusion of the beta lactamase 2 gene; (D) *Janthinobacterium* sp. HH102 carrying P*vioA*::*mCherry*, a promoter fusion of the *vioA* gene, the first gene of the violacein biosynthesis operon; (E) *S. fredii* NGR234 carrying P*traI*::*gfp* and P*ngrI*::*dsRed2* promoter fusions on the same plasmid. All images were extracted from unpublished work from our laboratory.

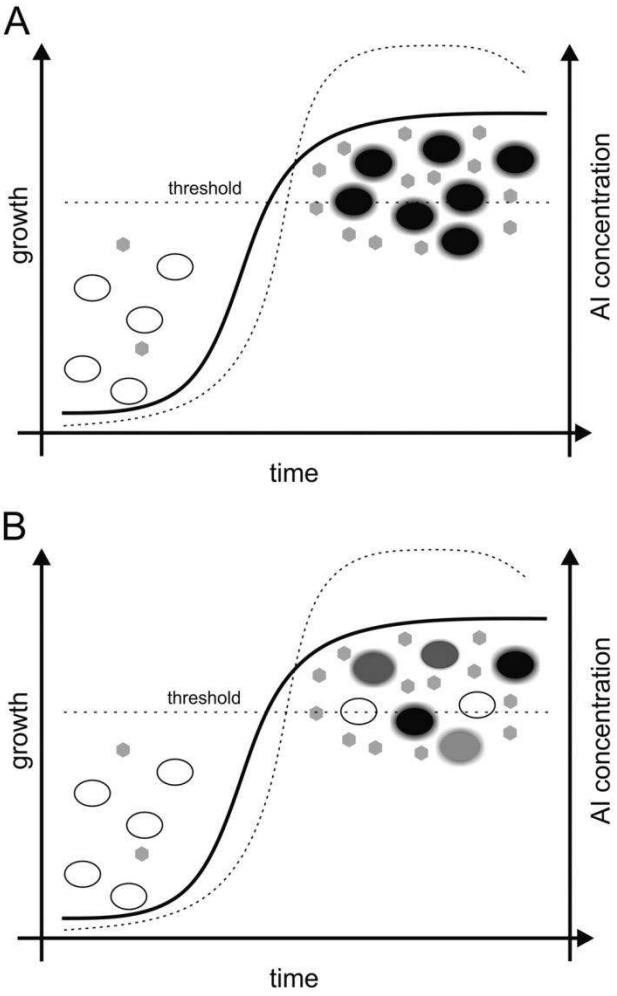

FIG 2 Two regulatory models that explain AI-dependent gene expression. (A) Classical textbook model of QS-dependent gene expression in *Vibrio* and other genera. Until recently, AI molecules were thought to be responsible for well-coordinated gene expression resulting in homogenous cell behavior. After reaching a certain threshold of AI concentration (dotted gray line) within a growing culture, all cells of the population turn on cell density-dependent genes like the *lux* genes in *Vibrio* and virtually all cells luminesce with the same signal intensity. (B) Only recently have independent research teams discovered a partly heterogeneous expression of QS-dependent genes in *V. fischeri* and *V. campbellii*. Not only did phenotypic heterogeneity in *Vibrio* result in the observation of on or off cells, cells also differed in the signal intensities throughout isogenic populations (13, 19, 24).

When a certain AI threshold concentration is reached, it is commonly accepted that the whole population collectively activates gene expression of QS-controlled genes in a well-coordinated manner (11, 12) (Fig. 2A). While this has been the classical view for many years, there is now growing evidence that QS-dependent responses of microorganism are not always homogenous (Fig. 2B).

One of the first examples of a heterogeneous QS response has been reported for the model organism *Vibrio fischeri* and its QS-dependent bioluminescence (13). *V. fischeri* is a Gram-negative marine bacterium that frequently colonizes the light organs of fish and squid species (14, 15). Cell-cell communication in this microorganism has been studied very extensively over the last 4 decades, and it is perhaps one of the best-understood systems with respect to QS (16). *V. fischeri* encodes a single LuxI synthase that is involved in the synthesis of a 3-oxo-C_6-HSL. Together with *luxI*, six genes are found in the same operon (*luxICDABEG*). Upstream of the *lux* operon, the corresponding regulator (i.e., *luxR*) is found (17). All together, these genes are involved in the biosynthesis of a luciferase protein, and their well-coordinated expression finally results in the observed bioluminescence through the reversible oxidation of a bacterially produced long-chain aldehyde and a reduced flavin mononucleotide. The expression of the *V. fischeri lux* operon is controlled through QS. At low cell densities, the *lux* genes are only weakly transcribed, whereas at high cell densities, the *lux* genes are transcribed at high levels. Light emission is observed only when a threshold concentration is reached, and until recently, it was assumed that virtually all cells would activate the *lux* operon's transcription, resulting in a cell density-dependent bioluminescence in isogenic populations (18) (Fig. 2A).

Interestingly, Perez and Hagan (13) provided evidence that individual cells differ widely in the onset of their bioluminescence and in their light intensities. Within their study, they convincingly demonstrated that on a time scale of 150 to 250 min, the bioluminescence of *V. fischeri* depends to a large extent on the exogenously added AI. However, the authors also described a large degree of cell-to-cell variability in their study. Obviously, many cells luminesced at very modest levels, while a small fraction of cells emitted much more brightly. The cells also responded on different time scales to the added AI. Based on these observations, the authors of that study postulate that the AI controls the overall behavior of the population but is less active at the single-cell level (13) (Fig. 2B).

One of the first studies reporting phenotypic heterogeneity in the QS-dependent regulatory network within the genus *Vibrio* was published by Anetzberger and colleagues for *Vibrio harveyi* (recently reclassified as *Vibrio campbellii*) (19). In *V. campbellii*, expression of the *lux* operon is regulated through the concentration of the AI, which is an *N*-(3-hydroxy-butanol)-L-homoserine lactone (20). The bioluminescence in *V. campbellii* is heterogeneous, similar to the above-made observation for *V. fischeri* and in contrast to the general belief that a high AI concentration leads to synchronized gene expression in virtually all cells of genetically identical populations. Anetzberger and colleagues observed that despite high cell densities, only 69% of the cells produce bioluminescence. Additionally, the strength of the bioluminescence varies throughout the glowing cells. In the background of a *luxO* mutant, all cells luminesce (19) (Fig. 3A). LuxO is known to collect all information of the three AI-specific sensor kinases in *V. campbellii* and thereby to regulate the synthesis of four small RNAs that are involved in destabilizing the *luxR* transcript. LuxR acts as a master regulator in the QS-responsive network (21–23). Further studies implied that other AI-dependent genes are heterogeneously expressed in *V. campbellii* as well. Among these are the *luxC*, *vscP*, and *vhp* genes. AI-independent genes appear to be mostly homogeneously expressed in *V. campbellii*, in contrast to the heterogeneous expression of AI-dependent genes (24).

