Universität Bremen

Molecular characterization of a novel quorum sensing system of the diazotrophic grass endophyte *Azoarcus* sp. strain BH72

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ABBREVIATIONS

2D	two-dimensional
3-OH-PAME	3-Hydroxypalmitic acid methyl ester
AHL	*N*-Acylhomoserine lactone
AI-2	Autoinducer-2
APS	ammoniumpersulfate
cAMP	cyclic adenosine monophosphate
COG	cluster of orthologous groups
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
DDT	dithiothreitol
DSF	Diffusible signal factor
EDTA	ethylenediaminetetraacetic acid
HSF	Hydrophilic signal factor
HSL	homoserine lactone
LC	liquid chromatography
MALDI-TOF	matrix assisted laser desorption/ionization time-of-flight
MS, MS/MS	mass spectrometry, tandem mass spectrometry
OD	optical density
PQS	P*seudomonas* quinolone signal
QS	quorum sensing
SDS	sodium dodecyl sulfate
Tris	tris(hydroxymethyl)aminomethane

SUMMARY

Quorum sensing (QS) is a cell density dependent regulatory mechanism which is involved in regulation of several processes in bacteria. *Azoarcus* sp. BH72 communicates via QS and conditioned culture supernatant, obtained from a culture grown until the stationary growth phase, shows inducing ability on QS target gene expression.

The presented study aimed to investigate the novel QS system of *Azoarcus* sp. BH72 by applying several methods: Comparative proteomic studies with *Azoarcus* wild type grown under QS conditions based on two-dimensional gel electrophoresis and mass spectrometry with an additional genome wide microarray approach were performed to detect QS regulated genes and proteins. Beside that, QS experiments were carried out with reporter gene studies by means of determination of β-glucuronidase activity encoded by the gene *uidA*. While the QS dependent regulation of the *pilAB* operon, involved in the formation of type IV pili, could be validated by reporter gene studies, it could be shown for the first time that expression of *azo3874*, encoding a conserved hypothetical secreted protein, was as well induced under QS conditions. Additionally, the proteome experiments revealed that 18 % of the detected proteins were differentially synthesized under QS conditions. Furthermore, transcriptome studies showed that the expression of around 10 % of all *Azoarcus* sp. BH72 genes was under QS control. The computational analyses of regulated genes and proteins showed that several cellular processes such as type IV pili formation/regulation, oxidative stress response, transport pathways (iron, nickel, and phosphate) and energy metabolism were altered under QS conditions. The expression of several genes, encoding regulatory proteins were under QS control indicating that quorum sensing regulation is organized in a complex hierarchy in the studied β-proteobacterium. Comparative proteomic studies with gel-based and gel-free methods with a *pilR*-mutant revealed that the responser regulator PilR is besides regulation of type IV pili biogenesis involved in activation as well as repression of a number of processes.

QS experiments with different bacterial species could clearly show that conditioned culture supernatants from *Azoarcus communis* SWub3 or *Azospira oryzae* 6a3 induced the *Azoarcus pilAB* as well as *azo3874* gene expression 2-fold. This observation indicates that *Azoarcus* sp. BH72 is capable of interspecies communication and that the novel quorum sensing system might be widespread in bacteria.

Concluding, the presented study demonstrates that density dependent regulation is important for this grass endophyte.

GERMAN SUMMARY – ZUSAMMENFASSUNG

Quorum sensing (QS) ist ein Zelldichte abhängiger Mechanismus zur Regulation verschiedener Prozesse in Bakterien. *Azoarcus* sp. BH72 kommuniziert via QS und konditionierter Kulturüberstand, der aus stationären Wildtypkulturen gewonnen wurde, besitzt die Fähigkeit zur Induktion der Expression von QS Zielgenen.

Ziel der vorliegenden Arbeit war die Untersuchung des neuartigen QS Systems von *Azoarcus* sp. BH72 mittels verschiedener Methoden: Es wurden sowohl vergleichende Proteomstudien, basierend auf zweidimensionaler Gelektrophorese und Massenspektrometrie, als auch Transkriptomstudien mittels Mikroarray mit *Azoarcus* Wildtyp unter QS Bedingungen durchgeführt. Weiterhin wurden QS Experimente mit Reportergenstudien, basierend auf der Bestimmung der β-Glucuronidaseaktivität, mit dem Reportergen *uidA* angefertigt. Die QS abhängige Regulation des *pilAB*-Operons, das an der Biogenese des Typ IV Pilus beteiligt ist, konnte durch Reportergenstudien validiert werden. Es konnte gezeigt werden, dass die Genexpression von *azo3874*, kodierend für ein hypothetisch sezerniertes Protein, ebenfalls unter QS induziert vorliegt. Zusätzlich verdeutlichten Proteomstudien, dass 18 % der detektierten Proteine unter QS Bedingungen differentiell synthetisiert wurden. Die Transkriptomstudien zeigten, dass die Expression von 10 % aller *Azoarcus* sp. BH72 Gene unter QS Kontrolle stand. Die computerunterstützte Analyse der regulierten Gene und Proteine ergab, dass diverse zelluläre Prozesse, wie z.B. die Bildung und Regulation des Typ IV Pilus, die Antwort auf oxidativen Stress, Transport (Eisen, Nickel und Phosphat) sowie Energiemetabolismus durch QS beeinflusst wurden. Die Expression von Genen, die für Regulatoren kodieren, lag unter QS Bedingungen vor allem aktiviert vor und deutet darauf hin, dass die Regulation durch QS einer komplexen Hierarchie unterliegt. Vergleichende Proteomstudien mit gel-basierten und gelfreien Methoden mit einer *pilR*-Mutante bewiesen, dass der Regulator PilR, neben der Regulation der Typ IV Pili Biogenese, eine aktivierende sowie reprimierende Rolle diverser Prozesse spielt.

QS Experimente mit verschiedenen Bakterien bewiesen, dass konditionierte Kulturüberstände von *Azoarcus communis* SWub3 und *Azospira oryzae* 6a3 die Genexpression von *pilAB* sowie *azo3874* induzieren können. Diese Beobachtung deutet darauf hin, dass *Azoarcus* sp. BH72 zu interspezifischer Kommunikation befähigt ist und dass das neuartige QS System möglicherweise weit verbreitet ist. Zusammenfassend konnte die Studie zeigen, dass die zelldichteabhängige Regulation für diesen Grasendopyten von großer Bedeutung ist.

I INTRODUCTION

I 1 Quorum sensing in the microbial world

Quorum sensing (QS) is a regulatory mechanism operating in response to cell density. This cell-to-cell communication is widespread in bacteria and in general involves the production and detection of quorum sensing signal molecules, termed autoinducers, followed by transcriptional gene regulation. This regulatory process was first described by Tomasz (1965) in the Gram-positive bacterium *Streptococcus pneumoniae* and in the marine Gram-negative organism *Vibrio fischeri* (Nealson et al. 1970).

Bacteria produce and release different quorum sensing signal molecules and their external concentration increases as a function of increasing cell density (Figure 1). At low cell densities the transcription of quorum sensing target genes is basal, but with high cell densities the concentration of QS signal molecules reaches a certain threshold. At this stage receptor proteins detect and bind the QS molecules and this complex leads to transcriptional activation or repression of quorum sensing target genes.

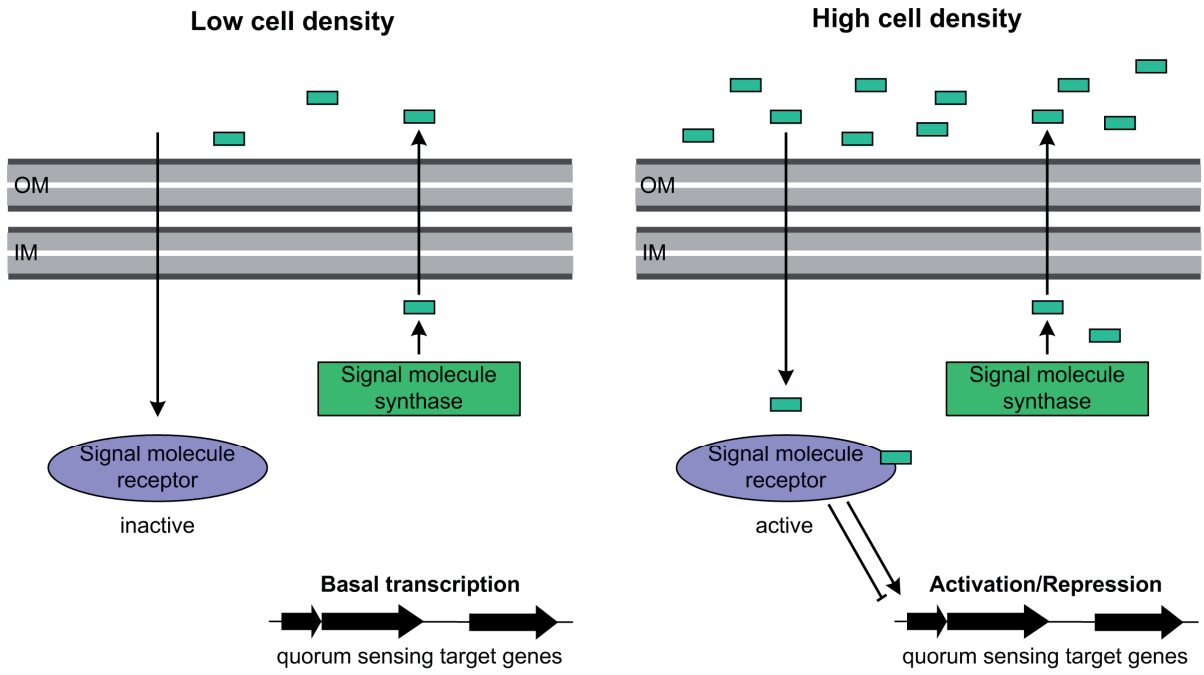

Figure 1: Simplified schematic representation of a quorum sensing system in a Gram-negative bacterium. The signal molecule synthase is responsible for basal production of quorum sensing signal molecules. At high cell densities these molecules accumulate and at a critical concentration interact with signal molecule receptor proteins. This leads to an activation of the receptor and subsequent alteration of quorum sensing target gene expression.

With this cell-to-cell communication process bacteria are capable to synchronize their behaviour and act as multicellular organisms in a changing environment (reviewed in Fuqua & Greenberg 1998 and Waters & Bassler 2005). In general, bacteria use quorum sensing to regulate a wide range of cellular processes such as biofilm formation, virulence, exopolysaccharide production, twitching and swarming motility as well as siderophore production among others (reviewed in Williams 2007). Moreover, plant-microbe interactions in several plant pathogens (*Erwinia carotovora* and *Burkholderia pseudomallei*), plant-associated bacteria (*Pseudomonas putida*) and symbionts (*Sinorhizobium meliloti*) are regulated in a cell density dependent manner (Marketon & González 2002, Bertani & Venturi 2004, Barnard & Salmond 2006, Eberl 2006).

I 1.1 Quorum sensing signal molecules

Bacteria produce chemically diverse quorum sensing signal molecules. In general, Gram-negative bacteria such as *Vibrio* spp., *Pseudomonas* spp, *Rhizobium* spp., *Burkholderia* spp., *Erwinia* spp. or *Serratia* spp. widely use *N*-Acylhomoserine lactones (AHL, Figure 2) as QS signal molecules (reviewed in Atkinson & William 2009). Acyl-homoserine lactones are composed of a fatty acyl chain ligated to a lactonized homoserine through an amide bond. The length of the acyl chain varies between 4 and 16 carbon atoms and the third carbon in the acyl chain can be fully oxidized, reduced or can carry a hydroxyl group. In general, AHLs are synthesized by special autoinducer synthesis proteins from the LuxI-type. This term is based on the luminescence genes from *Vibrio fischeri* where *luxI* is coding for the autoinducer synthase and *luxR* for the corresponding transcriptional activator protein (Engebrecht & Silverman 1984). LuxI-type proteins catalyse the production of AHLs and the acyl portion is derived from fatty acid precursors conjugated to the acyl carrier protein. The homoserine lactone (HSL) moiety is derived from S-adenosylmethionine. The QS signal molecules are detected by transcriptional regulators from the LuxR-type that show two functional domains: an autoinducer binding domain and a helix-turn-helix motif in their carboxyl terminus which is required for DNA binding (reviewed in Fuqua & Greenberg 2002).

Gram-positive bacteria communicate via peptide-based QS systems. Briefly, autoinducing peptides are ribosomally synthesized as precursor peptides and exported from the cell. During this process the precursor peptides are subjected to one or more posttranslational modification

events, until the active and stable autoinducer is produced (reviewed in Sturme et al. 2002). For example *Streptococcus pneumoniae*, *Bacillus subtilis* or *Staphylococcus aureus* use the peptides ComC (EMRLSKFFRDFILQRKK), ComX (ADPITRQWGD) or AgrD (YSTCDFIM) for communication, respectively (reviewed in Dunny & Leonard 1997).

But the categorization of signal molecules in Gram-negative and Gram-positive bacteria is not comprehensive: Several other autoinducers have been discovered and will be presented in the following.

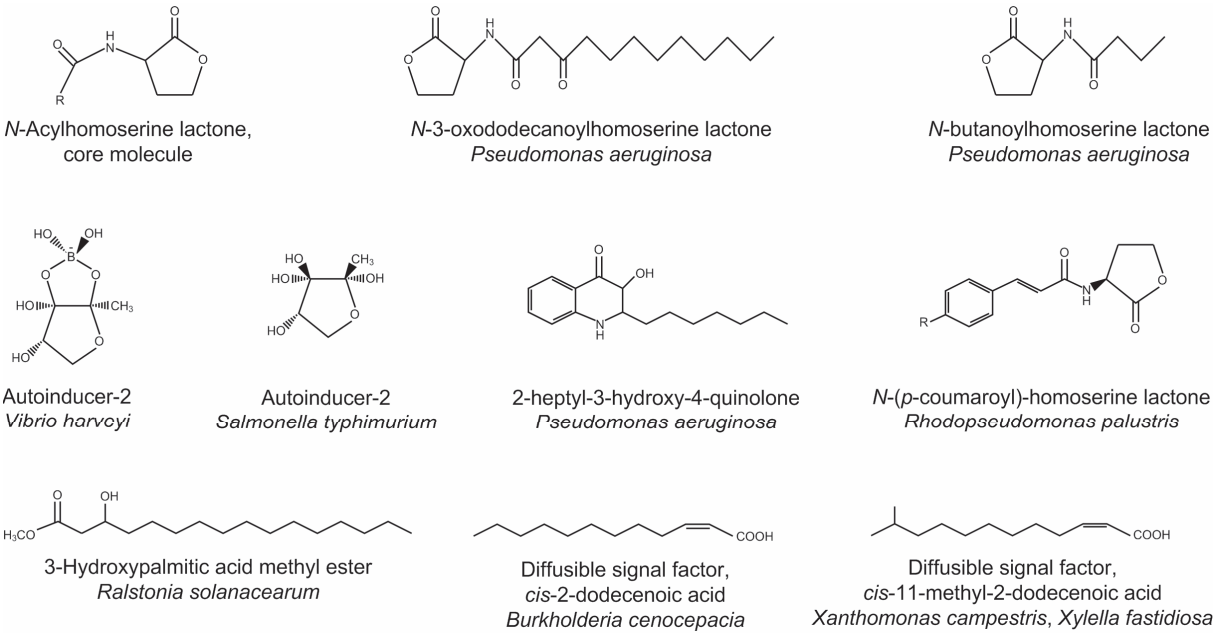

Figure 2: Chemical structures of several quorum sensing signal molecules.

The molecule Autoinducer-2 (AI-2, Figure 2) has been described in several bacteria (*Vibrio* spp., *Salmonella* spp.). This molecule has been proposed to enable interspecies communication and is based on the Autoinducer-2 synthase LuxS (S-ribosylhomocysteinase) that is widely conserved among Gram-negative and Gram-positive bacteria. AI-2 is produced from S-adenosylmethionine, which is hydrolysed by a nucleosidase yielding adenine and S-ribosylhomosysteine. This molecule is converted to 4,5-dihydroxy-2,3-pentanedione (DPD) and homocysteine with LuxS as a catalysator and the precursor DPD undergoes further rearrangements to form AI-2 (Chen et al. 2002).

Moreover, the Diffusible signal factor (DSF) has been described for *Xanthomonas campestris*, *Xylella fastidiosa*, *Stenotrophomonas maltophilia* and *Burkholderia cenocepacia*. In *X. campestris* production of the Diffusible signal factor (*cis*-11-methyl-2-dodecenoid acid,

Figure 2) is dependent on the enoyl-CoA hydratase RpfF and the long-chain fatty acyl CoA ligase RpfB. The signal molecule is detected by the two-component regulatory system RpfCG with RpfC as sensor kinase and RpfG as the corresponding response regulator. In this bacterium the QS system is responsible for pathogenicity gene expression (Barber et al. 1997, Slater et al. 2000). The plant pathogen *X. fastidiosa* also communicates via a *cis*-11-methyl-2-dodecenoid acid (Colnaghi et al. 2007), whereas *B. cenocepacia* uses *cis*-2-dodecenoic acid (Boon et al. 2008). The structure of the DSF of *S. maltophilia* was not examined but the genome encodes a cell-to-cell signalling system that is highly related to the one from *X. campestris* (Fouhy et al. 2007).

Some bacteria use very specific molecules for cell-to-cell communication: In the photosynthetic bacterium *Rhodopseudomonas palustris* a new class of homoserine lactone signal has been discovered, the *N*-(*p*-coumaroyl)-HSL (Figure 2, Schaefer et al. 2008). Furthermore, the plant symbiont *Bradyrhizobium japonicum* uses the signal molecule Bradyoxetin for density dependent gene regulation (Loh et al. 2002). Expression of virulence genes in the phytopathogenic bacterium *Ralstonia solanacearum* is controlled by a complex regulatory network that integrates the 3-Hydroxypalmitic acid methyl ester (3-OH-PAME, Figure 2) quorum sensing system. In this bacterium *phcB* encodes the signal molecule synthase that produces the fatty acid derivate 3-Hydroxypalmitic acid methyl ester and this molecule is detected by the two-component regulatory system PhcSR with PhcS as transmembrane sensor histidine kinase and PhcR as the corresponding transcription regulatory protein (Flavier et al. 1997, Clough et al. 1997). In *Pseudomonas aeruginosa* quorum sensing is controlled via two AHL-based systems, but a third quorum sensing molecule has been discovered, the *Pseudomonas* quinolone signal (PQS) 2-heptyl-3-hydroxy-4-quinolone (Figure 2, Pesci et al. 1999).

I 1.2 The complex quorum sensing hierarchy of *Pseudomonas*

The model organism for a complex quorum sensing hierarchy is the pathogen *Pseudomonas aeruginosa* where three QS systems with different signal molecules are interrelated. The complex QS cascade of *P. aeruginosa* with its several regulatory proteins that are involved in diverse cellular processes is depicted in Figure 3.

In this bacterium the AHL signal molecules *N*-3-oxododecanoylhomoserine lactone (3-oxo-C12-HSL) and *N*-butanoylhomoserine lactone (C4-HSL) are synthesized via the

autoinducer synthases LasI and RhlI, respectively. These autoinducers diffuse out of the cell and accumulate until a certain threshold is reached at high cell densities. In general, the *las*- and *rhl*-systems directly or indirectly regulate over 10 % of the *Pseudomonas* genome.

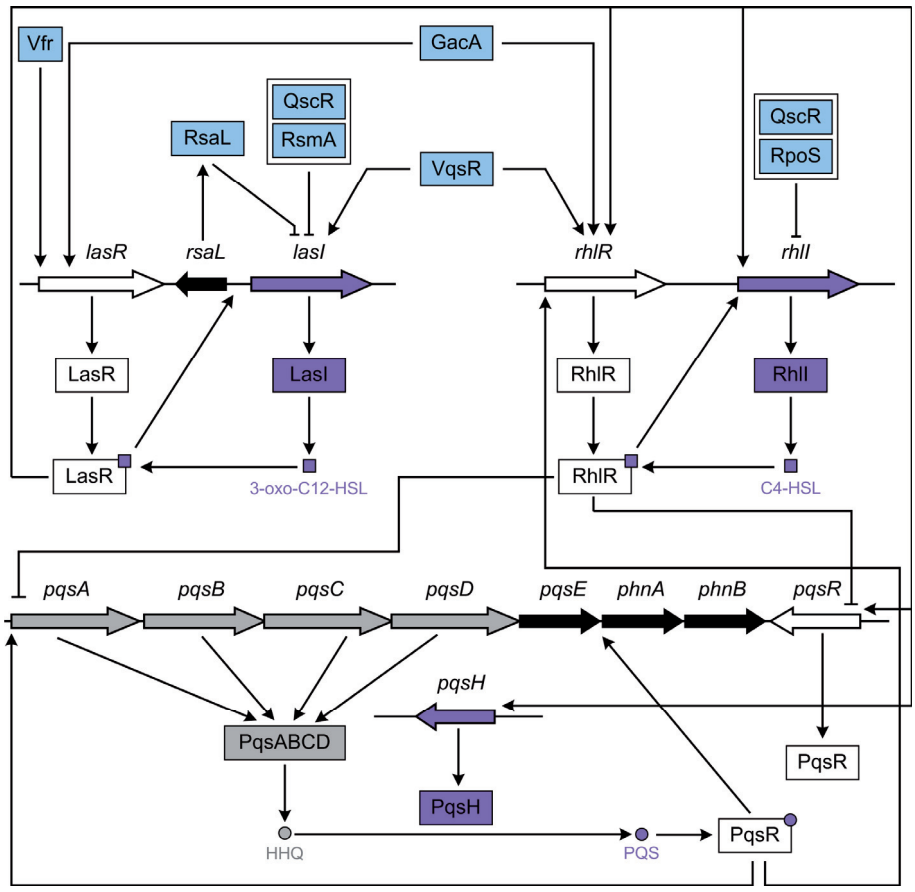

Figure 3: Representation of the complex quorum sensing network in *Pseudomonas aeruginosa* that controls the expression of virulence factors, secondary metabolites, swarming motility and biofilm development. 3-oxo-C12-HSL = *N*-3-oxododecanoylhomoserine lactone, C4-HSL = *N*-butanoyl-homoserine lactone, HHQ = 2-heptyl-4-quinolone, PQS = *Pseudomonas* quinolone signal, 2-heptyl-3-hydroxy-4-quinolone. Picture is adapted from Williams & Camara 2009.

These two AHL-based systems are organized in a hierarchy in which the LuxR-type transcriptional regulator LasR binds 3-oxo-C12-HSL and subsequently drives the expression of *lasI* as well as *rhlR* and *rhlI*. In this QS system the gene expression of *lasR* itself is positively regulated by the cyclic adenosinemonophosphate (cAMP) receptor protein Vfr and the global activator GacA. The negative regulator RsaL counteracts the LasR/3-oxo-C12-HSL positive feedback loop by direct repression of *lasI* gene expression. The complex of 3-oxo-C12-HSL bound to LasR activates the expression of several target genes: The genes encoding the second acyl homoserine lactone dependent system RhlIR and the genes coding for the *Pseudomonas* quinolone signal system. This system is based on 2-heptyl-3-hydroxy-4-quinolone which is synthesized by PqsABCD and PqsH. The activities

of both AHL-dependent quorum sensing systems are controlled at the transcriptional and posttranscriptional level by several regulatory proteins such as the LuxR-homologues QscR and VqsR. Moreover, the riboregulator RsmA negatively regulates *lasI* and *rhlI* expression and the alternative sigma factor RpoS represses expression of *rhlI*. In turn the *Pseudomonas* quinolone signal system positively regulates the RhlIR-system in *P. aeruginosa* (reviewed in Lazdunski et al. 2004, Juhas et al. 2005 and Williams & Camara 2009).

I 1.3 Quorum sensing is integrated in different regulatory networks

Studies of bacterial cell-to-cell communication are generally based on pure laboratory culture conditions. However, natural bacterial populations live in complex environments where biodiversity and abiotic factors are changing. The signalling processes are directly and indirectly influenced by abiotic factors such as pH-values, temperature or nutrient availability and biotic factors such as other members from the bacterial community or eukaryotes in the habitat. Therefore the mechanism of quorum sensing is integrated in a global regulatory network and besides intraspecies communication interspecies crosstalk and communication between bacteria and eukaryotes is of great interest (reviewed in Boyer & Wisniewski-Dyé 2009).

I 1.3.1 Interspecies communication in microbiomes

The microbiome is the entirety of bacterial organisms, their genome properties and all environmental interactions in a defined environment. Quorum sensing allows bacteria to communicate within species as depicted above but also between species (Figure 4). In one habitat signal molecules of several bacteria can be interrelated in cell-to-cell communication processes which lead to interspecies crosstalk. Different bacterial species that produce quorum sensing signals and coexist in the same environment can detect and respond to these signals. It has been shown that *Chromobacterium violaceum* produces the pigment violacein in response to C6-HSL irrespective of whether the *N*-Acylhomoserine lactone is supplied by another producer or by itself (McClean et al. 1997). Moreover, AHL-mediated communication occurs between *Pseudomonas aeruginosa* and *Burkholderia cenocepacia* in mixed biofilms, where signals produced by *P. aeruginosa* trigger the expression of several *Burkholderia* genes, including *cepI* coding for an autoinducer synthase (Riedel et al. 2001). Another example for interspecies communication has been described for the Autoinducer-2 system: AI-2 produced by *Streptococcus* sp. influences the expression of several QS

dependent genes in *P. aeruginosa* (Sibley et al. 2008). *Stenotrophomonas maltophilia* and *P. aeruginosa* can be found together in diverse niches including the rhizosphere of plants and lungs with cystic fibrosis: In mixed biofilms DSF produced by *S. maltophilia* influences the biofilm architecture of *Pseudomonas* (Ryan et al. 2008).

In natural environments bacteria form communities with bacteria that produce QS signal molecules and with bacteria that are capable of degrading such molecules. Such processes interfering with cell-to-cell communication are termed quorum quenching. Two categories of AHL-degrading enzymes have been described: AHL-lactonases of the AiiA family and AHL-acylases/amidohydrolases of the AiiD family.

AHL lactonases of the AiiA-family are present in several species like *Variovorax paradoxus* (Leadbetter & Greenberg 2000), *Bacillus thuringiensis* (Dong et al. 2002), *Arthrobacter* sp. (Park et al. 2003) and *Rhodococcus erythropolis* (Uroz et al. 2008). Experiments with *V. paradoxus* showed that this soil bacterium is capable of growth on different *N*-Acylhomoserine lactones and the molar growth yields correlated with the length of the acyl group (Leadbetter & Greenberg 2000). Another example is *Arthrobacter* sp. strain IBN110 that rapidly grows on AHLs and is able to degrade AHLs and utilize 3-oxo-C6-HSL as a sole carbon source. In co-cultures of *Arthrobacter* sp. and the plant pathogen *Erwinia carotovora* it has been shown that the level of AHL was significantly reduced, suggesting the possibility of using *Arthrobacter* sp. for controlling AHL-producing plant pathogenic bacteria (Park et al. 2003). An AHL-degrading enzyme of the AiiD-family is used by *Ralstonia* sp. strain XJ12B. It was isolated from mixed biofilms, and the enzyme responsible for AHL inactivation has been shown to be an acylase. This enzyme hydrolyses the AHL amide, releasing homoserine lactone and the corresponding fatty acid. Expression of AiiD in *Pseudomonas aeruginosa* PAO1 resulted in decreased swarming motility as well as elastase and pyocyanin production indicating quenching of the quorum sensing system (Lin et al. 2003).

Some bacteria contain LuxR-type transcriptional activator proteins without a corresponding LuxI-type synthase for AHL signal production. Such LuxR-orphans are used by *Escherichia coli*, *Salmonella typhimurium*, *Burkholderia cenocepacia*, *Pseudomonas putida* or *Sinorhizobium meliloti* among others to detect signals within a community followed by activation and repression of specific genes. *Salmonella* sp., *Escherichia* sp. and *Klebsiella* sp.

do not produce *N*-Acylhomoserine lactones but they encode the LuxR-orphan receptor SdiA that allows the response to exogenous AHLs from other species (Ahmer et al. 1998, Michael et al. 2001, Smith & Ahmer 2003, Wu et al. 2008). In *Pseudomonas putida* the unpaired LuxR-orphan PpoR binds 3-oxo-C6-HSL and plays therefore a role in detection and response to bacterial endogenous as well as exogenous signalling molecules (Subramoni & Venturi 2009). Furthermore, the two AHL-based systems CepIR and CciIR regulate virulence in *Burkholderia cenocepacia*, but this QS cascade includes a third component, the LuxR-orphan CepR2 that additionally regulates numerous genes (Malott et al. 2009).

I 1.3.2 Interkingdom signalling

Cell-to-cell communication not only occurs between bacteria from the same species and interspecifically but also between bacteria and their eukaryotic hosts. This communication system is termed interkingdom signalling and has been described for several interactions of bacteria with plants, fungi and animals.

Gene expression and protein synthesis in barrel clover (*Medicago trunculata*) roots is influenced by AHL signal molecules secreted by the nitrogen fixing symbiont *Sinorhizobium meliloti* and the pathogen *Pseudomonas aeruginosa*. It could be proven with proteomic studies that 150 proteins of *M. trunculata* showed altered levels after exposing seedlings to 3-oxo-C12-HSL or 3-oxo-C16-HSL, respectively. The affected proteins were assigned to several functions such as plant defense, stress response or protein degradation processes (Mathesius et al. 2003). Moreover, it was reported that morphology of the fungus *Candida albicans* is significantly affected by the presence of *Pseudomonas aeruginosa*. Secretion of the AHL molecule 3-oxo-C12-HSL inhibits filamentation of *C. albicans* without affecting fungal growth rates (Hogan et al. 2004).

Alongside plant responses, plants like pea (*Pisum sativum*) and rice (*Oryza sativa*) secrete AHL mimicking compounds which act as agonists or antagonists to bacterial AHL-based quorum sensing systems. Such AHL mimics could be found in exudates from *P. sativum* and it was stated that they affect bacterial behaviour. Experiments with well-characterized bacterial reporter strains revealed that pea exudates inhibit quorum sensing regulated behaviours like swarming in *Chromobacterium violaceum* and *Serratia liqufaciens* (Teplitski et al. 2000). Furthermore, *O. sativa* produces two different compounds that activate AHL biosensors. The detected molecules were shown to be sensitive to the AiiA lactonase

enzyme, a quorum quenching enzyme from *Bacillus* sp. that inactivates *N*-Acylhomoserine lactones. Therefore, they are either AHLs or have a related lactone structure. Probably such molecules can be used to interfere with bacterial signalling systems as a strategy to escape colonization processes of pathogenic organisms (Degrassi et al. 2007).

I 1.3.3 Communication in a specialized environment – the rhizosphere

The rhizosphere is a zone of active interchange between plants and bacteria that together secrete different molecules for specialized communication processes. The interplay of several partners in such a habitat is depicted in Figure 4 and it is obvious how complex the regulation is in this niche.

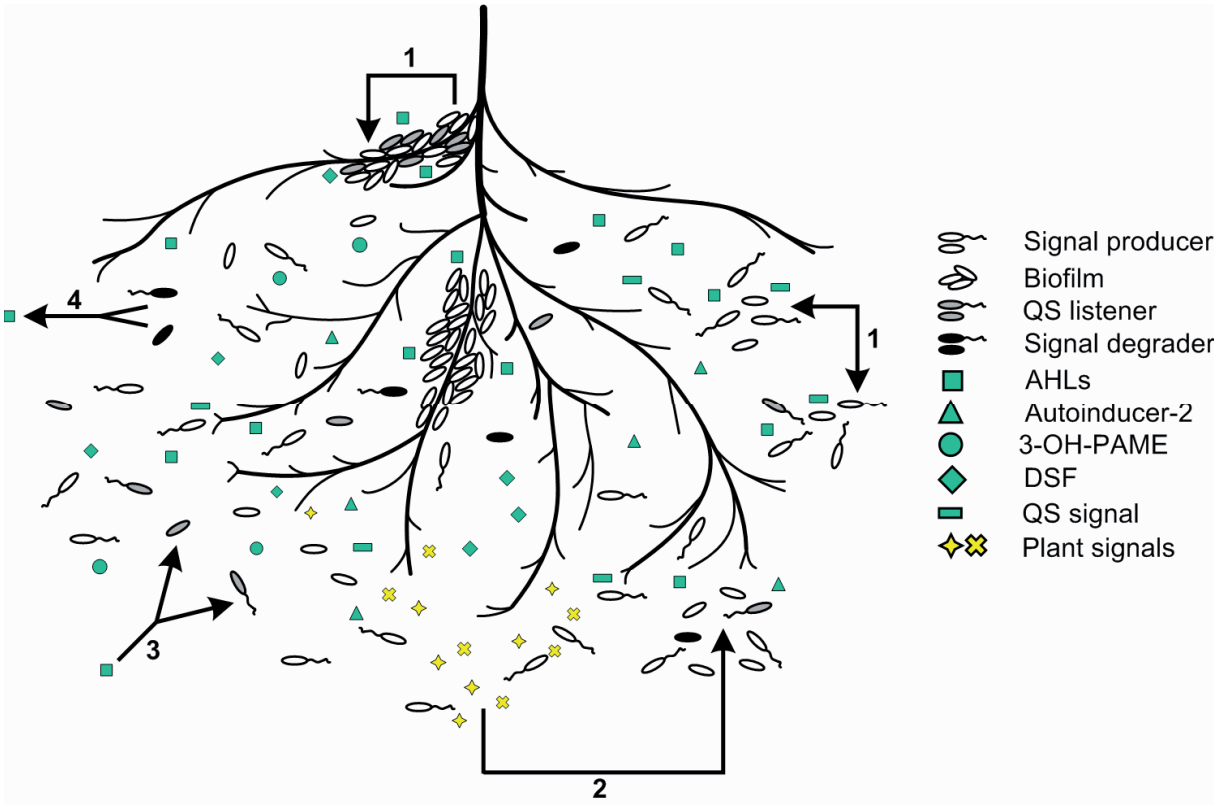

Figure 4: Schematic model of quorum sensing related interactions in the rhizosphere between plants and a bacterial community. Several bacterial species are present: Quorum sensing (QS) signal producing bacteria, bacteria organized in a biofilm, QS signal listening bacteria and QS signal degrading bacteria. Different bacterial signalling molecules (*N*-Acylhomoserine lactones, AHLs; Autoinducer-2, Diffusible signal factor, DSF, 3-Hydroxypalmitic acid methyl ester, 3-OH-PAME and unknown signals) as well as plant signals (e.g. AHL mimics) can be detected in the rhizosphere and are involved in communication processes like intra- and interspecies crosstalk (1), plant responses (2), interception by means of "listening" (3) and signal degradation (4).

On the one hand intraspecies as well as interspecies crosstalk (see I 1.3.1) is of great importance for plant pathogens, symbionts or endophytic bacteria for successful colonization

of their hosts. But on the other hand plants can circumvent such colonization processes by production of plant signalling compounds and therefore interfere with QS systems (see chapter I 1.3.2). Moreover, the fact that bacteria can listen to exogenous QS signal molecules suggests that these bacteria save energy linked to signal synthesis and therefore benefit from other bacteria in their community. Numerous bacterial species, possessing the ability to degrade *N*-Acylhomoserine lactones, have been isolated from the rhizosphere indicating that these bacteria can coexist with AHL-producing species within the same environment. The inactivation of molecules can not only offer a nutrient source but also prevent QS signalling in neighbouring bacteria. Such degrading enzymes may thus provide a competitive advantage for signal molecule degraders (reviewed in Boyer & Wisniewski-Dyé 2009).

The structural diversity of signal molecules and the huge variety of quorum sensing systems offers bacteria the opportunity to be dominant in a bacterial community. In the rhizosphere mainly *N*-Acylhomoserine lactones are produced by several soil- and plant-associated bacteria, therefore the synthesis of different molecules like the Diffusible signal factor or 3-Hydroxypalmitic acid methyl ester could offer a competitive advantage for *Xanthomonas* sp., *Burkholderia* sp. or *Ralstonia* sp. over their neighbouring bacteria. These QS molecules might be used to avoid interference of plants through AHL mimics as well as response of other bacteria to exogenous signals (reviewed in Gera & Srivastava 2006).

I 2 The grass endophyte *Azoarcus* sp. BH72

The β-proteobacterium *Azoarcus* sp. BH72 is a model endophyte which is able to colonize rice roots under laboratory conditions. The bacterium was originally isolated from its natural host Kallar grass (*Leptochloa fusca* (L.) Kunth), a pioneer plant on salt-affected alkaline soils in the Punjab of Pakistan. *Azoarcus* sp. BH72 is of agro biotechnical interest as it supplies biologically fixed nitrogen to its host and colonizes plants in high numbers without causing symptoms of plant disease (Reinhold et al. 1986, Hurek et al. 1994). *Azoarcus* sp. BH72 has a strictly respiratory type of metabolism and the major carbon sources used are dicarboxylic acids and ethanol (Reinhold-Hurek et al. 1993a).

The genome of *Azoarcus* sp. BH72 has been sequenced and it contains 3992 predicted protein-coding sequences (Krause et al. 2006). The plant-associated lifestyle of *Azoarcus* sp. requires several features such as type IV pili, which are crucial for adhesion to the root surface and host colonization. Strain BH72 harbours 41 genes coding for proteins putatively

involved in pilus assembly and regulation (Dörr et al. 1998, Krause et al. 2006). *Azoarcus* sp. BH72 appears to be disarmed concerning virulence and pathogenicity because only few cell wall degrading enzymes are present (Reinhold-Hurek et al. 2006). The endophyte lacks type III and IV secretion systems that are responsible for transport of various effector proteins involved in plant-pathogen interactions whereas the secretion systems type I, II and the newly described type VI secretion system are present (Krause et al. 2006, Shidore 2008).

I 2.1 Type IV pili and colonization

Type IV pili are filamentous cell appendages build from pilin subunits, that are located on the cell surface of several Gram-negative bacteria (reviewed in Strom & Lory 1993 and Mattick 2002). Besides in attachment and host colonization type IV pili are involved in twitching motility (Böhm et al. 2007), biofilm formation (O'Toole & Kolter 1998, Chiang & Burrows 2003) as well as virulence (Craig et al. 2004).

In *Azoarcus* sp. BH72 type IV pili are essential for adhesion to plant and fungal host surfaces and crucial for the colonization process. The *pilAB*-operon is required for the formation of type IV pili in this endophyte. The operon contains the genes *pilA* and *pilB*, with *pilA* encoding a prepilin precursor protein and *pilB* coding for the type IV pilus assembly protein. These genes are co-transcribed from a sigma-54 dependent promoter. It has been demonstrated that the capacity of *pil*-mutants to establish microcolonies on roots of rice seedlings differs significantly from *Azoarcus* wild type. In contrast to strain BH72, viable cells of single and double *pilAB*-mutants are only rarely detectable on the root surface (Dörr et al. 1998). Furthermore, the two-component response regulatory system PilSR has been shown to be involved in activation of pilin gene expression in *Azoarcus* sp. BH72. In this system, *pilS* encodes the sensor protein kinase PilS whereas the corresponding response regulator is encoded by *pilR*. The inactivation of *pilR* leads to a drastically decreased level of *pilAB* gene expression which demonstrates that the response regulator PilR is necessary for transcriptional activation of the *pilAB*-operon. (Dörr 1998, Böhm 2006, Friedrich 2010). Furthermore, it could be shown that the two-component regulatory system PilSR regulates the *pilAB* gene expression in dependence of nutrient availability. The sensor protein PilS is stimulated by carbon starvation: This stimulus leads to phosphorylation of the sensor kinase that subsequently transfers the phosphate residue to the corresponding response regulator PilR. PilR in turn activates *pilAB* gene expression. It was stated that a *pilS*-mutant is not able

to respond to carbon starvation (Plessl 2001, Friedrich 2010). In addition, it could be shown that the expression of *pilAB* is cell density dependent in *Azoarcus* sp. BH72 (Böhm 2006, see I 2.2).

I 2.2 *Azoarcus* sp. BH72 communicates via a novel quorum sensing system

The plant-associated bacterium *Azoarcus* sp. BH72 appears to use an unknown signalling molecule for cell-to-cell communication as there is no evidence for genes encoding the AHL autoinducer synthesis protein or corresponding receptor proteins. Genes coding for the AI-2 synthase are also lacking. Cross streak experiments with AHL monitor strains of *Escherichia coli* and *Pseudomonas putida* that sense *N*-Acylhomoserine lactones with short or long acyl chains did not give a positive response towards *Azoarcus* sp. BH72 (Krause et al. 2006). So far, the signal molecule of *Azoarcus* sp. BH72 could not be discovered but due to its chemical properties it was termed Hydrophilic signal factor (HSF). The hydrophilicity of the molecule was proven by ion-exchange chromatography and High Pressure Liquid Chromatography (HPLC). These experiments showed that the HSF is weakly binding to a cation exchange cartridge and positively charged at pH 6. Moreover, the molecule was already eluted from the HPLC column at 2 - 7 % acetonitrile (Böhm 2006).

There is strong evidence that *Azoarcus* sp. BH72 communicates through quorum sensing and that this regulatory system is very important for this strain. The *pilAB* genes are quorum sensing targets in strain BH72 as their gene expression is density dependent. In *Azoarcus* wild type the *pilAB* gene expression increases around 2-fold at high cell densities, whereas the gene expression is around 10-fold increased in a *pilS* deletion mutant. These experiments show that the sensor kinase PilS has a strong negative effect on the *pilAB* gene expression at high cell densities. Moreover, the *pilAB* gene expression can be around 2- to 3-fold induced by conditioned culture supernatant, obtained from cultures grown until the stationary growth phase (Böhm 2006). In addition, it has been shown that the protein pattern from *Azoarcus* sp. BH72 cells undergoes significant changes when grown under QS conditions (Hauberg 2006). These observations raise the hypothesis that quorum sensing might play a role in bacteria-plant interactions in the studied β-proteobacterium.

I 3 Objectives

The studies presented here aim to characterize the QS system of *Azoarcus* sp. BH72 with global approaches and to identify new QS targets in this β-proteobacterium. First, a proteome reference map for the grass endophyte *Azoarcus* sp. BH72 under aerobic standard growth conditions was constructed with two-dimensional gel (2D) electrophoresis and MALDI-TOF-MS (matrix assisted laser desorption/ionization time-of-flight mass spectrometry) analyses. Additionally, a gel-free approach with liquid chromatography tandem mass spectrometry (LC-MS/MS) was helpful to further increase the number of identified proteins synthesized under aerobic conditions.

To characterize the novel quorum sensing system in *Azoarcus* sp. BH72 a comparative proteomic approach was initiated with two-dimensional gel electrophoresis comparing the early exponential growth phase with growth under quorum sensing conditions. In parallel, a genome wide microarray approach was performed under the same conditions and the outcoming results were linked to proteomic data to get an overall idea of quorum sensing regulation in *Azoarcus* sp. BH72. This approach helped to detect quorum sensing targets and might be useful to identify genes or proteins that are required for synthesis of the quorum sensing signal molecule. Additionally, the construction of insertional mutants carrying transcriptional fusions of target genes to reportergenes like *gfp*, encoding the green fluorescent protein, or *uidA*, coding for β-glucuronidase was helpful to characterize target gene expression under different growth conditions.