Another interesting aspect of AI-linked heterogeneity was recently identified in the alphaproteobacterium *Dinoroseobacter shibae* (25). *D. shibae* is a member of the *Roseobacter* clade, which is highly abundant in marine habitats (26). Surprisingly, heterogeneous cell morphology in *D. shibae* is controlled in a QS-depen-

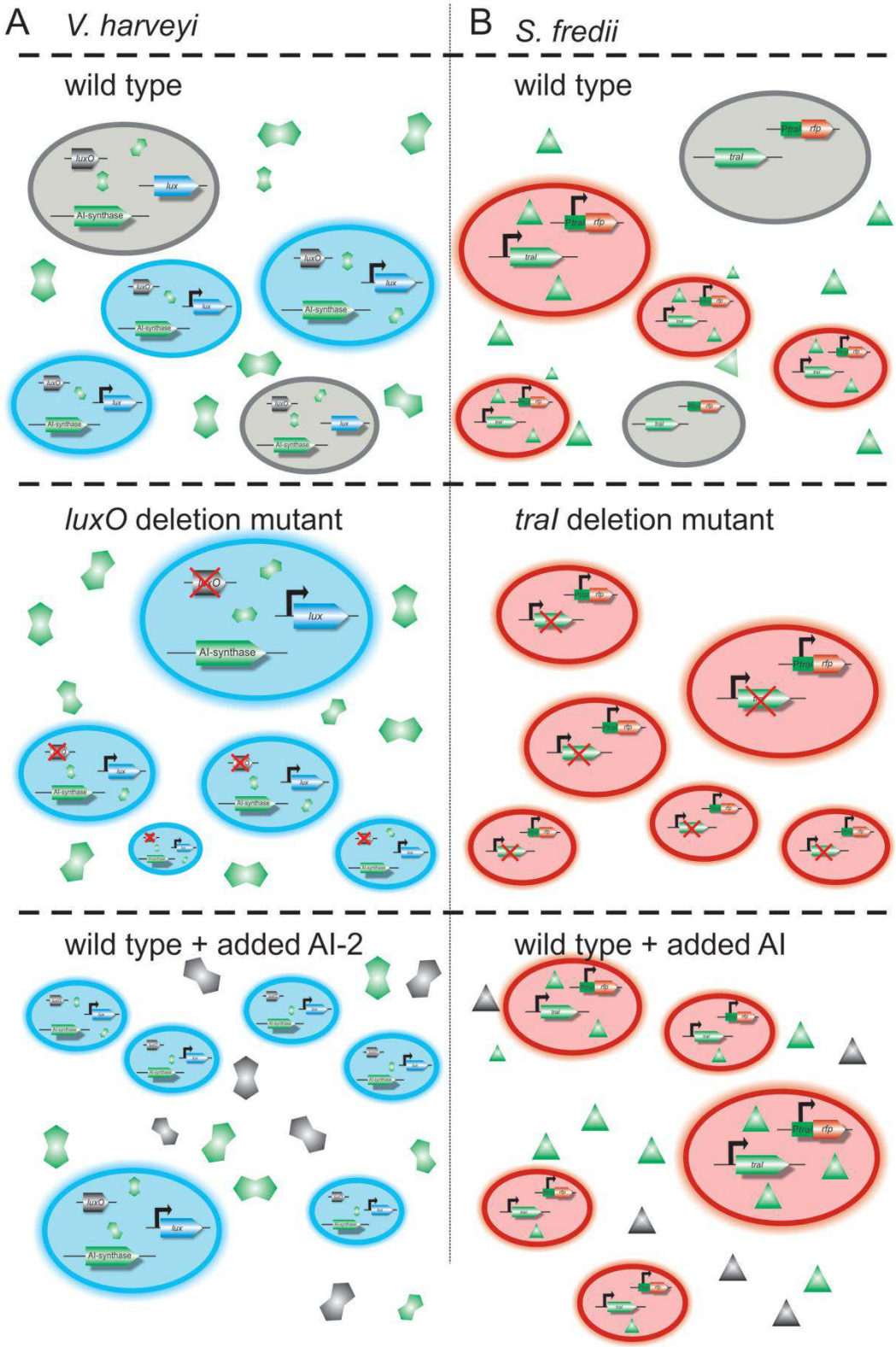

FIG 3 QS-dependent phenotypic heterogeneity observed in the two model organisms *Vibrio harveyi* and *Sinorhizobium fredii*. (A, top) The wild type of *V. harveyi* shows phenotypic heterogeneity with respect to AI-controlled *lux* gene expression. (Middle) If the regulator *luxO* is deleted, all cells turn on. (Bottom) When externally added AI is present, all cells turn on. (B, top) The wild type of *S. fredii* shows phenotypic heterogeneity with respect to the AI synthase gene *traI*. (Middle) After deletion of the AI synthase, all cells turn on. (Bottom) Once externally added AI is present, all cells turn on. For further details, see references 23, 24, and 30.

dent manner. The *D. shibae* cell shape is highly variable, as it ranges from variously sized ovoid rods to long filaments, and this goes in parallel with a high copy number of chromosomes. Cells divide either by binary fission or by budding from the cell poles. *D. shibae* encodes in its genome three AI synthases and *luxI* homologues, which produce AI molecules ranging from C_{14}- to C_{18}-HSL. Unexpectedly, the deletion of only one of the three *lux* genes resulted in highly homogeneous cell morphologies. The deletion of the *luxI1* gene abolished the synthesis of any AI molecule by this strain. Further, the authors of this study convincingly demonstrated that the phenotype of a *luxI1* mutant could be restored by the addition of the corresponding C_{18}-HSL at saturating concentrations. Additional transcriptome analyses in the background of this deletion mutant further supported the hypothesis of the AI-dependent morphological cell variability (25).

Within this framework, the QS-dependent DNA transfer and excision of a symbiotic island of *Mesorhizobium loti* has been observed. *M. loti* is a nitrogen-fixing alphaproteobacterium that is able to establish a symbiosis with legume plants. Its symbiotic genes (i.e., *nod*, *nif*, and *fix*) are found on a symbiotic island that has been designated ICE*M*/Sym^{R7A} (27). ICE*M*/Sym^{R7A} is excised and transferred in an AI concentration-dependent manner, during which the occurrence of excision events varies between single cells within an isogenic culture. In a laboratory culture of *M. loti*, the excision frequency of the symbiotic island was only 1% in the stationary growth phase compared to 40 to 70% in the exponential growth phase. The introduction of additional copies of *traR*, the regulator of the AI system, and the deletion of *qseM*, an antiactivator of the *M. loti* AI system, led to an upregulation of the symbiotic island excision to almost 100%. Additionally, the deletion of *qseC*, a modulator of *qseM*'s transcription, resulted in a reduced excision frequency in individual cells and an increase in the level of the synthesized AI molecules (28).

The AI systems in *Pseudomonas syringae* and *Xanthomonas campestris* are heterogeneously expressed over a long time period of growth in laboratory cultures. The heterogeneity is not influenced by the addition of external AI, which leads to the conclusion that the heterogeneity of the AI systems of these two strains is not regulated in response to the AI concentration. The authors provided evidence that even the addition of 20 to 35 μM AI or diffusible signaling factor to the culture media did not significantly reduce the fraction of non-QS-responsive cells present in the population. In fact, they observed that in both organisms, the number of uninduced cells ranged from 18 to 25% (29). This is intriguing, since in the majority of all other studied organisms, the expression frequency at the single-cell level was affected by the concentration of the available AI.

In a recent report from our lab, we provided evidence that plant-released molecules can override AI signals that are involved in the regulation of phenotypic heterogeneity. For these studies, the alphaproteobacterium *Sinorhizobium fredii* NGR234 was chosen as a model organism. This organism is a plant symbiont that is able to fix nitrogen in the root nodules of many legumes. In batch cultures, the two AI systems, TraI/R and NgrI/R, are heterogeneously expressed (Fig. 1A and B) (30). Further work from our laboratory thereby implies that some cells turn on the *traI* gene and others the *ngrI* gene; however, only a few cells turn on both genes at the same time (Fig. 1E).