The two-component regulatory system PilSR is involved in pilus formation in *Azoarcus* sp. BH72. As the genes *pilAB* are essential for endophytic colonization in *Azoarcus* sp., the characterization of the PilR regulon might reveal further insights in proteins required for this process. Beyond that, the genes for the structural pilin subunits *pilAB* are QS targets in this β-proteobacterium and this fact leads to the question how the response regulator PilR is involved in the QS regulatory cascade. Comparative proteomic studies with gel-based and gel-free methods with *Azoarcus* sp. BH72 wild type and a *pilR*-mutant (BH*pilR*K) were performed to study the influence of the regulatory protein PilR on the *Azoarcus* sp. proteome under standard growth conditions.

As strain BH72 colonizes rice roots, the Endophytic microbiome from Rice was compared with several selected metagenomes as well as endophytic bacterial genomes for the occurrence of quorum sensing systems with computational tools. These studies in general have helped to get a deeper insight into several interacting QS systems occurring in this special niche and their abundance. To address specifically how *Azoarcus* sp. BH72 communicates with other members of this community during its endophytic lifestyle, the impact of conditioned culture supernatants from several (closely related) bacteria were tested on its QS target gene expression stimulating its natural lifestyle. With such experiments it is possible to gain more insights into the specificity and distribution of the quorum sensing signal molecule. Moreover, the crucial question whether interspecies crosstalk exists in this grass endophyte can be answered.

II MATERIAL & METHODS

II 1 Chemicals and Buffers

Unless not stated, all chemicals and reagents were purchased from the following companies: Sigma Aldrich (Seelze, Germany), Merck (Darmstadt, Germany), Riedel de Häen (Seelze, Germany), Applichem (Darmstadt, Germany), Serva (Heidelberg, Germany), Roth (Karlsruhe, Germany) or GE Healthcare (Fairfield, CT, USA). Components for media and agar were from Difco/Becton Dickinson (Heidelberg, Germany) and Roth. All chemicals used had the analytical grade p.A. or were tested for their molecular biological use.

II 2 Oligonucleotides

Primers for real-time PCR experiments and oligonucleotides that were used for cloning experiments in this work are listed in Table 1. The oligonucleotides were purchased from Eurofins MWG GmbH (Ebersberg, Germany).

Table 1: Description of primers used in this study.

Application	Name	Sequence 5' - 3' [a)]	Product length	Annealing temperature
Real-time PCR	azo0156for azo0156rev	ATCAACGATCCCAAGCTTTC CGTGTTCGTTCTTCAGAGCA	169 bp	60℃
Real-time PCR	azo0673for azo0673rev	TCAGGAGGTGGGCAACTG ACAAGAACCGCCGTCCAC	154 bp	60℃
Real-time PCR	azo3294for azo3294rev	CACGCAAAGATGATCAGGAA TGATCTACACCCTGCTGCTG	151 bp	60℃
Real-time PCR	azo3412for azo3412rev	GAAACGCTTGAGGGTAGTGC GCTGAACATTCTGGCCTTCT	160 bp	60℃
Real-time PCR	azo3674for azo3674rev	AGTTCAAGGCCAAGGTGCT CGTAACGGAGTTTTCGAAGC	167 bp	60℃
Real-time PCR	azo3868RTfor azo3868RTrev	CACTCGCAGTGCCTGTACTC CCCTCGAAGTAGGACATCCA	176 bp	60℃
Real-time PCR	azo3874RTfor azo3874RTrev	CCTTCAAGTTCGAGGACGAC ACGTAGAAGGCCAGGTGATG	190 bp	60℃
Real-time PCR	16SRTfor 16SRTrev	CTTGACATGCCTGGAACCTT ATGACGTGTGAAGCCCTACC	245 bp	60℃
Cloning of BHazo3874	azo3874for azo3874rev	GCTCTAGA**TAA**TCCCTGCTCCACCGCTTG CCCAAGCTTGTGAAGGTGCTCGGCGTAT	456 bp	66℃
gfp-probe	gfppK18GGSTfor gfppK18GGSTrev	AGTGGAGAGGGTGAAGGTGA AAAGGGCAGATTGTGTGGAC	535 bp	61℃

[a)] *Hind*III recognition site is indicated with blue letters, *Xba*I recognition site with red letters and the stop codon (TAA) in bold black

II 3 Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table 2 and Table 3.

Table 2: Description of bacterial strains used in this study.

Strain	Description	Reference
***Azoarcus* sp.**		
BH72	wild type	Reinhold et al. 1986
BH*pilR*K	Km^r, BH72 *pilR*::Km-cassette at bp 247 of *pilR*	Dörr 1998
BHΔ*pilS*	Deletion of *pilS*	Plessl 2001
BHΔ*pilS*::pJBLP14	Ap^r, deletion of *pilS*, *pilAB*::*uidA*-fusion	Plessl 2001
BHΔ*pilS*::pJBLP1*gfp*	Ap^r,deletion of *pilS, pilAB*::*gfp*-fusion	Böhm 2006
BH72(pJLA*pilA-uidA*)	Tc^r, stable plasmid pJLA*pilA-uidA* in wild type BH72, *pilA*::*uidA*-fusion	Dörr 1998
BH*pilR*K (pJLA*pilA-uidA*)	Tc^r, stable plasmid pJLA*pilA-uidA* in mutant BH*pilR*K, *pilA*::*uidA*-fusion	Dörr 1998
BH72::pJBLP21	Ap^r, *pilSR*::*uidA*-fusion	Dörr 1998
BH*azo3874*	Km^r, pK18GGST*azo3874* chromosomally integrated at position 4246383 of *Azoarcus* sp. BH72 genome, *azo3874*::*gfpuidA*-fusion	This study
Escherichia coli		
DH5αF'	F´, ϕ80*lac*ZΔM15, Δ(*lac*ZYA-*arg*F), U169, *deo*R, *rec*A1, *end*A1, *hsd*R17, (r_k^-, m_k^+)*pho*A, *sup*E44, λ^-, *thi*-1, *gyr*A96, *rel*A1	Invitrogen (Karlsruhe, Germany)
S17-1	MM294,RP4-2-Tc::Mu-Km::Tn7 chromosomally integrated	Simon et al. 1983
Azoarcus communis SWub3	wild type	Reinhold-Hurek et al. 1993b
Azoarcus evansii KB740	wild type	Anders et al. 1995
Azonexus fungiphilus Bs5-8	wild type	Hurek et al. 1997
Azospira oryzae 6a3	wild type	Reinhold-Hurek et al. 1993b
Azospirillum brasilense Sp7	wild type	Tarrand et al. 1978
Azospirillum lipoferum Sp59b	wild type	Tarrand et al. 197
Azotobacter vinelandii MV521	wild type	Walmsley et al. 1994
Azovibrio restrictus S5b2	wild type	Reinhold-Hurek et al. 1993b
Chromobacterium violaceum ATCC31532	wild type	Cooper et al. 1995, McClean et al. 1997
Pseudomonas stutzeri DSM4166	wild type	Krotzky & Werner 1987
Pseudomonas syringae pv. *tomato* DC3000	wild type	Cuppels 1986
Xanthomonas oryzae PXO99	wild type	Hopkins et al. 1992

Table 3: Description of plasmids used in this study.

Plasmid	Description	Reference
pBK-CMV	Km^r, Neo^r, ColE1-Replicon, f1(-)origin, SV40ori	Stratagene (Basel, Schweiz)
pBluescript II SK(+/-)	Ap^r, ColE1 replicon	Stratagene (Basel, Schweiz)
pJBL1	Km^r, Neo^r, *pilAB* on a 5.4 kb chromosomal *Bam*HI-fragment of strain BH72 in pBK-CMV	Dörr et al. 1998
pJBL2	Km^r, Neo^r, *pilSR and pilAB* on a 6.5 kb chromosomal *Sau*3AI-fragment of strain BH72 in pBK-CMV	Hurek, unpublished
pJBLP1	Ap^r, *Eco*RI/*Sst*I-fragment of pJBL1 in EcoRI/SstI-site of pUC19	Dörr et al. 1998
pJBLP1*gfp*	Ap^r, *Eco*RI-fragment of pSKGFP in *Mfe*I-site of pJBLP1, *pilAB*::*gfp*-fusion	Böhm 2006
pJBLP14	Ap^r, 1.84 kb *Eco*RI-fragment of pSKGUS in *Mfe*I-site of pJBLP1, *pilAB*::*uidA*-fusion	Dörr 1998
pJBLP2	Ap^r, *Eco*RI-*Sst*I-fragment of pJBL2 in pUC19, containing pilSR	Dörr 1998
pJBLP21	Ap^r, 4.3 kb *Asp*718/*Xho*I-fragment of pJBLP2 in pSKGUS, *pilSR*::*uidA*-fusion	Dörr 1998
pSKGFP	Ap^r, 0.7 kb *gfp*-fragment in pBluescript SK(+/-)	Egener et al. 1998
pSKGUS	Ap^r, 1.84 kb *uidA*-fragment in pBluescript SK(+/-)	Egener 1999
pUC19	Ap^r, ColE1 origin	Yanisch-Perron et al. 1985
pLAFR3	Tc^r	Staskawicz et al. 1987
pJLA*pilA-uidA*	Tc^r, 3.5 kb *Pst*I/*Bgl*II-fragment of pJBLP14 in *Pst*I/*Bam*HI-site of pLAFR3, *pilAB*::*uidA*-fusion	Dörr 1998
pK18GGST	Km^r,derivative of the mobilizable cloning vector pK18mob2, promoterless *gfp* and *uidA*, T4 transcription terminator	Krause, unpublished
pK18GGST*azo3874*	Km^r, fragment of *azo3874* at *Hind*III/*Xba*I site in pK18GGST (14 – 470 bp of gene *azo3874*)	This study
pUC4K	Km^r, Neo^r, source of Km-cassette	GE Healthcare (Fairfield, CT, USA)
pRK2013	Km^r, RK2 *tra* gene cloned in ColE1 replicon	Figurski & Helinski 1979

II 4 Molecular weight marker and DNA size marker

The unstained protein Molecular Weight Marker with a size range of 14.4 kDa to 116.0 kDa (Fermentas, St. Leon-Rot, Germany) was used for protein gel electrophoresis. For agarose gel electrophoresis DNA of the lambda phage was digested with *Pst*I (Fermentas) and used as a base pair (bp) size marker with a range from 247 bp to 11501 bp.

II 5 Culture media and cultivation of microorganisms

All culture media were autoclaved at 121°C for 20 minutes and 2 bar. Non-autoclavable stock solutions were usually sterile filtered using filters with pore size 0.2 µm (Schleicher and Schuell, Dassel, Germany). For solid media usually 1.5 % to 1.7 % agar was added. In general, growth of bacterial cultures was monitored at optical density (OD) of 578 nm with an

Ultrospec 2000 UV-Visible spectrophotometer (formerly purchased from Amersham Pharmacia Biotech, Uppsala, Sweden).

II 5.1 Culture media

VM-Ethanol medium (Reinhold-Hurek et al. 1993a, modified), pH 6.8

KH_2PO_4	0.4	g/l
K_2HPO_4	0.6	g/l
NaCl	1.1	g/l
NH_4Cl	0.5	g/l
$MgSO_4$ x 7 H_2O	0.2	g/l
$CaCl_2$ x 2 H_2O	26.0	mg/l
$MnSO_4$ x 2 H_2O	10.0	mg/l
Na_2MoO_4 x 2 H_2O	2.0	mg/l
Fe(III)-EDTA	0.66	mg/l
Yeast extract	1.0	g/l
Bacto peptone	3.0	g/l
Ethanol	6.0	ml/l

VM-Malate medium

Equivalent to VM-Ethanol but ethanol was replaced by 5.0 g/l malic acid and 4.5 g/l KOH.

SM-Ethanol medium (Reinhold et al. 1986, modified), pH 6.8

KH_2PO_4	0.4	g/l
K_2HPO_4	0.6	g/l
NaCl	0.1	g/l
NH_4Cl	0.5	g/l
$MgSO_4$ x 7 H_2O	0.2	g/l
$CaCl_2$ x 2 H_2O	26.0	mg/l
$MnSO_4$ x 2 H_2O	10.0	mg/l
Na_2MoO_4 x 2 H_2O	2.0	mg/l
Fe(III)-EDTA	0.66	mg/l
Yeast extract	0.1	g/l
Ethanol	6.0	ml/l

SM-Malate medium

Equivalent to SM-Ethanol but ethanol was replaced by 5.0 g/l malic acid and 4.5 g/l KOH.

Selection medium, pH 6.8

NaCl	0.1	g/l
$MgSO_4$ x 7 H_2O	0.2	g/l
$CaCl_2$ x 2 H_2O	26.0	mg/l
$MnSO_4$ x 2 H_2O	10.0	mg/l
Na_2MoO_4 x 2 H_2O	2.0	mg/l
KNO_3	1.0	g/l
Fe(III)-EDTA	0.66	mg/l
Ethanol	6.0	ml/l

Conjugation medium, pH 6.8

KH_2PO_4	0.4	g/l
K_2HPO_4	0.6	g/l
NaCl	1.1	g/l
$MgSO_4$ x 7 H_2O	0.2	g/l
$CaCl_2$ x 2 H_2O	26.0	mg/l
$MnSO_4$ x 2 H_2O	10.0	mg/l
Na_2MoO_4 x 2 H_2O	2.0	mg/l
Fe(III)-EDTA	0.66	mg/l
Yeast extract	5.0	g/l
Malic acid	5.0	g/l
KOH	4.5	g/l

LB medium (Sambrook et al. 1989), pH 7.0

NaCl	10.0	g/l
Tryptone	10.0	g/l
Yeast extract	5.0	g/l

II 5.2 Antibiotics and other supplements

Selection of bacterial strains with antibiotic resistance was achieved by supplementing the media with the following final concentrations of sterile filtered (pore size 0.2 µm; Schleicher and Schuell, Dassel, Germany) antibiotics:

Kanamycin (Km)

Azoarcus sp.	30.0	µg/ml
E. coli	30.0	µg/ml

Ampicillin (Ap)

Azoarcus sp.	30.0	µg/ml
E. coli	150.0	µg/ml

Chloramphenicol (Chl)

Azoarcus sp.	12.5	µg/ml

Tetracyclin (Tc)

Azoarcus sp.	12.0	µg/ml

II 5.3 Cultivation of microorganisms

Escherichia coli liquid cultures were grown aerobically in LB medium at 30°C or 37°C with constant shaking at 180 – 200 rpm (HT; Infors AG, Bottmingen-Basel, Switzerland) until the desired optical density. For long term storage, *E. coli* strains were mixed in a ratio of 1:1 with 65 % glycerol/0.1 M $MgSO_4$/0.025 M Tris-HCl (tris(hydroxymethyl)aminomethane) (pH 8.0) and stored at –80°C in glass vials.

Liquid cultures of *Azoarcus* sp. were routinely grown aerobically at 37°C in VM-Ethanol medium with constant shaking at 180 – 200 rpm until the desired optical density. Early exponential growth was achieved at OD_{578nm} of 0.3, exponential growth included the optical densities OD_{578nm} from 0.4 to 0.6 and stationary growth of *Azoarcus* sp. starts at OD_{578nm} of 1.0. In general, only 1/10 of the Erlenmeyer flask was filled to assure sufficient oxygen aeration. For long term storage, the strains were kept in 10 % dimethylsulfoxid at -80°C or in a Dewar vessel in liquid nitrogen.

II 5.3.1 Growth under quorum sensing conditions

For collection of conditioned culture supernatants, containing the unknown quorum sensing signal molecule, cultures were grown in VM-Ethanol or SM-Ethanol medium with continuous shaking at 37°C or 30°C, respectively. After 24 h pre-cultures were diluted 1:6 with fresh medium and incubated for another 24 h. The optical density at 578 nm was measured and only supernatants from cultures that had an optical density above one were harvested by centrifugation with 12857 x g for 20 min at room temperature in 50 ml polyethylene tubes from Sarstedt (Sarstedt, Germany). This conditioned culture supernatant was always used directly after harvesting for growth of *Azoarcus* sp. under quorum sensing conditions and was not kept longer than few hours at room temperature under the clean bench.

The growth of *Azoarcus* sp. under QS conditions can be achieved by adding conditioned culture supernatants containing the unknown QS molecule to a culture in the early exponential growth phase with further incubation at 37°C. Therefore, the strain was routinely grown in VM-Ethanol medium until the early exponential growth phase ($OD_{578nm} \leq 0.3$), the culture was then 1:5 diluted with conditioned culture supernatant and was supplemented with 3 ml/l ethanol (abs.) to replace the depleted carbon source. For negative controls the bacterial culture was 1:5 diluted with fresh medium. The cultures were further incubated for one or four hours at 37°C rotary shaking, and then harvested under different conditions concerning the following experiment.

II 5.3.2 Quorum sensing bioassays

For studying the expression of QS target genes quorum sensing bioassays were carried out. Such bioassays involve the growth of *Azoarcus* sp. under QS conditions (see II 5.3.1) by means of incubation with conditioned culture supernatant in comparison to incubation with

respective medium as negative control. The gene expression was afterwards determined by measuring the β-glucuronidase activity (see II 6). Unless not stated differently, the production of conditioned culture supernatant was performed in 250 or 300 ml Erlenmeyer flasks with a total volume of 30 ml. The subsequent QS bioassays were carried out in 100 ml Erlenmeyer flasks with a final volume of 10 ml.

II 6 Determination of β-glucuronidase activity

To determine the β-glucuronidase activity 2 ml of bacterial culture was pelleted by centrifugation for 10 – 20 min at room temperature and 16100 x g, the supernatant was completely removed and cells were re-suspended in 2 ml of a buffer containing 60 mM Na_2HPO_4 and 40 mM NaH_2PO_4 (pH 7.0), 1 mM ethylenediaminetetraacetic acid (EDTA) and 14 mM β-mercaptoethanol. One millilitre of this suspension served as a control for optical density determination at OD_{600nm} and two aliquots of 500 µl were used for enzyme assays using a modified method of Jefferson et al. (1986). The cells were lysed by adding 50 µl of lysis buffer (5 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.05 % sodium dodecyl sulfate (SDS)) followed by an incubation for 10 min at 37°C. The enzyme reaction was started at 37°C by adding 100 µl 2 mM p-nitrophenyl β-D-glucuronide and after monitoring the colour change the reaction was stopped with 200 µl 2.5 M 2-amino-2-methylpropandiol. The absorbance of *p*-nitrophenol was measured at 420 nm and β-glucuronidase activity was calculated in Miller Units defined by:

$$\text{Miller Units} = \frac{A_{420nm} \times 1000}{t(\text{min}) \times OD_{600nm}}$$

Statistical analyses with a paired t-test were performed with GraphPad InStat version 3.01 (www.graphpad.com; San Diego, CA, USA).

II 7 Determination of fluorescence of the green fluorescent protein

The green fluorescent protein, encoded by the gene *gfp*, exhibits bright green fluorescence after exposed to blue light. With the help of a reporter strain, carrying a promoterless transcriptional fusion of the gene *gfp* to a target gene, the gene expression of this target gene can be studied under different growth conditions by detecting the green fluorescence of GFP with a fluorescence scanner (Typhoon 8600; GE Healthcare, Fairfield, CT, USA). Therefore,

the cultures were routinely grown at 37°C in VM-Ethanol medium with permanent shaking under aerobic conditions. The change of optical density was measured at 578 nm and 500 µl of the culture was transferred to 24 well plates. The green fluorescence of GFP was detected by scanning at 600 pmt with the filter 535WB35 at 535 nm. If enhancement of green fluorescence of GFP was necessary cultures were incubated for 30 min at 4°C prior to scanning. The relative fluorescence was determined by dividing the obtained fluorescence value by the respective OD_{578nm}.

II 8 Heterogeneity test and fluorescence microscopy

A test for heterogeneity in *Azoarcus* sp. was performed to address the question how single cells behave in a bacterial culture or within a quorum sensing activated community. For this experiment a reporter strain, carrying a transcriptional fusion of *gfp* to a target gene of *Azoarcus* sp., was grown in VM-Ethanol medium until different optical densities and under quorum sensing conditions. The heterogeneity of the *gfp* gene expression in those cultures was detected by fluorescence microscopy (HAL 100, Zeiss, Oberkochen, Germany). *Azoarcus* sp. cells were inspected on microscope glass slides in 40 x 1.6 fold magnification in phase contrast as well as fluorescence mode (long distance ocular Plan-NEOFLUAR; filter set 09, both Zeiss). Pictures were taken with a camera from Hamamatsu (color chilled 3CCD camera; Hamamatsu City, Japan) and for detection of fluorescent cells the pictures were merged with Photoshop CS3.

II 9 DNA techniques

All solutions and materials used for work with nucleic acids were autoclaved for 20 min at 121°C and 2 bar or baked for four hours at 180°C. Non-autoclavable stock solutions were usually sterile filtered using filters of pore size 0.2 µm (Schleicher and Schuell, Dassel, Germany). In general, DNA techniques were carried out according to Ausubel et al. 1987 with modified protocols.

II 9.1 Isolation of *E. coli* Plasmid DNA

The isolation of plasmid DNA was done as described in Birnboim & Doly (1979) and Ausubel et al. 1987 with following modifications. 3 ml over night culture from *E. coli* was harvested and re-suspended in 100 µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA,

supplemented with 1 mg/ml RNase A). Cell lysis was achieved by adding 200 µl 1 % SDS/200 mM NaOH and 5 min incubation on ice, followed by adding 150 µl 3 M potassium acetate (pH 5.2) and further incubation for 5 min on ice. Subsequently, cell debris, chromosomal DNA and proteins were removed by centrifugation (16100 x g, 10 min at room temperature) and the plasmid DNA in the aqueous supernatant was precipitated by adding the same volume of isopropanol. The DNA was pelleted by centrifugation and afterwards salts were removed with 70 % ethanol. The plasmid DNA was dissolved in TE and stored at -20°C until further processing.

II 9.2 Isolation of *Azoarcus* sp. chromosomal DNA

For isolation of chromosomal DNA 2 ml over night culture from *Azoarcus* sp. was harvested and re-suspended in 150 µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA,) and 300 µl extraction buffer (200 mM Tris-HCl, pH 7.0, 250 mM NaCl, 25 mM EDTA, 0.5 % SDS) supplemented with 1 mg/ml RNase A. This mixture was incubated for 30 min at 37°C, followed by proteinase K (Promega, Mannheim, Germany) digestion at 55°C with a final concentration of 50 µg/ml for at least one hour. Subsequently, the chromosomal DNA was extracted twice with the same volume of phenol-chloroform-isoamylalcohol (25:24:1, pH 8.0) followed by extraction with TE buffered chloroform. Finally, the DNA was precipitated with 1/10 potassium acetate (pH 5.2) and 2 volumes ethanol abs. at -20°C for 30 min and subsequent centrifugation at 4°C. Salts were removed with 70 % ethanol and the pellet was dissolved in TE and stored at -20°C until further processing.

II 9.3 Estimation of DNA concentration

The concentration of isolated DNA was determined by measuring its absorption at 260 nm with an Ultrospec 2000 UV-Visible spectrophotometer (formerly purchased from Amersham Pharmacia Biotech, Uppsala, Sweden). The DNA concentration was then calculated using the empirical formula for double stranded DNA:

$$\text{DNA in } \mu g/ml = \Delta E_{260nm} \times 50 \mu g/ml \times \text{dilution factor}$$

II 9.4 Cleavage of DNA with restriction endonucleases

The enzymes for restriction digestion of chromosomal DNA or plasmid DNA were purchased from Fermentas (St. Leon-Rot, Germany) and the reactions were performed according to manufacturer's instructions for three hours or over night with 1 - 5 µg DNA.

II 9.5 Agarose gel electrophoresis to separate DNA

The method of agarose gel electrophoresis was routinely used to separate DNA molecules by size. Briefly, separation was usually achieved in 0.8 - 1 % agarose gels in TAE buffer (40 mM Tris-Acetate, pH 8.0, 4 mM EDTA) with 3 – 5 V/cm. Prior to electrophoresis, the samples were mixed with 1/10 loading dye (20 % Ficoll 400, 0.1 % Bromphenolblue, 0.1 % Xylencyanol 0.1 % Orange G). Afterwards ethidiumbromide (0.5 µg/ml) was used for staining of nucleic acids inside the gel and the bands were detected under UV-light at 312 nm with the Image Master VDS camera (GE Healthcare, Fairfield, CT, USA).

II 9.6 Cloning procedures

The plasmid pK18GGST was used as a cloning vector in this study. The application of this plasmid offers the possibility to generate insertional mutations by homologous recombination in a gene of interest of *Azoarcus*. Moreover, the gene of interest is fused transcriptionally to the promoterless reporter genes *gfp* and *uidA*. This easy and convenient cloning strategy allows subsequent expression studies with the reporter genes *gfp* and *uidA*, coding for the green fluorescent protein or β-glucuronidase, in the respective assays (see II 6 and II 7).

II 9.6.1 Amplification of DNA by polymerase chain reaction

For amplification of DNA fragments for cloning procedures standard protocols for polymerase chain reaction (PCR) were routinely used. Briefly, a 50 µl reaction volume containing 100 – 300 ng DNA, 1.25 units of MolTaq polymerase (Molzym, Bremen, Germany), PCR buffer with 1.5 mM $MgCl_2$, 50 µM each dNTP (dATP, dCTP dGTP, and dTTP), 50 pmole forward and 50 pmole reverse primer was used. The standard programme for the amplification of DNA fragments was as follows: Initial denaturation for 10 min at 95°C linked to a loop of 40 cycles each having a denaturation step for 1 min at 95°C, 1 min at the specific annealing temperature of the used primers followed by an elongation step of

1 min at 72°C. After final elongation at 72°C for 5 min the PCR reaction was kept at 4°C to 8°C until further processing.

The annealing temperature was usually around 3°C higher than the melting temperature of the specific primers or the temperature was calculated with the standard formula where n refers to the number of bases in the primer (Sambrook et al. 1989):

$$\text{Annealing temperature} = 81.3 + (0.41 \times \%\ \text{GC content}) - (600/n) - 13.6$$

In general, the PCR products were verified by agarose gel electrophoresis and purified from the gel with the help of the NucleoSpin Extraction Kit II (Macherey & Nagel, Düren, Germany).

II 9.6.2 Construction of recombinant plasmids

For usual cloning procedures, the vector and the DNA fragment were digested with restriction endonucleases to yield compatible ends for ligation. Basically, a ratio of 4:1 of DNA fragment to cloning vector was used for the ligation set-up in a 20 µl reaction volume containing 5 units of T4 DNA ligase in standard buffer (both Fermentas, St. Leon-Rot, Germany). The ligation was carried out for two hours at room temperature or over night at 14 - 16 °C.

II 9.6.3 Transfer of plasmid DNA into *Escherichia coli*

With the help of $CaCl_2$ chemical competent cells it is possible to transfer DNA for cloning purposes via heat shock transformation into *E. coli*. The method, described by Kushner (1987) was applied to generate competent *E. coli* DH5aF' or S17-1 cells. Competent cells were stored at -80°C. Those cells were incubated with plasmid DNA (see II 9.6.2) for 10 min on ice, followed by a heat shock at 42°C for 2 min. The cells were immediately mixed with 1 ml LB medium, incubated for 45 min at 37°C with constant shaking and afterwards plated on LB agar containing proper antibiotics.

II 9.6.4 Conjugative transfer of plasmids into *Azoarcus* sp.

Conjugation of plasmids into *Azoarcus* sp. can be achieved in a biparental manner with the *E. coli* strain S17-1 that chromosomally carries the *tra* genes necessary for the transfer process. The *E. coli* donor strain, bearing the transferable plasmid pK18GGST and the recipient *Azoarcus* sp. BH72 were grown separately in respective medium with proper antibiotics over night. The cultures were pelleted by centrifugation (3220 x g, 15 min at room

temperature) re-suspended in SM medium without C-source or LB medium, adjusted to OD_{578nm} 1.0 and mixed in the ratio 1:3. This mixture was again centrifuged and re-suspended in 100 µl of SM medium followed by incubation on conjugation agar at 37°C over night. Finally, cells were scraped off the plate on the next day with SM medium. Afterwards the cells were plated in serial dilutions on VM-Ethanol agar with kanamycin and chloramphenicol. After two to four days the transconjugants were transferred to fresh VM-Ethanol plates (with kanamycin) and streaked out until pure single colonies of the respective strain were obtained.

II 9.7 DNA hybridization to test the correct plasmid integration

II 9.7.1 Preparation of the *gfp*-hybridization probe

For preparation of the *gfp*-probe, 60 ng plasmid DNA (pK18GGST) was used to amplify the *gfp*-fragment with PCR. Afterwards the PCR-fragment was denatured, mixed with 4 µl DIG-High Prime labelling mix (Roche, Basel, Switzerland) and incubated at 37°C over night. The reaction was stopped by heat inactivation at 65°C for 10 min and the probe was purified with the NucleoSpin Extraction Kit II (Macherey & Nagel, Düren, Germany). 5 µl aliquots of the digoxigenin-labelled probe and the unlabelled PCR-fragment were loaded on an agarose gel to check whether the labelling procedure was achieved. The successful incorporation of digoxigenin led to a shift in the fragment size from 535 bp to around 700 bp.

II 9.7.2 Transfer of DNA to a membrane

In general, DNA fragments were separated by agarose gel electrophoresis and transferred to a membrane by the help of capillary force according to Southern (1975). Briefly, the genomic DNA of *Azoarcus* sp., digested with the respective restriction endonucleases was separated on an agarose gel and later depurinated for 15 min in 0.78 % HCl, followed by two denaturation steps for 20 min in 1.5 M NaCl/0.5 M NaOH. Afterwards the DNA strands in the gel were neutralized twice for 20 min each in 0.5 M Tris-HCl/1.5 M NaCl/1 mM EDTA (pH 7.0); all steps were carried out at room temperature with constant shaking. Finally, the gel was placed below a nylon membrane (Hybond N; GE Healthcare, Fairfield, CT, USA) for transfer with high salt buffer 20 x SSC (3 M NaCl, 0.3 M Na_3Citrate) over night. After transfer, the DNA was covalently bound to the membrane by UV cross linking with 0.4 J/cm^2 at 312 nm.

II 9.7.3 DNA-DNA hybridization

Subsequently, the membrane was incubated for at least one hour in hybridization solution (6 x SSC, 5 x Denhardt's solution (100 x 2 % bovine serum albumin, 2 % ficoll, 2 % polyvinylpyrollidone), 0.5 % SDS) at 65°C, followed by hybridization with the digoxigenin-labelled probe in hybridization solution) over night at the same temperature. Prior to hybridization the probe was boiled for 15 min.

II 9.7.4 Probe detection

After hybridization the membrane was serially washed for 10 min in 20 x SSC, for 20 min in 2 x SSC/0.1 % SDS and for another 10 min in 0.1 x SSC/0.1 % SDS. All washing steps were carried out at 65°C. The digoxigenin-labelled probe, hybridized to DNA, was detected by a specific antibody against digoxygenin epitopes. Therefore the membrane was incubated for 30 min in blocking solution (0.1 M maleic acid, 0.15 M NaCl, 3 % milk powder, pH 7.5), followed by a 30 min incubation with the Anti-Digoxygenin-AP (1:25000 diluted), Fab fragments (Roche, Basel, Switzerland). The detection by chemiluminescence was achieved by using CDP-Star according to manufacturer's instructions (Roche) and CL Xposure films from Pierce (purchased from Thermo Scientific, Rockford, IL, USA) with all subsequent developing and fixing steps (Kodak developer: P7042, Kodak fixer: P7167 purchased from Sigma Aldrich, Seelze, Germany) of the film.

II 10 Proteome studies

II 10.1 Two-dimensional gel electrophoresis

II 10.1.1 Protein extraction

Azoarcus sp. BH72 was grown in 500 ml VM-Ethanol medium until the desired OD_{578nm} (see II 5.3) or under QS conditions (see II 5.3.1). Therefore, 100 ml of an early exponential culture were cultivated with 400 ml conditioned supernatant and further incubated for four hours. Afterwards, the cells were harvested by centrifugation for 15 min at 6300 x g and 4°C. Cell pellets were suspended in PBS (14 mM NaCl, 0.27 mM KCl, 1.65 mM Na_2HPO_4 and 0.15 mM KH_2PO_4, pH 7.0), centrifuged again and afterwards stored at -80°C until protein extraction.

The collected cells were re-suspended in extraction buffer (0.7 M sucrose, 0.5 M Tris-HCl, 30 mM HCl, 0.1 M KCl, 2 % β-mercaptoethanol, 1 mM phenylmethylsulfonylfluorid, pH 9.6). After cell disruption at 4°C by sonication (4 x 45 s with 50 W output and 1 min breaks on ice with the Branson sonifier 250; Branson, Danbury, CT, USA) and removal of cell debris by centrifugation (16220 x g, 5 min, 4°C), the proteins were extracted with phenol for 30 min on ice (pH 8.0). This step was followed by centrifugation (see above) and precipitation with five volumes of 0.1 M ammonium acetate in methanol over night at -20°C. The pellets were suspended once with precipitation solution, centrifuged again and afterwards dissolved in 2D-protein sample solution (5 M urea, 2 M thiourea, 1 % dithiothreitol (DTT), 2 % 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 2 % Sulfobetaine 3-10, 0.8 % BioLyte from Bio-Rad, Munich, Germany) and stored at -80°C until further processing. The protein concentration was determined with the protein RC DC protein assay kit from Bio-Rad.

II 10.1.2 Isoelectric focussing

The isoelectric focussing (IEF) was performed with 500 µg protein extract in 3.5 % acryl-bisacrylmide (30:1) gels using the Model 175 Tube Cell (Bio-Rad, Munich, Germany). The gels also contained 5.8 % ampholytes (Serva, Heidelberg, Germany) in a ratio of 2:1:1 of pH range 3.5 – 10, 4 – 6 and 5 – 8, 9 M urea, 2 % CHAPS, 0.3 % N,N,N',N'-tetramethylethylenediamine and 0.05 % ammoniumpersulfate (APS). Briefly, the protein solution was loaded on the IEF gels in glass tubes, covered with 20 µl 2D-protein sample solution (II 10.1.1) and 15 µl overlay solution (6 M urea, 0.4 % ampholytes (Serva) pH 3.5 – 10, 0.1 M DTT, 2 % CHAPS). The isoelectric focussing was performed for 10 min at 100 V, 10 min at 200 V, 2 h at 300 V and over night at 400 V with 20 mM NaOH as cathode solution and 10 mM phosphoric acid as anode solution. Prior to isoelectric focussing gels were pre focussed for 15 min at 200 V, 30 min at 300 V and 1 h at 400 V. After the run, the IEF gels were kept inside the glass tubes at -20°C until further processing.

II 10.1.3 SDS-Polyacrylamidgelelectrophoresis

The second dimension was performed on 12.5 % polyacrylamide gels in the Scie-Plas TV 400Y vertical electrophoresis system (Biostep, Jahnsdorf, Germany). The IEF gels were extruded from the glass tubes and equilibrated for 30 min in 60 mM Tris-HCl (pH 6.8), 1 % SDS, 20 % glycerol, 50 mM DTT and traces of bromphenolblue. Afterwards the gels

were loaded on the polyacrylamide gel with a composition previously described by Laemmli (1970).

II 10.1.4 Protein Staining

The proteins in the gels were stained with conventional Coomassie Brilliant Blue R 250 (45 % ethanol, 10 % acetic acid, 0.25 % Coomassie R-250) for 30 min at 37°C and afterwards destained in 30 % ethanol/10 % acetic acid until the background was colourless. The staining method with Colloidal Coomassie Brilliant Blue G 250 according to Candiano et al. 2004 with the exception that ethanol was used instead of methanol was also used. In general, gels were stored in 3 - 10 % glycerol at 4°C until excision of protein spots.

II 10.2 Analyses of protein gels with Image Master 2D

After staining procedures the protein gels were scanned (PowerLook III; Umax Data Systems Inc., Taipei, Taiwan), the images were saved as tagged image file format and analyzed with Image Master 2D v4.01 (GE Healthcare, Fairfield, CT, USA). Basically, all spots were detected, matched to the accordant spots in the parallel protein gels and their spot intensity volumes were calculated. After background correction with the lowest on boundary mode, total spot normalization and molecular weight calibration the intensity volumes of the distinct protein spots were compared between the conditions. Only those protein spots that showed at least 2.5-fold change in spot intensity between the tested conditions were considered to be differentially synthesized.

II 10.3 Protein identification by mass spectrometry

The proteins obtained by two-dimensional gel electrophoresis were identified by matrix assisted laser desorption/ionization time-of-flight (tandem) mass spectrometry whereas the proteins from the gel-free approaches were identified by liquid chromatography coupled to tandem mass spectrometry (MS/MS).

II 10.3.1 MALDI-TOF-MS with the Voyager-DE Pro Biospectrometry Workstation

Several proteins were identified by MALDI-TOF-MS analyses at „Fraunhofer-Institut für Fertigungstechnik und Angewandte Materialforschung“ (IFAM) in Bremen. Therefore, the protein spots were excised manually from the protein gels, transferred to LoBind tubes (Eppendorf, Hamburg, Germany) and washed three times for 15 min at room temperature

each in 25 mM bicarbonate/50 % acetonitrile to remove the Coomassie dye. After two steps of dehydration with acetonitrile and rehydration with 50 mM bicarbonate at room temperature the gel pieces were dried in a vacuum concentrator. Afterwards the proteins in the gels were reduced and alkylated with 10 mM DTT for one hour at 56°C and with 55 mM iodacetamide for 45 min at room temperature, respectively. The tryptic protein digest was carried out over night at 37°C in a solution of 25 mM bicarbonate containing trypsin (5 ng/µl; Roche, Basel, Switzerland). Peptide extraction was performed in 30 % acetonitrile/0.1 % trifluor acetic acid for 15 min in a ultrasonic water bath with maximum output (Transsonic T 420; Bender & Hobein, Singen, Germany) followed by a comparable step in 50 % acetronitrile/0.1 % trifluor acetic acid. The peptide extracts were dried in a vacuum concentrator, suspended in 10 µl trifluor acetic acid (0.1 %) and used for MALDI-TOF-MS analyses.

The MALDI-TOF-MS measurements were carried out with an α-cyano-4-hydroxycinnamic acid-matrix on a Voyager-DE Pro Biospectrometry Workstation (Applied Biosystems, Foster City, CA, USA). The peptides were mixed with an equal volume of matrix solution (50 % acetonitrile, 30 % isopropanol, 20 % formic acid), 0.5 µl of this mix was spotted on a 96 x 2 teflon coated target (Applied Biosystems) and air dried. The spectra were recorded in reflector mode in a mass range from 500 to 5000 Da with an acceleration voltage of 20 kV and 500 shots per spectrum (accumulation of three spectra per spot). External calibration was performed with the peptide calibration mix 2 (Applied Biosystems) containing distinct monoisotopic reference peptides. If the spectra were not satisfying $ZipTip_{C18}$ pipette tips (Millipore Corporation, Billerica, MA, USA) were used according to manufacturer's data to concentrate and purify peptides.

Database searches of MALDI-TOF mass spectra were done by using the Mascot search engine on http://www.matrixscience.com (Mascot Wizard) with the following settings: database - NCBInr, taxonomy - other proteobacteria, enzyme - trypsin with allowance of one missed cleavage, variable modification - oxidation M, peptide tolerance - 1.2 Da. Proteins with a Mowse score of at least 49 (Protein Score C.I. % ≥ 99.5 %) from *Azoarcus* sp. BH72 were regarded as positive identification.

II 10.3.2 MALDI-TOF-MS/MS with the 4700/4800-Proteomics-Analyzer

For the identification of proteins by MALDI-TOF-MS/MS at the University of Greifswald (Dr. Christian Scharf) the spots were excised manually from the protein gels and kept at

-80°C until MALDI-TOF measurements were performed. The proteins in the gel pieces were in gel digested with trypsin and the peptide solution was automatically spotted onto the MALDI-targets in an Ettan Spot Handling Workstation (GE Healthcare, Fairfield, CT, USA) using a modified standard protocol. Briefly, protein spots were incubated for digestion at 37°C for 120 min in a solution of 20 mM bicarbonate containing trypsin (4 ng/µl; Promega, Madison, WA, USA).

The MALDI-TOF-MS measurements were carried out with an α-cyano-4-hydroxycinnamic acid-matrix on a 4700- or 4800-Proteomics-Analyzer (Applied Biosystems, Foster City, CA, USA) and the spectra were recorded in reflector mode in a mass range from 900 to 4000 Da with a focus mass of 2000 Da. If the autolytical fragments of trypsin with the mono-isotopic $(M+H)^+$ m/z at 1045.5 or 2211.1 reached a signal to noise ratio (S/N) of at least 10, an internal calibration was automatically performed using these peaks for an one-point-calibration or a two-point calibration. A manual calibration was performed if the automatic calibration failed.

Database searches of MALDI-TOF mass spectra were performed with the *Azoarcus* database (azoarcus_pep_050621.fasta) using the Mascot search engine (Version 2.0, Matrix Science Ltd.) with 50 ppm precursor tolerance, 0.45 Da MS/MS fragment tolerance and Oxidation (M) and Carbamidomethylation (C) as variable modifications. Proteins with a Mowse score of at least 49 (Protein Score C.I. % ≥ 99.5 %) and a sequence coverage of at least 30 % were regarded as positive identification. Tandem mass spectrometry analysis was particularly useful for the identification of spots containing more than one component.

II 10.3.3 Protein identification by LC-MS/MS

For the gel-free approach, *Azoarcus* wild type or the mutant BH*pilR*K were respectively grown in 500 ml VM-Ethanol medium until the exponential growth phase. For each strain twenty optical density units of cells from three independent cultures and two technical replicates were harvested as follows: The desired volume of bacterial culture was mixed with half its volume of killing buffer (20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 20 mM NaN_3, frozen at -20°C) in centrifugation tubes and centrifuged at 4°C and 6300 x g for 10 min. The pellets were suspended in killing buffer, centrifuged again and cells were immediately frozen in liquid nitrogen and afterwards stored at -80°C until further processing.

For identification of proteins by LC-MS/MS at the University of Greifswald (Dr. Frank Schmidt) cell pellets were resuspended in 300 µl UT solution (8 M urea, 2 M thiourea). The

cells were disrupted by ultrasonication. Protein concentration was determined with the Coomassie Bradford assay kit (Pierce, Rockford, IL, USA). 10 µg proteins of each sample (in UT solution) were adjusted to a final volume of 1.3 µl using UT solution and further diluted 1:10 with 50 mM bicarbonate. For tryptic digestion trypsin (Promega, Madison, WA, USA) was added in a ratio 1:25 and samples were incubated for 15 h at 37°C. ZipTip$_{C18}$ pipette tips (Millipore Corporation, Billerica, MA, USA) were used to concentrate and purify peptides prior to LC-MS/MS analyses according to manufacturer's data. Samples were concentrated by vacuum centrifugation and 0.3 µg of peptide mixture was injected to the LC-MS/MS column.