In contrast to the heterogeneous behavior of *P. syringae* and *X. campestris*, the heterogeneous behavior of the two *S. fredii* AI systems can be quenched by either the external addition of 50 μM AI or the deletion of the corresponding AI synthase gene (30). The upregulation of the AI systems in the background of the AI synthase deletion mutants was in part verified by population-wide transcriptome data (31). Not only the AI themselves but also the addition of crude plant root exudates or octopine as a plant-released molecule caused a more homogenous expression of the AI systems (30). Part of this study was confirmed by tests with the close relative *Sinorhizobium meliloti* using a *sinI* reporter gene fusion (32). Further investigations in *S. fredii* NGR234 indicated that quorum quenching genes and a type IV pilus gene cluster showed phenotypic heterogeneity that was independent of the influence of cell-cell communication signals (30).

Within this context, a recently published study on single-cell analyses of *Pseudomonas putida* strain IsoF implied that QS induces the production of the biosurfactant putisolvin, which triggers asocial motility of induced cells out of microcolonies. The biosurfactant synthesis is turned on by the stochastic switch of the AI system of *P. putida*. These observations lead to the assumption that in early growth stages, the QS signals are not released but rather directly bind to the AI receptors of the producing cell, resulting in an individually regulated gene expression. This in turn causes individual-based behavior in younger biofilms and not, as assumed for QS regulatory networks, to a group-based behavior. Removing cells with the highest AI concentration exerted a negative feedback on the left-behind cells (33).

Finally, it is noteworthy that heterogeneity with respect to QS is not restricted to Gram-negative bacteria. In the Gram-positive bacterium *Listeria monocytogenes*, a similar QS-dependent phenotypic heterogeneity could be observed (34). *L. monocytogenes* is a facultative intracellular food-borne pathogen that can cause severe infections in humans and animals. It is widely spread in the environment but often occurs in processed food samples (35). In this bacterium, the autoinducer is a peptide encoded by the *agrD* gene. *agrD* is found together with the *agrB*, *-C*, and *-A* genes in an operon. *agrC* encodes a two-component histidine kinase, AgrA a response regulator, and AgrB a protein that is involved in the processing of the precursor peptide *agrD* into a mature autoinducing peptide. Fusing the *agr* promoter region with a green fluorescent protein (GFP), Garmyn and colleagues provided evidence that the *agr* system is in general highly heterogeneously expressed in *L. monocytogenes* (34). They employed five different strains in their study, in which heterogeneity was observed with respect to the expression of a fused reporter for the *agr* genes. Therefore, the authors concluded that the *agr* system is in general subject to heterogeneous regulation. However, in different isolates, the percentages of "on" Agr cells significantly differed and ranged from 15 to 73%. It appeared that the overall ratio of off to on cells was strain dependent. The growth conditions, i.e., liquid culture or growth in biofilm flow cells, had a significant impact on the degree of heterogeneity as well. In fact, biofilm lifestyle appeared to increase the observed heterogeneity of *L. monocytogenes* cells.

All together, these many examples of QS-dependent phenotypic heterogeneity may demand a careful reviewing of the classical model of QS-dependent cell-cell communication in which virtually all cells are believed to be in either the on or the off mode (Fig. 2A). Thereby, the model might need only a better definition for the level of homogeneity, which is thought to occur in QS-dependent regulons (Fig. 2B).

PERSISTER CELLS AS A CLASSICAL MODEL FOR PHENOTYPIC HETEROGENEITY

A well-studied system with respect to phenotypic heterogeneity is the phenomenon of persister cells. This phenomenon was already observed in the mid-40s of the last century, when Bigger described the occurrence of *Streptococcus pyogenes* persister cells in an exponentially growing culture that had been treated with penicillin (36). In these experiments, a small fraction of a homogeneous population entered a distinct physiological state in which they were not killed by antibiotic treatment. Usually, these were less than 0.1% of the whole population. Much research has been focused on the exploration of the molecular keys linked to the persister phenomenon (37–41). Bertram and colleagues recently provided evidence that toxin-antitoxin (TA) systems play a key role in the regulatory network of persisters (42, 43). These systems consist of a toxin, which is normally a stable protein that interferes with vital cellular functions, and a cognate antitoxin, an unstable protein or RNA molecule, which regulates the toxin level. The most prominent example of a TA system controlling persistence is the *Escherichia coli hipAB* TA system. The newest results imply an inactivation of the Glu-tRNA synthase by the toxin HipA, which leads to an activation of RelA-mediated (p)pp(G)pp synthesis. The increased (p)pp(G)pp levels indirectly result in multidrug tolerance (44). The inactivation of HipA is mediated through the binding to HipB, which additionally leads to a conformational change of HipA (45). Essential to the regulatory effect of the *hipAB* system is a threshold concentration of HipA. When the threshold is exceeded, cells turn into persisters. This on and off switch is driven by the proteolysis of the antitoxin, by stochastic fluctuation, or by any change in the growth rate (39). Recently, the *hipAB* system of *Shewanella oneidensis* was characterized, and a ternary HipAB-DNA complex different from the one from *E. coli* was observed (46). Since persister cells are highly problematic with respect to antibiotic treatments of infectious diseases, much emphasis has been placed on the development of drugs that suppress the development and survival of persister cells (47–53).

PHENOTYPIC HETEROGENEOUS GENE EXPRESSION IS A WIDELY OBSERVED PHENOMENON

Phenotypic heterogeneity has been observed in a wide array of Gram-negative and Gram-positive bacteria. To give a better overview, we have assembled many published examples of phenotypic heterogeneity in Table 1. In the following section, we address a few selected phenomena that are subject to phenotypic heterogeneous regulation and have recently been uncovered.

SPONTANEOUS PROPHAGE INDUCTION AS A DRIVER OF PHENOTYPIC HETEROGENEITY

Recent studies with *Corynebacterium glutamicum*, *Shewanella oneidensis*, *Salmonella* sp., and *Streptococcus pneumoniae* indicated that lysis of a small number of cells by spontaneous prophage induction is beneficial to the remainder of the population and increases the overall fitness of the population with respect to survival and biofilm formation (54–57). In the Gram-positive, biotechnologically important microorganism *C. glutamicum* ATCC 13032, a spontaneously induced SOS response is partly responsible for the induction of prophages (54). The authors of that study observed a positive correlation between the spontaneous activation of the SOS response and the spontaneous induction of the prophage CGP. Interestingly, not every cell that turns on the SOS response induces prophage excision at the same time. An exceeded threshold of a certain, yet unknown, SOS response regulator is probably responsible for prophage induction in this bacterium (54).

S. pneumoniae is a potent biofilm producer that colonizes the upper respiratory tracts of humans and is the cause of many infections (58, 59). The majority of genomes of pneumococcal isolates showed a high prevalence of lysogenic bacteriophages (60, 61). Carrolo et al. convincingly demonstrated that biofilms formed by phage-infected bacteria are characterized by a higher-than-normal biomass and cell viability. Their data suggest that the DNA release of individual cells that are lysed after phage induction results in more-robust biofilms (55).

Likewise, the biofilm of *S. oneidensis* MR-1 is stabilized by external DNA (eDNA) that is released by individual cells after phage lysis. *S. oneidensis* MR-1 encodes three prophages in its genome, which are activated through UV radiation and other stresses, like ionizing radiation (62). Spontaneous phage lysis produces significant amounts of free eDNA, which plays a major role during surface attachment and development of three-dimensional biofilm structures, with which the different prophages contribute to different stages of biofilm formation (56).

METABOLIC ACTIVITIES ARE NOT ALWAYS UNIFORM IN BATCH CULTURES

Yet another recent example for heterogeneous expression of genes linked to metabolic traits concerns the arabinose utilization system in *E. coli* (63, 64). With quantitative time-lapse microscopy and by challenging *E. coli* cells with very low concentrations of arabinose, a time delay in the response of single cells to arabinose was discovered. This delay corresponded directly to the arabinose concentration. In this study, cells were trapped in microfluidic devices, and the authors showed that heterogeneity was linked to the uptake of externally supplied arabinose. The established stochastic model allowed the conclusion that the heterogeneous timing of gene induction was related to a broad distribution of uptake proteins expressed by the cell at the time of arabinose addition. Further, they showed that the off switching triggered by the sudden removal of arabinose is quite homogeneous and fast. The rather quick off switch was independent of internal arabinose degradation. Within this context, it is noteworthy that the usual noise generated is a result not only of regulation of the level of mRNA production but also of the level of enzyme activity *per se*. Therefore, fluctuations during the expression and folding of catabolically active enzymes can propagate and cause significant growth fluctuations in *E. coli* when it is grown on lactulose (65).

SOME CELLS MOVE DIFFERENTLY THAN OTHERS

Chemotaxis and motility have, as well, been reported to be subject to phenotypic heterogeneity, and it was suggested that the behavior of cells exhibited larger temporal variations at time scales ranging from seconds to minutes (66, 67). Within this context, Bubendorfer and colleagues recently reported on the secondary *Shewanella putrefaciens* CN-32 flagellar system, which is induced under planktonic conditions within a distinct subpopulation. This results in the formation of at least one lateral flagellum in addition to the primary polar flagellum and is advantageous to the cell with respect to bacterial spreading. Cells that express the secondary filaments were more fit with respect to spreading on soft-agar plates and through medium-filled channels despite having

lower swimming speeds than their mono-flagellated counterparts. It is likely that the second flagellum is advantageous for directional movement and ultimately leads to a more efficient chemotaxis (68).

BACTERIAL COMPETENCE IS HETEROGENEOUS WITHIN ISOGENIC POPULATIONS

Bacterial competence is yet another process for which bistability and phenotypic heterogeneity can be observed. Natural competence is a complex process controlled by a regulatory network that ultimately allows the uptake of exogenous DNA from the environment. For detailed reviews, see references 69 and 70. *Bacillus subtilis*, *Streptococcus pneumoniae*, and *Streptococcus mutans* have been used as model organisms to characterize heterogeneity during this process. In all three bacterial strains, only a small fraction of the cells within an isogenic population will eventually be competent (71–74). For *B. subtilis*, a certain threshold concentration of ComK, the key competence-associated transcription factor, is responsible for an autostimulatory process that finally leads to genetic competence (74, 75).

Competence in *S. pneumoniae* is triggered by the secreted peptide pheromone competence-stimulating peptide (CSP), encoded by *comC*. CSP is bound by the receptor ComD, which forms a two-component signal transduction system with ComE and thereby responds to the external CSP concentration. Additionally, ComE activates the transcription of *comAB* and *comCDE*, inducing a positive-feedback loop that drives the cell toward the competent state. Cells that are not in the competent state are attacked and lysed by competent cells (reference 76 and references herein). This phenomenon is responsible for the observed heterogeneity.

Competence in *S. mutans* is regulated in a similar way. The competence-induced population of *S. mutans* segregates into autolysing cells and cells that become competent due to an imbalance in the CipB/CipI ratio. Besides these two cell fractions, a third fraction of cells that is not competent and carries low levels of ComE is observed (72, 73). The newest research results provide evidence that small peptides also play a key role in the competence regulation of *S. mutans* (77).

MEASURING PHENOTYPIC HETEROGENEITY AT A SINGLE-CELL LEVEL

Demonstrating the development of single cells within a population and thereby monitoring the fate of individual cells and their heterogeneous behavior over time are essential parameters for assessing the impact of phenotypic heterogeneity. Well-established methods for measuring phenotypic heterogeneity are time-lapse fluorescence microscopy and flow cytometric analyses or a combination of both. Additionally, the development of microfluidic devices and cell traps has been very helpful for data evaluation and single-cell tracking (72, 78–81).

With the exception of the studies on *Vibrio* in which naturally occurring bioluminescence was recorded, the majority of published studies have made use of fluorescence proteins (i.e., variants of the GFP) for the detection of gene expression at a single-cell level (Table 1). Mainly because of the high signal intensity needed for the detection of single cells for many of these studies, low-, medium-, or high-copy-number vectors were used. Therefore, the use of reporter genes in self-replicable plasmids might in part be problematic with respect to the regulation and expression of the target genes through the corresponding regulators, usually encoded on the bacterial chromosome in a single copy. This could be a problem especially when single-copy regulators are involved and their binding sites within the targeted promoter are outcompeted by the binding sites on the high-copy-number plasmids. Further, the maturation time of the fluorescing proteins employed will most likely have an impact on the outcome of the study (82, 83).

Besides the use of fluorescence proteins, the use of the fluorescing dyes Syto 9, propidium iodide, and 3,3′-diethyloxacarbocyanine iodide [DiOC2(3)] proved to be useful for the identification of subpopulations with reduced viability and membrane potential within *C. glutamicum* populations (84). Other studies employed *in situ* reverse transcription-PCRs at a single-cell level in combination with a single-cell beta-galactosidase assay to monitor heterogeneous expression of the *lac* operon in *Salmonella enterica* serovar Typhimurium (85). The detection of single mRNA molecules in individual cells might also be useful for analysis of phenotypic heterogeneity (86).

Within this context, culture conditions will also have a major impact on the outcome of any study, especially since it is well known that laboratory cultures cannot be considered truly adequate models for natural systems; besides, high variations of environmental parameters are known to occur with often-stable subpopulations in batch cultures. In particular, oxygen limitations and gradients of oxygen might affect the outcomes of the studies. This will be true for many of the GFP-based studies, as oxygenation of a tyrosine at position 67 is a rate-limiting step in its fluorescence (87). Further, is should be kept in mind that the bioluminescence of *Vibrio* also depends on oxygen availability due to the catalytic mechanism employed by the luciferase (88).

IS PHENOTYPIC HETEROGENEITY ALWAYS ONLY PHENOTYPIC?

With respect to the observation of phenotypic heterogeneity in a wide range of bacteria (Table 1), the question of how phenotypic the observed heterogeneity is may arise. It is likely that the observed phenomenon is in fact often related to genotypic changes caused by single nucleotide polymorphisms (SNPs) that potentially lead to a different phenotypic outcome for a certain subpopulation. One such example has been reported for the growth of *Staphylococcus aureus* on a laboratory medium with a high Mg^{2+} content (7). The parent strain divides into three subpopulations when growing on these plates: an orange center region (O) and a white (W) and a yellowish (Y) surrounding region. The transcriptional changes in the W strain match a point mutation in the RsbW anti-sigma factor. This leads to a loss of function of the kinase active residue, which in turn results in a nonfunctional σ^B complex. A σ^B deletion mutant shows the same QS hyperactivation phenotype as the W substrain, which leads to a growth advantage over the parent strain. Additionally, the Y strain revealed mutations in the GraRS and WalKR two-component systems causing a higher-than-normal resistance capacity against vancomycin and the bacteriocin Bsa (7). Yet another example of genomic mutations leading to phenotypic heterogeneity has recently been described for *E. coli* cultures that had been repeatedly treated with ampicillin. Sequencing of antibiotic-tolerant strains identified various mutations leading to stable subpopulations with altered growth behavior (8). These are only a very few and very recent examples of phenotypic heterogeneity that is caused by genetic changes instead of a stochastic or regulatory switch, and it

is likely that others that uncover phenotypic as genotypic heterogeneity will follow.