Prior to mass spectrometry analysis samples were fractionated using a nanoAcquity Ultra Performance Liquid Chromatography (UPLC, Waters, Eschborn, Germany) equipped with a C18 nanoAcquity column (100 µm × 100 mm, 1.7 µm particle size). Separation was achieved in a non-linear gradient within 300 min using 2 % acetonitrile in 0.05 % acetic acid in water (A) and 0.05 % acetic acid in 90 % acetonitrile (B) as eluents with a flow rate of 400 nL/min. Mass spectrometry data were generated by a LTQ-FT-ICR mass spectrometer (linear quadrupole ion trap fourier transform ion cyclotron resonance, Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with a nanoelectrospray ion source (PicoTip Emitter FS360-20-20-CE-20-C12, New Objective, Woburn, MA, USA). After a first survey scan in the FT-ICR (r = 50,000) tandem mass spectrometry data (MS2) were recorded for the five highest mass peaks in the linear ion trap at a collision induced energy of 35 %. The exclusion time was set to 90 sec and the minimal signal for MS2 was 1000.

Analysis of mass spectrometry data were performed using Rosetta Elucidator (http://www.rosettabio.com/products/elucidator/default.htm, Rosetta Biosoftware, Seattle, WA, USA). The frame and feature annotation was done using the following parameters: retention time minimum cut-off 40 min, retention time maximum cut-off 270 min, m/z minimum cut-off 300 and maximum 1600. An intensity threshold of 1000 counts, an instrument mass accuracy of 5 ppm and an alignment search distance of 10 min were applied. For quantitative analysis, the data were normalized. For identification, a subset of 3989 *Azoarcus* sp. BH72 FASTA sequence entries from NCBI (date 20.7.09) was used (Sorcerer version 3.5, Sage-N Research, Inc.). Mass spectrometry spectra were searched with precursor ion tolerance of 10 ppm and a fragment ion mass tolerance of 1.0 Dalton. Oxidation of methionine was specified as variable modification. Peptide identifications were accepted if they exceeded a peptide teller score of 0.95.

II 11 Transcriptome studies

The transcriptome studies presented in this work include oligonucleotide microarray and real-time PCR experiments. All microarray protocols used in this study were performed according to the "Oligonucleotide Microarray Manual: Bacteria V 1.5 October 2006" (CeBiTec, Bielefeld University) and to Becker et al. 2004, Brune et al. 2006 and Serrania et al. 2008 with modifications for the *Azoarcus* sp. BH72 oligonucleotide array.

While working with RNA special care was taken, all solutions and buffers were treated with 0.1 % diethylpyrocarbonate (DEPC) and stirred over night at 37°C. Moreover, all solutions and materials were autoclaved for 40 min at 121°C at 2 bar or baked at 180°C for four hours.

II 11.1 RNA isolation (Hot phenol procedure)

Azoarcus sp. BH72 was grown in VM-Ethanol medium until the early exponential growth phase and under quorum sensing conditions for one and four hours (see also III 7). The cells from two to three independent cultures were harvested quickly by centrifugation at room temperature and 6800 x g for 5 min, pellets were suspended in PBS (14 mM NaCl, 0.27 mM KCl, 1.65 mM Na_2HPO_4 and 0.15 mM KH_2PO_4, pH 7.0), centrifuged again and stored at -80°C until RNA isolation.

The cell pellets were re-suspended in a 1:1 mixture of pre-warmed phenol-chloroform-isoamylalcohol (25:24:1, pH 4.7) and NAES solution (50 mM sodium acetate, 10 mM EDTA, 1 % SDS, pH 5.1). This suspension was incubated for 5 min at 65°C in a water bath followed by 10 min incubation on ice. For phase separation the mixture was centrifuged for 10 min at 12°C and 8000 x g. The upper phase was transferred and mixed with the same volume of phenol-chloroform-isoamylalcohol. This phenol extraction was repeated three times (centrifugation see above) followed by an extraction with equal volume of chloroform-isoamylalcohol (24:1). RNA precipitation was performed with the same volume of isopropanol for 45 min on ice. The RNA was pelleted by centrifugation (12900 x g, 10 min at 4°C) and salts were removed with 1 ml of Ethanol (70 %). The dried RNA was dissolved in 100 µl 1 x RNAsecure (Applied Biosystems, Foster City, CA, USA), frozen in liquid nitrogen and kept at -80°C until further processing.

II 11.2 DNase I Treatment

Contaminating DNA was removed from RNA preparations by DNase I using Qiagen columns (RNeasy Mini Kit, Qiagen, Hilden, Germany) according to manufacturer's instructions. Briefly, 100 µl total RNA solution was mixed with 350 µl buffer RLT and 250 µl ethanol (98 %) and applied to the silica-based spin columns. After centrifugation (13000 x g, 30 sec, room temperature) 350 µl buffer RW1 was added to the column and again centrifuged as described before. A solution of 10 µl DNase I and 70 µl buffer RDD (RNase free DNase Set, Qiagen, Hilden, Germany) was applied to each column and they were incubated at room temperature for 15 min. 350 µl buffer RW1 was added to the column and after centrifugation (13000 x g, 30 sec, room temperature) washing procedures were carried out twice with 500 µl buffer RPE. The column was incubated for 1 min with 50 µl RNase free water for elution of RNA. The purified RNA was collected by centrifugation (13000 x g, 1 min, room temperature) and afterwards supplemented with 2 µl 25x RNAsecure (Applied Biosystems, Foster City, CA, USA), frozen in liquid nitrogen and kept at -80°C until further processing.

II 11.3 Determination of RNA concentration

The concentration of the isolated RNA was measured in 1 µl sample with NanoDrop ND-1000 UV/Vis Spectrophotometer (Peqlab, Erlangen, Germany). The RNA concentration was calculated by using the empirical formula:

$$\text{RNA in } \mu g/ml = \Delta E_{260nm} \times 40 \mu g/ml \times \text{dilution factor}$$

II 11.4 Reverse transcription of total RNA to yield aminoallyl-labelled first strand cDNA for microarray experiments

For reverse transcription, 20 µg total RNA was mixed with 2 µl amino modified random hexamers (5'MMT-NNNNNN-3', 5 µg/µl) purchased from Eurofins MWG GmbH (Ebersberg, Germany), filled up to 20.8 µl with RNase free water and incubated for 10 min at 70°C. Afterwards the primer annealing took place on ice for 5 min and was followed by reverse transcription (RT) at 42°C for 90 min with the below-mentioned mixture. The reverse transcriptase and the 5 x Reaction buffer were purchased from Bioline (Luckenwalde, Germany), the RNase Inhibitor from Invitrogen (Karlsruhe, Germany). The 25 x stock of dNTPs was a 4:1 mixture of aa-dUTP/dTTP with a final concentration of 12.5 mM each for

dATP, dCTP and dGTP, 2.5 mM for dTTP (Peqlab, Erlangen, Germany) and 10 mM for aminoallyl(aa)-dUTP (Fermentas, St. Leon-Rot, Germany).

Reverse transcription mixture		
RNAprimer-mix	20.8	µl
5 x Reaction buffer	6.0	µl
RNase inhibitor (40 U/µl)	0.5	µl
BioScript RT (200 U/µl)	1.5	µl
25 x dNTPs	1.2	µl

For hydrolysis of RNA 15 µl 0.2 M NaOH (Applichem, Darmstadt, Germany) was added to the mixture after reverse transcription, mixed by flicking and incubated for 10 min at 70°C. Shortly after neutralization of the sample with 15 µl of 0.2 M HCl (Applichem) the cleaning up of aminoallyl-labelled first strand cDNA with the CyScribe GFX Purification Kit (GE Healthcare, Fairfield, CT, USA) was performed with minor differences to the manufacturer's instructions. Instead of the washing buffer provided with the kit 80 % ethanol was used for all washing steps and for elution of the cDNA 60 µl 0.1 M sodium bicarbonate was used. The aminoallyl-labelled first strand cDNA was kept on ice until coupling of fluorescent dyes.

II 11.5 Coupling of fluorescent dyes to aminoallyl-labelled first strand cDNA

The monoreactive Cy5-NHS and Cy3-NHS esters were purchased from GE Healthcare (Fairfield, CT, USA), dissolved in 10 µl dimethylsulfoxid and divided in 1.5 µl aliquots. Those aliquots were dried in a vacuum concentrator, sealed in plastic bags with desiccation packs and stored until coupling to aminoallyl-labelled first strand cDNA.

For coupling of fluorescent dyes to the aminoallyl-labelled first strand cDNA aliquots of Cy3-NHS or Cy5-NHS esters were dissolved in 60 µl of first strand cDNA, mixed and incubated for 90 min at room temperature in the dark. Blocking of all remaining dyes (quenching) was necessary after labelling. This quenching was achieved by adding 4.5 µl hydroxylamine (4 M) to the sample solution followed by an incubation for 15 min at room temperature. Subsequent cleaning up of labelled cDNA from remaining dyes was performed with the CyScribe GFX Purification Kit (GE Healthcare) according to manufacturer's instructions. The Cy5- and Cy3-labelled cDNA that was used in one

hybridization experiment were cleaned up together. The labelled cDNA (60 µl) was stored at -20°C until microarray hybridization.

II 11.5.1 Checking of fluorescently labelled cDNA

An aliquot of the Cy3- and Cy5-labelled cDNA mix from the two conditions was loaded on a 1 % agarose gel (see II 9.5) to visually inspect whether the two dyes were incorporated equally into the samples. The gel electrophoresis was performed in TAE running buffer for 20 min at 80 V. Afterwards the gel was scanned (Typhoon 8600; GE Healthcare, Fairfield, CT, USA) at 800 pmt and medium sensitivity with the filters for Cy3 (green 532nm laser, 555 BP20) and Cy5 (red 633nm laser, 670 BP30) detection.

II 11.6 Oligonucleotide Microarray

II 11.6.1 Array layout

The bacterial 70mer oligonucleotide microarray was developed by the transcriptomic facility CeBiTec (Bielefeld University) under the guidance of Prof. Dr. Anke Becker.

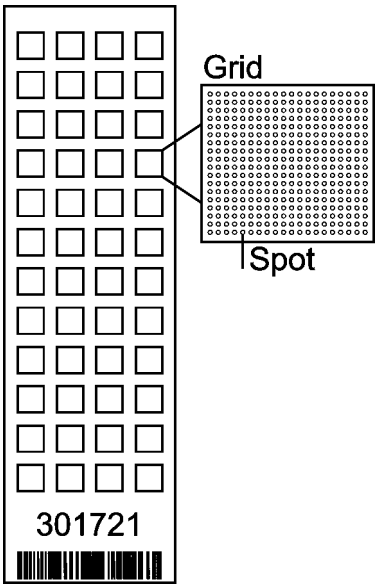

Figure 5: Oligonucleotide microarray layout, the array on the slide is shown with a close-up of the spot organization per grid. The array consists of 17280 single spots, spotted in rows of 20 x 18 in one grid which are arranged in 4 x 12 grids. Microarrays are indicated with slide numbers and barcode.

For the construction of the oligonucleotide microarray a collection of gene-specific oligonucleotide probes (Eurofins MWG GmbH; Ebersberg, Germany) for the 3992 predicted protein-coding genes were spotted on glass slides. Microarrays were visually inspected for irregular grid patterns and missing of groups of spots and one to two slides per printing series

were tested by hybridization to fluorescently labelled random nonamers to verify the printing and post-processing process. Each oligonucleotide (40 µM) was spotted in 1.5 M betaine/450 mM sodium chloride/45 mM sodium citrate (pH 7.0) in quadruplicates within one grid onto Nexterion E epoxy slides (Schott, Jena, Germany). Moreover, five internal controls were spotted in replicates (*azo1072*, *azo1081*, *azo2104*, *azo2837* and *azo2898*) and negative buffer controls were also included. The array layout is depicted in Figure 5, the array consists of 17280 single spots, spotted in rows of 20 x 18 in one grid which are arranged in 4 x 12 grids.

II 11.6.2 Nexterion E slide processing

Processing of slides is necessary to block free epoxy groups prior to hybridization. This processing is carried out in plastic slide holders and glass tanks on magnetic stirrers by transferring the slide holder from one solution to the next. Washing steps were performed at room temperature for 5 min in rinsing solution 1 (500 µl triton X-100 in 500 ml distilled water, dissolved at 80°C for 5 min and cooled to room temperature) and 2 min in rinsing solution 2 (100 µl 32 % HCl in 1 liter distilled water). Again washing was done for 2 min in rinsing solution 2, 10 min in rinsing solution 3 (50 ml 1 M KCl in 450 ml distilled water) followed by a rinsing step with distilled water. The glass slides were incubated for 15 min at 50°C in pre-warmed blocking solution (15 ml distilled water, 4.7 µl 32 % HCl, 5 ml Nexterion Block E solution (Schott, Jena, Germany)) with constant rotating. Afterwards the slides were washed with water and dried by centrifugation (7 min, 650 x g, room temperature). The processed slides were directly used for hybridization experiments.

II 11.6.3 Hybridization of labelled cDNA to oligonucleotide microarrays

The combined Cy3/Cy5-labelled targets were dried in a vacuum concentrator dissolved in 60 µl DIG Easy hybridization solution (Roche, Mannheim, Germany) and 1 µl of sonicated salmon sperm (5 µg/µl) was added. Prior to hybridization the targets were denatured at 65°C for 10 min. The hybridization of fluorescently labelled cDNA targets to oligonucleotide microarrays was performed at 42°C in a hybridization chamber (ArrayIt, Sunnyvale, CA, USA) in a water bath for 14 to 18 hours. It was crucial to apply stringent conditions throughout all preparative steps. Therefore, the chamber as well as the cover slip had to be pre-heated to 42°C before the target was applied.

II 11.6.4 Slide washing and scanning

After hybridization, microarray slides were washed once in 2 x SSC/0.2 % SDS for 5 min at 42°C, twice in 0.2 x SSC/0.1 % SDS for 1 min each at room temperature, twice in 0.2 x SSC for 1 min each at room temperature and for 1 min on 0.05 x SSC at 21°C. All washing steps were carried out in plastic slide holders and glass tanks. The glass slides were dried by centrifugation (7 min, 650 x g, room temperature) and the Cy3- and Cy5-fluorescence was scanned at 532 nm and 635 nm with the GenePix Scanner 4000A (Molecular Devices, Sunnyvale, CA, USA) with a pixel size of 10 µm.

II 11.7 Analyses of microarray data

With the help of microarray techniques it is possible to study gene expression in a large scale, but the enormous amount of data requires thorough analyses of data with specialized softwares. In this study image analysis was performed with the GenePix 4.1 program and the subsequent analyses were carried out with the open-source software Microarray Data Analysis System (MIDAS v2.19) of TM4 (Saeed et al. 2003, http://www.tm4.org/).

II 11.7.1 Image acquisition and spot intensity determination with GenePix

After scanning, raw data images of the slides that contain information about gene expression levels were obtained by the GenePix software. These images were analyzed by identifying each spot on the array (with the help of an array layout template) with measurements of its fluorescence intensity and the corresponding background intensity. With the file transformation tool Express Converter v.2.1 (http://www.tm4.org/utilities.html) data files generated from GenePix (*.gpr*) were converted to TM4 files (*.mev*) that could be uploaded to the MIDAS platform.

II 11.7.2 Data normalization with MIDAS

Before the intensity values that were measured with GenePix could be compared, normalization with MIDAS was necessary. This critical step compensated for variability by appropriately adjusting the data. In this study the LOWESS (locally weighted scattered plot smoothing) normalization was performed using the block mode as set-up parameter as this mode allowed correction of systematic spatial variation between the grids of the array.

II 11.7.3 Identification of differentially expressed genes

The normalized data for each spot were exported to Excel and aligned to the corresponding *Azoarcus* sp. BH72 gene name. The experiments were performed in triplicates and each gene was spotted in four replicates on every slide resulting in twelve intensity values per fluorescent dye. The expression ratios for each gene were calculated by dividing the corresponding intensity values from one condition by the intensity values from the other condition. To obtain an average expression fold the mean of the twelve replicate expression ratios was calculated. Moreover, a one-tailed paired t-test was performed and only genes that showed an expression fold of at least 1.8 and a P-value ≤ 0.05 were regarded as being differentially expressed.

II 11.8 Real-time PCR experiments

Some genes that were discovered to be differentially expressed by microarray experiments were further examined with the method of quantitative real-time PCR. The RNA samples of *Azoarcus* sp. BH72 which were harvested in the early exponential growth phase and under QS conditions for microarray experiments (see II 11.1) were also used for quantitative real-time PCR. The housekeeping gene for 16S rRNA was used as a reference gene in real-time PCR experiments.

II 11.8.1 Reverse transcription to yield cDNA for real-time PCR experiments

Synthesis of cDNA was achieved by using gene specific reverse primers (see Table 1) with the Verso 2-Step QRT-PCR Kit from ABgene (purchased through Thermo Fisher Scientific Inc., Rockford, IL, USA). Therefore, 30 ng total RNA was reverse transcribed in a final volume of 20 µl for 30 min at 42°C followed by a denaturation step for 2 min at 95°C in the Chromo 4 PTC-200 real-time PCR cycler (MJ Research, Waltham, MA, USA). The cDNA was kept at -20°C until further processing.

Reverse transcription mixture		
5 x cDNA synthesis buffer	4.0	µl
dNTP Mix	2.0	µl
Gene specific reverse primer (10 µM)	2.0	µl
Enhancer	1.0	µl
Verso Enzyme Mix	1.0	µl
Total RNA	30.0	ng

II 11.8.2 Quantitative PCR

From the cDNA synthesis mix 0.5 µl to 1.5 µl were used for the quantitative PCR (QPCR) step with 1 x 2-Step QPCR Mix (ABgene) and 0.5 x SYBR green I dye (Molecular Probes, Eugene, ME, USA) in a total volume of 25 µl. The QPCR was carried out in the Chromo 4 PTC-200 real-time PCR cycler (MJ Research, Waltham, MA, USA) with the following programme:

QPCR programme

15 min	95°C	
15 sec	95°C	40x and plate read of fluorescence
30 sec	60°C	
30 sec	72°C	
30 sec	95°C	
30 sec	60°C	

The QPCR programme was completed by a melting curve ranging from 60°C to 99°C with 0.5°C increasing steps for 10 sec. The resulting real-time PCR data obtained by the Opticon Monitor Analysis software (version 2.03, MJ Research) included the QPCR curves with corresponding C_T-values as well as melting curves with corresponding melting temperatures.

II 11.8.3 Analyses of real-time PCR curves

The analyses of real-time PCR data were performed with relative quantification and 16S as a reference gene for constitutive gene expression in *Azoarcus* sp. BH72 with the $2^{-\Delta\Delta C}{}_T$ method (Livak & Schmittgen 2001). This method includes the calculation of $\Delta\Delta C_T$ with the following equation:

$$\Delta\Delta C_T = \left(C_{T\,target} - C_{T\,reference}\right)_{QS} - \left(C_{T\,target} - C_{T\,reference}\right)_{exp}$$

Finally the expression ratio between the two conditions was calculated with $2^{-\Delta\Delta C}{}_T$. For the $\Delta\Delta C_T$ calculation to be valid, the amplification efficiencies of the target and reference gene must be approximately equal, assuming that the primer efficiency is ideal (= 2). In general, the real-time PCR experiments were performed with two independent sets of RNA and two technical replicates.

II 12 Computational based analyses

II 12.1 Analyses of quorum sensing regulated genes and different protein parameters

Several programs assisted in determination of protein parameters and protein functions: For classification of proteins the cluster of orthologous groups (COG) described by Tatusov et al. (1997) and the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) were used.

Furthermore, the enzyme database BRENDA (Chang et al. 2009, http://www.brenda-enzymes.org/) was used to search the EC numbers of proteins. The Pfam protein families database (Finn et al. 2010, http://pfam.sanger.ac.uk/) as well as the TIGRFAM database (Haft et al. 2003, http://blast.jcvi.org/web-hmm/) were used for identification of protein domains.

To study different protein parameters the following programs were employed: PSORTb (Gardy et al. 2005, http://www.psort.org/) for prediction of subcellular localization of proteins, the database TMHMM v2.0 (Krogh et al. 2001, http://www.cbs.dtu.dk/services/TMHMM-2.0/) to identify transmembrane helices in proteins. Proteomics tools from the ExPASy Server (Gasteiger et al. 2003, http://www.expasy.ch/tools/) were used to determine the GRAVY index (grand average of hydropathicity) as well as theoretical peptide masses resulting from theoretical tryptic digests of proteins: ProtParam (http://www.expasy.ch/tools/protparam.html) and Peptide Cutter (http://www.expasy.ch/tools/peptidecutter/), respectively.

General information about specific genes and proteins were taken from the database of the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) and identity searches were performed with the associated Basic Local Alignment Search Tool (BLAST) engine (Altschul et al. 1990, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

II 12.2 Analyses of microbiome samples and endophytic genomes

For the identification of quorum sensing systems in selected metagenomes and endophytic genomes pfam domain and COG classification searches were performed. Additionally,

identity searches with the associated BLAST engine blastp (default setting: e-value 1e-5) within the Integrated microbial genomes (IMG) system (Markowitz et al. 2006, http://img.jgi.doe.gov/cgi-bin/pub/main.cgi) from the DOE Joint Genome Institute (Walnut Creek, CA, USA) with specific sequences from well studied QS systems from different bacteria were carried out. This was necessary as not all proteins that are involved in quorum sensing have particular pfam domains.

The abundance of QS systems in the Endophytic microbiome from Rice was compared to the metagenomes Soil:Diverse Silage, Termite Gut, Human Gut Community (Subjects 7 and 8), Sludge/Australian Phrap assembly, Sludge/US Phrap assembly, *Olavius algarvensis* (gutless marine oligochaete, delta 1, delta 4, gamma 1 and gamma 3) Whalefall (sample 1, 2 and 3) as well as Acid mine drainage. A short description of the microbiome samples and the IMG taxon object identification numbers are given in Table 4.

Moreover, QS systems of the following endophytes were analyzed with computational tools: *Azoarcus* sp. BH72, *Burkholderia phytofirmans* PsJN, *Enterobacter* sp. 638, *Gluconacetobacter diazotrophicus* PAl 5, *Klebsiella pneumoniae* 342, *Methylobacterium populi* BJ001, *Pseudomonas putida* W619 and *Pseudomonas stutzeri* A1501. In Table 4 the IMG taxon object identification numbers and a brief description about host plants of the studied endophytes are listed.

Table 4: Short description of metagenome samples and endophytic genomes with IMG taxon object identification numbers.

Sample		Object ID	Description
Metagenome sample			**Isolation site**
Endophytic microbiome from Rice		2010549000	*O. sativa* cv. Apo, Los Baños, Philippines
Soil:Diverse Silage		2001200001	Farm silage surface soil (0-10 cm), Waseca County, MN, USA
Termite Gut		2004080001	Gut microbiome of termites, Costa Rica
Human Gut Community	Subject 7	2004002000	Fecal specimen of female healthy human, age 28, Rockville , MD, USA
	Subject 8	2004002001	Fecal specimen of female healthy human, age 37, Rockville , MD, USA
Sludge/Australian, Phrap assembly		2000000001	Laboratory bioreactor, Brisbane, Australia
Sludge/US, Phrap assembly		2000000000	Laboratory bioreactor, Madison, WI, US
O. algarvensis	delta 1	2004178001	Gutless worm, Capo di Sant' Andrea, Elba, Italy
	delta 4	2004178002	
	gamma 1	2004178003	
	gamma 3	2004178004	
Whalefall	sample 1	2001200002	Sunken whale skeleton carcass, section of a rib bone
	sample 2	2001200003	Sunken whale skeleton carcass
	sample 3	2001200004	Sunken whale skeleton carcass
Acid mine drainage		2001200000	Richmond mine, biofilm, Iron Mountain, CA, USA
Endophytic genome			**Host plants**
Azoarcus sp. BH72		639633007	Kallar grass, rice
B. phytofirmans PsJN		642555113	onion, tomato, potato, cucumber, chickpea, watermelon
Enterobacter sp. 638		640427113	poplar
G. diazotrophicus PAl 5		641228493	sugarcane
K. pneumoniae 342		643348560	rice, sweet potato, sugarcane, banana, maize
M. populi BJ001		642555139	poplar
P. putida W619		641522646	poplar
P. stutzeri A1501		640427133	wheat

III RESULTS

The presented study aimed to investigate the novel quorum sensing system of *Azoarcus* sp. BH72 with a combination of several methods and the results of these approaches will be presented in the following chapter. To gain better insights into abundance and distribution of QS systems in the natural environment, a computational approach with nine selected metagenome samples and eight endophytic genomes was performed. With this method the abundance of genes encoding proteins that are responsible for QS signal molecule synthesis and detection was analyzed. Moreover, gene expression studies with QS target genes were carried out by means of reporter gene studies. Therefore, gene expression of the genes *pilAB*, coding for type IV pilus biogenesis proteins, as well as *azo3874* encoding a conserved hypothetical secreted protein, was examined under QS conditions in comparison to early exponential growth. In addition, the impact of conditioned culture supernatants obtained from different bacterial species like *Azospira oryzae*, *Azoarcus communis* or *Azovibrio restrictus* on the QS target gene expression was tested and the results will be introduced.

Beside that, a proteome reference map of the grass endophyte *Azoarcus* sp. BH72 under standard aerobic growth conditions was constructed as this was the basis for subsequent comparative studies. To detect QS regulated genes and proteins such comparative proteomic studies with *Azoarcus* sp. BH72 grown under QS conditions and an additional genome wide oligonucleotide microarray approach were performed. The obtained gene products and proteins were categorized according to their functions and the QS regulation of *Azoarcus* sp. BH72 will be presented. Furthermore, the characterization of the PilR regulon with the aid of gel-based and gel-free approaches was achieved by comparative proteomic studies with a *pilR*-mutant. Finally, the PilR regulated proteins were compared to genes and proteins that were shown to be QS regulated and the results will be discussed at the end of this chapter.

III 1 Quorum sensing systems in microbiome samples

A metagenome is the entirety of bacterial organisms and their genome poperties within a natural habitat. But most bacterial species are difficult to study under laboratory conditions and therefore sequencing of DNA directly from the environment, a technique commonly referred to as metagenomics, is an important tool for cataloging microbial life (Morgan et al. 2010). The term metagenome was first described by Handelsman (1998) and this relative new field of research provides the opportunity to study organisms in their natural environment

with culture-independent methods. Metagenomics might also offer insights into population dynamics of microbial communities and individual roles of their members (Handelsman et al 1998, Morgan et al. 2010). Quorum sensing plays an important role for cellular communication and subsequent gene regulation processes within a habitat. Different quorum sensing systems are integrated in a complex regulatory network and therefore it is of high interest to analyze the occurrence of well studied QS systems in metagenomes. In the presented study a computational approach of nine selected metagenome samples was performed and the abundance of genes encoding proteins that are responsible for quorum sensing signal synthesis as well as detection was compared.

For the Endophytic microbiome from Rice, DNA was extracted from the endophytic community of *Oryza sativa* cv. APO plants grown on IRRI (International rice research institute, Los Baños, Philippines) experimental fields. The isolated DNA was used for shotgun library construction, ribosomal 16S and 18S RNA gene PCR library construction as well as Sanger sequencing. Sanger sequencing lead to 47 Mb of bacterial DNA sequences and the metagenomic assembly was loaded into the integrated microbial genomes platform for gene predictions and annotation.

The abundance of QS systems in the Endophytic microbiome from Rice was compared to the metagenomes Soil:Diverse Silage, Termite Gut, Human Gut Community (Subjects 7 and 8), Sludge/Australian Phrap assembly, Sludge/US Phrap assembly, *Olavius algarvensis* (marine oligochaete, delta 1, delta 4, gamma 1 and gamma 3) Whalefall (sample 1, 2 and 3) as well as Acid mine drainage. A short description of the selected metagenomes and their sampling sites is provided in Table 4 of chapter II 12.2.

In Figure 6 the abundance of genes coding for quorum sensing signal molecule synthesis and detection proteins within the nine selected metagenomes is depicted, the sequences of characteristic proteins that were found in the microbiomes are listed in Table B 1 of Appendix B. Microbial communication by means of quorum sensing appears to be of high importance for the rice endophytic community as genes encoding proteins for autoinducer synthesis and detection were most abundant in the Endophytic microbiome from Rice. All together, four different systems could be detected in the nine metagenome samples and these systems are depicted in Figure 7.

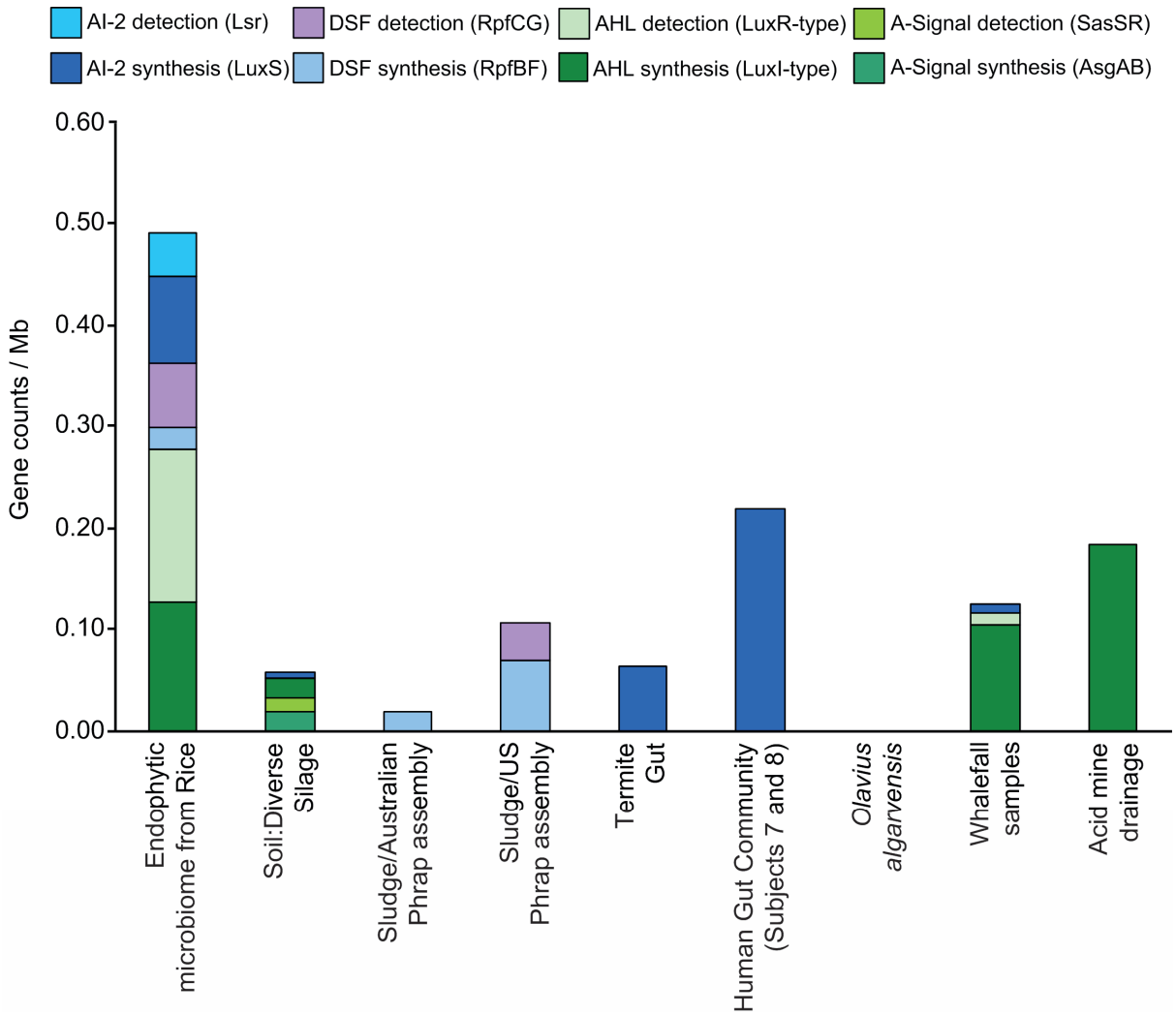

Figure 6: Comparison of abundance of genes coding for quorum sensing signal molecule synthesis and detection proteins in different metagenomes, normalized as gene counts per Mb. AHL = *N*-acylhomoserine lactone system, AI-2 = Autoinducer-2 system and DSF = Diffusible signal factor system.

The Autoinducer-2 system, the Diffusible signal factor system and the *N*-Acylhomoserine lactone system were identified in the Endophytic microbiome from Rice. The AI-2 system was also present in metagenome samples isolated from soil, whalefall, termite gut and human gut. This system, based on the S-ribosylhomocysteinase LuxS, is well studied in both Gram-negative and Gram-positive bacteria, where it acts as a global signal molecule for interspecies communication (Williams et al. 2007). All LuxS sequences found in the Endophytic microbiome from Rice (4 sequences) and the Whalefall metagenome (1 sequence) were from γ-proteobacteria, whereas the metagenome samples from Soil:Diverse Silage (1 sequence), Human Gut Community (8 sequences) and Termite Gut (4 sequences) contained only sequences from Gram-positive bacteria like *Actinobacteria* and *Firmicutes* (see Table B 1 of Appendix B).

The *N*-Acylhomoserine lactone system seems to be very important for communication in the endophytic rice community as six LuxI-type AHL synthetases and seven LuxR-type autoinducer binding proteins could be identified. Blast analyses with protein sequences of AHL-synthesis proteins revealed that they mostly belonged to α-proteobacteria whereas the sequences of autoinducer binding domains were from α- as well as γ-proteobacteria. The general phylogenetic distribution in the Endophytic microbiome from Rice shows that *Proteobacteria* were dominant in the endophyte community, among this γ-proteobacteria and α-proteobacteria were most abundant.

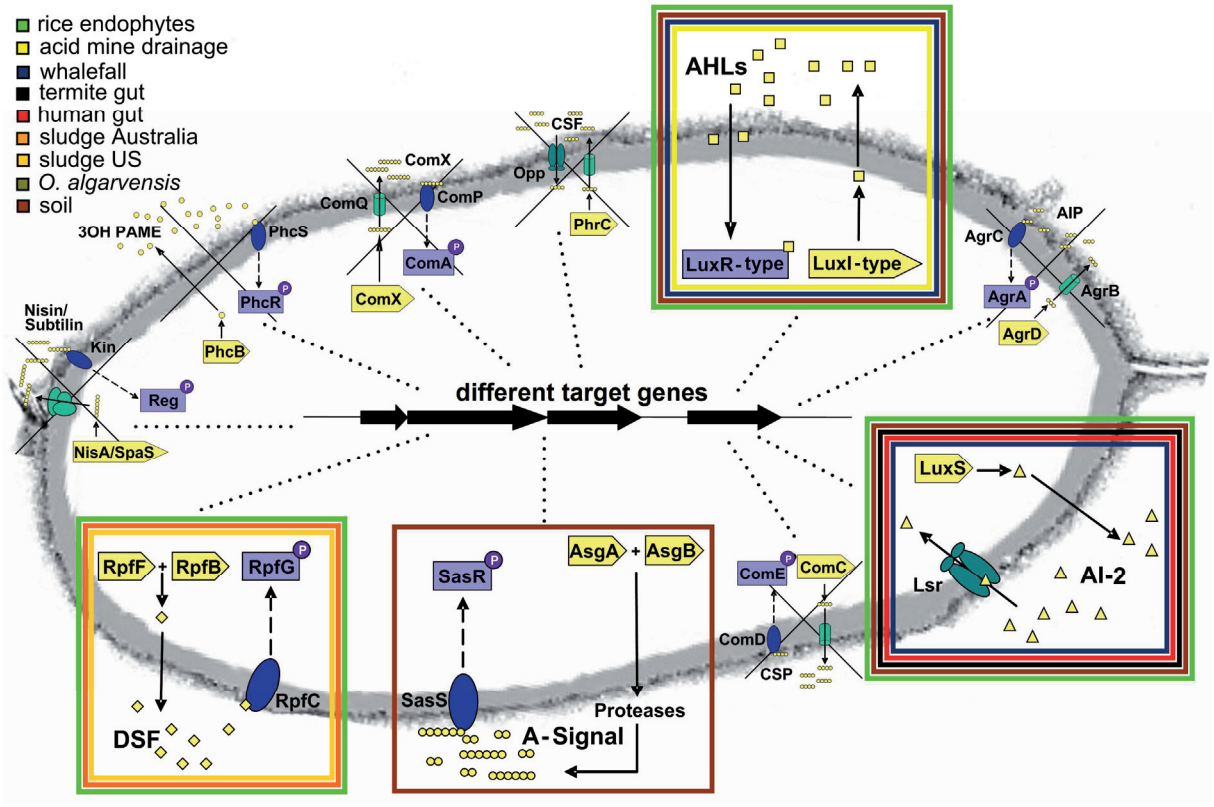

Figure 7: Quorum sensing systems detected in selected metagenomes. Proteins for signal molecule synthesis and detection that were present in the microbiome are boxed in the respective colours. Known quorum sensing systems for which no evidence was found in any of the tested samples are crossed out. The *N*-acylhomoserine lactone system (AHL), Autoinducer-2 system (AI-2), Diffusible signal factor system (DSF), A-signal system, Sporulation factor system (CSF), ComX system, Competence stimulating peptide system (CSP), Autoinducing peptide system (AIP), 3-OH-PAME system and Nisin/Subtilisin system were analyzed. Proteins that are involved in signal molecule synthesis (yellow arrows), detection (blue ellipses and blue boxes) and transport (green ellipses and barrels) are indicated with the particular protein name.

In comparison, the *N*-Acylhomoserine lactone system could be also detected in Soil:Diverse Silage, Whalefall and Acid mine drainage metagenomes. The LuxI-type and LuxR-type sequences in the whalefall and soil samples were mainly from α-proteobacteria and sequences

of *Nitrospira* represent the AHL-based system in the Acid mine drainage metagenome. Interestingly, the DNA-binding transcriptional activator SdiA (a LuxR-orphan) from *Citrobacter koseri* was detected in the Endophytic microbiome from Rice. It has been shown that *sdiA* encodes a receptor that exclusively detects signal molecules of other species (Michael et al. 2001), indicating the importance of quorum sensing molecules and their receptors in a diverse bacterial community.

Moreover, the Diffusible signal factor system was also found in both sludge samples. The A-Signal system was only detected in the soil metagenome sample. Neither the Sporulation factor system (CSF), ComX system, Competence stimulating peptide system (CSP), Autoinducing peptide system (AIP) nor the Nisin/Subtilisin system could be detected in any of the analyzed metagenomes. This observation is contradictory to the expectations. Many Gram-positive bacteria were detected in the microbiome samples but no QS system that is usually present in such bacteria could be found with the methods applied. In general, genes encoding the responsible peptide signal molecules are very short and might therefore be missed in metagenome sequencing approaches. Furthermore, the Bradyoxetin system as well as the 3-OH-PAME system was not identified in the metagenomes, although the bacteria *Bradyrhizobium japonicum* and *Ralstonia* sp., which are known to communicate via the mentioned systems, could be found in the Endophytic microbiome from Rice. Unfortunately, the synthesis protein for the signal molecule Bradyoxetin remains unknown and therefore the detection of this quorum sensing system is not possible with the applied pfam-, COG- or Blast-analyses. However, it can not be excluded that such quorum sensing system might play a role in the bacterial community as *Bradyrhizobium* sp. appeared in high abundance in the Endophytic microbiome from Rice.

III 2 Quorum sensing systems in several endophytes

In addition to metagenome studies it is important to investigate the abundance of genes encoding proteins that are responsible for quorum sensing signal synthesis as well as detection in selected endophytic genomes. In general, comparison of available endophytic genomes offers the opportunity to get better insights into the endophytic lifestyle and might answer the question which sets of functions are required for such a lifestyle. Moreover, it is possible to understand how communication via quorum sensing is achieved in endophytes and how the different QS systems are distributed among the bacterial species.

In the presented study the occurrence of QS systems within the genomes of selected endophytes that were originally isolated from plants like rice, poplar, maize or sugarcane was analyzed and compared. The genomes of the endophytes *Azoarcus* sp. BH72, *Burkholderia phytofirmans* PsJN, *Enterobacter* sp. 638, *Gluconacetobacter diazotrophicus* Pal5, *Klebsiella pneumoniae* 342, *Methylobacterium populi* BJ001, *Pseudomonas putida* W619 and *Pseudomonas stutzeri* A1501 were investigated by means of pfam-domain searches and Blast-analyses of genome sequences on the Integrated Microbial Genomes database. A short description about host plants of the studied endophytes is provided in chapter II 12.2, Table 4.

The computational analyses revealed that proteins involved in the *N*-Acylhomoserine lactone system, the Autoinducer-2 system, the Diffusible signal factor system as well as the 3-OH-PAME system could be detected in the endophytic genomes and the results are shown in Figure 8.

In *Burkholderia phytofirmans* PsJN two AHL-based systems were detected on chromosome 1 and chromosome 2 with Bphyt_4275 and Bphyt_0126 as LuxI-type AHL-synthesis proteins and Bphyt_4277 and Bphyt_0127 as the corresponding LuxR-type autoinducer binding proteins. This system has already been described for strain PsJN by Sessitsch et al. 2005. Moreover, this strain harbours a LuxR-orphan (Bphyt_6042) on chromosome 2. Surprisingly, all three important components for the 3-OH-PAME system were discovered in one cluster (Bphyt_1287/1288/1289) on chromosome 1. So far, this system has only been described for *Ralstonia* sp. where the synthesis of 3-Hydroxypalmitic acid methyl ester requires the methyl-accepting chemotaxis protein methyltransferase PhcB (Bphyt_1287). The signal molecule is detected by the periplasmic sensor signal transduction histidine kinase PhcS (Bphyt_1288) followed by gene regulation through the response regulator PhcR (Bphyt_1289).

The Autoinducer-2 system could be detected with the important enzyme for Autoinducer-2 synthesis S-ribosylhomocysteinase (LuxS, Ent638_3167) in *Enterobacter* sp. 638. All components that are important for detection and transport of the molecule back into the cell could also be discovered in one large cluster, the Autoinducer-2 ABC transporter (Ent638_3536), the putative periplasmic solute-binding protein (Ent638_3533) and the inner-membrane translocators (Ent638_3535 and Ent638_3534). Additionally, the LuxR-orphan Ent638_2503 could be identified.

For the sugarcane endophyte *Gluconacetobacter diazotrophicus* Pal5 communication through an AHL-based system has been already described (Bertalan et al. 2009) and the autoinducer synthesis protein GDI_2836 as well as two transcriptional regulators of the LuxR-type (GDI_2837 and GDI_2838) could be detected in one cluster.

Klebsiella pneumoniae 342 encodes the *N*-Acylhomoserine lactone receptor SdiA (KPK_1867) and might therefore be able to respond to exogenous QS molecules in its habitat. The well studied Autoinducer-2 system was also detected with the S-ribosylhomocysteinase LuxS (KPK_1109) for synthesis and the Lsr-cluster (KPK_0605, KPK_0608, KPK_0606, KPK_0607) for detection and transport.