Independently from the mechanisms through which phenotypic heterogeneity arises within a population, the many examples that have been published over the last 5 years indicate the wide occurrence and general importance of phenotypic heterogeneity for coordinated bacterial behavior. All together, these examples have significantly advanced our knowledge of the molecular keys involved in generating phenotypic heterogeneity within isogenic and homogeneous cultures. Because of the frequent occurrence of phenotypic heterogeneity, we may need to ask if truly homogenous and isogenic cultures exist at all. Within this framework, it is perhaps safe to speculate that phenotypic heterogeneity is a very common phenomenon that occurs in virtually any bacterial culture even though it is believed to be homogeneous, as this was the case for the QS-dependent processes.

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REFERENCES

1. **Davidson CJ, Surette MG.** 2008. Individuality in bacteria. Annu Rev Genet **42:**253–268. http://dx.doi.org/10.1146/annurev.genet.42.110807.091601.
2. **Dubnau D, Losick R.** 2006. Bistability in bacteria. Mol Microbiol **61:**564–572. http://dx.doi.org/10.1111/j.1365-2958.2006.05249.x.
3. **Dhar N, McKinney JD.** 2007. Microbial phenotypic heterogeneity and antibiotic tolerance. Curr Opin Microbiol **10:**30–38. http://dx.doi.org/10.1016/j.mib.2006.12.007.
4. **Smits WK, Kuipers OP, Veening JW.** 2006. Phenotypic variation in bacteria: the role of feedback regulation. Nat Rev Microbiol **4:**259–271. http://dx.doi.org/10.1038/nrmicro1381.
5. **de Jong IG, Haccou P, Kuipers OP.** 2011. Bet hedging or not? A guide to proper classification of microbial survival strategies. Bioessays **33:**215–223.
6. **Veening JW, Smits WK, Kuipers OP.** 2008. Bistability, epigenetics, and bet-hedging in bacteria. Annu Rev Microbiol **62:**193–210. http://dx.doi.org/10.1146/annurev.micro.62.081307.163002.
7. **Koch G, Yepes A, Förstner KU, Wermser C, Stengel ST, Modamio J, Ohlsen K, Foster KR, Lopez D.** 2014. Evolution of resistance to a last-resort antibiotic in *Staphylococcus aureus* via bacterial competition. Cell **158:**1060–1071. http://dx.doi.org/10.1016/j.cell.2014.06.046.
8. **Fridman O, Goldberg A, Ronin I, Shoresh N, Balaban NQ.** 2014. Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. Nature **513:**418–421. http://dx.doi.org/10.1038/nature13469.
9. **Siebring J, Elema MJH, Vega FD, Kovács ÁT, Haccou P, Kuipers OP.** 2014. Repeated triggering of sporulation in *Bacillus subtilis* selects against a protein that affects the timing of cell division. ISME J **8:**77–87. http://dx.doi.org/10.1038/ismej.2013.128.
10. **Kussell E, Kishnoy R, Balaban NQ, Leibler S.** 2005. Bacterial persistence: a model of survival in changing environments. Genetics **169:**1807–1814. http://dx.doi.org/10.1534/genetics.104.035352.
11. **Waters CM, Bassler BL.** 2006. The *Vibrio harveyi* quorum-sensing system uses shared regulatory components to discriminate between multiple autoinducers. Genes Dev **20:**2754–2767. http://dx.doi.org/10.1101/gad.1466506.
12. **Ng WL, Bassler BL.** 2009. Bacterial quorum-sensing network architectures. Annu Rev Genet **43:**197–222. http://dx.doi.org/10.1146/annurev-genet-102108-134304.
13. **Perez PD, Hagen SJ.** 2010. Heterogeneous response to a quorum-sensing signal in the luminescence of individual *Vibrio fischeri*. PLoS One **5:**e15473. http://dx.doi.org/10.1371/journal.pone.0015473.
14. **Visick KL, Foster J, Doino J, McFall-Ngai MJ, Ruby EG.** 2000. *Vibrio fischeri lux* genes play an important role in colonization and development of the host light organ. J Bacteriol **182:**4578–4586. http://dx.doi.org/10.1128/JB.182.16.4578-4586.2000.
15. **Visick KL, McFall-Ngai MJ.** 2000. An exclusive contract: specificity in the *Vibrio fischeri-Euprymna scolopes* partnership. J Bacteriol **182:**1779–1787. http://dx.doi.org/10.1128/JB.182.7.1779-1787.2000.
16. **Ruby EG.** 1996. Lessons from a cooperative, bacterial-animal association: the *Vibrio fischeri-Euprymna scolopes* light organ symbiosis. Annu Rev Microbiol **50:**591–624. http://dx.doi.org/10.1146/annurev.micro.50.1.591.
17. **Ruby EG, Urbanowski M, Campbell J, Dunn A, Faini M, Gunsalus R, Lostroph P, Lupp C, McCann J, Millikan D, Schaefer A, Stabb E, Stevens A, Visick K, Whistler C, Greenberg EP.** 2005. Complete genome sequence of *Vibrio fischeri*: a symbiotic bacterium with pathogenic congeners. Proc Natl Acad Sci U S A **102:**3004–3009. http://dx.doi.org/10.1073/pnas.0409900102.
18. **Miyashiro T, Ruby EG.** 2012. Shedding light on bioluminescence regulation in *Vibrio fischeri*. Mol Microbiol **84:**795–806. http://dx.doi.org/10.1111/j.1365-2958.2012.08065.x.
19. **Anetzberger C, Pirch T, Jung K.** 2009. Heterogeneity in quorum sensing-regulated bioluminescence of *Vibrio harveyi*. Mol Microbiol **73:**267–277. http://dx.doi.org/10.1111/j.1365-2958.2009.06768.x.
20. **Cao JG, Meighen EA.** 1989. Purification and structural identification of an autoinducer for the luminescence system of *Vibrio harveyi*. J Biol Chem **264:**21670–21676.
21. **Freeman JA, Bassler BL.** 1999. A genetic analysis of the function of LuxO, a two-component response regulator involved in quorum sensing in *Vibrio harveyi*. Mol Microbiol **31:**665–677. http://dx.doi.org/10.1046/j.1365-2958.1999.01208.x.
22. **Tu KC, Bassler BL.** 2007. Multiple small RNAs act additively to integrate sensory information and control quorum sensing in *Vibrio harveyi*. Gene Dev **21:**221–233. http://dx.doi.org/10.1101/gad.1502407.
23. **Anetzberger C, Reiger M, Fekete A, Schell U, Stambrau N, Plener L, Kopka J, Schmitt-Kopplin P, Hilbi H, Jung K.** 2012. Autoinducers act as biological timers in *Vibrio harveyi*. PLoS One **7:**e48310. http://dx.doi.org/10.1371/journal.pone.0048310.
24. **Anetzberger C, Schell U, Jung K.** 2012. Single cell analysis of *Vibrio harveyi* uncovers functional heterogeneity in response to quorum sensing signals. BMC Microbiol **12:**209. http://dx.doi.org/10.1186/1471-2180-12-209.
25. **Patzelt D, Wang H, Buchholz I, Rohde M, Gröbe L, Pradella S, Neumann A, Schulz S, Heyber S, Münch K, Münch R, Jahn D, Wagner-Döbler I, Tomasch J.** 2013. You are what you talk: quorum sensing induces individual morphologies and cell division modes in *Dinoroseobacter shibae*. ISME J **7:**2274–2286. http://dx.doi.org/10.1038/ismej.2013.107.
26. **Wagner-Döbler I, Biebl H.** 2006. Environmental biology of the marine *Roseobacter* lineage. Annu Rev Microbiol **60:**255–280. http://dx.doi.org/10.1146/annurev.micro.60.080805.142115.
27. **Sullivan JT, Ronson CW.** 1998. Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-tRNA gene. Proc Natl Acad Sci U S A **95:**5145–5149. http://dx.doi.org/10.1073/pnas.95.9.5145.
28. **Ramsay JP, Major AS, Komarovsky VM, Sullivan JT, Dy RL, Hynes MF, Salmond GPC, Ronson CW.** 2013. A widely conserved molecular switch controls quorum sensing and symbiosis island transfer in *Mesorhizobium loti* through expression of a novel antiactivator. Mol Microbiol **87:**1–13. http://dx.doi.org/10.1111/mmi.12079.
29. **Pradhan BB, Chatterjee S.** 2014. Reversible non-genetic phenotypic heterogeneity in bacterial quorum sensing. Mol Microbiol **92:**557–569. http://dx.doi.org/10.1111/mmi.12575.
30. **Grote J, Krysciak D, Schorn A, Dahlke RI, Soonvald L, Müller J, Hense BA, Schwarzfischer M, Sauter M, Schmeisser C, Streit WR.** 2014. Evidence of autoinducer-dependent and -independent heterogeneous gene expression in *Sinorhizobium fredii* NGR234. Appl Environ Microbiol **80:**5572–5582. http://dx.doi.org/10.1128/AEM.01689-14.
31. **Krysciak D, Grote J, Rodriguez Orbegoso M, Utpatel C, Förstner K, Li L, Schmeisser C, Krishnan HB, Streit WR.** 2014. RNA sequencing analysis of the broad-host-range strain *Sinorhizobium fredii* NGR234 identifies a large set of genes linked to quorum sensing-dependent regulation in the background of a *traI* and *ngrI* deletion mutant. Appl Environ Microbiol **80:**5655–5671. http://dx.doi.org/10.1128/AEM.01835-14.