The poplar endophyte *Methylobacterium populi* BJ001 harbours two genes encoding an *N*-Acylhomoserine lactone based QS system with Mpop_5028 as respective LuxI-type autoinducer synthesis proteins and the corresponding LuxR-type regulator Mpop_5029.

Only a small number of *Pseudomonas putida* strains posess AHL-based systems. The LuxR-orphan PpoR is well studied and the homologue PputW619_0791 could also be detected in the genome of *P. putida* W619.

In the endophytes *Azoarcus* sp. BH72 and *Pseudomonas stutzeri* A1501 no known QS system could be identified with the computational analyses applied here. *Azoarcus* sp. BH72 harbours the LuxI-type synthesis protein (azo3178), but until now no function was stated for this conserved hypothetical protein. There is no evidence for a corresponding LuxR-type regulator and therefore an AHL-based quorum sensing system is not likely.

Taking all these observations together, it was obvious that cell-to-cell communication via AHL-based systems as well as Autoinducer-2 systems is of great importance in endophytic bacteria. Moreover, *K. pneumoniae*, *P. putida* and *Enterobacter* sp. have established QS systems which enable them to respond to exogenous signals present in their environment with LuxR-orphans. Such an adaption or the variety of quorum sensing systems offers bacteria the opportunity to be dominant in a bacterial community.

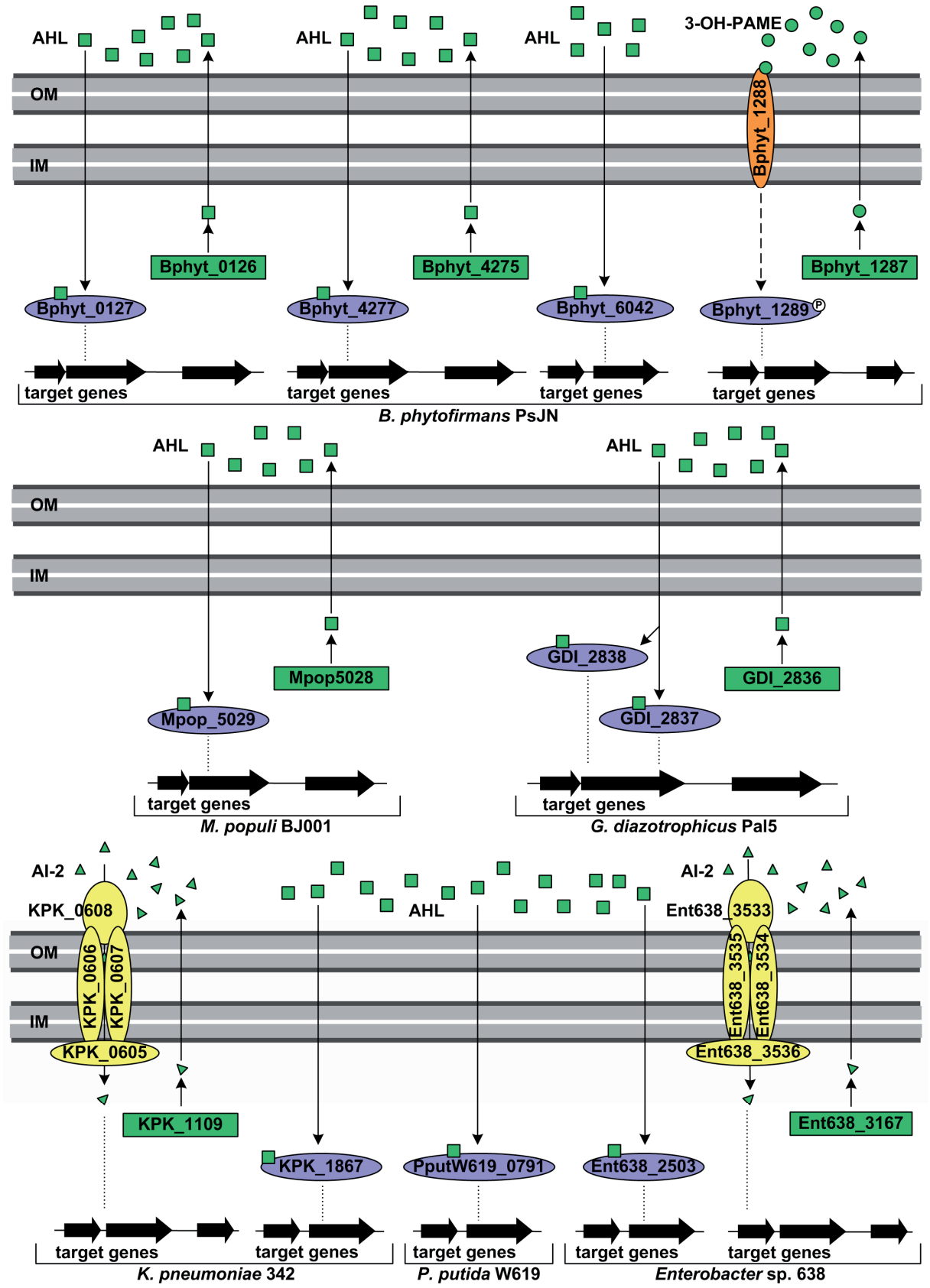

Figure 8: Quorum sensing systems detected in genomes of several endophytic bacteria. Proteins that are involved in signal molecule synthesis (green), detection (blue and orange ellipses) and transport (yellow ellipses) are indicated with the particular locus ID in the genome. AHL = *N*-Acylhomoserine lactone, AI-2 = Autoinducer-2, 3-OH-PAME = 3-Hydroxypalmitic acid methyl ester.

III 3 Quorum sensing bioassays and gene expression studies

The characterization of the novel QS system of *Azoarcus* sp. BH72 can be achieved with different methods. Gene expression studies allow the investigation of quorum sensing targets under different growth conditions. In the presented work, reporter gene studies with the gene *uidA*, encoding β-glucuronidase were carried out with *Azoarcus* sp. reporter strains. Such reporter strains carry a transcriptional fusion of the promoterless gene *uidA* to a QS target gene, in this case *pilAB* and *azo3874*. The QS target genes will be introduced in more detail in the respective chapter. With this transcriptional fusion the promoter activity and therefore also the gene expression of the target gene is directly linked to the reporter gene expression and this expression can be determined by calculating the β-glucuronidase activity in an enzyme assay.

For studying target gene expression under QS conditions the production of conditioned culture supernatant of *Azoarcus* sp. BH72 is necessary. This supernatant was obtained from *Azoarcus* wild type after growth in VM-Ethanol medium until the stationary phase and it could be already shown that conditioned culture supernatant contains the so far unknown Hydrophilic signal factor for QS communication (Böhm 2006). Growth under QS conditions was achieved by incubation of *Azoarcus* sp. grown until the early exponential phase with conditioned culture supernatant for one, two, three or four hours. To detect the gene expression change of QS target genes due to the influence of the HSF, the mentioned β-glucuronidase activity assay was carried out. Respective medium instead of supernatant served as a negative control. In the following chapter such studies will be displayed and P-values of statistical analyses of β-glucuronidase activity assays are listed in Table A 1 of Appendix A.

III 3.1 The genes *pilAB* are quorum sensing targets in *Azoarcus* sp.

It has been already shown that the genes *pilA* and *pilB*, which are crucial for the formation of type IV pili, are target genes for the QS cascade in *Azoarcus* sp. as their gene expression is density dependent. The *pilAB* gene expression increases with high cell densities and can be induced by adding conditioned culture supernatant to a culture in the exponential growth phase. Moreover, *pilA* and *pilB* are co-transcribed and their expression is controlled by the

two component regulatory system PilSR, with PilS as sensor kinase and PilR as the corresponding response regulator (Dörr et al. 1998, Böhm 2006).

The reporter strain *Azoarcus* sp. BHΔ*pilS*::pJBLP14 carries a transcriptional fusion of the reporter gene *uidA* to the QS target genes *pilAB* and can therefore be used for the detection of *pilAB* gene expression under different growth conditions. In Figure 9 it is shown that the *pilAB* gene expression increased over the time after incubation with conditioned culture supernatant. After one hour incubation of the reporter strain BHΔ*pilS*::pJBLP14 with conditioned culture supernatant the expression was almost unchanged (1.29 ± 0.22), whereas an increase of gene expression could be already measured after two hours (1.65 ± 0.06). After four hours the gene expression of *pilAB* was induced 2.37-fold (± 0.32) compared to the negative control with medium. This observation revealed that the conditioned culture supernatant from *Azoarcus* sp. BH72 has a great impact on the *pilAB* gene expression, due to an unknown QS signal molecule in the bacterial exudate. So far, the increase of *pilAB* gene expression after adding conditioned culture supernatant could only be measured with the β-glucuronidase activity assay in the Δ*pilS*-mutant background, where the pilin genes already show higher expression rates.

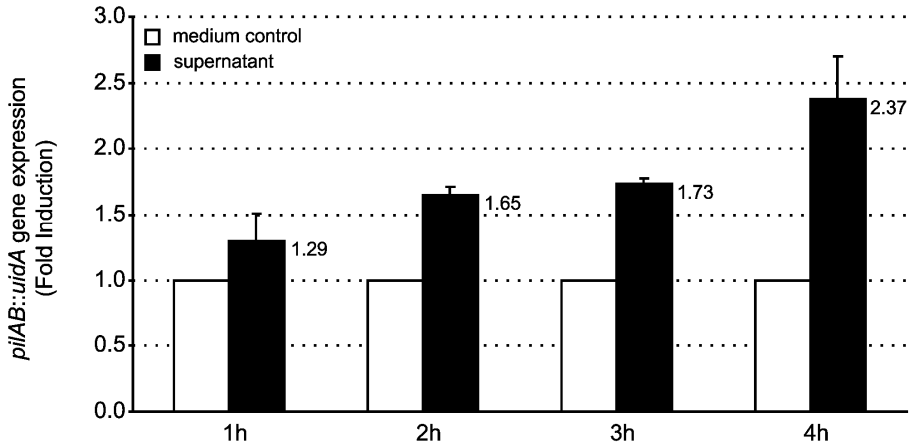

Figure 9: Fold induction of *pilAB* gene expression after incubation of *Azoarcus* sp. BHΔ*pilS*::pJBLP14 with conditioned culture supernatant from *Azoarcus* wild type. The induction factors were obtained by quorum sensing bioassays and are based on determination of β-glucuronidase activity. VM-Ethanol medium was used instead of supernatant as negative control and the values were set to one for comparison of induction factors after incubation for one, two, three or four hours, respectively. Standard deviation was calculated from two independent experiments with eight technical replicates.

In general, the growth of *Azoarcus* sp. under QS conditions was decreased in QS bioassays when compared to growth in the negative control with respective medium. This observation indicates that the HSF in the conditioned culture supernatant might also influence expression of several other genes which in turn led to growth inhibition. Moreover, it is possible that

metabolites or secondary metabolites in the conditioned culture supernatant inhibit growth of the grass endophyte.

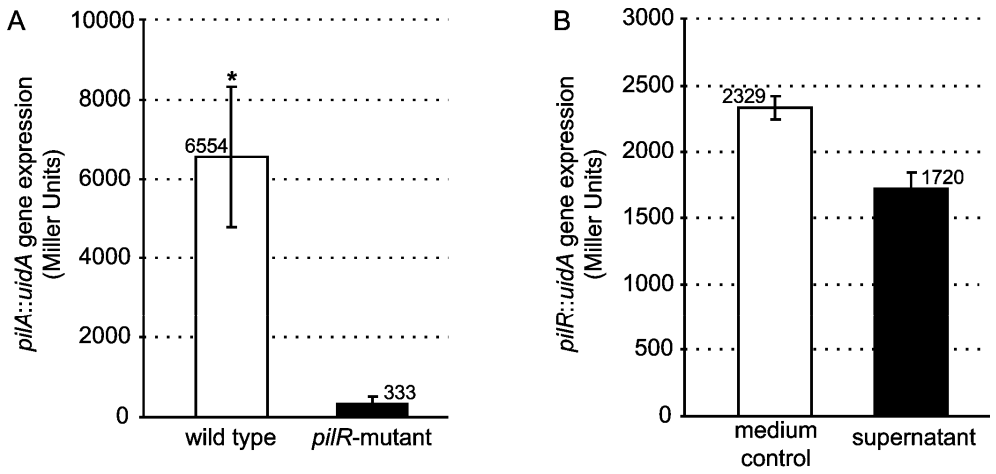

Figure 10: A: Comparison of *pilAB* gene expression of *Azoarcus* wild type (BH72(pJLA*pilA-uidA*) and mutant strain BH*pilR*K(pJLA*pilA-uidA*). Both strains were incubated aerobically until the exponential growth phase in VM-Ethanol medium and the Miller Units were measured by β-glucuronidase activity assay. Significance is indicated with * and standard deviation was calculated from five independent experiments with twelve technical replicates. **B:** Display of *pilR* gene expression after incubation of *Azoarcus sp. BH72*::pJBLP21 with conditioned culture supernatant from *Azoarcus* wild type. Miller Units were obtained by quorum sensing bioassays and are based on determination of β-glucuronidase activity with VM-Ethanol medium instead of supernatant as negative control. Standard deviation was calculated from one experiment with four technical replicates.

As mentioned above, the *pilAB* gene expression is controlled by the two-component regulatory system PilSR. Therefore, it was of high interest to examine, how the *pilAB* gene expression is altered in a *pilR*-mutant. The reporter strain BH*pilR*K(pJLA*pilA-uidA*) used in this experiment carries a transcriptional fusion of the reporter gene *uidA* to the QS target genes *pilAB* on a stable plasmid in the *pilR*-mutant background and this plasmid in the wild type background served as a control strain (BH72(pJLA*pilA-uidA*)). In Figure 10 (A) it is depicted that the *pilAB* gene expression was significantly decreased in the *pilR*-mutant strain (333 ± 175 Miller Units) in comparison to the *Azoarcus* wild type with 6554 ± 1780 Miller Units. This observation clearly shows that the response regulator PilR is responsible for the activation of *pilAB* gene expression. Furthermore, the *pilR* gene expression could be determined with the reporter strain BH72::pJBLP21 which carries a transcriptional fusion of the reporter gene *uidA* to the *pilR* gene in the wild type background.The *pilR* gene expression could not be induced after four hours incubation of *Azoarcus* sp. BH72::pJBLP21 with conditioned culture supernatant in a QS bioassay (Figure 10 B). This bioassay was only performed with one culture and technical replicates and therefore needs further validation.

III 3.2 The gene a*zo3874* is a newly discovered quorum sensing target

The transcriptome profiling of *Azoarcus* sp. BH72 under quorum sensing conditions led to the detection of new QS target genes. The expression of the gene *azo3874*, coding for a conserved hypothetical secreted protein, was up-regulated under the influence of conditioned culture supernatant (see III 7).

For studying the *azo3874* gene expression in quorum sensing bioassays with the reporter gene *uidA*, the insertional mutant *Azoarcus* sp. BH*azo3874* was constructed. Therefore, the plasmid pK18GGST*azo3874*, carrying a fusion of the promoterless reporter genes *gfp* and *uidA* to the gene *azo3874*, was conjugated into *Azoarcus* wild type by homologous recombination. The quorum sensing experiments revealed that after one hour of incubation with conditioned culture supernatant the *azo3874* gene expression could be significantly induced (1.97 ± 0.37). After four hours, the *azo3874* gene expression was 2.66-fold (± 0.44) higher compared to the negative control with VM-Ethanol medium instead of supernatant (Figure 11).

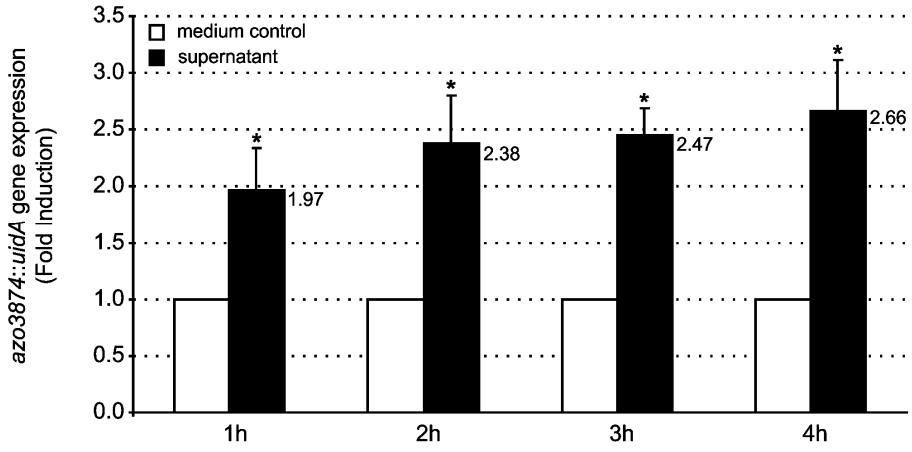

Figure 11: Fold induction of *azo3874* gene expression after incubation of *Azoarcus* sp. BH*azo3874* with conditioned culture supernatant from *Azoarcus* wild type. The induction factors were obtained by quorum sensing bioassays and are based on determination of β-glucuronidase activity. VM-Ethanol medium was used instead of supernatant as negative control and the values were set to one for comparison of induction factors after incubation for one, two, three or four hours, respectively. The average gene expression of *azo3874* in the medium control (factor 1.0) was 93 ± 25 Miller Units. Significance is indicated with * and standard deviation was calculated from three independent experiments with twelve technical replicates.

The microarray data showed that the induction of gene expression was high after one hour incubation with conditioned culture supernatant and decreased to 2.0-fold after four hours. This observation could not be validated by reporter gene studies with β-glucuronidase activity measurements as the gene expression of *azo3874* increased over the time. In general, the

experiments showed high variations between the independent experiments and induction factors ranging from 2.0- to 3.5-fold could be observed (Figure 11, see also Figure 13 and Figure 15).

In contrast to the *pilAB* gene expression studies with the reporter strain *Azoarcus* sp. BHΔ*pilS*::pJBLP14, the experiments with *Azoarcus* sp. BH*azo3874* revealed that the gene expression of *azo3874* could be induced by conditioned culture supernatant in the wild type background. This proofs for the first time that *Azoarcus* sp. supernatant containing the unknown QS signal molecule is capable to alter gene expression independently of the histidine kinase PilS which is also stimulated by carbon starvation.

III 3.3 Influence of growth medium on quorum sensing target gene expression

Usually, the QS bioassays were carried out with conditioned culture supernatants obtained from stationary cultures of *Azoarcus* sp. BH72 grown in VM-Ethanol medium. But growth in this complex medium might cause problems in subsequent experiments for identification of the unknown QS signal molecule. The undefined chemical compounds from yeast extract and peptone in this medium might interfere with analytical analyses like gas chromatography and mass spectrometry. Because of this, *Azoarcus* sp. BH72 was grown in the minimal medium SM-Ethanol. In this medium the generation time of the wild type and the mutant BH*azo3874* was longer, but cultures reached the stationary phase after over night incubation at 37°C. The generation time of *Azoarcus* sp. wild type in VM-Ethanol medium was 1.5 ± 0.03 hours compared to 2.2 ± 0.02 hours in minimal medium, whereas strain BH*azo3874* showed a generation time of 2.2 ± 0.15 hours in SM-Ethanol and 1.6 ± 0.16 hours in complex medium.

Quorum sensing bioassays with conditioned culture supernatants harvested from cultures grown in different media were performed with the reporter strain *Azoarcus* sp. BHΔ*pilS*::pJBLP14 and the results are shown in Figure 12. It was obvious that the *pilAB* gene expression could be induced by adding conditioned culture supernatant obtained from *Azoarcus* sp. cultures grown in VM-Ethanol medium as well as grown in SM-Ethanol medium. The induction factors were with 2.42 ± 0.27 and 2.30 ± 0.43 comparable and even the detected Miller Units as an indicator for *pilAB* gene expression were equal under both conditions.

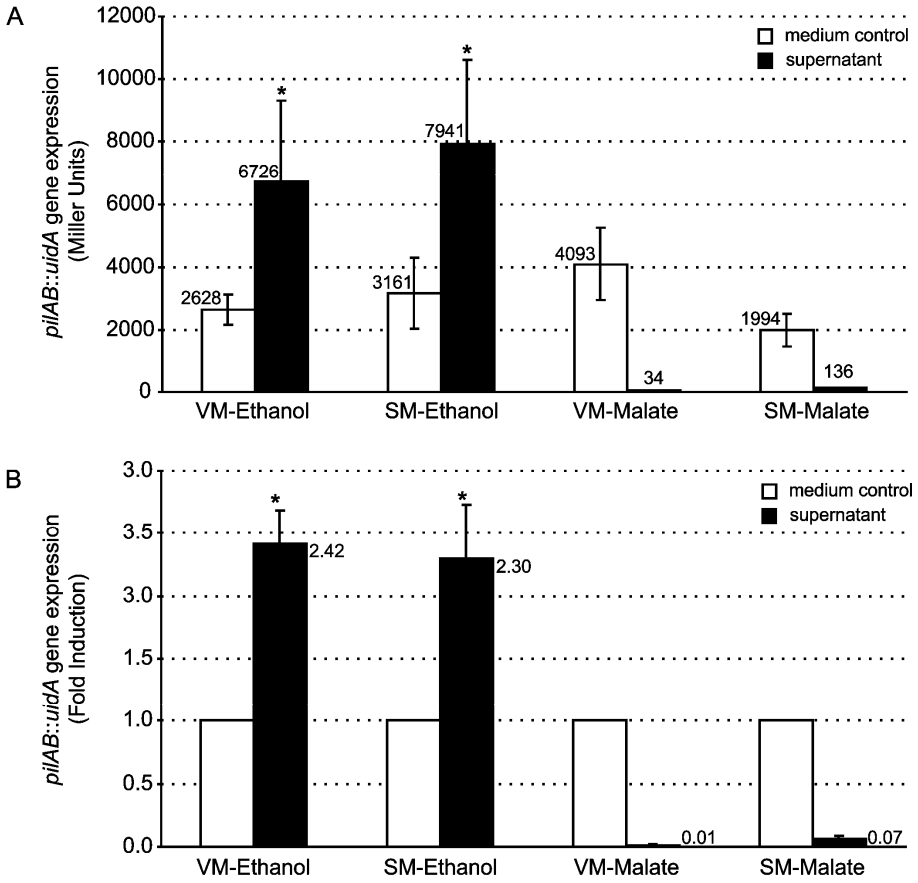

Figure 12: Display of *pilAB* gene expression after incubation of *Azoarcus* sp. BHΔ*pilS*::pJBLP14 with conditioned culture supernatant from *Azoarcus* wild type grown in different media. A: Miller Units were obtained by quorum sensing bioassays and are based on determination of β-glucuronidase activity with respective medium instead of supernatant as negative control. B: Fold induction of *pilAB* gene expression, induction factors were obtained by quorum sensing bioassays and are based on determination of β-glucuronidase activity. Respective medium instead of supernatant was used as negative control and the values were set to one for comparison of induction factors after incubation for four hours. Significance is indicated with * and standard deviation was calculated from seven independent experiments with 28 technical replicates (VM-Ethanol and SM-Ethanol medium) or two independent experiments with eight technical replicates (VM-Malate and SM-Malate medium).

Interestingly, almost no β-glucuronidase activity could be observed in the quorum sensing bioassay with conditioned culture supernatants harvested from cultures grown in SM-Malate and VM-Malate medium, only 136 ± 11 and 34 ± 19 Miller Units were detected in this experiments (Figure 12). This observation in VM-Malate medium has already been shown by Böhm (2006). In the former study it was assumed that the QS molecule is not stable in extreme pH-values due to the fact that the medium containing malate becomes alkaline after growth of *Azoarcus* sp. BH72 for several hours.

The pH-values of conditioned culture supernatants were measured: The growth in malate containing medium led to a shift to an alkaline pH (~9.5) in the supernatant in contrast to pH 6.0 after grown in ethanol containing medium. Furthermore, it could be observed that the

growth of *Azoarcus* sp. during the QS bioassay was decreased in experiments with conditioned culture supernatants obtained from cultures grown in malate containing media. A slight decrease in growth was also detected after incubation with conditioned culture supernatant obtained from cultures grown in SM-Malate medium.

The gene expression of *pilAB* that was observed after incubation of *Azoarcus* sp. BHΔ*pilS*::pJBLP14 with conditioned culture supernatants from malate containing cultures was much lower than the expression detected in the control with Malate medium (Figure 12, 30 to 130 Miller Units). Actually, equal expression was expected in both samples because the signal molecule is not stable in alkaline supernatants and therefore the *pilAB* gene expression could not be induced but should remain unchanged. The fact that the expression of *pilAB* was highly reduced indicates a direct effect on the pilin gene expression because of the pH change. Moreover, it is possible, that the enzyme test for β-glucuronidase activity is not stable and reliable in such alkaline conditions, but this hypothesis needs further validation.

Comparable studies were performed with the reporter strain *Azoarcus* sp. BH*azo3874* and supernatants harvested from cultures of *Azoarcus* sp. wild type grown in VM-Ethanol, SM-Ethanol, VM-Malate as well as SM-Malate. Surprisingly, the *azo3874* gene expression was induced after incubation of *Azoarcus* sp. with conditioned culture supernatants from cultures grown in all four media (Figure 13) compared to the medium control.

However, the gene expression detected by β-glucuronidase activity assays was extremely low with supernatants from malate containing media compared to ethanol containing media (Figure 13, 30 to 200 Miller Units) indicating a lower *azo3874* gene expression independent from QS conditions. The induction factors of gene expression due to supernatants from cells grown in malate medium were not significant.

It could be shown that the *pilAB* gene expression was more or less equal in the different media tested (medium control in Figure 12, 2000 to 4000 Miller Units) However, the experiments with the reporter strain *Azoarcus* sp. BH*azo3874* demonstrated that the expression of *azo3874* varied from 19 to 125 Miller Units in the different media (medium control in Figure 13). In addition, the gene expression detected after incubation of the reporter strain with an alkaline medium was comparable or even higher than the control with respective medium. This shows that the *azo3874* gene expression is not negatively affected by

alkaline pH conditions. One could even speculate that the gene expression of *azo3874* was induced in the presence of ethanol.

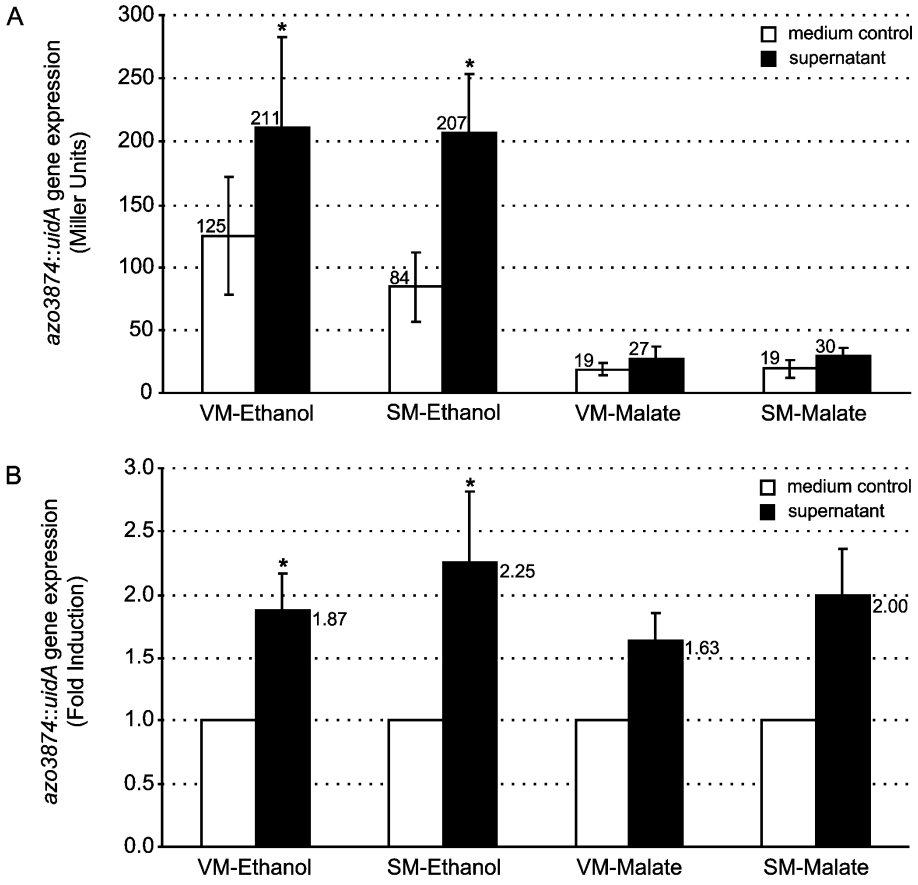

Figure 13: Display of *azo3874* gene expression after incubation of *Azoarcus* sp. BH*azo3874* with conditioned culture supernatant from *Azoarcus* wild type grown in different media. A: Miller Units were obtained by quorum sensing bioassays and are based on determination of β-glucuronidase activity with respective medium instead of supernatant as negative control. B: Fold induction of *azo3874* gene expression, induction factors were obtained by quorum sensing bioassays and are based on determination of β-glucuronidase activity. Respective medium was used as a negative control and the values were set to one for comparison of induction factors after incubation for four hours. Significance is indicated with * and standard deviation was calculated from five independent experiments with 20 technical replicates (VM-Ethanol and SM-Ethanol medium) or three independent experiments with twelve technical replicates (VM-Malate and SM-Malate medium).

At this point it can not be clarified why the *pilAB* gene expression was repressed after incubation with alkaline supernatants compared to the medium control and why the expression of *azo3874* was unaffected.

Taking all these observations together, the presented QS bioassays have clearly demonstrated that monitoring of QS regulation in *Azoarcus* sp. can be achieved with the two used robust reporter strain systems.

III 3.4 Possible distribution of the novel quorum sensing signal molecule

Within a given habitat bacteria are capable of interspecies communication with diverse chemical signal molecules outside the general intraspecies communication. This interspecies crosstalk has been shown for a wide range of bacteria and leads to the question: Does *Azoarcus* sp. BH72 also communicate with other bacterial species in the environment? Additionaly, the possible distribution of the novel *Azoarcus* QS signal molecule is of high interest. To test this, the *uidA* reporter strains were used to examine the impact of conditioned culture supernatants from different species on the *pilAB* and *azo3874* gene expression in strain BH72.

For such experiments closely related bacteria like *Azoarcus communis*, *Azoarcus evansii*, *Azospira oryzae* or *Azovibrio restrictus*, other plant associated bacteria such as *Azospirillum brasilense*, *Azospirillum lipoferum* and *Pseudomonas stutzeri* were used as well as pathogenic bacteria (*Pseudomonas syringae*, *Xanthomonas oryzae* and *Chromobacterium violaceum*) and soil bacteria (*Azonexus fungiphilus* and *Azotobacter vinelandii*). Conditioned culture supernatants from the mentioned bacteria were produced as described in chapter II 5.3.1 and all bacterial strains were culturable on VM-Ethanol medium. *Pseudomonas* sp., *Xanthomonas* sp., *Chromobacterium* sp. and *Azotobacter* sp. were grown at 30°C, whereas all other strains were incubated at 37°C.

III 3.4.1 Influence of conditioned culture supernatants from different bacterial strains on the *pilAB* gene expression of *Azoarcus* sp.

The inducing ability of conditioned culture supernatants from different bacterial species was tested in quorum sensing bioassays with the *Azoarcus* sp. BHΔ*pilS*::pJBLP14 reporter strain system with supernatants from the mentioned bacteria.

The experiments revealed that the *pilAB* gene expression of *Azoarcus* sp. could be induced significantly by supernatants from the β-proteobacteria *Azospira oryzae* strain 6a3 or *Azoarcus communis* strain SWub3 2.38-fold (± 0.42) or 2.30-fold (± 0.41), respectively. Surprisingly, the incubation with supernatants from *Pseudomonas stutzeri* strain DSM4166, isolated from the rhizosphere of *Sorghum mutan*s, led to an induction of the *Azoarcus* sp. *pilAB* gene expression (1.75 ± 0.41) as well (Figure 14, columns 5, 7 and 13).

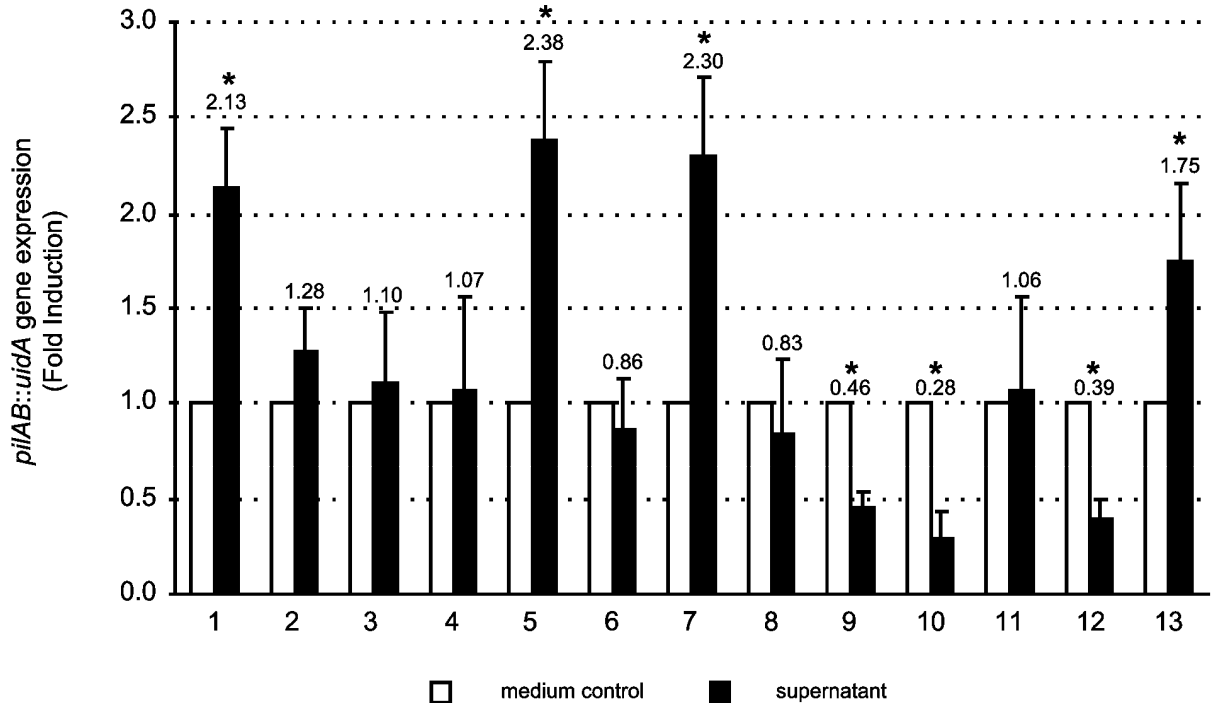

Figure 14: Fold induction of *pilAB* gene expression after incubation of *Azoarcus* sp. BHΔ*pilS*::pJBLP14 with conditioned culture supernatants from different bacterial species. The induction factors were obtained by quorum sensing bioassays and are based on determination of β-glucuronidase activity. VM-Ethanol medium was used instead of supernatant as negative control and the values were set to one for comparison of induction factors after incubation with diverse supernatants (1: *Azoarcus* sp. BH72, 2: *Azoarcus evansii* KB740, 3: *Azospirillum brasilense* Sp7, 4: *Azospirillum lipoferum* Sp59b, 5: *Azospira oryzae* 6a3, 6: *Azonexus fungiphilus* BS5-8, 7: *Azoarcus communis* SWub3, 8: *Azovibrio restrictus* S5b2, 9: *Chromobacterium violaceum* ATCC31532, 10: *Xanthomonas oryzae* PXO99, 11: *Azotobacter vinelandii*, 12: *Pseudomonas syringae* pv. *tomato* DC3000, 13: *Pseudomonas stutzeri* DSM4166). Significance is indicated with * and standard deviation was calculated from at least three independent experiments with at least twelve technical replicates.

The incubation of *Azoarcus* sp. BHΔ*pilS*::pJBLP14 with conditioned culture supernatants from *Chromobacterium violaceum* ATCC31532, *Xanthomonas oryzae* PXO99 and *Pseudomonas syringae* pv. *tomato* DC3000 significantly decreased the expression of *pilAB* genes, indicating a negative effect of those supernatants on the QS target gene transcription (Figure 14, columns 9, 10 and 12). This negative effect could be due to toxin production by those pathogenic bacteria or expression of type IV pili might be regulated via mechanisms other than quorum sensing. Furthermore, the pH of the supernatants from *C. violaceum* and *P. syringae* was around 8.5 to 9.0 and as described in chapter III 3.3, the *pilAB* gene expression was altered in alkaline conditions.

Incubation of *Azoarcus* sp. with supernatants from *Azoarcus evansii*, *Azospirillum lipoferum*, *Azospirillum brasilense*, *Azonexus fungiphilus*, *Azovibrio restrictus* as well as *Azotobacter vinelandii* did not show a significant impact on the pilin gene expression.

Based on experimental results one can speculate that *Azoarcus* sp. BH72 probably uses a similar type of QS signal molecule like *A. oryzae*, *A. communis* and *P. stutzeri.* However, further evidence by analytical identification of the chemical compounds in the different bacterial exudates is required to justify this speculation.

III 3.4.2 Influence of supernatants from different bacterial strains on the gene expression of *azo3874*

The inducing ability of conditioned culture supernatants from closely related bacteria needed further validation to exclude that this observation is only true for the target genes *pilAB*. Hence, supernatants from *A. communis* and *A. oryzae* were harvested and used for QS bioassays with the reporter strain BH*azo3874*. In Figure 15 the induction factors are represented which showed that the *azo3874* gene expression could also be induced by supernatants from these closely related bacteria and not only by *Azoarcus* sp. BH72.

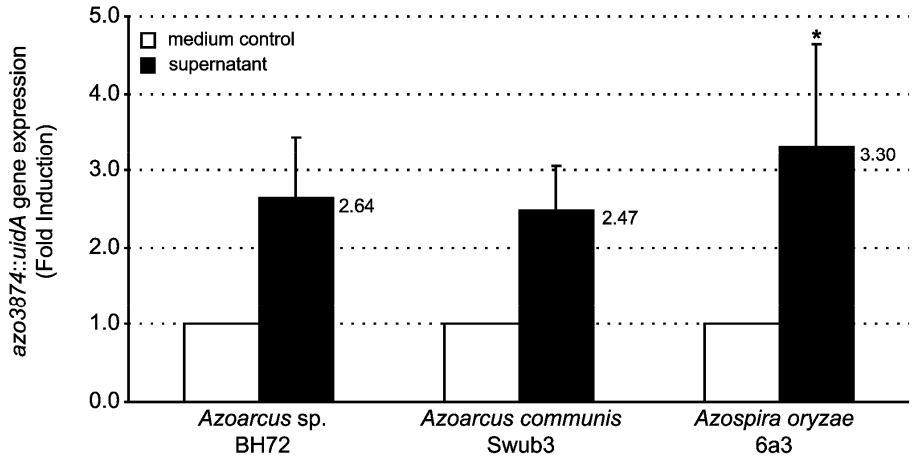

Figure 15: Fold induction of *azo3874* gene expression after incubation of *Azoarcus* sp. BH*azo3874* with conditioned culture supernatants from different bacterial strains grown in VM-Ethanol medium. The induction factors were obtained by quorum sensing bioassays and are based on determination of β-glucuronidase activity. VM-Ethanol medium instead of supernatant was used as negative control and the values were set to one for comparison of induction factors after incubation for four hours. Significance is indicated with * and standard deviation was calculated from three independent experiments with six technical replicates.

The fact that supernatants from *Azoarcus* sp. BH72, *A. oryzae* and *A. communis* induced the expression of *pilAB* as well as *azo3874* justifies the ability of strain BH72 in responding to QS signals from closely related bacteria. This leads to the hypothesis that an interspecies communication system is likely in the plant rhizosphere.

III 3.4.3 Influence of supernatants from different bacterial strains grown in SM-Ethanol medium on the *pilAB* gene expression

Azoarcus communis and *Azospira oryzae* were grown in SM-Ethanol medium and the resulting conditioned culture supernatants were used for QS bioassays with the reporter strain *Azoarcus* sp. BHΔ*pilS*::pJBLP14 (Figure 16).

The inducing ability of *A. communis* conditioned culture supernatant (2.06 ± 0.05) was comparable to the ability of *Azoarcus* sp. wild type (2.86 ± 0.05), whereas the supernatant from *A. oryzae* could only induce the *pilAB* gene expression 1.74-fold (± 0.35). This observation shows that the induction of this QS target genes of strain BH72 is independent from the growth medium (see III 3.3).

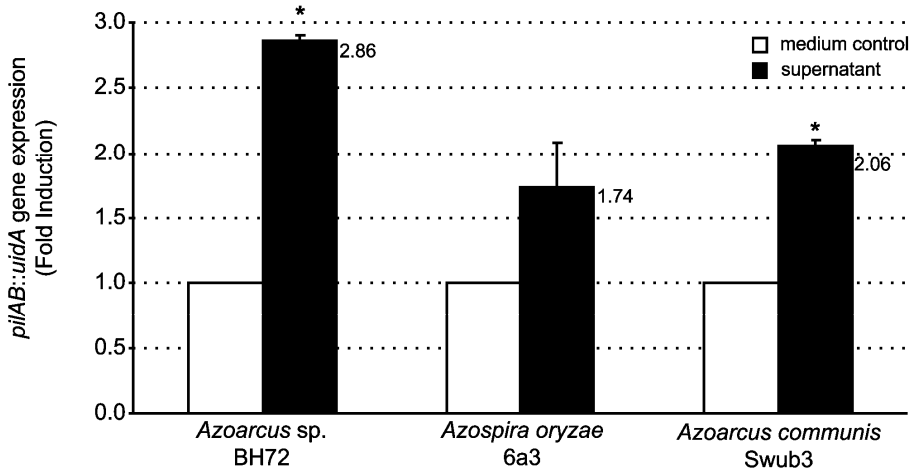

Figure 16: Fold induction of *pilAB* gene expression after incubation of *Azoarcus* sp. BHΔ*pilS*::pJBLP14 with conditioned culture supernatants from different bacterial strains grown in SM-Ethanol medium. The induction factors were obtained by quorum sensing bioassays and are based on determination of β-glucuronidase activity. SM-Ethanol medium was used instead of supernatant as negative control and the values were set to one for comparison of induction factors after incubation for four hours. Significance is indicated with * and standard deviation was calculated from two independent experiments with eight technical replicates.

III 4 Heterogeneity in *Azoarcus* sp. cultures

The test for heterogeneity in *Azoarcus* sp. is important to answer the question how single cells behave in a bacterial culture or within a quorum sensing activated community. For *Vibrio harveyi* it could be shown that only 69 % of the cells of a population produced bioluminescence even at high cell densities (Anetzberger et al. 2009). In this strain bioluminescence is controlled via a quorum sensing regulated mechanism.

The reporter strain *Azoarcus* sp. BHΔ*pilS*::pJBLP1*gfp* carries a *gfp*-fusion to the QS target genes *pilAB*. It is possible to measure the *pilAB-gfp* gene expression of *Azoarcus* sp. at different growth stages and under QS conditions by detecting the fluorescence by fluorescence microscopy. It could be shown that the green fluorescence of GFP, and therefore the *pilAB* gene expression, of *Azoarcus* sp. cells was not homogenous (Figure 17). Only 40 % to 60 % of single cells in a culture showed green fluorescence when inspected by fluorescence microscopy. This result is very interesting as in general intracellular communication in bacteria is thought to be a synchronic behaviour of all members of the group. The observations in *Vibrio* sp. and *Azoarcus* sp. indicate that bacterial cultures form subpopulations. Unfortunately, this experiment could not be performed with the QS target gene *azo3874* as the gene expression and consequently the *gfp* expression was too low to be detected by fluorescence microscopy.

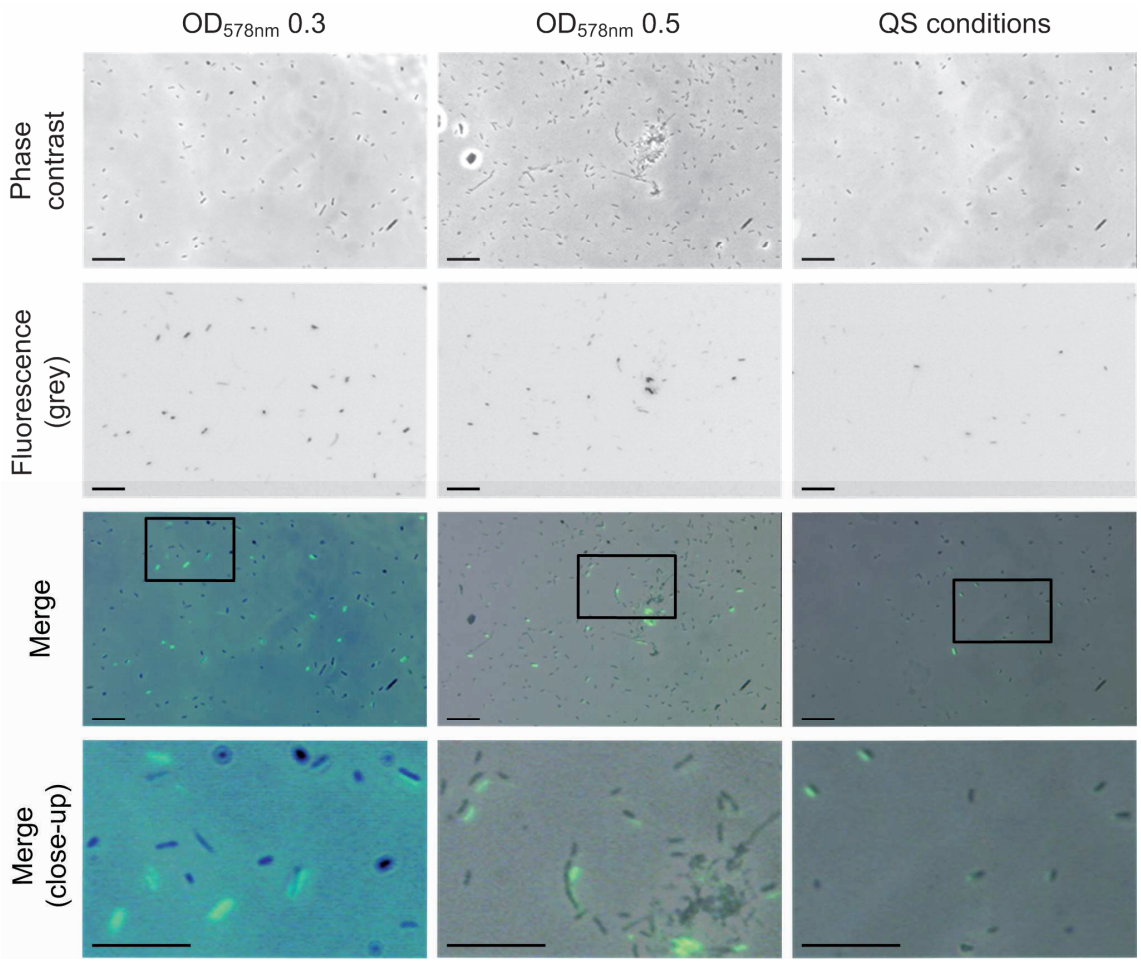

Figure 17: Heterogeneity of *pilAB-gfp* gene expression in cultures of *Azoarcus* sp. BHΔ*pilS*::pJBLP1*gfp* grown until the early exponential growth phase (OD_{578nm} 0.3), the exponential growth phase (OD_{578nm} 0.5) and under quorum sensing conditions (4 h) detected by fluorescence microscopy. Exposure time 0.08 s for phase contrast and 0.32 s for fluorescence, magnification 40 x 1.6 (ocular Plan-NEOFLUAR, Zeiss, long distance), bars = 10 µm.

III 5 The proteome of *Azoarcus* sp. BH72 under aerobic standard growth conditions

The term proteome evolved in the year 1995 and describes the entirety of all proteins in an organism. The proteome of a bacterial cell is subjected to extensive changes in protein expression patterns due to changes of environmental conditions, stress adaptation or culture conditions. Those alterations are controlled through complex regulatory processes. Many different experimental procedures were developed to study proteome changes and still proteomics is an upcoming field in molecular biology. In this study, gel-based methods like two-dimensional gel electrophoresis and gel-free approaches have been combined with mass spectrometry. Two-dimensional gel electrophoresis is a robust method to separate hundreds of proteins and subsequently analyze them by mass spectrometry. However, common limitations are isoelectric focus range, molecular mass (Mr) and the hydrophobicity of proteins. Therefore, it is prudent to combine gel-based approaches with gel-free studies like LC-MS/MS to increase the number of detected proteins and thereby enhancing the chance to identify low abundant proteins.

The construction of a proteome reference map was the first step of comparative proteomic studies of *Azoarcus* sp. BH72. It allowed subsequent approaches to discover basic and fundamental processes in this β-proteobacterium. The reference map is based on aerobic exponential growth in complex medium since such growth conditions were further applied to study the impact of quorum sensing on protein synthesis and gene expression in *Azoarcus* sp. BH72. In nature *Azoarcus* sp., being an endophyte of grasses, lives in a microaerobic habitat and therefore studies under nitrogen fixation are of high importance. The proteomic studies of Oetjen et al. (2009) show that the protein pattern, with respect to most abundant proteins and locations of proteins on 2D-gels, were comparable to the protein pattern described in this chapter.

III 5.1 Protein identification with gel-based and gel-free approaches

A proteome reference map with 431 protein spots was constructed and 140 spots corresponding to 126 different proteins were identified by MALDI-TOF-MS (Figure 18). With a complementing gel-free proteomic approach the number of detectable and identified proteins could be increased to 785 proteins. Almost all proteins, except twelve, detected with

the gel-based method were also covered by the gel-free approach. All identified proteins, their functions and detailed protein parameters are provided in Table B 2 and Table B 3 of Appendix B.

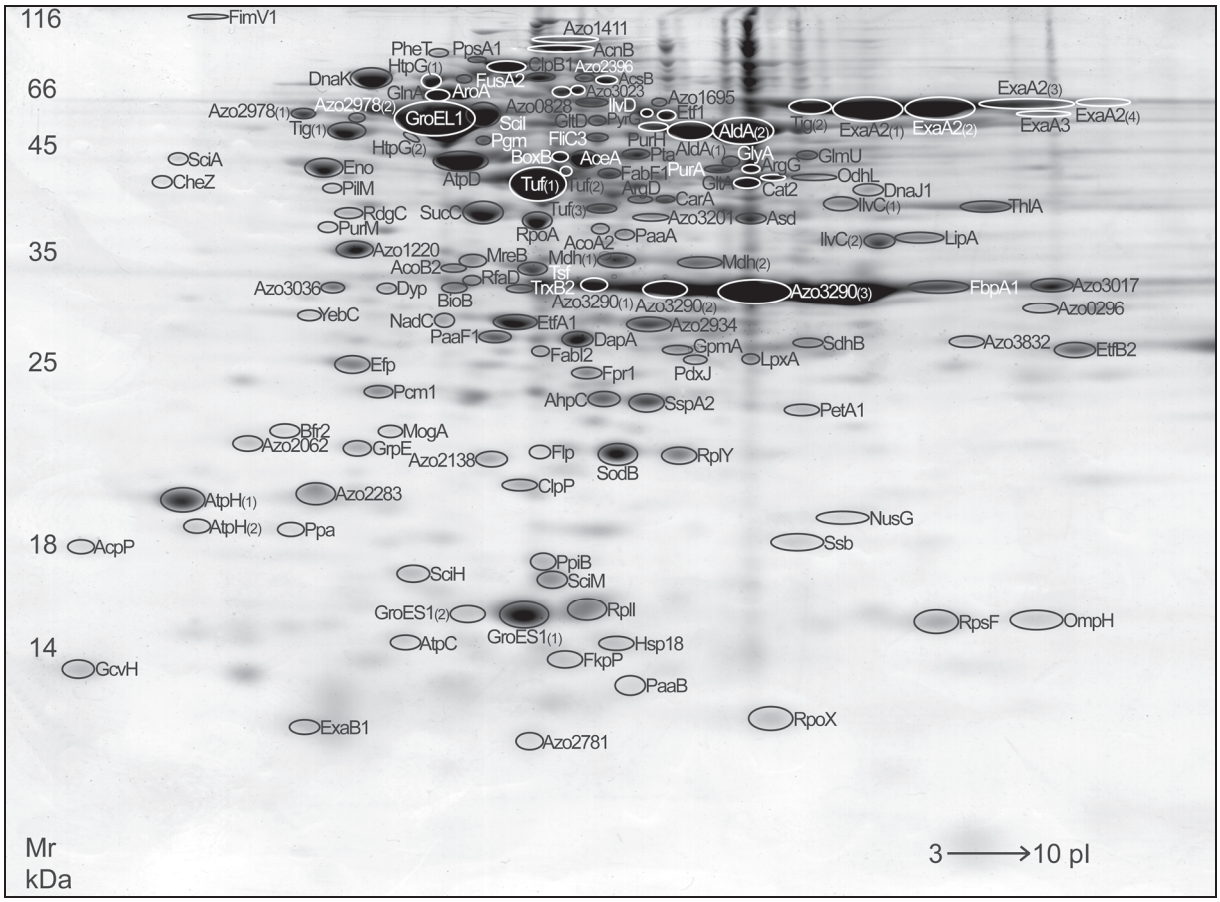

Figure 18: Proteome reference map of cellular proteins of *Azoarcus* sp. BH72. Cells were grown aerobically in VM-Ethanol-medium at 37℃. The indic ated proteins were identified by mass spectrometry and proteins represented by more than one spot are shown with numbers in parenthesis.

In general, the detected proteins in the reference gel covered the whole isoelectric point range and molecular weight spectrum, but the majority of protein spots could be observed between pI 5 and pI 6. In Figure 19 the theoretical distribution of the isoelectric point and the molecular weight of all *Azoarcus* sp. BH72 proteins in comparison to the identified proteins are displayed. Apparently, it was very difficult to detect proteins with high isoelectric points and with very high or low molecular masses with gel-based as well as gel-free methods.

Eleven proteins could be identified as different protein species in more than one spot of the reference gel (Figure 18 and Table B 2 of Appendix B). A major change in the protein mass of those multiple spots might result from different posttranslational modifications or proteolysis. In general, changes in the pI can be caused by modifications such as

phosphorylation (Büttner et al. 2001), methylation (Pethe et al. 2002), cysteine oxidation (Schmidt et al. 2006), glycosylation or acetylation (Görg et al. 2004) of proteins. Further studies are necessary to distinguish between those possible types of protein modification.

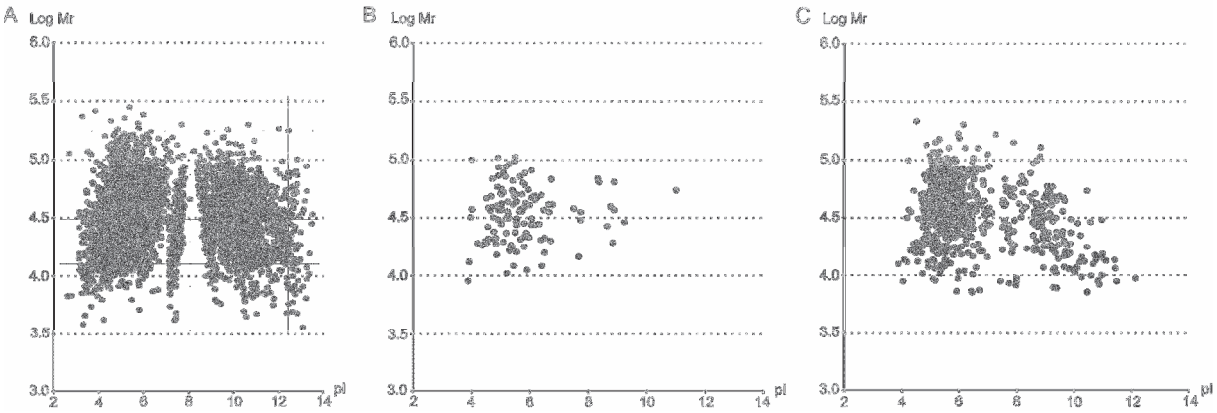

Figure 19: Plot of isoelectric point (pI) and molecular mass (Mr) of all *Azoarcus* sp. BH72 proteins (A) in comparison to proteins detected by two-dimensional gel electrophoresis (B) as well as gel-free (LC-MS/MS) studies (C). The molecular mass is displayed with logarithmic values of the respective mass in Dalton. All values are based on theoretical values obtained from genome annotation.

III 5.2 Most abundant proteins of *Azoarcus* sp. BH72

In Table 5 the 30 most abundant proteins of the protein reference map that were identified by MALDI-TOF-MS are listed, including proteins involved in energy metabolism such as AldA (aldehyde dehydrogenase), SucC (succinyl-CoA synthetase, beta subunit), Eno (enolase) and Mdh (malate dehydrogenase). General chaperones (GroEL1, GroES1, DnaK, HtpG), ribosomal proteins and enzymes for protein biosynthesis (RplI, TufA) were also found in high abundance, reflecting their fundamental importance for cell survival.

The most abundant protein that could be identified is one protein species of the outer membrane porin protein precursor Azo3290. The protein ExaA2, a putative quinoprotein ethanol dehydrogenase, was highly abundant under the conditions studied as ethanol served as the carbon source. With less abundance, another putative quinoprotein ethanol dehydrogenase (ExaA3) was also identified. Position six and ten on the most abundant protein list are held by the beta and delta subunit of the ATP synthase, AtpD and AtpH, respectively. The same observation has been made in *Rickettsia conorii* (Renesto et al. 2005), which substantiates the importance of oxidative phosphorylation required for ATP-synthesis, necessary to compensate for the lack of glycolysis.

Interestingly, two conserved hypothetical proteins are among the most abundant proteins (Azo1220 and Azo2934), further studies are essential to discover their biochemical function. So far, these proteins were annotated as a conserved hypothetical alkene monooxygenase (Azo1220) and a conserved hypothetical amino acid binding protein (Azo2934). In addition, the proteins AhpC (alkyl hydroperoxide reductase subunit C) and SodB (superoxide dismutase) seem to be crucial for *Azoarcus* sp. BH72, because all known aerobic bacteria synthesize enzymes that convert toxic superoxide (O_2^-) and hydrogen peroxide (H_2O_2) to harmless products.

Table 5: Most abundant proteins in the *Azoarcus* sp. BH72 proteome reference map.

Acc. No.[a)]	Protein[b)]	Protein description	Mean Volume in % ± SD[c)]
azo3290(3)		outer membrane porin protein precursor	15.10 ± 4.54
azo0974	GroEL1	chaperonin	11.48 ± 2.71
azo2939	AldA(2)	aldehyde dehydrogenase (NAD^+)	8.69 ± 3.18
azo2972	ExaA2(1)	quinoprotein ethanol dehydrogenase	7.08 ± 2.01
azo3419	TufA(1)	elongation factor Tu	6.10 ± 0.94
azo0159	AtpC	F_0F_1 ATP synthase subunit beta	3.79 ± 0.45
azo1063	DnaK	chaperone protein	3.35 ± 1.38
azo0973	GroES1(1)	chaperonin	3.32 ± 1.73
azo1425	HtpG(1)	heat shock protein 90	2.94 ± 1.29
azo0156	AtpH(1)	putative ATP synthase delta chain	2.57 ± 0.54
azo3333	SucC	succinyl-CoA synthetase subunit beta	2.50 ± 1.12
azo0721	RplI	50S ribosomal protein L9	2.43 ± 1.14
azo2072	Tig(2)	trigger factor	2.42 ± 1.01
azo2172	ThlA	acetyl-coa acetyltransferase	2.27 ± 0.73
azo2205	FbpA1	putative iron transport system substrate-binding protein	2.24 ± 1.46
azo1534	AcnB	aconitate hydratase	2.14 ± 0.44
azo3420	FusA2	elongation factor EF-G	1.97 ± 0.68
azo1466	SodB	putative superoxide dismutase	1.90 ± 0.31
azo2144	Eno	enolase	1.87 ± 0.66
azo0769	AhpC	alkyl hydroperoxide reductase. subunit C	1.86 ± 1.15
azo1554	GltA	citrate synthase	1.75 ± 0.76
azo0738	GlnA	glutamine synthetase I	1.66 ± 0.39
azo0963	SspA2	stringent starvation protein A	1.66 ± 0.49
azo1700	EtfA1	electron transfer flavoprotein subunit alpha	1.58 ± 0.44
azo1220		conserved hypothetical aromatic/ alkene monooxygenase subunit gamma	1.54 ± 0.53
azo3391	RpoA	DNA-directed RNA polymerase alpha chain	1.49 ± 0.96
azo2934		conserved hypothetical amino acid-binding protein	1.21 ± 0.33
azo1117	AceA	isocitrate lyase	1.14 ± 0.39
azo1096	DapA	dihydropicolinate synthase	1.13 ± 0.47
azo1547	Mdh(1)	malate dehydrogenase	0.80 ± 0.17

[a)] Acc. No. = accession number/locus tag of *Azoarcus* sp. BH72 genome
[b)] List only shows the most abundant proteins that were identified by mass spectrometry, numbers in parenthesis show the identified protein species of the proteins
[c)] Mean volume was calculated as follows: Spot volume/Sum of most abundant spots volumes x 100 of five replicate gels

III 5.3 Characterization of *Azoarcus* sp. BH72 protein parameters

III 5.3.1 GRAVY value analyses

The overall hydropathy of proteins is expressed by the GRAVY index (grand average of hydropathicity), which in general assigns positive values to hydrophobic proteins and negative GRAVY values to hydrophilic proteins. Figure 20 displays high differences in numbers for predicted and identified proteins within the given intervals. In agreement with the expectations, hydrophobic proteins are underrepresented in the gel-based approach, reflecting that these proteins do not easily enter conventional 2D-gels. The use of gel-free methods increases the number of hydrophobic proteins, but still the majority of detected proteins have negative GRAVY values.

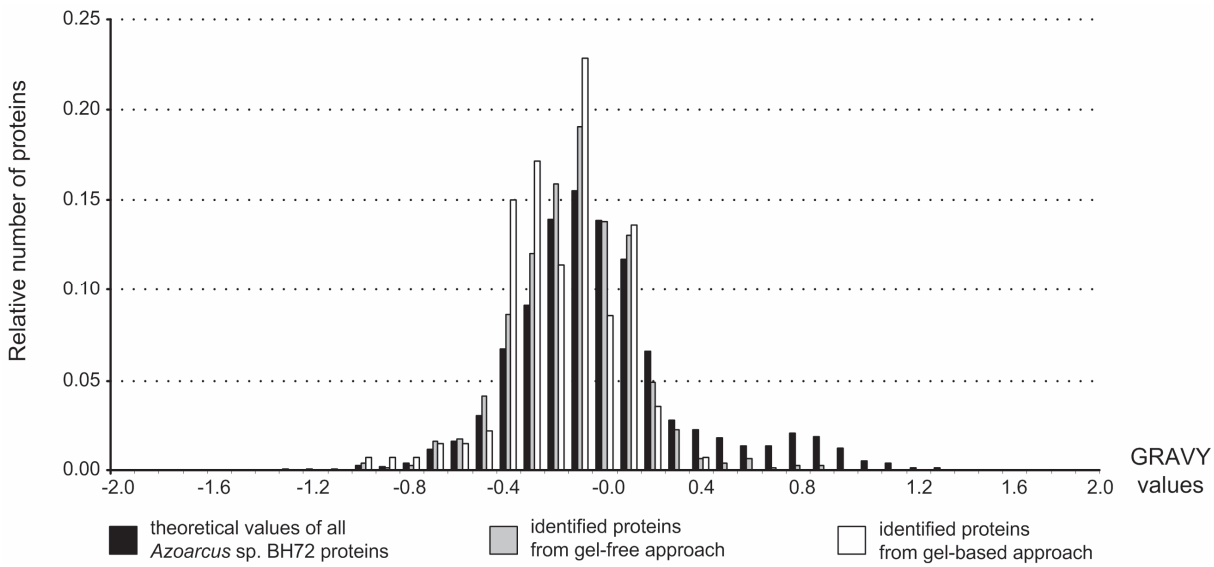

Figure 20: Hydropathy of the identified (grey and white) and predicted (black) proteins of *Azoarcus* sp. BH72. The GRAVY values were calculated according to Kyte & Doolittle 1982. The chart represents the number of proteins per interval divided by the total number of proteins.

III 5.3.2 Distribution of proteins according to their subcellular localization

The subcellular localization of proteins can be predicted by using the tool PSORTb v2.0. (Gardy et al. 2005). In this study, most of the proteins were predicted to occur in the cytoplasm (55.2 % and 48.6 %), but there were differences in the two detection methods. The partition of the proteins identified by gel-free proteomics was comparable to the theoretical localization profile of all predicted *Azoarcus* sp. BH72 proteins (Figure 21). Moreover, the percentage of proteins in the two proteomic studies that were predicted to occur extracellularly, in the outer membrane or in the periplasm is more or less equal to the

theoretical distribution of proteins. Proteins putatively localized in the cytoplasmic membrane were underrepresented in the gel-based approach (0.8 %). This reflects the observation that only few hydrophobic proteins could be detected by two-dimensional gel electrophoresis (see III 5.3). Around 35 % of the proteins were predicted to have an unknown localization site; most of those proteins did not show helix-turn-helix motifs, signal peptides or transmembrane helix domains, concluding that they might be cytoplasmatic too. The fact that the program PSORTb is designed for confident prediction rather than any prediction could explain the high number of unknown located proteins.

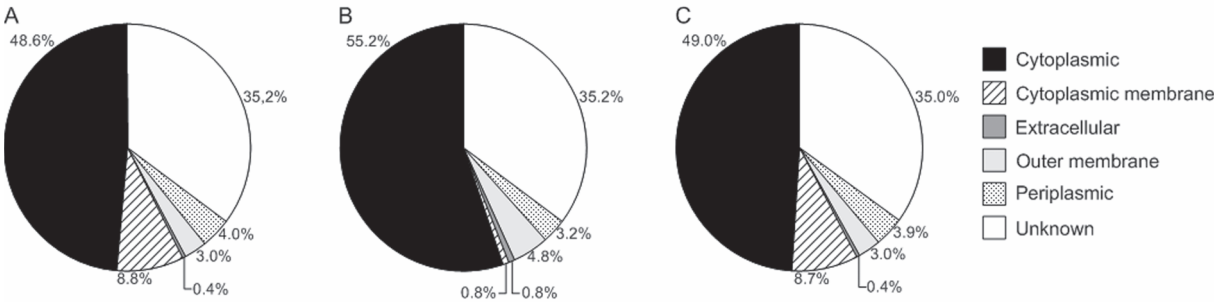

Figure 21: Distribution of the predicted subcellular localization of the identified proteins in the gel-free (A) and the gel-based (B) approach compared to the distribution of all *Azoarcus* sp. BH72 proteins (C). The localization site was predicted with PSORTb v2.0 according to Gardy et al. 2005.

III 5.3.3 Distribution of proteins according to COG

The identified proteins were classified according to their biological functions using the clusters of orthologous groups (COG) described by Tatusov et al. in 1997 and the experimental results were compared to the theoretical distribution of all predicted proteins from *Azoarcus* sp. BH72.

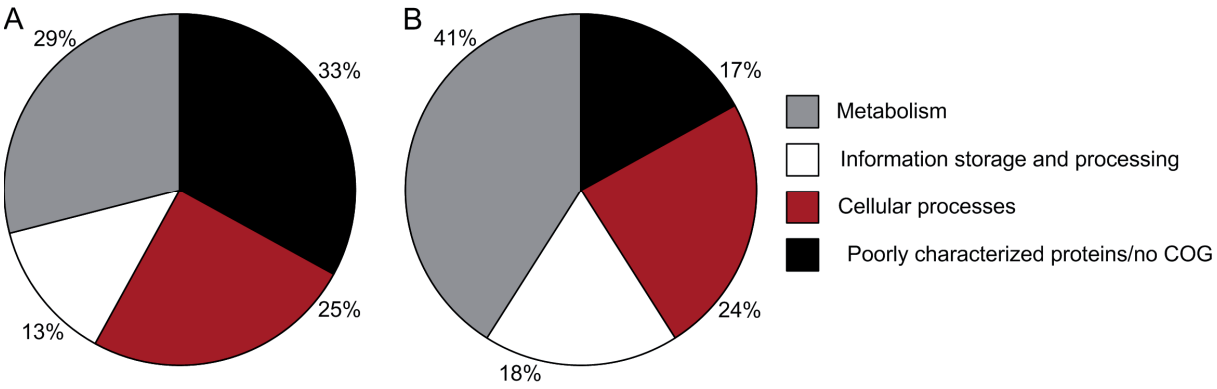

Figure 22: Distribution of proteins in COG-classes of gel-based and gel-free identified proteins (B) compared to the theoretical partition of all *Azoarcus* sp. BH72 proteins (A) according to the annotation of gene products.

The partition of 785 proteins in four classes is shown in Figure 22. In general, the theoretical partition of *Azoarcus* sp. proteins is comparable to the COG-classification of the identified proteins. Proteins involved in metabolic processes are overrepresented (41 %) in this approach, whereas poorly characterized proteins and such without COG-class are less abundant. Among the metabolic proteins, the ones involved in amino acid transport and metabolism (12 %) as well as energy production and conversion (10 %) are most important in *Azoarcus* sp. BH72 under the chosen growth conditions. In the class of information storage and processing the majority is held by proteins involved in translation, ribosomal structure and biogenesis (11 %).

III 5.3.4 Distribution of proteins according to metabolic functions

The EC numbers of the identified proteins give information about their functions as classified in the Kyoto Encyclopedia of Genes and Genomes (KEGG) for metabolic pathways. With respect to this, 325 of the proteins, detected by gel-based as well as gel-free methods, could be classified in 98 different pathways. Their importance for metabolic processes is shown in Figure 23.

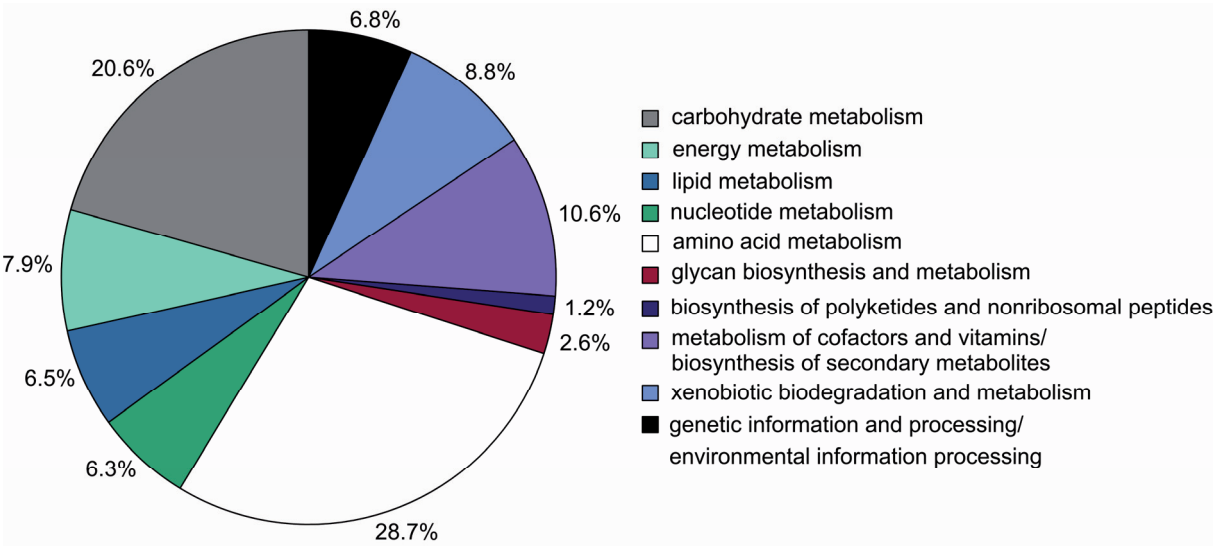

Figure 23: Distribution of proteins identified by gel-based and gel-free studies according to their metabolic functions in KEGG pathways.

Most of the detected proteins belong to housekeeping pathways such as amino acid metabolism (28.7 %) or carbohydrate metabolism (20.6 %). Moreover, proteins involved in metabolism of cofactors and vitamins and in biosynthesis of secondary metabolites (10.6 %) are also important for *Azoarcus* sp. BH72 under chosen growth conditions. 460 proteins could not be classified according to KEGG-classes, indicating lack of sufficient information for

classification or otherwise they might have another function beyond metabolism. Those proteins were mainly chaperones, ribosomal proteins or hypothetical.

Under aerobic growth of *Azoarcus* sp. BH72 in complex medium VM-Ethanol, proteins involved in different metabolic pathways were actively synthesized; a reconstruction of such pathways is depicted in Figure 24. Major carbon metabolism as well as biosynthesis and metabolism of fatty acids were also represented.

All enzymes of the tricarboxylic acid cycle could be identified in the proteomic study under the chosen growth condition. The enzyme converting glucose-6-phosphate to glucose (EC 2.7.1.2) in the gluconeogenesis/glycolysis pathway is lacking, although the genes coding for the protein glucokinase are present in *Azoarcus* sp. BH72 (*glk1* and *glk2*). Moreover, the 6-phosphofructokinase (EC 2.7.1 1, encoded by the *pfk*-gene) that is responsible for fructose-1,6-bisphosphate synthesis was missing among the detected proteins. Two enzymes 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) and the oleoyl-[acyl-carrier-protein] hydrolase (EC 3.1.2.14) of the fatty acid metabolism pathway could not be detected with this approach.

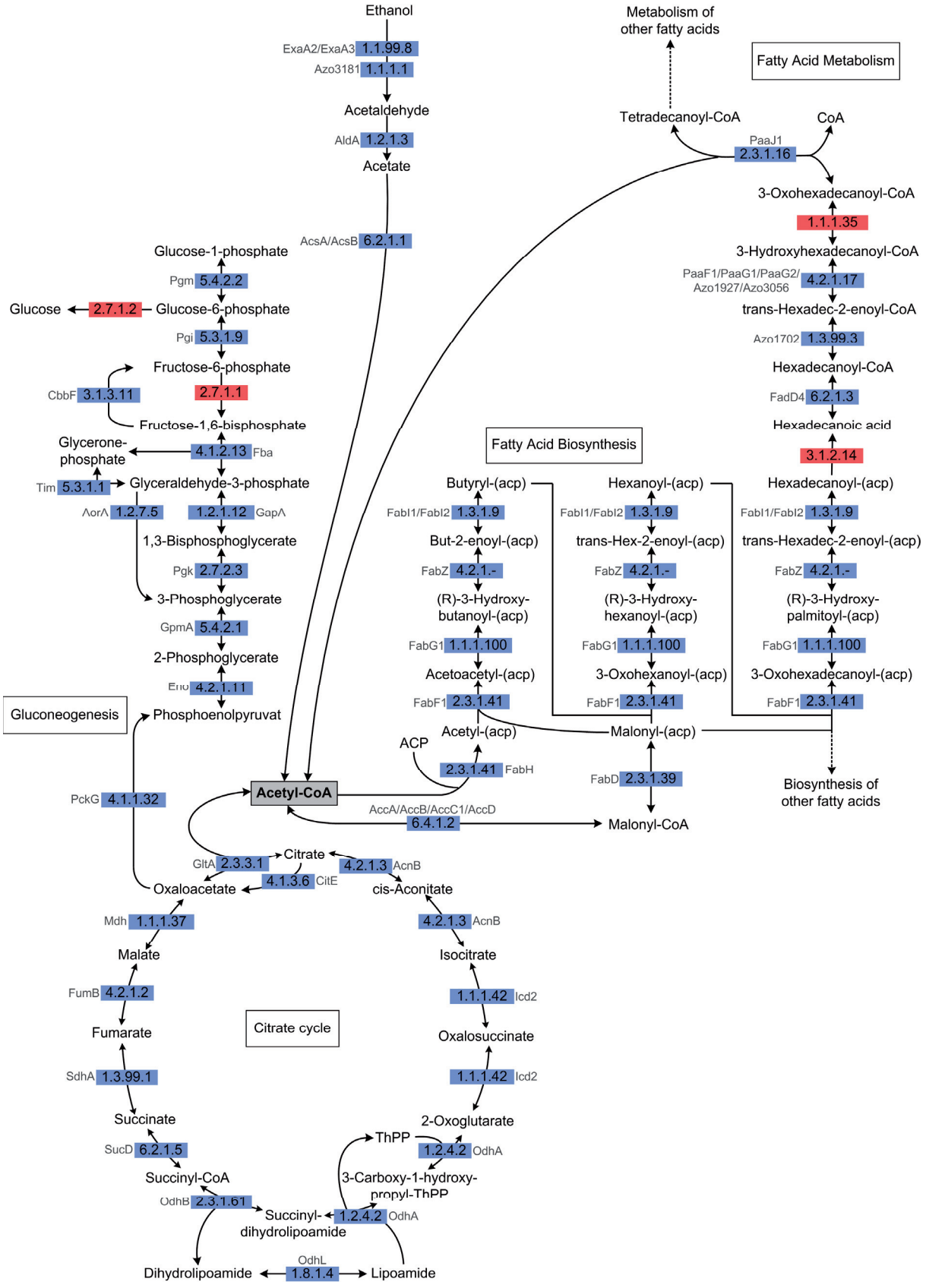

Figure 24: Reconstruction of different metabolic pathways (according to the Kyoto Encyclopedia of Genes and Genomes database, KEGG) detected in *Azoarcus* sp. BH72 in the exponential growth phase under aerobic conditions with ethanol as carbon source. Blue boxes indicate proteins with their respective EC-number identified with gel-based and gel-free approaches. Proteins with EC-numbers in red boxes could not be detected.

III 6 The *Azoarcus* sp. BH72 proteome under quorum sensing conditions

The proteome of *Azoarcus* sp. BH72 undergoes significant changes after incubation of cells grown until the early exponential growth phase with conditioned culture supernatant. But so far only few proteins that were shown to be differentially synthesized have been identified by mass spectrometry (Hauberg 2006). The reference map of the grass endophyte was useful to increase the number of identified proteins that are under QS control.

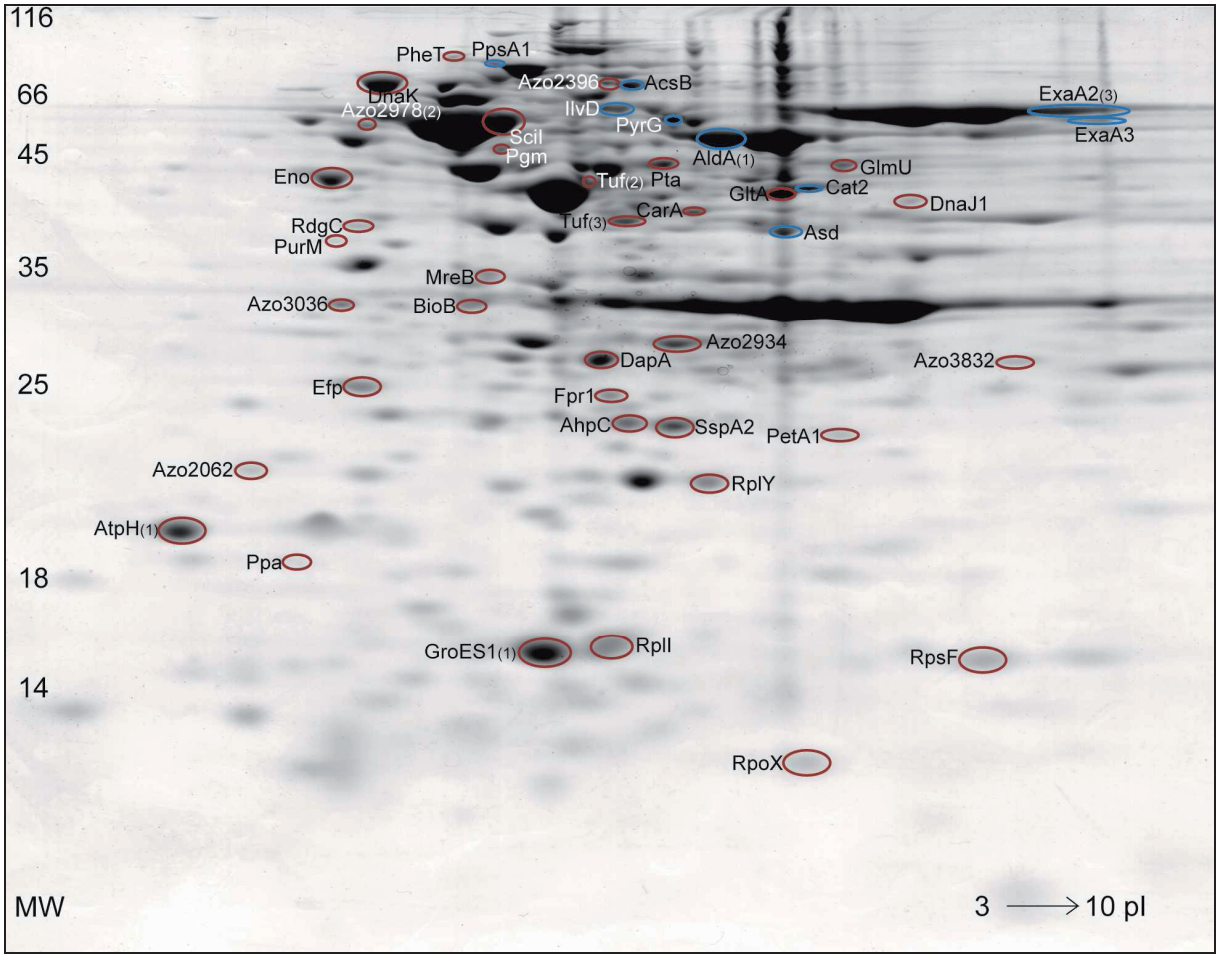

Figure 25: Protein pattern of *Azoarcus* sp. BH72 grown aerobically under standard conditions. Encircled spots have been analyzed by MALDI-TOF-MS and proteins that are marked with blue circles show increased intensity under quorum sensing conditions, red circles indicate proteins with lower levels.

To identify additional QS targets and to characterize the QS cascade in *Azoarcus* sp. BH72 the proteome of the strain grown in presence of the QS signal molecule and in the early exponential growth phase were compared. Therefore, two-dimensional gel electrophoresis

was performed and the protein patterns were compared with the Image master software package for 2D gel electrophoresis. Fold changes were determined from mean intensity values of four parallel gels each and only changes in intensity of at least 2.5 fold were taken into consideration. These experiments showed that 18.0 % of all detected proteins in the 2D-gels were under QS control, around 14.0 % showed decreased intensities (49 protein spots) whereas 4.0 % (12 protein spots) appeared to be present with higher intensities under QS conditions. Forty-four protein spots could be identified by mass spectrometry and proteins that were detected with different levels under QS conditions are displayed in the comparative protein gel (Figure 25).

Proteins that were increased in intensity under QS conditions are encircled in blue, the ones with lower protein levels are encircled in red. Several protein parameters such as GRAVY values, subcellular localization as well as the occurrence of signal peptides from the under QS conditions differentially synthesized proteins were analyzed and detailed protein information is listed in Table 6. It was surprising that much more proteins were repressed under quorum sensing conditions. It is possible that proteins were missed with the proteomic approach based on 2D-gels as the detection of proteins with high/low isoelectric points and very hydrophobic ones is difficult. Moreover, hypothetical secreted proteins could not be detected with the applied methods (see III 5.1 and III 5.3). The proteins that are under quorum sensing control will be discussed in more detail in chapter III 8.

Table 6: Detailed parameters and mass spectrometry data for quorum sensing regulated proteins in *Azoarcus* sp. BH72 discovered by MALDI-TOF-MS.