32. **Schlüter JP, Czuppon P, Schauer O, Pfaffelhuber P, McIntosh M, Becker A.** 2015. Classification of phenotypic subpopulations in isogenic bacterial cultures by triple promoter probing at single cell level. J Biotechnol **198:**3–14. http://dx.doi.org/10.1016/j.jbiotec.2015.01.021.
33. **Cárcamo-Oyarce G, Lumjiaktase P, Kümmerli R, Eberl L.** 2015. Quorum sensing triggers the stochastic escape of individual cells from *Pseudomonas putida* biofilms. Nat Commun **6:**5945. http://dx.doi.org/10.1038/ncomms6945.
34. **Garmyn D, Gal L, Briandet R, Guilbaud M, Lemaître JP, Hartmann A, Piveteau P.** 2011. Evidence of autoinduction heterogeneity via expression of the Agr system of *Listeria monocytogenes* at the single-cell level. Appl Environ Microbiol **77:**6286–6289. http://dx.doi.org/10.1128/AEM.02891-10.
35. **Schuppler M, Loessner MJ.** 2010. The opportunistic pathogen *Listeria monocytogenes*: pathogenicity and interaction with the mucosal immune system. Int J Inflamm **2010:**704321.
36. **Bigger JW.** 1944. Treatment of staphylococcal infections with penicillin by intermittent sterilisation. Lancet **244:**497–500. http://dx.doi.org/10.1016/S0140-6736(00)74210-3.
37. **Conlon BP.** 2014. *Staphylococcus aureus* chronic and relapsing infections: evidence of a role for persister cells. Bioessays **36:**991–996. http://dx.doi.org/10.1002/bies.201400080.
38. **Helaine S, Kugelberg E.** 2014. Bacterial persisters: formation, eradication, and experimental systems. Trends Microbiol **22:**417–424. http://dx.doi.org/10.1016/j.tim.2014.03.008.
39. **Maisonneuve E, Gerdes K.** 2014. Molecular mechanisms underlying bacterial persisters. Cell **157:**539–548. http://dx.doi.org/10.1016/j.cell.2014.02.050.
40. **Prax M, Bertram R.** 2014. Metabolic aspects of bacterial persisters. Front Cell Infect Microbiol **4:**148.
41. **Lewis K.** 2010. Persister cells. Annu Rev Microbiol **64:**357–372. http://dx.doi.org/10.1146/annurev.micro.112408.134306.
42. **Bertram R, Schuster CF.** 2014. Post-transcriptional regulation of gene expression in bacterial pathogens by toxin-antitoxin systems. Front Cell Infect Microbiol **4:**6.
43. **Schuster CF, Bertram R.** 2013. Toxin-antitoxin systems are ubiquitous and versatile modulators of prokaryotic cell fate. FEMS Microbiol Lett **340:**73–85. http://dx.doi.org/10.1111/1574-6968.12074.
44. **Germain E, Castro-Roa D, Zenkin N, Gerdes K.** 2013. Molecular mechanism of bacterial persistence by HipA. Mol Cell **52:**248–254. http://dx.doi.org/10.1016/j.molcel.2013.08.045.
45. **Schumacher MA, Piro KM, Xu W, Hansen S, Lewis K, Brennan RG.** 2009. Molecular mechanisms of HipA-mediated multidrug tolerance and its neutralization by HipB. Science **323:**396–401. http://dx.doi.org/10.1126/science.1163806.
46. **Wen Y, Behiels E, Felix J, Elegheert J, Vergauwen B, Devreese B, Savvides SN.** 2014. The bacterial antitoxin HipB establishes a ternary complex with operator DNA and phosphorylated toxin HipA to regulate bacterial persistence. Nucleic Acids Res **42:**10134–10147. http://dx.doi.org/10.1093/nar/gku665.
47. **Conlon BP, Nakayasu ES, Fleck LE, LaFleur MD, Isabella VM, Coleman K, Leonard SN, Smith RD, Adkins JN, Lewis K.** 2013. Activated ClpP kills persisters and eradicates a chronic biofilm infection. Nature **503:**365–370. http://dx.doi.org/10.1038/nature12790.
48. **Briers Y, Walmagh M, Grymonprez B, Biebl M, Pirnay JP, Defraine V, Michiels J, Cenes Q, Aertsen A, Miller S, Lavigne R.** 2014. Art-175 is a highly efficient antibacterial against multidrug-resistant strains and persisters of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother **58:**3774–3784. http://dx.doi.org/10.1128/AAC.02668-14.
49. **Murima P, McKinney JD, Pethe K.** 2014. Targeting bacterial central metabolism for drug development. Chem Biol **21:**1423–1432. http://dx.doi.org/10.1016/j.chembiol.2014.08.020.
50. **Lebeaux D, Chauhan A, Létoffé S, Fischer F, de Reuse H, Beloin C, Ghigo JM.** 2014. pH-mediated potentiation of aminoglycosides kills bacterial persisters and eradicates *in vivo* biofilms. J Infect Dis **210:**1357–1366. http://dx.doi.org/10.1093/infdis/jiu286.
51. **Marques CNH, Morozov A, Planzos P, Zelaya HM.** 2014. The fatty acid signaling molecule *cis*-2-decenoic acid increases metabolic activity and reverts persister cells to an antimicrobial-susceptible state. Appl Environ Microbiol **80:**6976–6991. http://dx.doi.org/10.1128/AEM.01576-14.