Acc. No.[a]	Protein	Fold	Score		Peptide	exp.[b]		the.[c]	SP[d]	GRAVY[e]	PSL[f]
			Protein	Ion	Match	Mr (Da)	pI	Mr (Da)			
azo0082	RdgC	-3.7	429	321	16	43448	4.7	36216	0	-0.258	C
azo0086	Efp	-9.1	446	318	15	29343	4.6	20730	0	-0.322	C
azo0156	AtpH	-4.6	120	56	9	18792	4.2	18803	0	0.121	C
azo0174	MreB	-6.9	580	347	26	36938	5.0	36659	0	0.146	C
azo0503	RpoX	-8.1	327	215	11	12124	6.4	12132	0	-0.486	C
azo0632	IlvD	3.2	167		20	70000	5.5	65693	0	-0.073	C
azo0718	RpsF	-7.6	420	319	12	14455	7.7	14464	0	-0.536	C
azo0721	RplI	-3.8	261	205	9	16299	5.3	16308	0	-0.126	C
azo0754	RplY	-2.9	630	450	19	21458	6.0	21471	0	-0.170	U
azo0769	AhpC	-2.5	360	272	11	27771	5.7	20595	0	-0.150	C
azo0960	PetA1	-15.8	68	16	7	26908	6.5	20987	1	-0.253	CM
azo0963	SspA2	-5.2	234	175	9	26025	5.9	23164	0	-0.249	C
azo0973	GroES1	-5.2	248	140	10	15832	5.2	10411	0	-0.194	C
azo1042	Asd	3.1	239	169	11	45002	6.0	40451	0	0.055	U
azo1062	DnaJ1	-7.9	367	296	15	49498	6.8	40839	0	-0.696	C
azo1063	DnaK	-2.7	921	749	26	68718	4.7	68760	0	-0.351	C
azo1084	PheT	-3.0	582	336	35	86215	4.9	85860	0	0.077	C
azo1096	DapA	-3.0	601	496	14	31860	5.6	30960	0	0.217	U
azo1280	Fpr1	-7.5	724	488	24	29524	5.6	29165	0	-0.293	U
azo1358	Ppa	-3.5	140	43	12	18714	4.6	19182	0	-0.052	C
azo1381	CarA	-5.0	172	115	11	45545	6.0	40432	0	0.051	U
azo1554	GltA	-4.2	313	176	22	46998	6.1	48295	0	-0.226	C
azo2062		-2.8	331	308	4	28525	4.5	19560	0	-0.235	C
azo2144	Eno	-2.7	761	614	21	45917	4.5	45945	0	-0.102	C
azo2146	PyrG	5.1	398	237	25	65682	5.8	63193	0	-0.170	C
azo2167	PpsA1	4.7	577	366	33	83498	4.9	103784	0	-0.097	C
azo2396		-3.3	163		18	79000	6.1	80001	0	-0.28	U
azo2414	AcsB	24.3	432	283	25	71528	5.7	71572	0	-0.170	C
azo2778	Pgm	-3.0	623	326	33	56483	5.0	49840	0	-0.101	U
azo2817	BioB	-5.2	301	243	11	35384	5.0	36900	0	-0.173	U
azo2934		-3.6	675	439	25	32719	6.1	30860	1	-0.018	U
azo2939	AldA	2.8	116	19	17	55086	6.0	55120	0	-0.032	C
azo2972	ExaA2	3.3	504	310	28	67939	8.4	67980	1	-0.437	P
azo2975	ExaA3	3.7	494	350	23	65000	8.9	64149	1	-0.291	P
azo2978		-4.3	647	425	33	62391	5.2	86888	1	-0.432	OM
azo3036		-3.2	544	496	10	35796	4.9	39983	0	0.078	U
azo3136	PurM	-3.4	633	529	15	42216	5.0	39223	0	0.154	U
azo3419	Tuf	-3.3	92		8	47048	5.2	43116	0	-0.148	C
azo3419	Tuf	-2.8	524	454	13	44783	5.2	43116	0	-0.148	C
azo3544	Cat2	6.5	571	399	22	51471	6.2	46381	0	-0.060	U
azo3637	GlmU	-4.0	496	305	25	54890	6.2	48486	0	-0.054	C
azo3638	Pta	-2.9	245	148	17	58711	5.8	50411	0	0.095	C
azo3832		-3.0	508	308	22	31000	9.2	28647	1	0.080	U
azo3896	SciI	-3.4	838	639	25	64787	5.0	55618	0	-0.457	U

[a] Acc. No. = accession number/locus tag of *Azoarcus* sp. BH72 genome
[b] exp. Mr (Da) = experimental molecular mass in Dalton
[c] the. Mr (Da) = theoretical molecular mass in Dalton and isoelectric point
[d] The index for grand average of hydropathicity (GRAVY) was calculated with ProtParam (http://www.expasy.ch/tools/protparam.html)
[e] The presence of signal peptides (SP) was predicted with SignalP 3.0 (Emanuelsson et al. 2007)
[f] The subcellular localization (PSL) was predicted with PSORTb v.2 (Gardy et al. 2005)
C = Cytoplasm, CM = Cytoplasmic membrane, OM = Outer membrane, P = Periplasm, U = Unknown

III 7 The *Azoarcus* sp. BH72 transcriptome under quorum sensing conditions

The transcriptome describes the set of all RNA molecules such as mRNA, rRNA, tRNA and non coding RNA and it reflects the genes that are expressed in one organism at a certain time point. Like the proteome, the transcriptome varies with many different environmental conditions. With the methods of transcriptomics like microarray or real-time PCR it is possible to study gene expression profiles. A 70mer oligonucleotide DNA microarray was developed by the transcriptomic facility CeBiTec (Bielefeld University) to analyse global gene expression under QS conditions and to detect new QS target genes in the grass endophyte *Azoarcus* sp. BH72. For the construction of the oligonucleotide microarray a collection of gene-specific oligonucleotide probes for the 3992 predicted protein-coding genes was spotted in quadruplicates on glass slides (see II 11.6).

For the transcriptomic study of *Azoarcus* sp. BH72 two different experiments (Table 7 and Figure 26) were performed for studying gene expression in the early exponential growth phase in comparison to gene expression under QS conditions.

Table 7: Experimental set-up for microarray experiments.

Experiment	Microarray slide	Incubation	QS condition	Early exponential	Comment
1	302725	1 h	Cy3	Cy5	
1	302942	1 h	Cy3	Cy5	
1	302943	1 h	Cy5	Cy3	dye flip
2	301713	4 h	Cy5	Cy3	
2	301715	4 h	Cy3	Cy5	dye flip
2	301721	4 h	Cy5	Cy3	

In detail, *Azoarcus* wild type was grown in 200 ml VM-Ethanol medium until the early exponential growth phase, 100 ml of this culture was harvested and the total RNA was isolated with a Hot-Phenol method as described in II 11.1. From the remaining culture 20 ml was diluted with 80 ml conditioned culture supernatant obtained from a stationary *Azoarcus* sp. BH72 culture (see II 5.3.1) and this “quorum sensing culture” was further incubated for one hour (experiment 1) or for four hours (experiment 2). Afterwards the cultures were harvested and the total RNA was isolated as described. Experiment 1 is based on three independent cultures from each growth conditions, whereas experiment 2 was performed with

two independent cultures. For the microarray hybridization cDNA was synthesized and subsequently labelled with cyanine fluorescent dyes Cy3 and Cy5. The cDNA targets from the samples that should be directly compared were hybridized together on one microarray slide and for experiment 1 and 2 three slides were used each (Table 7 and Figure 26). In summary, two experiments (1 h and 4 h) were performed with three independent microarray hybridizations each resulting in twelve technical spot replicates per experiment and gene.

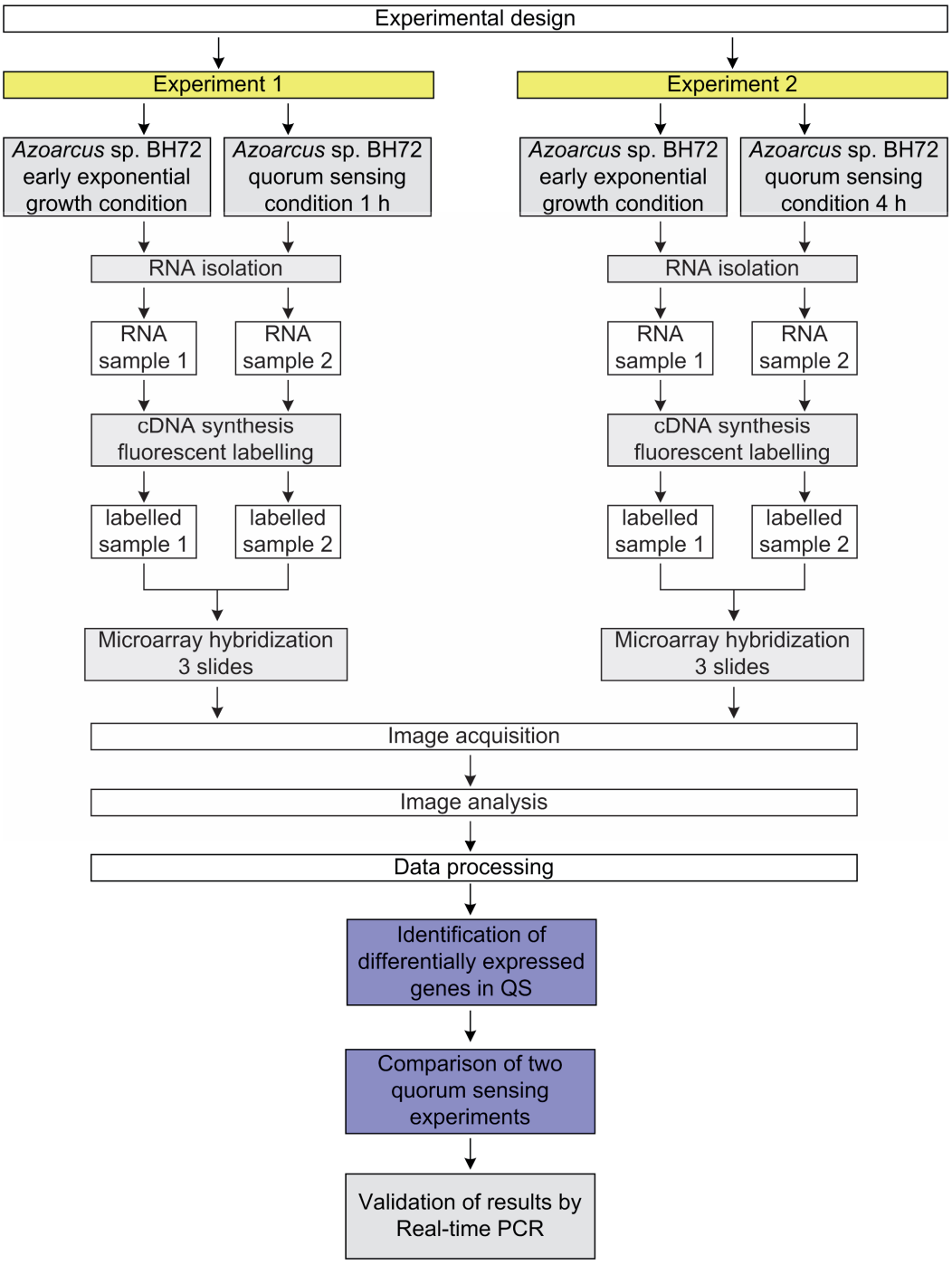

Figure 26: Experimental set-up of the genome-wide expression study of *Azoarcus* sp. BH72.

III 7.1 Genome-wide expression profile of *Azoarcus* sp. BH72

A whole genome microarray approach under standard growth conditions in comparison to quorum sensing conditions was applied. In general, it was expected that the expression of most of the *Azoarcus* sp. BH72 genes would not be affected under the studied growth conditions. The experiment revealed that the expression of around 10 % of all *Azoarcus* sp. BH72 genes was influenced by conditioned culture supernatant containing the unknown quorum sensing signal molecule. This clearly shows that quorum sensing is an important gene regulation process in this grass endophyte.

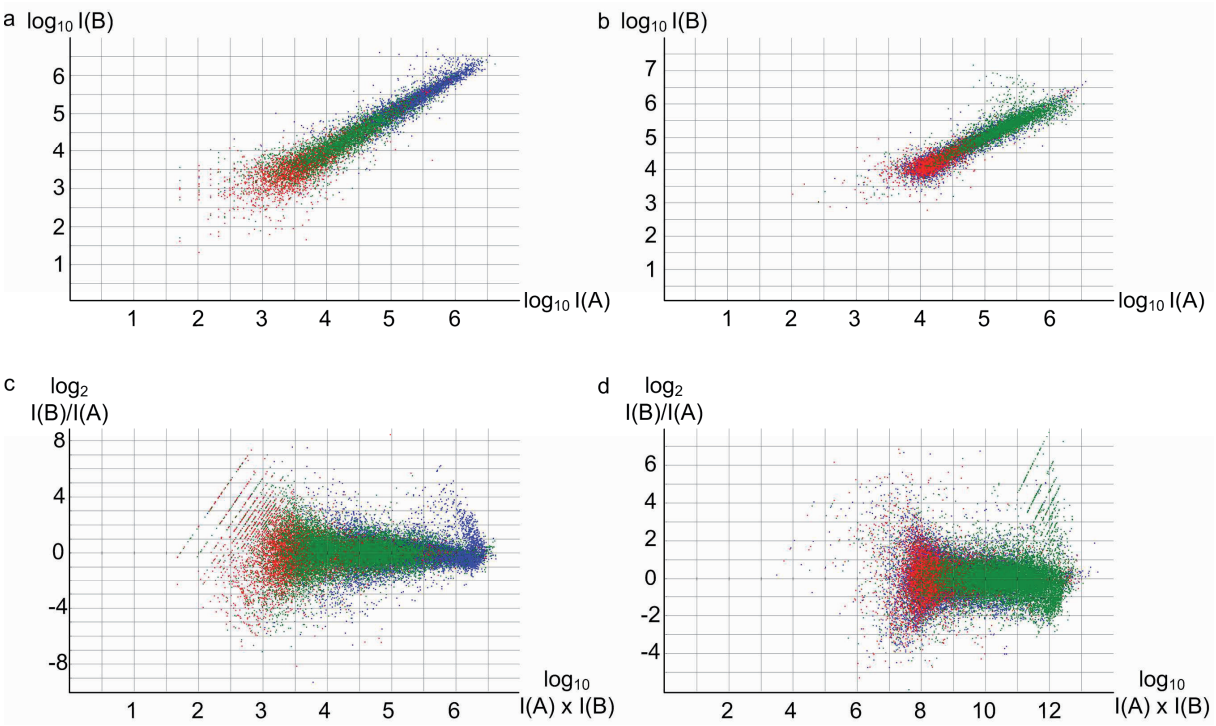

Figure 27: Schematic representation of the genome-wide transcriptional profiling of *Azoarcus* sp. BH72 grown under quorum sensing conditions for one hour (a and c) and for four hours (b and d). In the upper part the intensity graphs show the relation between the $\log_{10}$ intensity values (I) of channel A (Cy3) and channel B (Cy5) for the four replicate spots per gene in each slide. In the lower part the RI graphs are depicted for all spots in the microarray. For comparison of experimental replicates the spots on the three microarray slides are shown with different colours: In experiment 1 (1 h) blue dots represent slide 302725, red dots slide 302942 and green dots slide 302943. In experiment 2 (4 h) blue dots represent slide 301713, red dots slide 301715 and green dots slide 301721.

The scatter plots of the microarray experiments are depicted in Figure 27 and the relation between the $\log_{10}$ intensity values of the colour channel A (Cy3) and B (Cy5) for replicate spots per gene in a slide is shown in the upper panel. Every gene was spotted in quadruplicates within each microarray slide and in this graphs the intensity values of the four replicate spots are given as average intensities. As the intensity values of the two channels

(A and B) and not the intensity values of the two conditions (early exponential amd QS) are plotted, the fluorescent dyes that were used for labelling of cDNA targets are listed in Table 7.

In the lower part the RI graph is plotted based on $\log_2$ I(B)/I(A) versus $\log_{10}$ (I(A) x I(B)) data sets on the slides, where I(A) denotes the intensity value of each single spot in channel A (Cy3) and I(B) displays the respective values in channel B (Cy5). Most spots cluster around zero, which shows that their expression is not changed between the two tested conditions.

The gene expression intensity values in the early exponential growth phase compared to values from QS conditions for one hour are represented on the left (experiment 1, a and c). The three replicate slides for each experiment are shown in red (302942), green (302943) and blue (302725), respectively. The similar experiment with applying four hours QS conditions is depicted on the right (experiment 2, b and d) and the three replicates for each experiment are shown in red (301715), green (301721) and blue (301713), respectively.

Additional data are shown in Appendix A: Figure A 1 and Figure A 2 show the box graphs and intensity graphs of all independent microarray experiments separately. In this figures the LOWESS normalized data were compared to raw data in each microarray slide. Moreover, dye flip (see Table 7) experiments were performed to account for dye bias in microarray experiments and the results are depicted in Figure A 3. In Table B 4 of Appendix B M-values of all independent micrarray experiments are listed.

III 7.2 Monitoring differential gene expression of *Azoarcus* sp. BH72 grown under quorum sensing conditions

Genes were considered as being differentially expressed if the expression fold between the tested conditions was at least 1.8 and the P-value ≤ 0.05. From all regulated genes, the expression of 211 genes (5.3 %) was activated, whereas 191 genes (4.8 %) showed decreased expression under quorum sensing conditions. The expression of eight genes were repressed after one hour QS conditions whereas the expression of the same genes showed activation through quorum sensing after four hours incubation with conditioned culture supernatant.

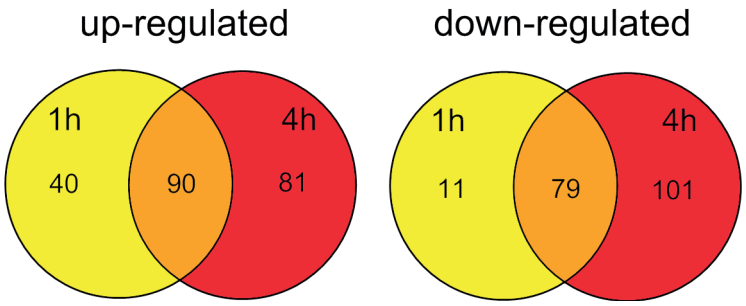

Figure 28: Venn diagram of quorum sensing regulated genes in *Azoarcus* sp. BH72 detected by microarray. The comparisons between experiment 1 (1 h) and experiment 2 (4 h) revealed 90 and accordingly 79 genes that were quorum sensing regulated at both time points.

Out of the up-regulated genes detected by microarray, 90 genes were found to be activated after one as well as four hours of incubation of *Azoarcus* sp. BH72 with conditioned culture supernatant, whereas the other 121 genes were only regulated at one of the two chosen incubation times. From all down-regulated genes, 79 genes showed repression after one and four hours applying quorum sensing conditions. However, the 112 remaining genes were only down-regulated at one of the time points (Figure 28). A list of all differentially expressed genes of Azoarcus sp. BH72 grown under QS conditions is provided in Table A 2 of Appendix B. A detailed description of such genes is listed in Table B 5 of Appendix B.

In general, it would be expected that nearly all genes in one operon show differential gene expression in the microarray approach after incubation of *Azoarcus* sp. cells with the conditioned culture supernatant containing the QS molecule. Indeed, this assumption can be observed in several gene clusters and five such clusters are depicted in Figure 29.

The *paa*-gene (E) cluster responsible for phenyl acetic acid degradation appeared to be down-regulated after one hour incubation with conditioned culture supernatant and up-regulated after four hours. The *atp*-operon (C), coding for the subunits of ATP synthase, as well as the *nuo*-cluster (A) encoding the NADH-ubiquione oxidoreductase chains was repressed under QS conditions at both time points. The *nap*-genes (D), coding for the subunits of the periplasmic nitrate reductase complex, were activated by quorum sensing. However, several genes that were down-regulated under QS conditions encode ribosomal proteins and proteins involved in general translation processes. Those genes have different localization sites in the *Azoarcus* genome and one huge cluster (B) is depicted. This cluster is delimited with three terminator structures, leading to four probable operons from which only three were

under QS control, namely the operons ranging from *azo3390* to *azo3422*, from *azo3425* to *azo3428* as well as from *azo3429* to *azo3431*.

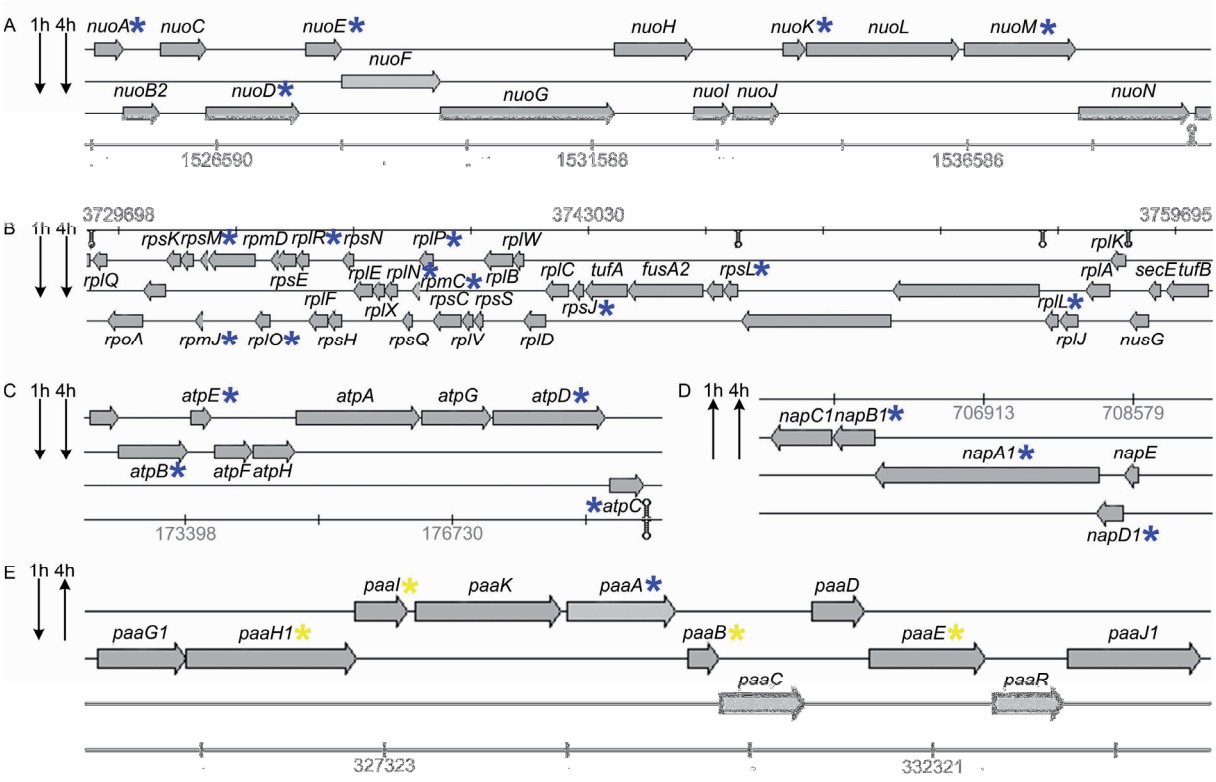

Figure 29: Quorum sensing regulated gene clusters in *Azoarcus* sp. BH72 identified by microarray. Activated clusters are depicted with arrows showing upwards and repressed clusters with arrows showing downwards detected at the distinct time point of one and four hours, respectively. Stars indicate that gene expression in the cluster was not changed after one hour (blue *) or four hours (yellow *) incubation with conditioned culture supernatant. Bold grey arrows refer to the direction of transcription with the respective gene name in black letters. Locations in the genome are depicted with grey numbers.

III 7.3 Validation of microarray results with real-time PCR

Some results obtained by the microarray approach were further examined with real-time PCR. The expression of seven genes that showed differential expression under QS conditions were analyzed with the 16S rRNA gene as a reference. Therefore, quantitative PCR with the fluorescent dye SYBR green I was performed and the $2^{-\Delta\Delta C}{}_T$ method was used for analyses of gene expression changes.

The fold induction factors of the seven examined genes obtained by real-time PCR analyses in comparison to the microarray results are depicted in Figure 30. Detailed quantitative PCR results such as C_T values and curves are shown in Table A 3 (C_T values and melting temperatures) and Figure A 4 (real-time PCR curves and melting curves) of Appendix A.

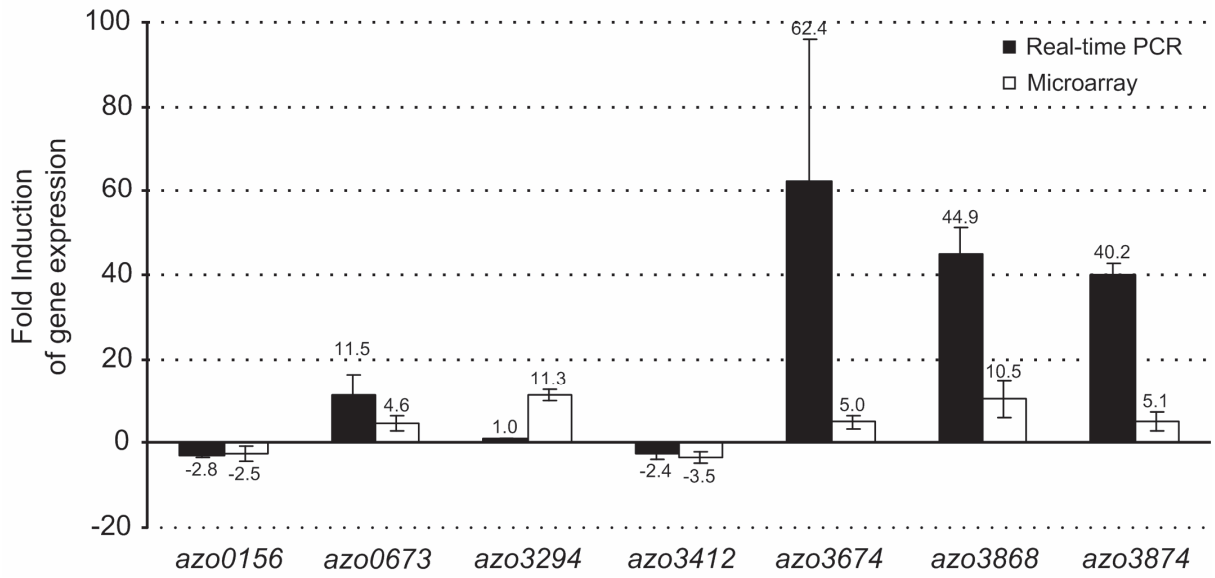

Figure 30: Comparison of fold induction factors of expression of several genes in the early exponential growth phase and under quorum sensing conditions detected by real-time PCR and microarray experiments. The expression ratios were calculated from gene expression studies with RNA isolated from cultures grown under quorum sensing conditions for one hour (*azo0156*, *azo3674*, *azo3868* and *azo3874*) and four hours (*azo3412*, *azo3294*, and *azo0673*).

The real-time PCR results could prove that the genes *azo0156*, coding for the δ-subunit of ATPase, and *azo3412*, encoding the ribosomal protein L22P, were indeed down-regulated under QS conditions with the factors -2.8 ± 0.8 and -2.4 ± 1.3, respectively. Moreover, the genes *azo0673* (11.5 ± 4.6), *azo3674* (62.4 ± 33.5), *azo3868* (44.9 ± 6.2) as well as *azo3874* (40.2 ± 2.5) were found to be up-regulated under the mentioned growth conditions. The gene *azo3294* (1.0 ± 0.1) was not differentially expressed under QS conditions as detected by real-time PCR studies, whereas its expression was shown to be highly up-regulated in the microarray approach.

The fold induction factors obtained by microarray and real-time experiments show differences to some extent. This observation was not unexpected since a bias towards underestimating the magnitude of mRNA change has previously been described for oligonucleotide microarray data (Yuen et al. 2002). The direction of regulation agreed between the results detected by microarray and quantitative PCR, this substantiates the cDNA microarray technology. Accordingly, these results clearly demonstrate that the used microarray was applicable for monitoring gene expression changes in *Azoarcus* sp. BH72.

III 8 Gene expression and protein synthesis of *Azoarcus* sp. BH72 are altered under quorum sensing conditions

The combination of transcriptomic and proteomic approaches revealed that growth of *Azoarcus* sp. BH72 under quorum sensing conditions had a big impact on gene expression and protein synthesis. All together, 440 genes or proteins of *Azoarcus* sp. BH72 were regulated under QS conditions and the heat map of regulation is shown in Figure 31.

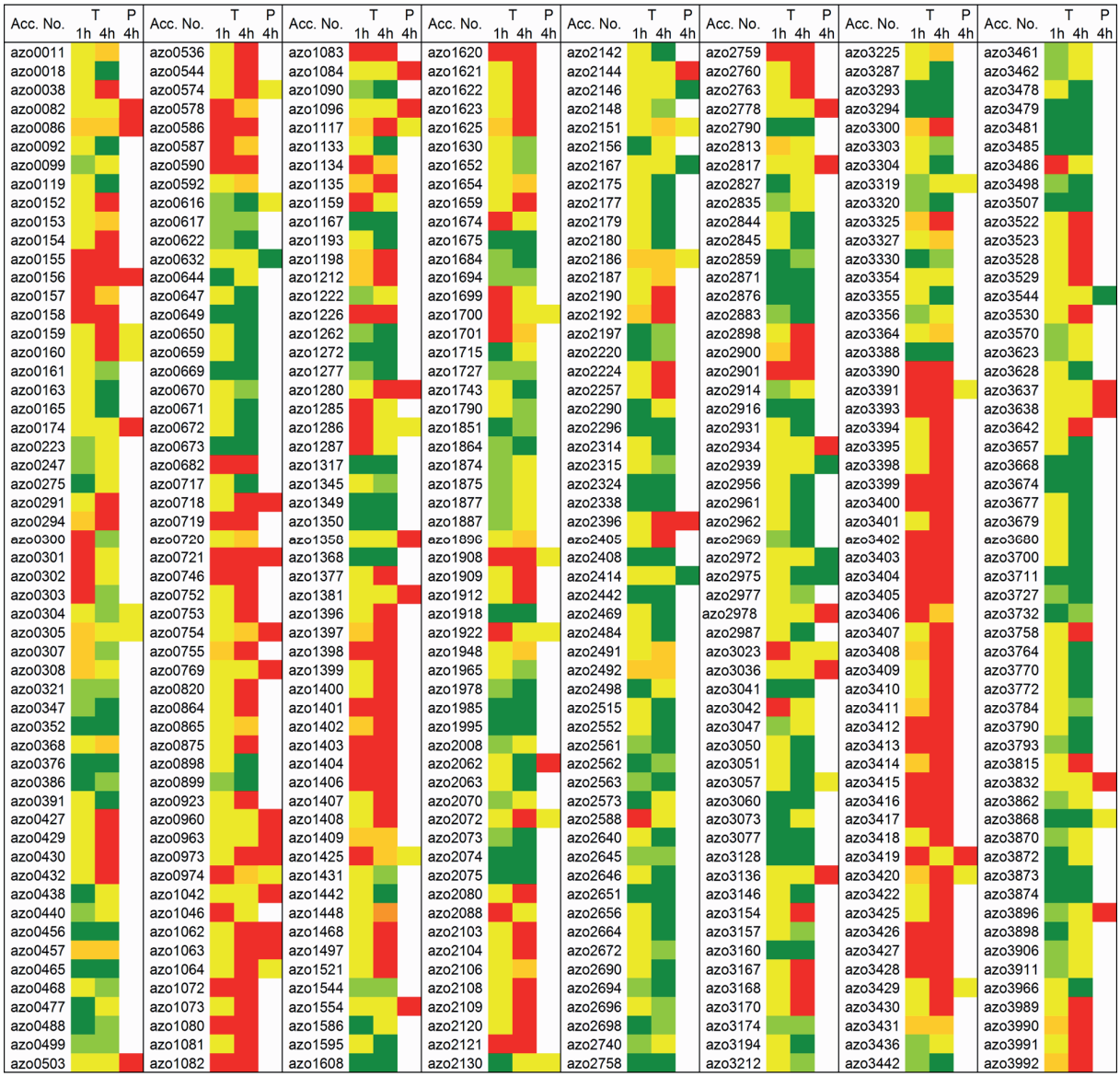

Figure 31: Comparison of quorum sensing regulation in *Azoarcus* sp. BH72 discovered by transcriptomic (T) and proteomic (P) approaches. Colours indicate the fold-change in gene expression or protein synthesis, respectively. Colour code: light green = ≥ +1.8, dark green ≥ +2.0 for transcriptomic study and ≥ +2.5 for proteomic study, orange ≤ -1.8, red ≤ -2.0 for transcriptomic study and ≤ -2.5 for proteomic study, yellow = no change, white = not detected in 2D-gels).

Only eleven of the proteins that showed lower synthesis levels under quorum sensing conditions in the 2D-gels (azo0086, azo0156, azo0718, azo0721, azo0754, azo0973, azo1062, azo1063, azo1280, azo2396, azo3419) were also down-regulated on the gene expression level as depicted in Figure 31 and Figure 32. Some discrepancies between the two approaches appeared: The genes *azo2062*, *azo3628* and *azo3896* were found to be up-regulated in the transcriptome approach, whereas the corresponding proteins showed decreased synthesis under QS condition in the protein gels.

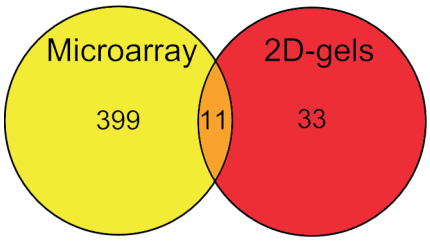

Figure 32: Venn diagram of quorum sensing regulated genes/proteins in *Azoarcus* sp. BH72. 410 genes were detected to be differentially expressed in the transcriptome, whereas 44 proteins could be identified to be differentially synthesized under quorum sensing conditions in two-dimensional gels. The comparison revealed 11 quorum sensing regulated genes/proteins in both approaches.

III 8.1 Different cellular processes are under quorum sensing control

COG-categories are used for characterization of proteins to gain better insights into their functions. This categorization was performed for quorum sensing regulated gene products and proteins that were detected by microarray and 2D-gels. In Figure 33 it is depicted that several cellular processes were affected by density dependent mechanisms in *Azoarcus* sp. BH72. In general, energy production and conversion (C) as well as translation, ribosomal structure and biogenesis (J) were down-regulated under QS conditions. Moreover, hypothetical proteins (R, S and no COG) and signal transduction mechanisms (T) appeared to be up-regulated after incubation with conditioned culture supernatants.

III 8.1.1 Replication and transcription factors

Five proteins that play a role in replication were influenced by conditioned culture supernatant and nine transcription factors were found to be quorum sensing regulated: The sigma-38 (RpoS), sigma-24 (AlgU), SigZ as well as the sigma-54 (RpoN2) RNA polymerase sigma factors were around 2.0-fold up-regulated under QS conditions, whereas RpoA (DNA-directed RNA polymerase, α-subunit) was after one hour incubation with conditioned culture supernatant -3.4-fold repressed. Moreover, the transcription accessory protein Tex and

the antitermination factors NusA and NusG were also down-regulated under QS conditions after four hours (-2.1-, -2.8- and 2.1-fold).

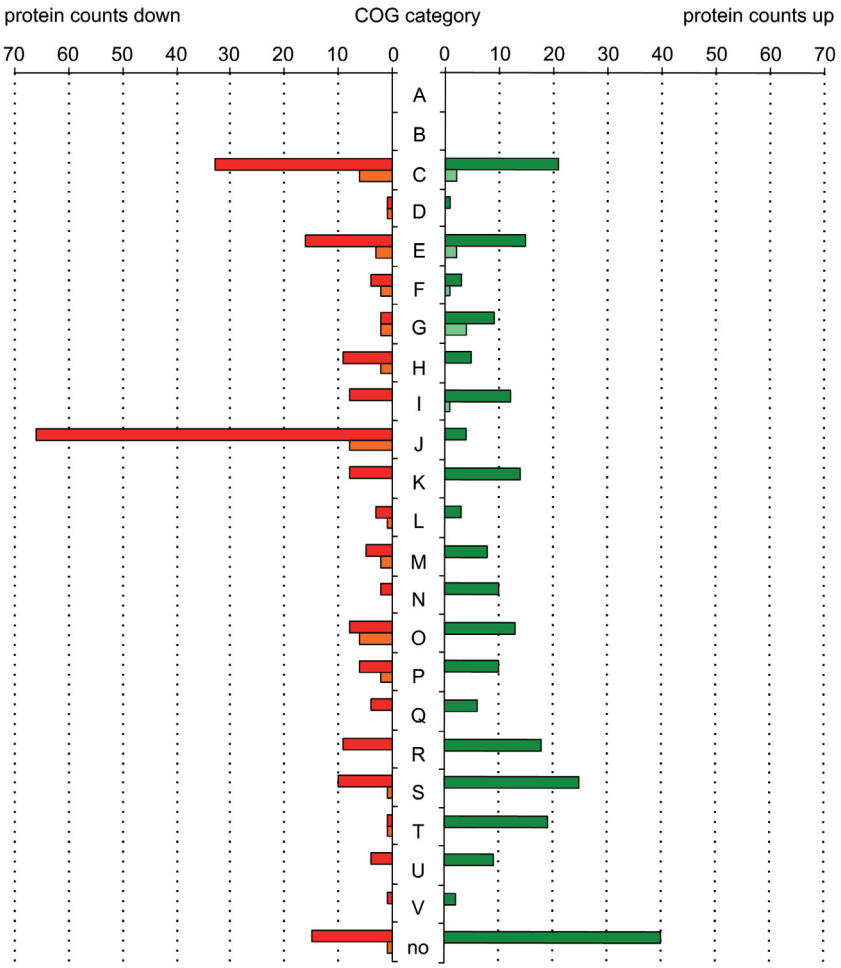

Figure 33: Distribution of quorum sensing regulated proteins detected by two-dimensional gel electrophoresis (orange and light green bars) and gene products identified by microarray (red and dark green bars) in COG-categories, absolute protein counts are shown. Activation due to quorum sensing is indicated with “up” and repression with “down”. A: RNA processing and modification, B: Chromatin structure and dynamics, C: Energy production and conversion, D: Cell cycle control, mitosis and meiosis, E: Amino acid transport and metabolism, F: Nucleotide transport and metabolism, G: Carbohydrate transport and metabolism, H: Coenzyme transport and metabolism, I: Lipid transport and metabolism, J: Translation, K: Transcription, L: Replication, recombination and repair, M: Cell wall/membrane biogenesis, N: Cell motility, O: Posttranslational modification, protein turnover, chaperones, P: Inorganic ion transport and metabolism, Q: Secondary metabolites biosynthesis, transport and catabolism, R: General function prediction only, S: Function unknown, T: Signal transduction mechanisms, U: Intracellular trafficking and secretion, no: not in COG.

III 8.1.2 Translation, ribosomal structure and biogenesis

Many proteins that are involved in translation processes were under quorum sensing control, among that the bacterial translation initiation factor 3, elongation factors such as Efp, Tsf, TufA and TufB. Fifty-one out of 53 genes coding for ribosomal proteins were found to be repressed under QS conditions in the *Azoarcus* sp. BH72 microarray. Three of these proteins

(RpsF, RplI and RplY) were also decreased in spot intensity in the 2D-gels. The fact that only few ribosomal proteins could be detected with the proteomic approach is probably due to the high pI, ranging from 10 to 11, of such proteins.

III 8.1.3 Signal transduction and regulatory systems

Several proteins that are involved in regulatory processes such as the putative serine threonine kinase PrkA or the two component response regulators PilR, RagA and FlcA were affected by quorum sensing in *Azoarcus* sp. BH72. Interestingly, the genes coding for the ferric uptake regulator (*azo0644*), the nickel responsive regulator (*nikR*, *azo3128*) and the phosphate regulon sensor proteins PhoR as well as the phosphate uptake regulator PhoU were around 2- to 3-fold up-regulated under QS conditions in the microarray approach.

III 8.1.4 Energy metabolism

The inhibitory influence of the conditioned culture supernatant was particularly obvious for energy metabolic processes, for example all subunits of the ATP synthase were down-regulated. Furthermore, almost all factors from the Nuo-cluster, responsible for the formation of the NADH dehydrogenase complex in the respiratory chain, were found to be repressed and six electron transfer flavoproteins (EtfA1, EtfA2, EtfB1, EtfB2, EtfB3 and Etf1) were also negatively QS regulated.

III 8.1.5 Carbohydrate and amino acid transport and metabolism

Quorum sensing also seemed to be involved in amino acid and carbohydrate metabolism, because 30 proteins that are somehow involved in amino acid transport and metabolism were activated as well as repressed by incubation of *Azoarcus* sp. BH72 with conditioned culture supernatant. Furthermore, fifteen carbohydrate transport and metabolism proteins such as ExaA1, ExaA2 and ExaA3, encoding ethanol dehydrogenases, were up-regulated under quorum sensing conditions.

III 8.1.6 Posttranslational modification and chaperones

The chaperones GroES1, GroEL1, DnaJ1, DnaK and HtpG and also the heat shock proteins GrpE and Hsp15 as well as the cold shock proteins CspA and CspE were repressed under the influence of conditioned culture supernatant. Moreover, the serine proteases MucD1 and MucD4 appeared to be activated under quorum sensing conditions.

III 8.1.7 Type IV pili and flagella

Nine proteins encoded in the *Azoarcus* sp. *pil*-clusters were found to be activated under QS conditions: the putative type IV pili biogenesis proteins Azo1608, PilY1A and PilW, the prepilin like proteins Azo2179, Azo2180 and PilV, the type IV pilus assembly protein PilX as well as the twitching motility protein PilU1. The QS target *pilA* was also detected among the regulated genes in the microarray, with a fold expression of 2.9 after four hours incubation with conditioned culture supernatant. Furthermore, genes encoding the flagellar motor protein MotA2 and FlgB, a protein of the basal-body were also controlled by quorum sensing.

III 8.1.8 Iron metabolism and storage

The bacterioferritins Bfr1 and Bfr2 and the bacterioferritin associated ferredoxin Bfd were activated under QS conditions. Interestingly, four TonB-dependent receptors were also regulated under QS conditions: Azo2156 was activated wherease Azo2396, Azo2978 and Azo3023 were repressed under QS conditions in *Azoarcus* sp. BH72.

III 8.1.9 Oxidative stress response

The two bacterioferritin comigratory proteins Bcp1 and Bcp2 showed activation after growth under quorum sensing conditions. Moreover, the alkyl hydroperoxide reductase AhpC, the organic hydroperoxide resistance protein Ohr and the thioredoxin disulfide reductase TrxC2 were also found to be QS regulated.

III 8.1.10 Secondary metabolites biosynthesis, transport and catabolism

Interestingly, several genes from the *paa*-cluster with genes coding for phenylacetic acid degradation proteins (*paaA*, *paaB*, *paaD*, *paaE* and *paaI*), phenylacetate-CoA ligase (*paaK*), 3-hydroxyacyl-CoA dehydrogenase (*paaH1*) and enoyl-CoA hydratase (*paaG1*) were regulated under QS conditions. In this case the microarray experiments revealed that the genes were mainly down-regulated after one hour and up-regulated after four hours incubation with conditioned culture supernatant.

III 8.1.11 Hypothetical and poorly characterized proteins

Interestingly, more than hundred proteins that were under quorum sensing control belonged to the group of (conserved) hypothetical proteins or proteins that were poorly characterized. Some of those proteins were highly regulated such as the conserved hypothetical membrane

protein Azo2876 and the hypothetical secreted protein Azo0456 that showed 6.8-fold as well as 13.1-fold expression after four hours of incubation with conditioned culture supernatant, respectively. Moreover, the gene expression of *azo1684* was 10.9-fold up-regulated in the microarray approach, the gene product showed a domain that is required for attachment to host cells in *Agrobacterium tumefaciens* and is therefore of high interest in the plant-associated bacterium *Azoarcus* sp. BH72.

Interestingly, thirteen (conserved) hypothetical secreted proteins were found to be activated under QS conditions. Protein blast analyses (see Table B 6 of Appendix B) revealed that Azo0223, Azo0275, Azo0347, Azo2962, Azo3478 and Azo3784 show only poor sequence similarities to known bacterial proteins and these proteins were therefore of high interest. The genome of *Azoarcus* sp. BH72 has been sequenced and annotated several years ago. New blast analyses showed that to some hypothetical proteins new functions could be assigned. These blast analyses might be helpful to further characterize hypothetical proteins.

III 9 Role of the response regulator PilR in the quorum sensing system of *Azoarcus* sp. BH72

The response regulator PilR is responsible for activation of *pilAB* gene expression. As the genes *pilAB* are essential for endophytic colonization in *Azoarcus* sp., a characterization of the PilR regulon might reveal further insights in proteins required for this process. The *pilAB* genes are also regulated through quorum sensing in *Azoarcus* sp. BH72 and *pilR* gene expression itself is slightly activated after induction with conditioned culture supernatant. Therefore, it is likely that the response regulator PilR is involved in the QS cascade and it seems to be promising to perform comparative studies with the *Azoarcus* wild type and the regulatory mutant BH*pilR*K.