52. **Starkey M, Lepine F, Maura D, Bandyopadhaya A, Lesic B, He J, Kitao T, Righi V, Milot S, Tzika A, Rahme L.** 2014. Identification of anti-virulence compounds that disrupt quorum-sensing regulated acute and persistent pathogenicity. PLoS Pathog **10:**e1004321. http://dx.doi.org/10.1371/journal.ppat.1004321.
53. **Kim JH, O'Brien K, Sharma R, Boshoff HIM, Rehren G, Chakraborty S, Wallach JB, Monteleone M, Wilson DJ, Aldrich CC, Barry CE, III, Rhee KY, Ehrt S, Schnappinger D.** 2013. A genetic strategy to identify targets for the development of drugs that prevent bacterial persistence. Proc Natl Acad Sci U S A **110:**19095–19100. http://dx.doi.org/10.1073/pnas.1315860110.
54. **Nanda AM, Heyer A, Krämer C, Grünberger A, Kohlheyer D, Frunzke J.** 2014. Analysis of SOS-induced spontaneous prophage induction in *Corynebacterium glutamicum* at the single-cell level. J Bacteriol **196:**180–188. http://dx.doi.org/10.1128/JB.01018-13.
55. **Carrolo M, Frias MJ, Rodrigues Pinto F, Melo-Cristino J, Ramirez M.** 2010. Prophage spontaneous activation promotes DNA release enhancing biofilm formation in *Streptococcus pneumoniae*. PLoS One **5:**e15678. http://dx.doi.org/10.1371/journal.pone.0015678.
56. **Gödeke J, Paul K, Lassak J, Thormann KM.** 2011. Phage-induced lysis enhances biofilm formation in *Shewanella oneidensis* MR-1. ISME J **5:**613–626. http://dx.doi.org/10.1038/ismej.2010.153.
57. **Bossi L, Fuentes JA, Mora G, Figuerosa-Bossi N.** 2003. Prophage contribution to bacterial population dynamics. J Bacteriol **185:**6467–6471. http://dx.doi.org/10.1128/JB.185.21.6467-6471.2003.
58. **Sleeman KL, Griffiths D, Shackley F, Diggle L, Gupta S, Maiden MC, Moxon ER, Crook DW, Peto TEA.** 2006. Capsular serotype-specific attack rates and duration of carriage of *Streptococcus pneumoniae* in a population of children. J Infect Dis **194:**682–688. http://dx.doi.org/10.1086/505710.
59. **Walker CL, Rudan I, Liu L, Nair H, Theodoratou E, Bhutta ZA, O'Brien K, Campbell H, Black RE.** 2013. Global burden of childhood pneumonia and diarrhoea. Lancet **381:**1405–1416. http://dx.doi.org/10.1016/S0140-6736(13)60222-6.
60. **Ramirez M, Severina E, Tomasz A.** 1999. A high incidence of prophage carriage among natural isolates of *Streptococcus pneumoniae*. J Bacteriol **181:**3618–3625.
61. **Severina E, Ramirez M, Tomasz A.** 1999. Prophage carriage as a molecular epidemiological marker in *Streptococcus pneumoniae*. J Clin Microbiol **37:**3308–3315.
62. **Heidelberg JF, Paulsen IT, Nelson KE, Gaidos EJ, Nelson WC, Read TD, Eisen JA, Seshadri R, Ward N, Methe B, Clayton RA, Meyer T, Tsapin A, Scott J, Beanan M, Brinkac L, Daugherty S, DeBoy RT, Dodson RJ, Durkin AS, Haft DH, Kolonay JF, Madupu R, Peterson JD, Umayam LA, White O, Wolf AM, Vamathevan J, Weidman J, Impraim M, Lee K, Berry K, Lee C, Mueller J, Khouri H, Gill J, Utterback TR, McDonald LA, Feldblyum TV, Smith HO, Venter JC, Nealson KH, Fraser CM.** 2002. Genome sequence of the dissimilatory metal ion-reducing bacterium *Shewanella oneidensis*. Nat Biotechnol **20:**1118–1123. http://dx.doi.org/10.1038/nbt749.
63. **Megerle JA, Fritz G, Gerland U, Jung K, Rädler JO.** 2008. Timing and dynamics of single cell gene expression in the arabinose utilization system. Biophys J **95:**2103–2115. http://dx.doi.org/10.1529/biophysj.107.127191.
64. **Fritz G, Megerle J, Westermayer S, Brick D, Heermann R, Jung K, Rädler JO, Gerland U.** 2014. Single cell kinetics of phenotypic switching in the arabinose utilization system of *E. coli*. PLoS One **9:**e89532. http://dx.doi.org/10.1371/journal.pone.0089532.
65. **Kiviet DJ, Nghe P, Walker N, Boulineau S, Sunderlikova V, Tans SJ.** 2014. Stochasticity of metabolism and growth at the single-cell level. Nature **514:**376–379. http://dx.doi.org/10.1038/nature13582.
66. **Korobkova E, Emonet T, Vilar JMG, Shimizu TS, Cluzel P.** 2004. From molecular noise to behavioural variability in a single bacterium. Nature **428:**574–578. http://dx.doi.org/10.1038/nature02404.
67. **Spudich JL, Koshland DE.** 1976. Non-genetic individuality: chance in the single cell. Nature **262:**467–471. http://dx.doi.org/10.1038/262467a0.
68. **Bubendorfer S, Koltai M, Rossmann F, Sourjik V, Thormann KM.** 2014. Secondary bacterial flagellar system improves bacterial spreading by increasing the directional persistence of swimming. Proc Natl Acad Sci U S A **111:**11485–11490. http://dx.doi.org/10.1073/pnas.1405820111.
69. **Mell JC, Redfield RJ.** 2014. Natural competence and the evolution of DNA uptake specificity. J Bacteriol **196:**1471–1483. http://dx.doi.org/10.1128/JB.01293-13.
70. **Seitz P, Blokesch M.** 2013. Cues and regulatory pathways involved in

natural competence and transformation in pathogenic and environmental Gram-negative bacteria. FEMS Microbiol Rev **37:**336–363. http://dx.doi.org/10.1111/j.1574-6976.2012.00353.x.

71. **Hamoen LW, Venema G, Kuipers OP.** 2003. Controlling competence in *Bacillus subtilis*: shared use of regulators. Microbiology **149:**9–17. http://dx.doi.org/10.1099/mic.0.26003-0.
72. **Son MJ, Ahn SJ, Guo Q, Bume RA, Hagen SJ.** 2012. Microfluidic study of competence regulation in *Streptococcus mutans*: environmental inputs modulate bimodal and unimodal expression of *comX*. Mol Microbiol **86:**258–272. http://dx.doi.org/10.1111/j.1365-2958.2012.08187.x.
73. **Lemme A, Gröbe L, Reck M, Tomasch J, Wagner-Döbler I.** 2011. Subpopulation-specific transcriptome analysis of competence-stimulating-peptide-induced *Streptococcus mutans*. J Bacteriol **193:**1863–1877. http://dx.doi.org/10.1128/JB.01363-10.
74. **Smits WK, Eschervins CC, Susanna KA, Bron S, Kuipers OP, Hamoen LW.** 2005. Stripping *Bacillus*: ComK auto-stimulation is responsible for the bistable response in competence development. Mol Microbiol **56:**604–614. http://dx.doi.org/10.1111/j.1365-2958.2005.04488.x.
75. **van Sinderen D, Luttinger A, Kong L, Dubnau D, Venema G, Hamoen L.** 1995. *comK* encodes the competence transcription factor, the key regulatory protein for competence development in *Bacillus subtilis*. Mol Microbiol **15:**455–462. http://dx.doi.org/10.1111/j.1365-2958.1995.tb02259.x.
76. **Johnsborg O, Havarstein LS.** 2009. Regulation of natural genetic transformation and acquisition of transforming DNA in *Streptococcus pneumoniae*. FEMS Microbiol Rev **33:**627–642. http://dx.doi.org/10.1111/j.1574-6976.2009.00167.x.
77. **Ahn SJ, Kaspar J, Kim JN, Seaton K, Burne RA.** 2014. Discovery of novel peptides regulating competence development in *Streptococcus mutans*. J Bacteriol **196:**3735–3745. http://dx.doi.org/10.1128/JB.01942-14.
78. **Probst C, Grünberger A, Wiechert W, Kohlheyer D.** 2013. Microfluidic growth chambers with optical tweezers for full spatial single-cell control and analysis of evolving microbes. J Microbiol Methods **95:**470–476. http://dx.doi.org/10.1016/j.mimet.2013.09.002.
79. **Probst C, Grünberger A, Wiechert W, Kohlheyer D.** 2013. Polydimethylsiloxane (PDMS) sub-micron traps for single-cell analysis of bacteria. Micromachines **4:**357–369. http://dx.doi.org/10.3390/mi4040357.
80. **Unthan S, Grünberger A, van Ooyen J, Gätgens J, Heinrich J, Paczia N, Wiechert W, Kohlheyer D, Noack S.** 2014. Beyond growth rate 0.6: what drives *Corynebacterium glutamicum* to higher growth rates in defined medium? Biotechnol Bioeng **111:**359–371. http://dx.doi.org/10.1002/bit.25103.
81. **Stratz S, Eyer K, Kurth F, Dittrich PS.** 2014. On-chip enzyme quantification of single *Escherichia coli* bacteria by immunoassay-based analysis. Anal Chem **86:**12375–12381. http://dx.doi.org/10.1021/ac503766d.
82. **Bevis BJ, Glick BS.** 2002. Rapidly maturing variants of the *Discosoma* red fluorescent protein (DsRed). Nat Biotechnol **20:**83–87. http://dx.doi.org/10.1038/nbt0102-83.
83. **Zhang J, Campbell RE, Ting AY, Tsien RY.** 2002. Creating new fluorescent probes for cell biology. Nat Rev Mol Cell Biol **3:**906–918. http://dx.doi.org/10.1038/nrm976.
84. **Neumeyer A, Hübschmann T, Müller S, Frunzke J.** 2013. Monitoring of population dynamics of *Corynebacterium glutamicum* by multiparameter flow cytometry. Microb Biotechnol **6:**157–167. http://dx.doi.org/10.1111/1751-7915.12018.
85. **Tolker-Nielsen T, Holmstrøm K, Boe L, Molin S.** 1998. Non-genetic population heterogeneity studied by *in situ* polymerase chain reaction. Mol Microbiol **27:**1099–1105. http://dx.doi.org/10.1046/j.1365-2958.1998.00760.x.
86. **Skinner SO, Sepúlveda LA, Xu H, Golding I.** 2013. Measuring mRNA copy number in individual *Escherichia coli* cells using single-molecule fluorescent *in situ* hybridization. Nat Protoc **8:**1100–1113. http://dx.doi.org/10.1038/nprot.2013.066.
87. **Cubitt AB, Heim R, Adams SR, Boyd AE, Gross LA, Tsien RY.** 1995. Understanding, improving and using green fluorescent proteins. Trends Biochem Sci **20:**448–455. http://dx.doi.org/10.1016/S0968-0004(00)89099-4.
88. **Makemson JC.** 1986. Luciferase-dependent oxygen consumption by bioluminescent vibrios. J Bacteriol **165:**461–466.
89. **Kamenšek S, Podlesek Z, Gillor O, Žgur-Bertok D.** 2010. Genes regulated by the *Escherichia coli* SOS repressor LexA exhibit heterogenous expression. BMC Microbiol **10:**283. http://dx.doi.org/10.1186/1471-2180-10-283.
90. **Kesel S, Mader A, Höfler C, Mascher T, Leisner M.** 2013. Immediate and heterogeneous response of the LiaFSR two-component system of *Bacillus subtilis* to the peptide antibiotic bacitracin. PLoS One **8:**e53457. http://dx.doi.org/10.1371/journal.pone.0053457.
91. **Hense BA, Müller J, Kuttler C, Hartmann A.** 2012. Spatial heterogeneity of autoinducer regulation systems. Sensors **12:**4156–4171. http://dx.doi.org/10.3390/s120404156.
92. **Ratcliff WC, Denison RF.** 2011. Bacterial persistence and bet hedging in *Sinorhizobium meliloti*. Commun Integr Biol **4:**98–100. http://dx.doi.org/10.4161/cib.14161.
93. **Stewart MK, Cummings LA, Johnson ML, Berezow AB, Cookson BT.** 2011. Regulation of phenotypic heterogeneity permits *Salmonella* evasion of the host caspase-1 inflammatory response. Proc Natl Acad Sci U S A **108:**20742–20747. http://dx.doi.org/10.1073/pnas.1108963108.
94. **Miyazaki R, Minoia M, Pradervand N, Sulser S, Reinhard F, van der Meer JR.** 2012. Cellular variability of RpoS expression underlies subpopulation activation of an integrative and conjugative element. PLoS Genet **8:**e1002818. http://dx.doi.org/10.1371/journal.pgen.1002818.
95. **Minoia M, Gaillard M, Reinhard F, Stojanov M, Sentchilo V, van der Meer JR.** 2008. Stochasticity and bistability in horizontal transfer control of a genomic island in *Pseudomonas*. Proc Natl Acad Sci U S A **105:**20792–20797. http://dx.doi.org/10.1073/pnas.0806164106.
96. **Chung JD, Stephanopoulos G, Ireton K, Grossman AD.** 1994. Gene expression in single cells of *Bacillus subtilis*: evidence that a threshold mechanism controls the initiation of sporulation. J Bacteriol **176:**1977–1984.
97. **Veening JW, Stewart EJ, Berngruber TW, Taddei F, Kuipers OP, Hamoen LW.** 2008. Bet-hedging and epigenetic inheritance in bacterial cell development. Proc Natl Acad Sci U S A **105:**4393–4398. http://dx.doi.org/10.1073/pnas.0700463105.
98. **De Angelis G, Moschinoi M, Muzzi A, Pezzicoli A, Cesini S, Delany I, Lo Sapio M, Sinisi A, Donati C, Masignani V, Barocchi MA.** 2011. The *Streptococcus pneumoniae* pilus-1 displays a biphasic expression pattern. PLoS One **6:**e21269. http://dx.doi.org/10.1371/journal.pone.0021269.
99. **Kröger C, Srikumar S, Ellwart J, Fuchs TM.** 2011. Bistability in *myo*-inositol utilization by *Salmonella enterica* serovar Typhimurium. J Bacteriol **193:**1427–1435. http://dx.doi.org/10.1128/JB.00043-10.
100. **Grantcharova N, Peters V, Monteiro C, Zakikhany K, Römling U.** 2010. Bistable expression of CsgD in biofilm development of *Salmonella enterica* serovar Typhimurium. J Bacteriol **192:**456–466. http://dx.doi.org/10.1128/JB.01826-08.
101. **Chai Y, Chu F, Kolter R, Losick R.** 2008. Bistability and biofilm formation in *Bacillus subtilis*. Mol Microbiol **67:**254–263.
102. **Mehra S, Charaniya A, Takano E, Hu WS.** 2008. A bistable gene switch for antibiotic biosynthesis: the butyrolactone regulon in *Streptomyces coelicolor*. PLoS One **3:**e2724. http://dx.doi.org/10.1371/journal.pone.0002724.

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