III 9.1 Comparison of protein patterns of the wild type strain BH72 and the regulatory mutant BH*pilR*K

The proteome reference map was utilized to comparatively profile the wild type strain and the regulatory mutant *Azoarcus* sp. BH*pilR*K with two-dimensional gel electrophoresis. In addition, the gel-based approach was complemented with a gel-free study to increase the number of proteins that were shown to be differentially synthesized in the *pilR*-mutant. For the gel-free study the ratio data were merged with the peptide information. Proteins were considered to be influenced by the *pilR* mutation when one of the peptides was unique for the protein, the regulation factors had to be $\geq$ 1.5-fold and the P-value of the t-test had to be $\leq$ 0.01. The gel-free study is based on three independent cultures from each strain.

For the gel-based approach 2D-gels were constructed and the spot intensity was compared between all parallel gels with the software package for 2D-gel electrophoresis analysis Image master. Fold changes were determined from mean intensity values of six parallel gels each and only changes in intensity of at least 2.5 fold were taken into consideration.

All together, 26 protein spots in the 2D-gels showed higher intensities in the mutant strain BH*pilR*Kwhereas 24 proteins spots appeared to be present with lower intensity (Figure 34). Not all protein spots were analyzed by MALDI-TOF-MS, but 21 proteins could be identified, so far.

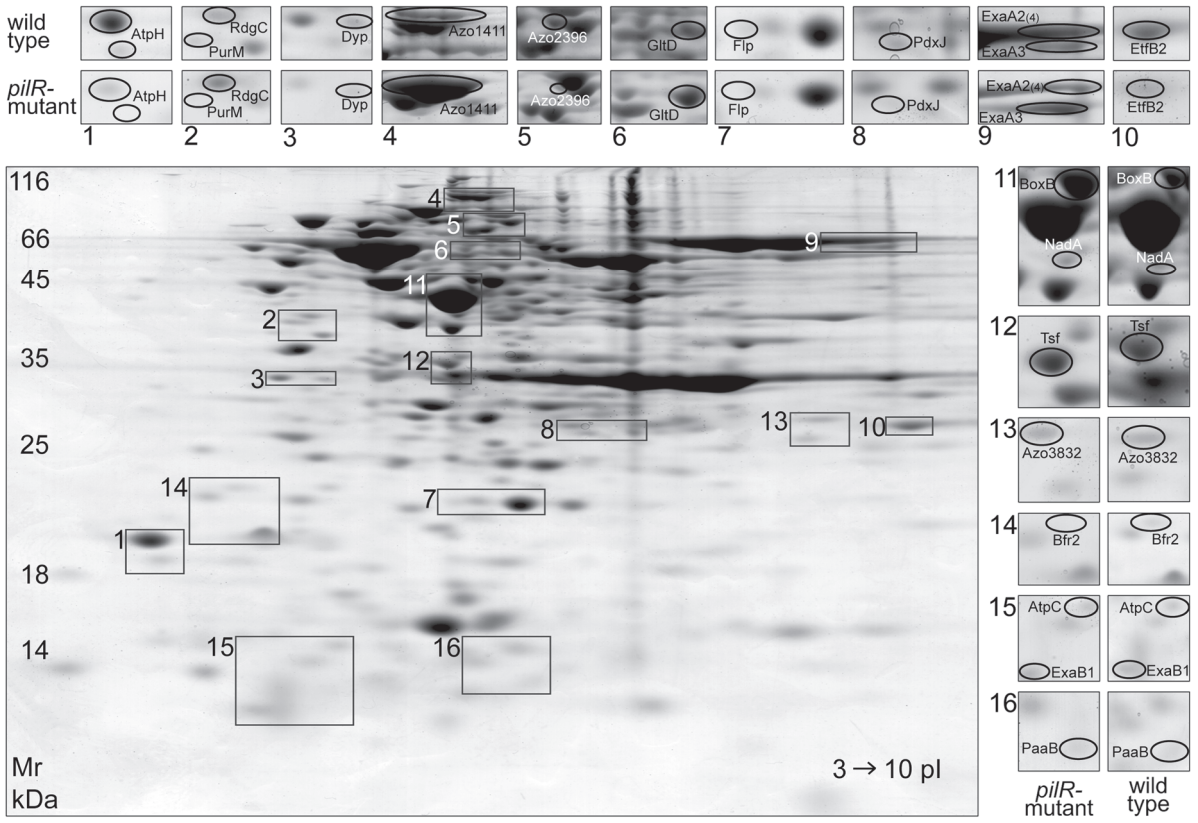

Figure 34: Comparative two-dimensional protein gels of total cellular proteins of *Azoarcus* wild type and the mutant BH*pilR*K grown under standard conditions to the exponential growth phase. The wild type proteome pattern is shown with detailed sections (numbered) of those proteins which were differentially synthesized in the *pilR*-mutant. Numbered close-ups show proteins of strainBH72 and BH*pilR*K.

In addition, only 61 out of 773 proteins detected by LC-MS/MS analyses were considered to be differentially synthesized in the mutant strain *Azoarcus* sp. BH*pilR*K. In Figure 35 the correlation plot of 28 up-regulated and 33 down-regulated proteins in the PilR regulatory mutant is shown. In the correlation plot the LC-MS/MS intensity values of all detected proteins of *Azoarcus* sp. BH72 are compared to the values of the mutant strain BH*pilR*K. Most of the proteins were constitutively synthesized by means of regulation factors below the treshold of 1.5-fold. Red crosses represent proteins with higher synthesis rates in strain BH*pilR*K; whereas green crosses indicate proteins with lower synthesis rates in the *pilR*-mutant.

The analyses revealed that the inactivation of *pilR* did not dramatically change the protein pattern in general. The amount of regulated proteins showed that PilR is likely to participate in transcriptional activation as well as repression. In Table 8 the proteins which were detected with significantly different levels in the wild type strain and the *pilR*-mutant are displayed.

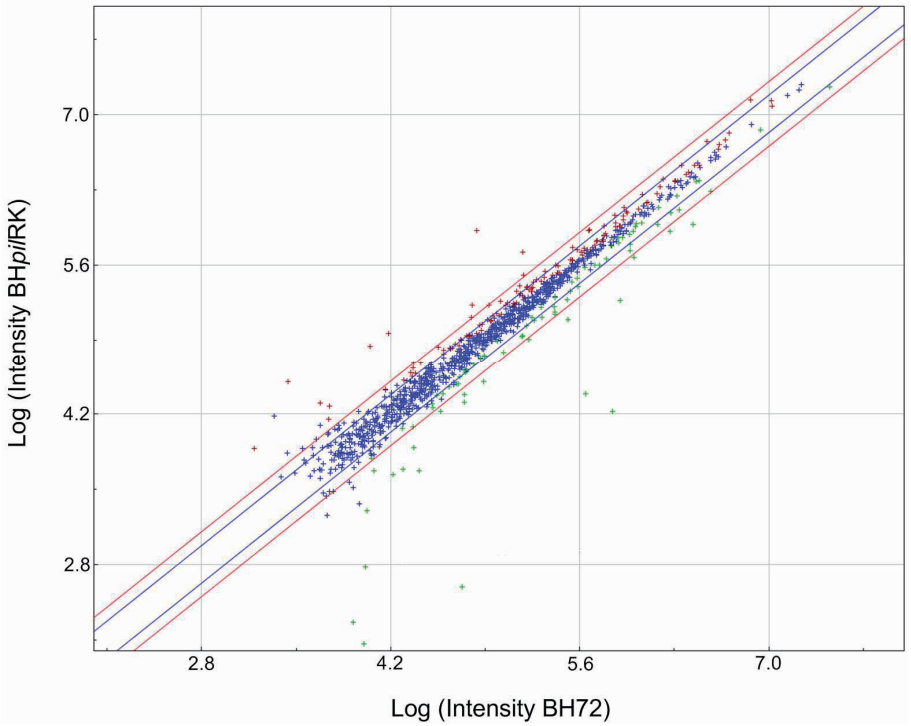

Figure 35: Correlation plot of differentially synthesized proteins identified by comparative proteomic studies via LC-MS/MS of the *Azoarcus* sp. wild type and the regulatory mutant BH*pil*RK. Red crosses indicate proteins with higher synthesis in the *pilR*-mutant while green crosses represent proteins with lower synthesis. Most of the proteins are constitutively synthesized in the proteomic study (blue crosses).

PilR itself was not detected by any of the methods; probably expression levels of this regulator were too low. Neither PilA nor PilB could be detected on the 2D-gels, but with a theoretical pI of 9.7 and a mass of only 6403 Da PilA would not be expected in the analytical window employed. Moreover, only two theoretical peptides of PilA were shown by *in silico* digestion with trypsin to fall into the analytical window from 300 - 3000 Da, which makes it difficult to detect the protein by mass spectrometry due to ion suppression effects. PilB could be detected by western blot analysis (data not shown), but identification of the corresponding spot on the protein gel by MALDI-TOF failed. Similar to PilA, PilB would only be represented by three tryptic peptides in the analytical range of 300 - 3000 Da.

In the gel-free approach the two proteins PilA and PilB could be identified with only one peptide each, but since those peptides were present at -42.8-fold and -23.1-fold lower levels in the *pilR*-mutant, it can be assumed that the response regulator PilR showed the expected effect on pilin gene expression also at protein level. Besides the pilin biogenesis proteins, the type IV pilus assembly protein PilY1B was also down-regulated (-4.1-fold) in the regulatory mutant.

Table 8: List of proteins that are differentially synthesized in the *pilR*-mutant compared to the wild type.

Acc. No.[a)]	Protein	Fold[b)]	Protein Description	Method[c)]	COG[d)]
azo0082	RdgC	3.0	recombination associated protein	GB	L
azo0091		-2.8	probable methyl-accepting chemotaxis protein	GF	N, T
azo0098		1.6	conserved hypothetical membrane protein	GF	S
azo0156	AtpH	nd/-9.8	putative ATP synthase delta chain	GB	C
azo0160	AtpC	-2.9	F_0F_1 ATP synthase subunit epsilon	GB	C
azo0184	LipA	-1.5	lipoyl synthase	GF	H
azo0299	PaaZ	1.5	dehydrogenase	GF	C
azo0305	PaaB	-3.8	phenylacetic acid degradation protein	GB	Q
azo0344		-1.7	hypothetical protein	GF	X
azo0357		-2.3	hypothetical protein	GF	X
azo0438	AnsB2	2.0	periplasmic L-asparaginase II	GF	E, J
azo0439	GlnQ1	1.5	glutamine transport ATP-binding protein	GF	E
azo0442	GlnH	1.8	putative glutamine-binding protein	GF	E, T
azo0504	RpoN1	-1.5	RNA polymerase sigma-54 factor	GF	K
azo0507	RecA	-1.8	recombinase A	GF	L
azo0584	MetF	-1.8	5,10-methylenetetrahydrofolate reductase ($FADH_2$)	GF	E
azo0587	AcyH	-3.0	S-adenosyl-L-homocysteine hydrolase	GF	H
azo0616	Bfr2	-2.8	putative bacterioferritin	GB	P
azo0682	ActP	1.5	acetate permease	GF	R
azo0741	NadA	2.5	quinolinate synthetase	GB	H
azo0752	YchF	1.8	conserved hypothetical protein	GF	J
azo0923	Ndk	2.1	nucleoside diphosphate kinase	GF	F
azo0973	GroES1	-1.6	chaperonin	GF	O
azo0974	GroEL	-1.6	chaperonin	GF	O
azo1003	PntAA	1.5	NAD(P) transhydrogenase subunit alpha part 1	GF	C
azo1005	PntB	2.0	NAD(P) transhydrogenase subunit beta	GF	C
azo1034	IlvB	-2.1	acetolactate synthase catalytic subunit	GF	E, H
azo1062	DnaJ1	-1.7	chaperone protein	GF	O
azo1063	DnaK	-2.0	chaperone protein	GF	O
azo1064	GrpE	-1.9	probable heat shock protein	GF	O
azo1071	AroA	1.6	3-phosphoshikimate 1-carboxyvinyltransferase	GF	E
azo1280	Fpr1	-18.5	ferredoxin-$NADP^+$ reductase	GF	C
azo1286	GcvH	1.6	glycine cleavage system protein H	GF	E
azo1411		7.0	conserved hypothetical ribonucleoside reductase	GB	F
azo1425	HtpG	-2.3	heat shock protein 90	GF	O
azo1566	ClpB1	-2.2	ATP-dependent Clp protease, ATP-binding subunit	GF	O
azo1580	GuaB	-1.5	IMP dehydrogenase	GF	F
azo1609		-1.7	conserved hypothetical ATP-dependent protease	GF	O
azo1645	PdxJ	-4.4	pyridoxal phosphate biosynthetic protein	GB	H
azo1699	EtfB1	1.6	electron transfer flavoprotein beta subunit	GF	C
azo1702		1.8	probable acyl-CoA dehydrogenase	GF	I
azo1908	Tsf	3.0	elongation factor EF-Ts	GB	J
azo1922	EtfB2	-5.0	electron transfer flavoprotein subunit beta	GB	C
azo2062		-1.5	conserved hypothetical peptidyl-prolyl cis-trans isomerase	GF	O
azo2064	LexA	-3.1	repressor protein	GF	K, T
azo2069	Lon	12.0	ATP-dependent protease Lon	GF	O
azo2150		1.6	conserved hypothetical protein	GF	R
azo2151	Etf1	1.6	electron transfer flavoprotein-ubiquinone oxidoreductase	GF	C
azo2153		2.6	conserved hypothetical secreted protein	GF	S
azo2155		6.1	conserved hypothetical secreted protein	GF	X
azo2156		3.4	TonB-dependent receptor	GF	P
azo2231		3.3	putative mannose-1-phosphate guanylyltransferase	GF	M
azo2232	UdgH	1.5	putative UDP-glucose 6-dehydrogenase	GF	M
azo2255		-5.5	conserved hypothetical secreted protein	GF	X
azo2396		-2.7	putative TonB-dependent receptor	GB	P
azo2415	FumB	-1.8	putative fumarate hydratase	GF	C

azo2548	Dyp	-2.7	putative iron-dependent peroxidase	GB	P
azo2669		1.8	conserved hypothetical cytochrome c family protein	GF	C
azo2795		-1.6	putative periplasmic substrate binding protein	GF	Q
azo2917	PilY1B	-4.1	putative type IV pilus assembly protein	GF	N, U
azo2933	ExaB1	-2.8	cytochrome c550	GB	C
azo2972	ExaA2	-3.6	quinoprotein ethanol dehydrogenase	GB	G
azo2975	ExaA3	-1.7	quinoprotein ethanol dehydrogenase	GF	G
azo2975	ExaA3	4.4	quinoprotein ethanol dehydrogenase	GB	G
azo3050	LivJ	1.5	putative leucine-specific binding protein	GF	E
azo3057	BoxB	5.1	benzoyl-CoA oxygenase component B	GB	S
azo3084	Flp	nd	putative transcriptional regulator	GB	T
azo3136	PurM	nd	phosphoribosylformylglycinamidine cyclo-ligase	GB	F
azo3237		-1.6	conserved hypothetical protein	GF	S
azo3243	Hsp18	-4.3	putative small heat shock protein	GF	O
azo3370	AcpD	-1.5	acyl carrier protein phosphodiesterase	GF	I
azo3443		1.5	conserved hypothetical ABC-type branched-chain amino acid transport system periplasmic component	GF	E
azo3523	LeuA2	-3.9	2-isopropylmalate synthase	GF	E
azo3544	Cat2	1.5	4-hydroxybutyrate coenzyme A transferase	GF	C
azo3600	PolA	-1.8	DNA polymerase I	GF	L
azo3641	GltD	2.8	glutamate synthase (small chain) oxidoreductase	GB	E, R
azo3674	MucD4	-1.6	serine protease MucD	GF	O
azo3726	MetH	-2.7	B12-dependent methionine synthase	GF	E
azo3782	PtsI	1.7	putative phosphoenolpyruvate-protein phosphotransferase	GF	G
azo3815		1.5	phasin	GF	X
azo3832		-3.4	ABC-type tungstate transport system permease component	GB	H

[a] Acc. No. = accession number/locus tag of *Azoarcus* sp. BH72 genome
[b] nd = not detected
[c] GB = gel-based approach, GF = gel-free approach
[d] COG-categories: A: RNA processing and modification, B: Chromatin structure and dynamics, C: Energy production and conversion, D: Cell cycle control, mitosis and meiosis, E: Amino acid transport and metabolism, F: Nucleotide transport and metabolism, G: Carbohydrate transport and metabolism, H: Coenzyme transport and metabolism, I: Lipid transport and metabolism, J: Translation, K: Transcription, L: Replication, recombination and repair, M: Cell wall/membrane biogenesis, N: Cell motility, O: Posttranslational modification, protein turnover, chaperones, P: Inorganic ion transport and metabolism, Q: Secondary metabolites biosynthesis, transport and catabolism, R: General function prediction only, S: Function unknown, T: Signal transduction mechanisms, U: Intracellular trafficking and secretion, no: not in COG.

III 9.2 Different cellular processes are under control of the response regulator PilR

As depicted in Figure 36 and Table 8 many different cellular processes are regulated in *Azoarcus* sp. BH*pilR*K. In general, energy production and conversion (C) as well as amino acid transport and metabolism (E) were controlled by the response regulator PilR. Moreover, many proteins that are involved in posttranslational modification and protein turnover as well as chaperones (O) and proteins responsible for coenzyme transport and metabolism (H) were repressed in strain BH*pil*RK. Several proteins that are poorly characterized (R and S) appeared to show higher synthesis levels when PilR was inactivated.

III 9.2.1 Replication, recombination and repair proteins

The recombination associated protein RdgC was 3.0-fold increased in level in the *pilR*-mutant. The DNA polymerase I (PolA) as well as the recombinase A (RecA) was with -1.8-fold down-regulated when the response regulator was disrupted.

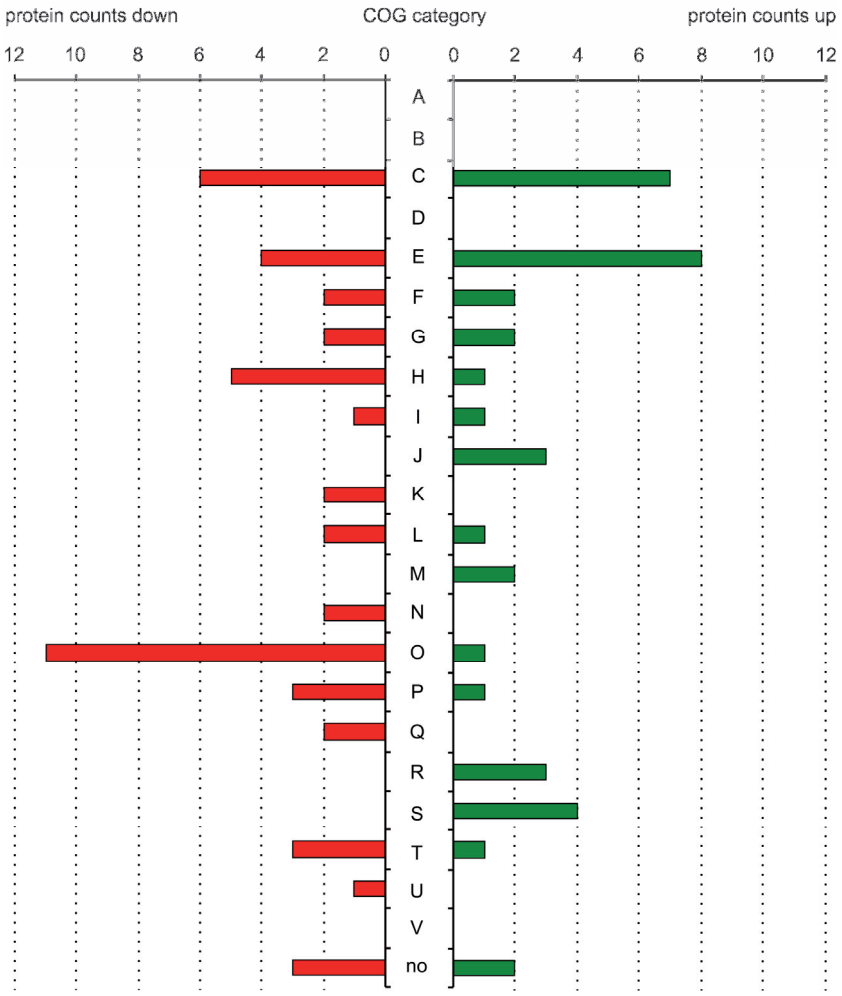

Figure 36: Distribution of PilR-regulated proteins detected by two-dimensional gel electrophoresis in COG-categories, absolute protein counts are shown. Activation in the mutant BH*pilR*K is indicated with up (green) and repression with down (red). A: RNA processing and modification, B: Chromatin structure and dynamics, C: Energy production and conversion, D: Cell cycle control, mitosis and meiosis, E: Amino acid transport and metabolism, F: Nucleotide transport and metabolism, G: Carbohydrate transport and metabolism, H: Coenzyme transport and metabolism, I: Lipid transport and metabolism, J: Translation, K: Transcription, L: Replication, recombination and repair, M: Cell wall/membrane biogenesis, N: Cell motility, O: Posttranslational modification, protein turnover, chaperones, P: Inorganic ion transport and metabolism, Q: Secondary metabolites biosynthesis, transport and catabolism, R: General function prediction only, S: Function unknown, T: Signal transduction mechanisms, U: Intracellular trafficking and secretion, no: not in COG.

III 9.2.2 Signal transduction and regulatory proteins

The RNA polymerase sigma-54 factor RpoN1 was 1.5-fold down-regulated in the *pilR*-mutant. Moreover, regulatory proteins such as the methyl-accpeting chemotaxis protein

Azo0091 (-2.8-fold) and the repressor protein LexA (-3.1-fold) were repressed in strain BH*pilR*K. The putative transcriptional regulator Flp could not be detected in the *pilR*-mutant. This observation shows a strong impact of the response regulator PilR on the expression of another transcription factor.

III 9.2.3 Energy, carbohydrate and amino acid metabolism

The comparative proteomic studies revealed that the ATP synthase was down-regulated in the *pilR*-mutant; while the amount of the ε-chain (AtpC) was only slightly reduced (-2.9-fold), the δ-chain (AtpH) was -9.8-fold down-regulated when the gene for *pilR* was disrupted. In accordance, also the synthesis of many proteins involved in carbohydrate and energy metabolism was reduced. As iron is essential for many of these processes like respiration, the observed down-regulation in iron metabolism (see above) might be involved in this effect. Interestingly, the ferredoxin-$NADP^+$ reductase (Fpr1) was -18.5-fold down-regulated in the mutant. These observations were in agreement with growth behaviour experiments under aerobic conditions in complex medium. In the *pilR*-mutant the generation time was longer (2.3 ± 0.3 hours) than in the wild type (g = 1.9 ± 0.1). Furthermore, twelve proteins that are involved in amino acid metabolism and transport were influenced by the response regulator PilR.

III 9.2.4 Iron metabolism and storage

Interestingly, five proteins that are related to iron metabolism, iron-storage or proteins that contain iron were down-regulated about -3.0-fold in the *pilR*-mutant, the putative bacterioferritin Bfr2, a putative iron-dependent peroxidase DyP, a probable cytochrome c550 and two putative TonB-dependent receptors. The TonB-dependent receptors Azo2396 and Azo2156 also seem to be directly or indirectly PilR-regulated; such proteins are part of the TonB-dependent transport system, which is an important iron-uptake system in bacteria.

III 9.2.5 Posttranslational modification, protein turnover and chaperones

The ATP-dependent protease Lon was 12.0-fold up-regulated in the *pilR*-mutant indicating a repression by the response regulator PilR. The serine protease MucD4, the conserved hypothetical ATP-dependent protease Azo1609 as well as the ATP-dependent ClpB1 protease was down-regulated in the regulatory mutant. Moreover, the synthesis of several chaperones

like GroES1, GroEL1, DnaJ1, DnaK, HtpG and Hsp18 was around -2.0 to -4.0-fold decreased.

III 9.2.6 Hypothetical and poorly characterized proteins

Twelve hypothetical and poorly characterized proteins were under control of the regulatory protein PilR. For example, the (conserved) hypothetical proteins Azo0344 (-1.7-fold), Azo0357 (-2.3-fold) and Azo2150 (1.6-fold) showed differential protein synthesis in strain BH*pilR*K. Interestingly, the conserved hypothetical secreted proteins Azo2153 (2.6-fold), Azo2155 (6.1-fold) and Azo2255 (-5.5-fold) were highly influenced by the response regulator PilR.

III 9.3 The response regulator PilR is part of the quorum sensing hierarchy in *Azoarcus* sp. BH72

It could be shown that 440 genes and proteins were under quorum sensing control in the grass endophyte *Azoarcus* sp. BH72 (see Figure 31). The comparison of PilR regulated proteins with the newly identified QS targets revealed that 35 proteins were influenced in both approaches (Figure 37), i.e. they were differentially synthesized in the *pilR*-mutant as well as under QS conditions. The fact that almost half of the PilR regulated proteins was also influenced by conditioned culture supernatant indicates that this response regulator might be part of the QS cascade in *Azoarcus* sp. BH72.

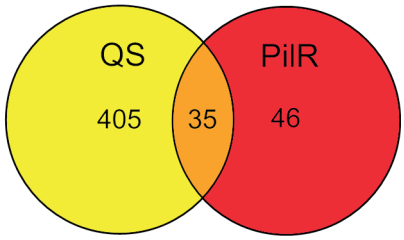

Figure 37: Venn diagram of quorum sensing targets and PilR-regulated proteins in *Azoarcus* sp. BH72. Thirty-five PilR-regulated proteins were also influenced under quorum sensing conditions.

All proteins and their type of regulation are listed in Table 9. It was obvious that several chaperones like GroES1, GroEL1, DnaJ1, DnaK, GrpE as well as HtpG were regulated in both studies. Moreover, proteins involved in energy metabolism such as the electron transfer flavoproteins EtfB1/EtfB2 and the electron transfer flavoprotein-ubiquinone oxidoreductase Etf1 were affected in the *pilR*-mutant and under QS conditions. Two subunits of the ATP

synthase (AtpH and AtpC) and the ferredoxin-$NADP^+$ reductase Fpr1 were also found to be down-regulated in the compared studies. Interestingly, two TonB-dependent receptors were affected: Azo2156 was activated in both approaches, whereas Azo2396 was repressed under QS conditions as well as in the regulatory mutant.

Table 9: List of differentially synthesized proteins in the *pilR*-mutant and genes/proteins affected under quorum sensing conditions.

Acc. No.[a)]	Gene	Gene product description	Regulation[b)]	
			BH*pilR*K	QS
azo0082	*rdgC*	recombination associated protein	up	down
azo0156	*atpH*	putative ATP synthase delta chain	down	down
azo0160	*atpC*	F_0F_1 ATP synthase subunit epsilon	down	down
azo0305	*paaB*	phenylacetic acid degradation protein	down	down/nc
azo0438	*ansB2*	periplasmic L-asparaginase II	up	up
azo0587	*acyH*	S-adenosyl-L-homocysteine hydrolase	down	down
azo0616	*bfr2*	putative bacterioferritin	down	up
azo0752	*ychF*	conserved hypothetical protein	up	down
azo0923	*ndk*	nucleoside diphosphate kinase	up	down
azo0973	*groES1*	chaperonin	down	down
azo0974	*groEL*	chaperonin	down	down
azo1062	*dnaJ1*	chaperone protein	down	down
azo1063	*dnaK*	chaperone protein	down	down
azo1064	*grpE*	probable heat shock protein	down	down
azo1280	*fpr1*	ferredoxin-$NADP^+$ reductase	down	down
azo1425	*htpG*	heat shock protein 90	down	down
azo1699	*etfB1*	electron transfer flavoprotein beta subunit	up	down
azo1908	*tsf*	elongation factor EF-Ts	up	down
azo1922	*etfB2*	electron transfer flavoprotein subunit beta	down	down
azo2062		conserved hypothetical peptidyl-prolyl cis-trans isomerase	down	ambiguous
azo2151	*etf1*	electron transfer flavoprotein-ubiquinone oxidoreductase	up	down
azo2156		TonB-dependent receptor	up	up
azo2396		putative TonB-dependent receptor	down	down
azo2972	*exaA2*	quinoprotein ethanol dehydrogenase	down	up
azo2975	*exaA3*	quinoprotein ethanol dehydrogenase	up	up
azo3050	*livJ*	putative leucine-specific binding protein	up	up
azo3057	*boxB*	benzoyl-CoA oxygenase component B	up	up
azo3136	*purM*	phosphoribosylformylglycinamidine cyclo-ligase	down	up
azo3354	*pilB*	type IV pilus assembly protein	down	up
azo3355	*pilA*	Type IV pilus structural protein	down	up
azo3523	*leuA2*	2-isopropylmalate synthase	down	up
azo3544	*cat2*	4-hydroxybutyrate coenzyme A transferase	up	down
azo3674	*mucD4*	serine protease MucD	down	up
azo3815		phasin	up	down
azo3832		ABC-type tungstate transport system permease component	down	down

[a)] Acc. No. = accession number/locus tag of *Azoarcus* sp. BH72 genome

[b)] down/nc = down-regulated under quorum sensing conditions after one hour of incubation with conditioned culture supernatant and no change (nc) after four hours of incubation, ambiguous = up-regulated in microarray approach and down-regulated in protein gels

IV DISCUSSION

Quorum sensing is an important mechanism in bacteria for intra- as well as interspecies communication. This cell-to-cell communication allows bacteria to regulate their gene expression in response to quorum sensing signal molecules in their environment. Studies from several bacteria have shown that different cellular processes such as pathogenesis, exopolysaccharide production, oxidative stress response as well as twitching and swarming motility are regulated through quorum sensing (von Bodman et al. 1998, Lindum et al. 1998, Sperandio et al. 1999, Glessner et al. 1999, Lumjiaktase et al. 2006). In addition, interaction with plants in pathogens, symbionts and plant-associated bacteria are under quorum sensing control (Loh et al. 2001, González & Marketon 2003, Soto et al. 2009). The study presented here aimed to characterize the novel quorum sensing system in the grass endophyte *Azoarcus* sp. BH72. By applying comparative proteomic and transcriptomic studies combined with reporter gene studies it could be shown that quorum sensing plays an important role in several cellular processes in this β-proteobacterium.

IV 1 The *Azoarcus* sp. BH72 proteome under standard growth conditions

Proteome studies proved to be a valuable tool for functional genomic analyses of *Azoarcus* sp. BH72. Construction of the proteome reference map combined with gel-free studies via LC-MS/MS gave interesting insights into the abundance of proteins under standard growth conditions. 785 proteins were detected and identified when *Azoarcus* sp. was cultivated aerobically in complex medium. This shows that almost one quarter of all predicted *Azoarcus* sp. proteins was synthesized under the chosen conditions. As expected, the quinoprotein ethanol dehydrogenase ExaA2 was among the most abundant proteins as ethanol served as the carbon source. Moreover, proteins involved in energy metabolism, protein biosynthesis and transcription/translation were dominant in the proteome reference map as they are crucial for cell survival and required in high abundance for their functionality. Comparable experimental studies with other *Proteobacteria* like *Shigella* (Liao et al. 2003) *Herbaspirillum* (Chaves et al. 2007) and *Burkholderia* (Riedel et al. 2006) showed that the same or similar proteins were among the most abundant proteins.

Hundred conserved hypothetical proteins could be identified and the fact that the synthesis of such proteins with unknown functions was confirmed with the proteomic approach indicates their importance under the chosen growth conditions. Further studies might help to assign functions to these proteins and thus complement genome annotation.

The proteins PhbC1 and PhbC2, which are responsible for the synthesis of poly-beta-hydroxybutyrate (PHB), were synthesized under standard growth conditions. Moreover, the regulatory protein PhbF, which is involved in regulating PHB accumulation in bacteria, was also present (reviewed in Anderson & Dawes 1990 and Reddy et al. 2003). In general, PHB serves as an energy and carbon reserve during starvation conditions. PHB and glycogen are major carbon storage compounds in rhizobia: The ability of *Sinorhizobium meliloti* to synthesize PHB leads to increased infection of *Medicago truncatula* and is important for nitrogen fixation. Prior to symbiosis, *S. meliloti* maintains a storage of carbon in the form of PHB when excess carbon is available, so that this compound can provide energy for cell division and host invasion (Wang et al. 2007). In *Rhizobium* sp. strain CC 1192 the polymer PHB accumulates to a large extent in free-living cells but not in bacteroids during nitrogen-fixing symbiosis with chickpea (*Cicer arietinum* L.) plants (Chohan & Copeland 1998). *Azoarcus* sp. BH72 produces poly-beta-hydroxybutyrate and it was stated that the cell content of PHB increases in nitrogen-fixing cultures with increasing oxygen concentrations (Hurek et al. 1987). It is possible that PHB is synthesized under standard aerobic growth conditions to provide a carbon storage reservoir for the colonization process and the endophytic lifestyle.

Several proteins (SciA, SciE, SciH, SciI and SciM) from the newly described type VI secretion system were identified in *Azoarcus* sp. BH72. As these proteins were detectable under standard growth conditions and as *Azoarcus* sp. lacks type III and type IV secretion systems, type VI secretion might constitute important secretion machinery in strain BH72.

Moreover, seven out of twenty-two putative TonB-dependent receptors, related to the uptake of ferric siderophore complexes and other substrates, were detected under the chosen conditions with Fe(III)-EDTA as an iron source. This observation and the fact that the iron storage protein Bfr2 and the putative ferric uptake regulation protein Fur were present, highlights the importance of iron acquisition and storage for *Azoarcus* sp. BH72 under the chosen aerobic growth conditions.

IV 2 The grass endophyte *Azoarcus* sp. BH72 communicates via a novel quorum sensing system

Former studies revealed that the expression of the *pilAB* operon which is crucial for the formation of type IV pili is cell density dependent (Plessl 2001, Böhm 2006). This raised the hypothesis that quorum sensing might act as a mechanism for gene regulation in *Azoarcus* sp. BH72. Although the genome lacks genes for known quorum sensing signal molecule synthases (Krause et al. 2006) it could be stated for certain that conditioned culture supernatant of *Azoarcus* sp. BH72 contains an unknown signal factor. The chemical structure of this molecule could not be discovered, but due to its chemical properties it was termed Hydrophilic signal factor (Böhm 2006).

The gene expression studies under QS conditions were performed with *Azoarcus* sp. strain BHΔ*pilS*::pJBLP14 which carries a reporter gene fusion of *uidA*, coding for β-glucuronidase, to the QS target genes *pilAB* in a *pilS*-deletion strain. In general, *pilAB* gene expression in *Azoarcus* sp. is induced under carbon starvation conditions and this is sensed by the kinase PilS. Moreover, the cell-density dependent induction of the *pilAB* gene expression is much higher in the *pilS*-mutant strain compared to the wild type (Plessl 2001, Böhm 2006). This observation indicates that the sensor kinase PilS has an inhibitory effect on the pilin gene expression under QS conditions (Plessl 2001, Friedrich 2010). The activated *pilAB* expression is helpful for studying the induction of *pilAB* gene expression under quorum sensing conditions.

It could be clearly shown that the *pilAB* gene expression was 2- to 3- fold induced after four hours of incubation of the reporter strain with conditioned culture supernatant of *Azoarcus* wild type. This result indicates that components in the conditioned culture supernatant affected the *Azoarcus* sp. gene expression. To exclude that this effect was only true for the pilin gene expression, which are already subjected to complex regulatory processes (Plessl 2001, Böhm 2006, Friedrich 2010), reporter gene studies with another QS target gene (*azo3874*) were performed. The gene *azo3874* encodes a conserved hypothetical secreted protein and its QS regulation was detected by transcriptome studies. Reporter gene studies with *Azoarcus* sp. BH*azo3874* showed that this gene was induced in QS bioassays, too. This demonstrates that the conditioned culture supernatant from *Azoarcus* sp. BH72 shows inducing ability on the expression of several target genes.

The structure of the QS signal molecule remains unclear and only little is known about its synthesis. It is likely that medium compounds or side products from metabolic pathways are necessary for production of the Hydrophilic signal factor. Therefore, it was necessary to test the inducing ability of conditioned culture supernatants obtained from *Azoarcus* cultures grown in different media. It was demonstarted that the growth of *Azoarcus* sp. in complex medium (VM) as well as minimal medium (SM) with ethanol as a carbon source led to activation of *pilAB* and *azo3874* gene expression under QS conditions in the same manner.

In contrast, growth of *Azoarcus* sp. under QS conditions with malate as a carbon source led to ambiguous results: Whereas the *pilAB* gene expression was abolished, expression of *azo3874* was still induced after incubation with conditioned culture supernatant compared to the medium control. In these experiments the absolute gene expression of *azo3874* determined by Miller Units was very low compared to the studies with ethanol containing medium indicating that malate is not the favoured carbon source for *azo3874* gene expression. Further studies are necessary to prove whether the expression of *azo3874* is stimulated under different growth conditions and carbon sources.

The fact that *pilAB* gene expression cannot be induced by supernatants obtained from malate containing media has already been observed (Böhm 2006). But in former studies it was stated that this effect is only due to the lability of the QS signal molecule under alkaline conditions. This hypothesis is opposed to the presented results because *azo3874* gene expression could still be induced under QS conditions indicating that the HSF is functional. Therefore it is likely that the *pilAB* gene expression is influenced by the alkaline milieu that arises after growth with malate. To test this hypothesis, the *pilAB* gene expression after growth in fresh medium with an alkaline pH (complex medium adjusted to pH 9) could be examined with a *pilAB-uidA* reporter strain in the wild type background.

The dependence of pilus formation on pH conditions has been shown for *Escherichia coli* (Schwan et al. 2002) and *Burkholderia cenocepacia*. In *B. cenocepacia* the *cblBACDS* locus, encoding components of the cable pilus biogenesis pathway, is influenced by various environmental cues: The activity of the *cblB* promoter is sensitive to pH, induced by acidic conditions (pH 6.0) and repressed in more acidic (pH 5.0) and basic (pH 8.0) environments (Tomich & Mohr 2004).

IV 2.1 Interspecies crosstalk

Bacteria use cell-to-cell communication to regulate their gene expression in response to signal molecules produced by the same species and by other partners in their habitat. This latter interspecies crosstalk has been shown for a wide range of bacteria. Accordingly, it was interesting to examine whether *Azoarcus* sp. BH72 communicates with other bacterial species in its environment.

To test this, *Azoarcus* sp. was grown under QS conditions with conditioned culture supernatants from various plant-associated bacteria, plant pathogens and soil bacteria. The impact of these supernatants on the *pilAB* as well as *azo3874* gene expression was detected by determination of β-glucuronidase activity in reporter gene studies. The experiments revealed that the expression of *pilAB* genes and *azo3874* could be induced by supernatants of *Azoarcus communis* SWub3 or *Azospira oryzae* 6a3. This demonstrates that bacterial exudates from these species contain signal molecules with inducing ability. Moreover, it is likely that *A. oryzae*, *A. communis* as well as strain BH72 produce the same or a similar QS signal molecule that enables them to communicate with each other in their natural environment. Furthermore, the results raise the hypothesis that the Hydrophilic signal factor is possibly widespread among plant-associated bacteria. *A. communis* and *A. oryzae* were isolated from roots of Kallar grass in Pakistan like *Azoarcus* sp. BH72 (Reinhold-Hurek et al. 1993b, Reinhold-Hurek et al. 2000). This shows that these three species occur naturally in the same habitat and might live in close cooperation and in association with their host plant. Nothing is known about QS systems in these close relatives of strain BH72, but it would be interesting to examine the inducing ability of conditioned culture supernatant from *Azoarcus* sp. BH72 on *A. oryzae* and *A. communis* gene expression.

Furthermore, the incubation of *Azoarcus* sp. with conditioned culture supernatant from the endophyte *Pseudomonas stutzeri* DSM4166 also led to enhanced *pilAB* gene expression. This was very surprising as *P. stutzeri* is not closely related to *Azoarcus* sp. BH72. Usually *Pseudomonas* species communicate via *N*-Acylhomoserine lactones or the specific *Pseudomonas* quinolone signal, and it has been shown that *Azoarcus* sp. does not respond to such molecules (Krause et al. 2006). So far nothing is known about quorum sensing in *Pseudomonas stutzeri* and the comparison of QS systems in several endophytes showed that *P. stutzeri* A1501 has no homologues for LuxI- or LuxR-type quorum sensing factors. Taking

these observations together, *P. stutzeri* appears to communicate via a different, so far unknown, QS system. *Pseudomonas stutzeri* strains were isolated from diverse habitats, including soil, marine sediments, sewage and groundwater, tunicates (*Didemnum* sp.) and plants (Nishino et al. 1992, Rosselló-Mora et al. 1994, Sikorski et al. 2002, Mulet et al. 2008). The strain used in this study (DSM4166) was isolated from the rhizosphere of *Sorghum mutans* (Krotzky & Werner 1987), whereas strain A1501 was isolated from rice plants (Xie et al. 2006) and both strains are able to fix nitrogen. The settlement of *P. stutzeri* in diverse environments makes the development of different survival strategies necessary. Therefore, it might be also useful to establish novel QS systems. The fact that *Azoarcus* sp. BH72 and *P. stutzeri* DSM4166 both live in a plant-associated lifestyle raises the hypothesis that the two organisms might regulate their gene expression in response to interspecies communication signals or use similar signal molecules for habitat-specific reasons.

IV 3 Quorum sensing in microbiomes and endophytes

The computational characterization of several microbiome samples and selected endophytic genomes revealed that quorum sensing systems based on *N*-Acylhomoserine lactones, Diffusible signal factors and Autoinducer-2 play a dominant role in microbe-host interactions. These systems appear to be especially important in plant-associated bacteria and endophytes. The endophytic bacteria *Methylobacterium populi* BJ001, *Gluconacetobacter diazotrophicus* Pal5 and *Burkholderia phytofirmans* PsJN communicate via AHL-based systems whereas *Pseudomonas putida* W619 only harbours the respective genes for listening to AHL-signals (LuxR-orphan). In *Klebsiella pneumonia* 342 and *Enterobacter* sp. 638 two communication systems are present: the Autoinducer-2 system as well as a LuxR-orphan for response to AHLs. The fact that *Azoarcus* sp. BH72 and *Pseudomonas stutzeri* A1501 did not show good homology to known QS factors indicates that these bacteria have established novel QS systems (see IV 2.1). It seems likely that the development of novel QS systems and therefore the production of signal molecules that are not widespread among bacterial species allow these strains to be highly competitive within their ecological niche.

IV 4 Characterization of the quorum sensing regulon of *Azoarcus* sp. BH72

The combination of comparative proteomic studies based on two-dimensional gel electrophoresis and mass spectrometry and expression profiling with whole genome microarray proved to be valuable for analyses of the quorum sensing regulon in *Azoarcus* sp. BH72 (see Figure 38). In the presented study, many targets of the QS cascade of the grass endophyte could be detected. The comparative studies revealed that 18 % of the detected proteins and around 10 % of the transcriptome were regulated under QS conditions. This indicates that quorum sensing is a global regulatory mechanism in *Azoarcus* sp. BH72.

Comparable studies were performed in several bacteria under QS conditions as well as with quorum sensing deficient mutants with similar results. In *Pseudomonas aeruginosa* PAO1 around 6 % of the genome is regulated by quorum sensing: Schuster et al. (2003) showed that 315 genes are activated and 38 genes are repressed by quorum sensing. Moreover, transcriptome analysis revealed that more than 300 genes are under control of the quorum sensing LuxR-type regulator LasR (Gilbert et al. 2009). The studies from Wagner et al. (2003) depicted that as much as 10 % of the *Pseudomonas aeruginosa* PAO1 genome is affected in the QS signal generation deficient mutant *Pseudomonas* sp. ΔlasI Δ*rhlI*. Furthermore, in *Escherichia coli* W3110 5.6 % of the genome is shown to be regulated in a l*ux*S-mutant strain deficient in production of Autoinducer-2 (DeLisa et al. 2001), whereas around 10 % of all *E. coli* 0157:H7 genes were regulated by AI-2 dependent quorum sensing (Sperandio et al. 2001). An interesting observation was made for the hyperthermophilic bacterium *Thermotoga maritima* where 22 % of the genome is differentially expressed when transcription levels of cells isolated from high cell density co-cultures with *Methanococcus jannaschii* were compared with the lower density pure cultures. *T. maritima* growth is enhanced in mixed biofilms with the archaeon *Methanococcus* sp. indicating interspecies communication (Johnson et al. 2005).

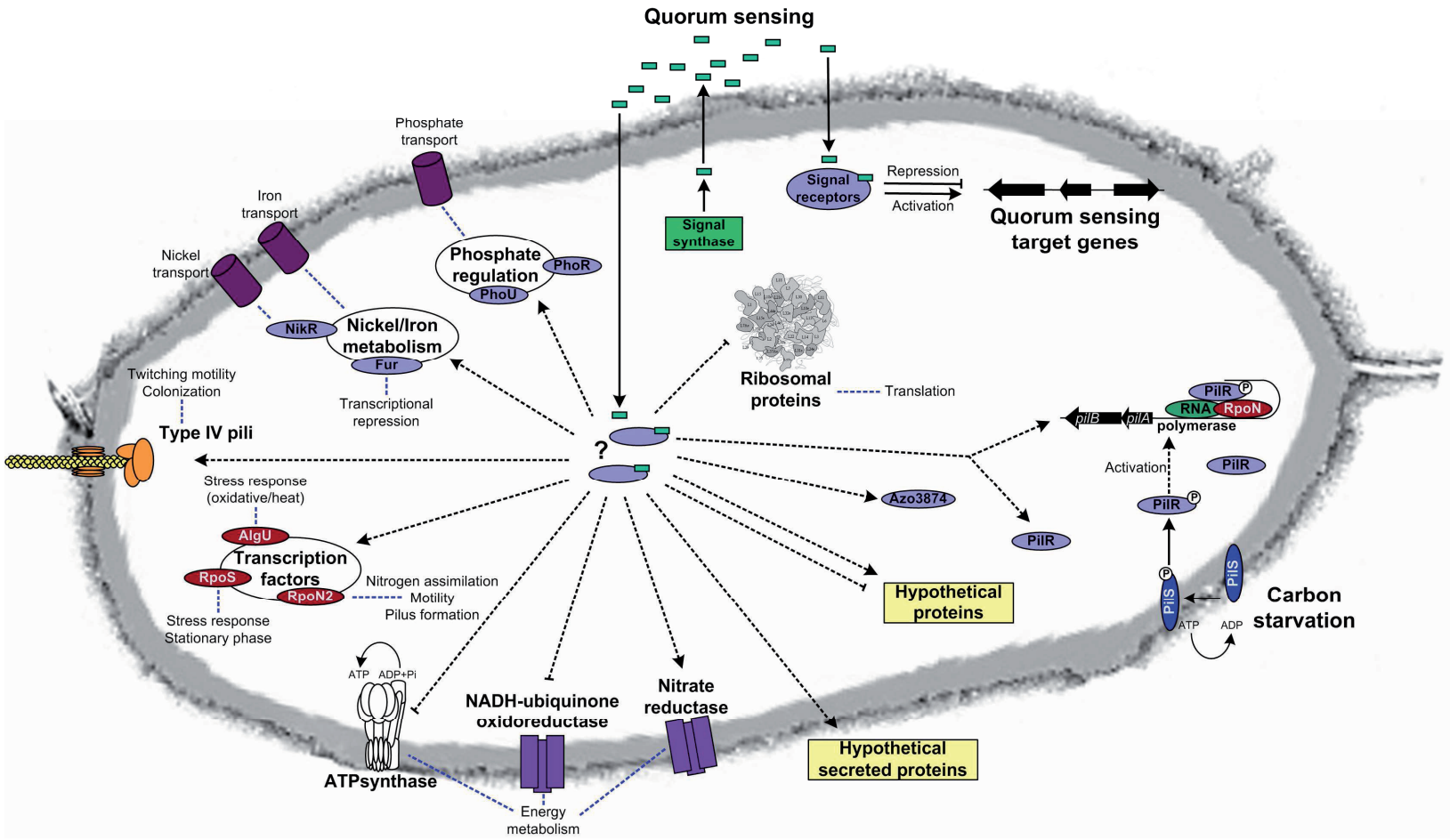

Figure 38: Schematic representation of quorum sensing regulation in *Azoarcus* sp. BH72. Several processes like nickel/iron metabolism, phosphate regulation or energy metabolism are under quorum sensing control. Genes encoding subunits of the nitrate reductase, ATP synthase, NADH-ubiquinone oxidoreductase as well as transcription factors, ribosomal proteins and hypothetical proteins are affected under QS conditions. The *pilAB* genes are activated in response to carbon starvation and QS and the involvement of the two-component regulatory system PilSR is depicted. Activation of gene expression is indicated by dotted black arrows whereas repression is shown with blocked dotted lines. The activation and repression of QS factors leads in turn to further regulation processes, e.g. through regulatory proteins such as Fur, PilR or transcription factors. Blue dotted lines indicate processes that might further be affected by QS regulation.

In the presented study it could be shown that the response regulator PilR is involved in the *pilAB* gene expression of *Azoarcus* sp. BH72 and therefore plays a role in type IV pili formation. In general, pili are involved in plant-microbe interactions. They are responsible for twitching motility and adhesion to eukaryotic cells and therefore play an important role in colonization processes (Dörr et al. 1998, Böhm et al. 2007). In the study presented here, it could be demonstrated that nine proteins involved in type IV pili formation, assembly or twitching motility and the response regulator PilR itself were under positive quorum sensing control. These observations connect the regulation mechanism of quorum sensing to type IV pili mediated processes in this β-proteobacterium. In *Pseudomonas aeruginosa* it could also be shown that type IV pili assembly and function is dependent on the three interrelated quorum sensing systems (Beatson et al. 2002, Wagner et al. 2003, Whitchurch et al. 2005).

Moreover, several proteins that are involved in iron metabolism and iron storage (Bfr1, Bfr2 and Bfd) were under quorum sensing control. This regulatory link between iron and quorum sensing has already been shown in *Pseudomonas aeruginosa* (Oglesby et al. 2008). The ferric uptake regulator Fur (Azo0644) was activated by quorum sensing in *Azoarcus* sp. BH72. Typically, Fur acts as a transcriptional repressor by binding to regulatory Fur box sequences in the promoters of iron-regulated genes under iron repleted conditions. This protein also acts as a global regulator controlling the expression of iron acquisition and storage genes as well as the expression of genes involved in the oxidative stress response, virulence genes and small, iron-repressible regulatory RNAs (Ernst et al. 2005, Boulette & Payne 2007, Massé et al. 2007). The activation of the Fur-protein under QS conditions would repress the expression of Fur-dependent genes in *Azoarcus* sp. BH72 and this process would in turn lead to more indirect regulation processes integrated in a complex QS regulon. Furthermore, four TonB-dependent receptors were under QS control in *Azoarcus* sp. BH72, Azo2396, Azo2978 and Azo3023 were repressed, whereas Azo2156 was activated under QS conditions.

Nickel is an essential trace element for prokaryotes as it forms the active centre of metalloenzymes. When present at high concentrations nickel inhibits growth and exhibits a toxic effect. Therefore, the expression of the nickel-specific transport system is under tight control of the metallo-regulatory protein NikR that specifically responds to nickel (De Pina et al. 1999). In the presented study NikR was shown to be up-regulated under QS conditions. Furthermore, the phosphate regulon sensor protein PhoR and the phosphate uptake regulator PhoU were also under positive quorum sensing control. Phosphorus is an essential nutrient for

bacterial cells and is required for biosynthesis of nucleotides, DNA and RNA, but also for the regulation of protein activity by phosphorylation of amino acid residues in sensor kinase proteins. A common phosphorus source is inorganic phosphate and several mechanisms for acquisition, assimilation and storage of phosphate have been developed in bacteria (Schaaf & Bott 2007). In *Escherichia coli* the cellular response to phosphate availability is mediated via the two-component regulatory system PhoRB: Under phosphate limitation conditions the sensor kinase PhoR is autophosphorylated and then PhoR activates its cognate response regulator PhoB. PhoB in turn activates expression of several genes of the *pho*-regulon responsible for transport of inorganic phosphate (Yamada et al. 1989). The gene *phoU* is a part of the *pstSCAB-phoU*-operon and functions as a negative regulator of the *pho*-regulon. Moreover, it was stated that PhoU enhances the ability of the pathogenic *E. coli* strain CFT073 to colonize the urinary tract of its host. It serves as a virulence factor by indirectly affecting the expression of virulence-related genes (Buckles et al. 2006). The fact that PhoR and PhoU are under QS control and the presence of the two-component regulatory system PhoBR and genes of the *pho*-regulon (e.g. *pstS2CAB*) in *Azoarcus* sp. BH72, raise the hypothesis that quorum sensing is linked to phosphate metabolism in the studied organism. In *Pseudomonas aeruginosa* it was demonstrated that the production of the blue antibiotic pigment pyocyanin is influenced by QS systems as well as by iron and phosphate availability. The phosphate regulon is involved in transcriptional activation of *rhlR* and the augmentation of the P*seudomonas* quinolone signal and pyocyanin production under phosphate limitation (Bazire et al. 2005, Jensen et al. 2006). In the presented study it can not be excluded that the growth of *Azoacrus* sp. under QS conditions is also influenced by phosphate limitation. The conditioned culture supernatant was not supplemented with an additional phosphate source like it was done for the depleted carbon source ethanol.

Taken all these observations together, quorum sensing appears to control diverse transport processes in *Azoarcus* sp. BH72.

Interestingly, proteins that are important for the oxidative stress response were under quorum sensing control in *Azoarcus* sp. BH72. The genes for two bacterioferritin comigratory proteins Bcp1/Bcp2 and the thioredoxin-disulfide reductase TrxC2 were activated under QS conditions. In contrast, the alkyl hydroperoxide reductase AhpC and the hydroperoxide resistance protein Ohr were repressed. Peroxiredoxins like bacterioferritin comigratory proteins and alkyl hydroperoxide reductases catalyze the reduction of hydrogen peroxide and

organic hydroperoxides and therefore protect bacteria from damage by reactive oxygen species. The bacterioferritin comigratory protein Bcp is involved in oxidative stress resistance and colonization processes in *Helicobacter pylori* (Wang et al. 2005) and *Staphylococcus aureus* (Horsburgh et al. 2001). For *Vibrio cholerae* it has been shown that quorum sensing enhances the stress response (Joelsson et al. 2007). In *Burkholderia pseudomallei* quorum sensing regulates expression of *dpsA* encoding a non-specific DNA-binding protein which plays a key role in protection against oxidative stress (Lumjiaktase et al. 2006). Oxidative stress is a challenge that bacteria have to overcome to establish infection in eukaryotic hosts as mammals and plants use hydrogen peroxide to create an oxidative environment to protect themselves against pathogens (Benjamin et al. 2010). It might be useful for *Azoarcus* sp. BH72 to regulate its oxidative stress response in a cell density dependent manner to prepare for its endophytic lifestyle.

Comparison of the transcriptome and proteome studies of the QS regulon of *Azoarcus* sp. BH72 with other bacteria revealed that similar sets of genes and proteins were under QS control (DeLisa et al. 2001, Sperandio et al. 2001, Wagner et al. 2003, Schuster et al. 2003, O'Grady et al. 2009). For example, the *nap*-genes, encoding subunits of the periplasmic nitrate reductase were up-regulated in this study and in *Pseudomonas aeruginosa* (Wagner et al. 2003, Schuster et al. 2003), where the periplasmic nitrate reductase is encoded within the *napEFDABC* operon. In general, nitrate serves as a terminal electron acceptor under anaerobic conditions and reduction of nitrate to nitrite generates a transmembrane proton motive force allowing ATP synthesis and anaerobic growth (van Alst et al. 2009). This indicates that quorum sensing is involved in the regulation of different metabolic pathways and with this cell-to-cell communication system bacteria are generally capable to switch to preferential usage of cellular pathways or energy generation processes. In the presented study almost all genes encoding ribosomal proteins of the grass endophyte were down-regulated under QS conditions. A similar regulation has been observed in *Escherichia coli* where several ribosomal proteins were also under QS control (Sperandio et al. 2001).

The RNA polymerase sigma factors RpoS (sigma-38), AlgU (sigma-24) and RpoN2 (sigma-54) were found to be quorum sensing regulated in *Azoarcus* sp. BH72. In general, sigma factors act as prokaryotic transcription initiation factors and they can be activated in response to different environmental stimuli (Potvin et al. 2007). For *Pseudomonas aeruginosa* it was stated that quorum sensing is negatively regulated by RpoN (Heurlier et al. 2003). The

interplay of RNA polymerase together with the sigma-54 factor RpoN and the response regulator PilR is necessary for activation of *pilAB* gene expression. Therefore it is not surprising that the genes *pilR* as well as *rpoN2* are also activated under quorum sensing conditions as the *pilAB* genes expression is also enhanced. Moreover, the alternative sigma factor RpoS represses *rhlI* gene expression and therefore plays a role in QS regulation in *P. aeruginosa* (Whiteley et al. 2000). Therefore it is likely that quorum sensing regulation is also linked to such transcription factors in *Azoarcus* sp. BH72.

Many regulatory proteins, several posttranslational modification proteins and transcription factors were regulated under QS conditions. This leads to the assumption that quorum sensing consists of a complex regulon in *Azoarcus* sp. BH72. In the future such regulatory proteins need to be studied in more detail to investigate their position in the QS hierarchy.

The fact that more than hundred proteins with unknown functions are under quorum sensing control in many different organisms, including *Azoarcus* sp., demonstrates that several cellular processes remain unclear within the complex quorum sensing cascades.

IV 5 The response regulator PilR is involved in the regulation of several cellular processes in *Azoarcus* sp. BH72

PilR is the response regulator of the two-component regulatory system PilSR which activates the expression of the *pilAB* operon, required for the formation of type IV pili. The comparative proteomic studies demonstrate that a large set of proteins is differentially synthesized in the *Azoarcus* wild type compared to the regulatory mutant BH*pilR*K, indicating an important role of this response regulator. In total, 35 up-regulated proteins in the *pilR*-mutant were repressed through PilR, whereas the 46 down-regulated proteins in *Azoarcus* sp. BH*pilR*K were activated via the response regulator in the wild type.

As expected, the synthesis of the proteins PilA and PilB and the *pilAB* gene expression was drastically decreased in strain BH*pilR*K as shown by proteomic studies (see III 9.1) and reporter gene studies (see III 3.1). A direct regulation of *pilA*/*fimA*-gene expression by the response regulator PilR was shown in other bacteria (Boyd & Lory 1996, Parker et al. 2006), but so far there is no evidence for PilR control of other genes involved in type IV pilin biogenesis. In this study it could be shown that besides the pilin biogenesis proteins, the type IV pilus assembly protein PilY1B was also down-regulated in the regulatory mutant. PilY1 is

known to be involved in the formation of type IV pili in *Pseudomonas aeruginosa* and is homologous to PilC2 of *Neisseria meningitidis*, which is a major adhesion protein and able to regulate PilT-mediated pilus retraction. Furthermore, PilT expression can be switched off and on by phase variation. This combination of features indicates that PilY1 might be a protein with multiple functions (Bohn et al. 2009).

The transcription of pilin genes in *Pseudomonas aeruginosa* requires the RNA polymerase alternative sigma factor RpoN (Ishimoto & Lory 1989). This sigma factor is also required for transcription of several genes including those encoding proteins for nitrogen assimilation, amino acid uptake, degradation of organic compounds and cell-to-cell signalling (Totten et al. 1990, Heurlier et al. 2003). *Azoarcus* sp. BH72 harbours two genes encoding the RNA polymerase sigma-54 factor (RpoN1 and RpoN2) and RpoN1 was repressed -1.5-fold in the *pilR*-mutant. This observation indicates that the response regulator might be involved in direct or indirect activation of this sigma factor. The result also raises the hypothesis that RpoN1 is not required to a great extent in the *pilR*-mutant because the interplay of RNA polymerase, sigma-54 factor and PilR is not possible for activation of *pilAB* gene expression. But as RpoN1 might also be necessary for transcription of several other genes, this sigma factor is still present in the *pilR*-mutant.

Some proteins that are involved in replication, recombination and repair were affected by the response regulator PilR: The recombination associated protein RdgC was increased 3.0-fold in level in the *pilR*-mutant. RdgC is a DNA-binding protein that is associated with recombination and replication fork repair in *Escherichia coli* and with the virulence associated pilin antigenic variation in *Neisseria* species (Briggs et al. 2007). *Neisseria gonorrhoeae* pilin undergoes high-frequency changes in primary amino acid sequence that aid in the avoidance of the host immune response and alter pilus expression. In this organism the pilus is composed primarily of the *pilE* gene product, and pilin and pilus antigenic variation occur when DNA sequences from one of several silent pilin gene copies (*pilS*) are transferred unidirectionally to replace variable sequences within the *pilE* gene (Mehr et al. 2000). However, a silent *pilA* copy was not detected in strain BH72.

The response regulator PilR also seems to be involved in regulation of other regulatory proteins such as the putative transcriptional regulator Flp indicating that PilR might be part of a hierarchical regulatory cascade in *Azoarcus* sp. BH72. Flp belongs to the superfamily of

Crp-Fnr regulators with an N-terminally located nucleotide-binding domain similar to the cyclic adenosine monophosphate receptor protein and a C-terminally located helix-turn-helix motif for DNA binding. The Crp-Fnr regulators can mediate or control responses to a broad spectrum of intracellular and exogenous signals such as cyclic adenosine monophosphate, anoxia, the redox state, oxidative and nitrosative stress, nitric oxide, carbon monoxide, 2-oxoglutarate or temperature (Körner et al. 2003). Cyclic AMP is used as a second messenger and this signalling molecule is important in many bacteria for regulation of gene expression in response to a variety of environmental stimuli. It is synthesized by adenylyl cyclase from adenosinetriphosphate (ATP) and the level of cAMP inside the cell is dependent on growth conditions (reviewed in Baker & Kelly 2004 and Lory et al. 2004). The cAMP receptor protein CRP forms a complex with cyclic adenosine monophosphate and binds in this activated form to DNA. The complex of CRP and cAMP regulates the transcription of several genes (Körner et al. 2003). For *Vibrio cholerae* it could be shown that the cyclic AMP receptor protein (CRP) modulates quorum sensing, motility and virulence in this pathogenic bacterium (Liang et al. 2007).

Interestingly, the ATP-dependent protease Lon was highly up-regulated in *Azoarcus* sp. BH*pilR*K indicating repression by PilR. Lon activity is essential for cellular homeostasis. The protease is involved in protein quality control and metabolic regulation. Additionally, Lon is required in certain pathogenic bacteria for rendering pathogenicity and host infectivity (Lee & Suzuki 2008).

The bacterioferritin Bfr2 and two TonB-dependent receptors (Azo2156 and Azo2396) were found to be differentially synthesized in the *pilR*-mutant compared to the wild type, indicating a link between the response regulator PilR and iron related mechanisms in *Azoarcus* sp. In *Geobacter sulfurreducens* it has already been shown that the two component regulatory system PilSR is involved in transcriptional regulation of *pilA* and moreover PilR also affects the formation of an important set of cytochromes involved in the electron transfer to soluble and insoluble Fe(III) (Juarez et al. 2009). Iron is an essential element for organisms and it is involved in a variety of bacterial cell processes. Bacterioferritins function as iron-storage proteins in bacteria, however, the mechanism of iron entry and exit remain unclear. Bfr2 of *Azoarcus* sp. BH72 shows 71% similarity to the bacterioferritin of *Azotobacter vinelandii*, which is speculated to be a specific iron-storage depot for nitrogenase and an electron storage for nitrogen fixation (Liu et al. 2004, Stiefel & Watt 1979).

Several (conserved) hypothetical proteins were under control of the response regulator PilR, among thess Azo2155 and Azo2255 are of hight interest as they are with fold changes of 6.1-fold and -5.5-fold highly regulated. Further studies are necessary to understand their role in *Azoarcus* sp. BH72 and to gain more insights into their regulation via the response regulator PilR.

The comparison of PilR-regulated proteins with the newly identified quorum sensing targets revealed that 35 proteins that were differentially synthesized in the *pilR*-mutant were also under QS control. The fact that almost half of all PilR-regulated proteins was also influenced by the unknown QS signal molecule and that PilR itself was up-regulated under QS conditions leads to the hypothesis that this response regulator is part of the QS cascade in *Azoarcus* sp. BH72. At this point it is not possible to state how the regulation network of the response regulator PilR and the quorum sensing cascade is connected. As excpected the studies with *Azoarcus* sp. BH*pilR*K as well as the QS approach can not provide information about direct or indirect regulation processes.

IV 6 Concluding remarks and outlook

The construction of the proteome reference map of *Azoarcus* sp. BH72 combined with gel-free methods for identification of synthesized proteins gave essential insights into cellular processes and metabolism under standard aerobic growth conditions. This knowledge is the basis for comparative proteomic studies and might further help to examine protein composition under specialized growth conditions.

The presented study is the first global approach that characterizes the novel quorum sensing system in *Azoarcus* sp. BH72. Although this bacterium does not communicate via known quorum sensing signal molecules it could be clearly shown that a density dependent regulation in conjunction with conditioned culture supernatant occurs in this grass endophyte. In Figure 38 (see IV 4) several processes that are under quorum sensing control are depicted and summarized. It is obvious that cell-to-cell communication is important for activation as well as repression of cellular actions. Transcription, translation and transport (nickel, iron, and phosphate) are regulated via quorum sensing. Moreover, energy metabolism and the oxidative stress response in *Azoarcus* sp. BH72 are affected by the unknown QS signal molecule. The formation and regulation of type IV pili and twitching motility is also under QS control

indicating that quorum sensing might be important for colonization of host plants by this endophyte. The repression of energy metabolism and translation under QS conditions can be further confirmed with the observation from the QS bioassays where *Azoarcus* sp. showed decreased growth after incubation with conditioned culture supernatant.

The comparative proteomic studies with the regulatory mutant *Azoarcus* sp. BH*pilR*K revealed that the response regulator PilR is involved in activation as well as repression of a number of processes besides regulation of type IV pilus biogenesis. Moreover, this regulatory protein is part of the quorum sensing cascade in *Azoarcus* sp. BH72. Several regulatory proteins, including PilR and Fur as well as transcription factors such as RpoN, RpoS, and AlgU are under positive QS control and therefore lead to a complex QS hierarchy. The generation of new regulatory mutants followed by subsequent comparative studies under QS conditions with microarrays would give more insights about the position of these regulatory proteins in the quorum sensing cascade.

The molecular characterization of the novel QS system of *Azoarcus* sp. BH72 is important for understanding fundamental gene regulation processes in this grass endophyte. But beside that, the chemical identification of the quorum sensing signal molecule and the proteins involved in its synthesis is unavoidable for further studies. The structural identification of the Hydrophilic signal factor can be achieved by separation of supernatant compounds with gas/liquid chromatography coupled to tandem mass spectrometry. For such studies comparisons of HSF containing supernatants with exudates without QS signal molecules as negative controls are necessary. Therefore, the results from QS experiments with closely related bacteria will be helpful as supernatants from *Azoarcus evansii* KB740, *Azonexus fungiphilus* BS5-8 or *Azovibrio restictus* S5b2 obviously do not contain the *Azoarcus* sp. BH72 signal molecule. Moreover, *Azoarcus communis* SWub3 and *Azospira oryzae* 6a3 can serve as positive controls as their supernatants show inducing ability.

Subsequently, it is crucial to test the inducing ability of either fractionated supernatant or the purified HSF on quorum sensing target genes with reporter gene studies in a quorum sensing bioassay. In this study the inducing ability of supernatant was determined by measuring the β-glucuronidase activity as the QS induction factors of the genes *pilAB* and *azo3874* were only around two to three. The mentioned method is very time consuming and therefore it would be advantageous to use *gfp*-reporter strains instead. The quorum sensing activation of target genes fused to *gfp*, encoding the green fluorescent protein can be detected more easily

by fluorimetric methods. This method is convenient and even possible with low amounts of bacterial culture but the induction factors of the QS target genes must be very high to discriminate between background fluorescence and a real increase of green fluorescence resulting from GFP. The detection of QS target genes with the whole genome microarray approach gives the opportunity to generate new mutant strains carrying a transcriptional fusion of reporter the gene *gfp* to genes that are highly up-regulated under QS conditions.

In addition, a transposon mutagenesis strategy with *Azoarcus* sp. BH72 might be useful to detect genes, encoding proteins responsible for HSF production. The analysis of mutants that are not able to produce supernatant with inducing ability will be of high interest. Transposon mutagenesis studies need to be carried out in a reporter gene mutant background to allow convenient screening of transposon mutants by green fluorescence QS bioassays.

Several differentially synthesized proteins were detected with two-dimensional gel-electrophoresis with *Azoarcus* sp. BH72 grown under QS conditions. The fact that around 400 genes of *Azoarcus* sp. BH72 are under QS control (identified by microarray) leads to the question whether these factors are also regulated on protein level. To increase the number of detectable proteins, gel-free studies based on LC-MS/MS with *Azoarcus* sp. BH72 grown under QS conditions are useful. These experiments are already on the way in cooperation with the University of Greifswald and will help to elucidate the QS system of this grass endophyte.

In the presented study the genome wide microarray analyses were performed under QS conditions for one and four hours, but unfortunately it was not possible to examine a real time series for quorum sensing in *Azoarcus* sp. BH72. Several studies have shown that the expression of QS responsive genes and proteins is altered at different time points of quorum sensing (Chen et al. 2003, Schuster et al. 2003, Wagner et al. 2003). Therefore it is necessary to carry out further microarray experiments based on incubation of *Azoarcus* wild type with conditioned culture supernatant for two and three hours.

Special proteins are required for detection of QS signal molecules and subsequent signal transduction processes. In *Vibrio harveyi* three quorum sensing systems are present: The proteins LuxM (Acyl-homoserine lactone synthesis protein), LuxS (S-ribosylhomocysteinase) and CqsA (CAI-1 autoinducer synthesis protein) are responsible for synthesis of the different autoinducer molecules HAI-1, AI-2 and CAI-1, respectively. These molecules are detected at

the cell surface by the membrane-bound, two-component receptor proteins LuxN, LuxQ and CqsS (reviewed in Defoirdt et al. 2008). Subsequently, a common phosphorylation cascade via the phosphorelay protein LuxU is initiated and LuxU alters the phosphorylation state of the response regulator LuxO. In turn LuxO controls expression of several QS target genes (Taga & Bassler 2003). In *Azoarcus* sp. BH72 it is likely that the expression of genes, coding for such quorum sensing proteins, would be activated under QS conditions as QS leads to autoinduction. Among QS up-regulated genes obtained by microarray nine genes could be candidate genes, encoding probable proteins for detection of the Hydrophilic signal factor or signal transduction. These proteins and their functions are listed in Table 10 and all of these proteins contain typical domains for sensor and regulatory proteins.

Table 10: List of proteins that might be involved in detection of the Hydrophilic signal factor and subsequent signal transduction processes in *Azoarcus* sp. BH72.

Acc. No.[a)]	Protein description	Pfam	TIGRfam	SP[b)]	TMH[c)]	PSL[d)]
azo0622	putative signalling protein, diguanylate cyclase/phosphodiesterase	PF00563 PF00672 PF00990 PF08447	TIGR00229 TIGR00254	Yes	Yes	CM
azo0898	putative serine/threonine protein kinase	PF00069 PF00672		No	Yes	CM
azo1544	diguanylate cyclase/ phosphodiesterase with PAS/PAC sensor(s), GGDEF/EAL/PAC/PAS-domain containing protein	PF00563 PF00989 PF00990 PF08448	TIGR00229 TIGR00254	No	No	C
azo2073	putative serine protein kinase	PF06798 PF08298		No	No	C
azo2408	hypothetical sensor protein	PF00990 PF11845	TIGR00229 TIGR00254	Yes	No	U
azo2672	conserved hypothetical sensor histidine kinase	PF00512 PF02518		Yes	No	CM
azo3330	FHA-domain containing protein	PF00498		No	No	C
azo3436	two component transcriptional regulator (LuxR family) DNA-binding response regulator	PF00072 PF00196		No	No	C
azo3498	histidine kinase, putative sensor protein	PF02518		Yes	No	CM

[a)] Acc. No. = accession number/locus tag of *Azoarcus* sp. BH72 genome
[b)] The presence of signal peptides (SP) was predicted with SignalP 3.0 (Emanuelsson et al. 2007)
[c)] The presence of transmembrane helices was predicted with TMHMM v.2.0 (Krogh et al. 2001)
[d)] The subcellular localization (PSL) was predicted with PSORTb v.2 (Gardy et al. 2005)
C = Cytoplasm, CM = Cytoplasmic membrane, OM = Outer membrane, P = Periplasm, U = Unknown

Four of these proteins (Azo0622, Azo0898, Azo2672, and Azo3498) were predicted to occur in the cytoplasmic membrane; in this compartiment they could sense external signal molecules. Moreover, one can speculate that the two-component response regulator Azo3436

might be responsible for transcriptional regulation of QS target genes in *Azoarcus* sp. BH72. But further experiments are crucial to prove such hypothesis.

Concluding, the molecular characterization of the novel quorum sensing system in the diazotrophic grass endophyte *Azoarcus* sp. BH72 was achieved with the performed experiments. The presented study demonstrates that density dependent regulation is important for *Azoarcus* sp. BH72. The question whether QS is essential for this grass endophyte can only be examined with QS deficient mutant strains. Moreover, the investigation of the cell-to-cell communication system is a complex and challenging task that needs further research to fully elucidate the hierarchical interrelations.

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VI APPENDIX

To minimize the number of printed pages some additional tables are supplied in Appendix B on a CD-ROM. These tables are not necessarily required to understand the presented experiments but crucial for completeness of the studies.

VI 1 Appendix A

Table A 1: Statistical analyses of quorum sensing bioassays and determination of β-glucuronidase activity with GraphPad InStat version 3.01.

Experiment	Incubation	Gene	Supernatant	Medium[a)]	Significant	P-value[b)]
QS bioassay	4 h	*pilAB*	*Azoarcus* sp. BH72	VME	yes	0.0001
QS bioassay	4 h	*pilAB*	*Azoarcus evansii*	VME	no	0.2500
QS bioassay	4 h	*pilAB*	*Azospirillum brasilense*	VME	no	0.9871
QS bioassay	4 h	*pilAB*	*Azospirillum lipoferum*	VME	no	0.6896
QS bioassay	4 h	*pilAB*	*Azospira oryzae*	VME	yes	0.0033
QS bioassay	4 h	*pilAB*	*Azonexus fungiphilus*	VME	no	0.2796
QS bioassay	4 h	*pilAB*	*Azoarcus communis*	VME	yes	0.0104
QS bioassay	4 h	*pilAB*	*Azovibrio restrictus*	VME	no	0.4609
QS bioassay	4 h	*pilAB*	*Chromobacterium violaceum*	VME	yes	0.0135
QS bioassay	4 h	*pilAB*	*Xanthomonas oryzae*	VME	yes	0.0007
QS bioassay	4 h	*pilAB*	*Azotobacter vinelandii*	VME	no	0.3813
QS bioassay	4 h	*pilAB*	*Pseudomonas syringae*	VME	yes	0.0024
QS bioassay	4 h	*pilAB*	*Pseudomonas stutzeri*	VME	yes	0.0004
QS bioassay	4 h	*azo3874*	*Azoarcus* sp. BH72	VME	not quite	0.0531
QS bioassay	4 h	*azo3874*	*Azospira oryzae*	VME	yes	0.0465
QS bioassay	4 h	*azo3874*	*Azoarcus communis*	VME	no	0.1207
time curve	1 h	*pilAB*	*Azoarcus* sp. BH72	VME	no	0.2326
time curve	2 h	*pilAB*	*Azoarcus* sp. BH72	VME	not quite	0.0850
time curve	3 h	*pilAB*	*Azoarcus* sp. BH72	VME	no	0.1374
time curve	4 h	*pilAB*	*Azoarcus* sp. BH72	VME	no	0.1400
time curve	1 h	*azo3874*	*Azoarcus* sp. BH72	VME	yes	0.0070
time curve	2 h	*azo3874*	*Azoarcus* sp. BH72	VME	yes	0.0009
time curve	3 h	*azo3874*	*Azoarcus* sp. BH72	VME	yes	0.0040
time curve	4 h	*azo3874*	*Azoarcus* sp. BH72	VME	yes	0.0015
QS bioassay	4 h	*pilAB*	*Azoarcus* sp. BH72	VME	yes	0.0036
QS bioassay	4 h	*pilAB*	*Azoarcus* sp. BH72	SME	yes	0.0001
QS bioassay	4 h	*pilAB*	*Azoarcus* sp. BH72	VMM	no	0.1490
QS bioassay	4 h	*pilAB*	*Azoarcus* sp. BH72	SMM	no	0.1208
QS bioassay	4 h	*azo3874*	*Azoarcus* sp. BH72	VME	yes	0.0019
QS bioassay	4 h	*azo3874*	*Azoarcus* sp. BH72	SME	yes	0.0024
QS bioassay	4 h	*azo3874*	*Azoarcus* sp. BH72	VMM	not quite	0.0954
QS bioassay	4 h	*azo3874*	*Azoarcus* sp. BH72	SMM	no	0.1455
PilR-analyses	4 h	*pilR*	*Azoarcus* sp. BH72	VME	yes	0.0016

[a)] VME = VM-Ethanol medium, SME = SM-Ethanol medium, VMM = VM-Malate medium, SMM = SM-Malate medium

[b)] Two-tailed P-values were calculated with paired t-test.

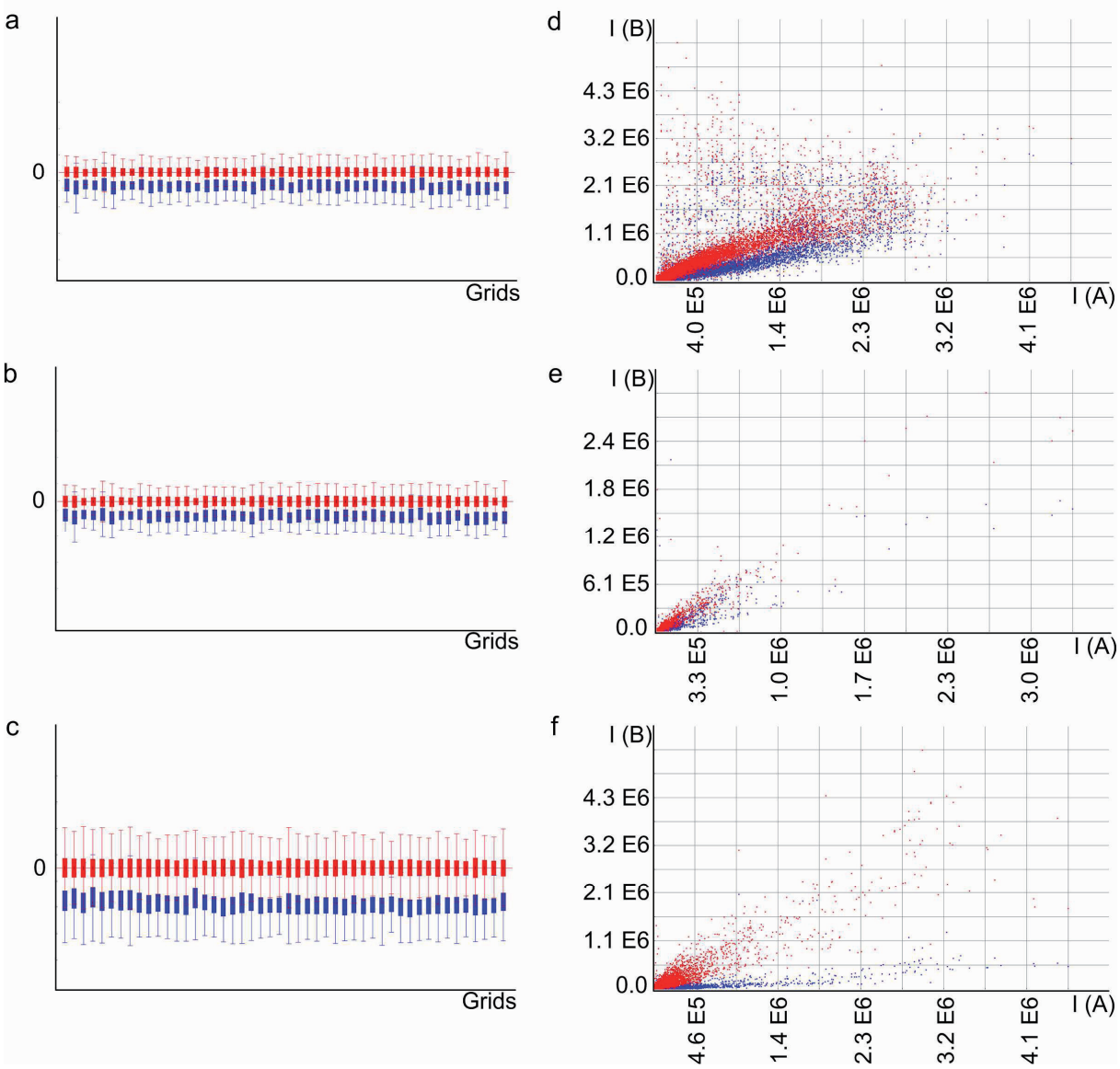

Figure A 1: Schematic representation of the genome-wide transcriptional profiling of *Azoarcus* sp. BH72 grown under quorum sensing conditions for one hour (experiment 1). The box graphs (left) show how the distribution of expression values of all spots varies among 48 different blocks/grids within a slide. The intensity graphs (right) show the relations between the intensity values of channel A (Cy3) and B (Cy5) for each spot in a slide. Blue data represent raw data and red data show LOWESS normalized values. a/d: slide 302725, b/e: slide 302942 and c/f: slide 302943.

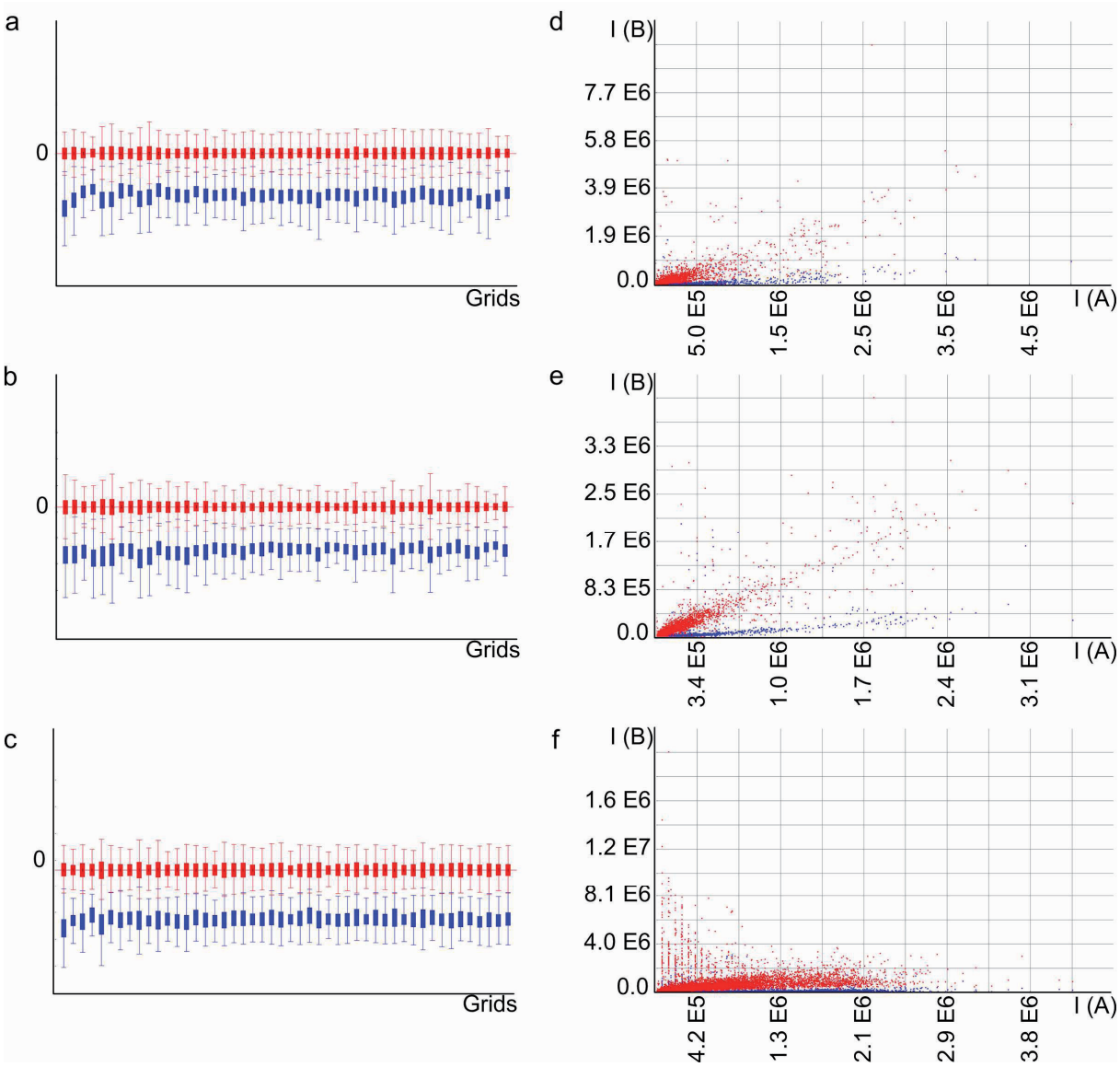

Figure A 2: Schematic representation of the genome-wide transcriptional profiling of *Azoarcus* sp. BH72 grown under quorum sensing conditions for four hours (experiment 2). The box graphs (left) show how the distribution of expression values of all spots varies among 48 different blocks/grids within a slide. The intensity graphs (right) show the relations between the intensity values of channel A (Cy3) and B (Cy5) for each spot in a slide. Blue data represent raw data and red data show LOWESS normalized values. a/d: slide 301713, b/e: slide 301715 and c/f: slide 301721.

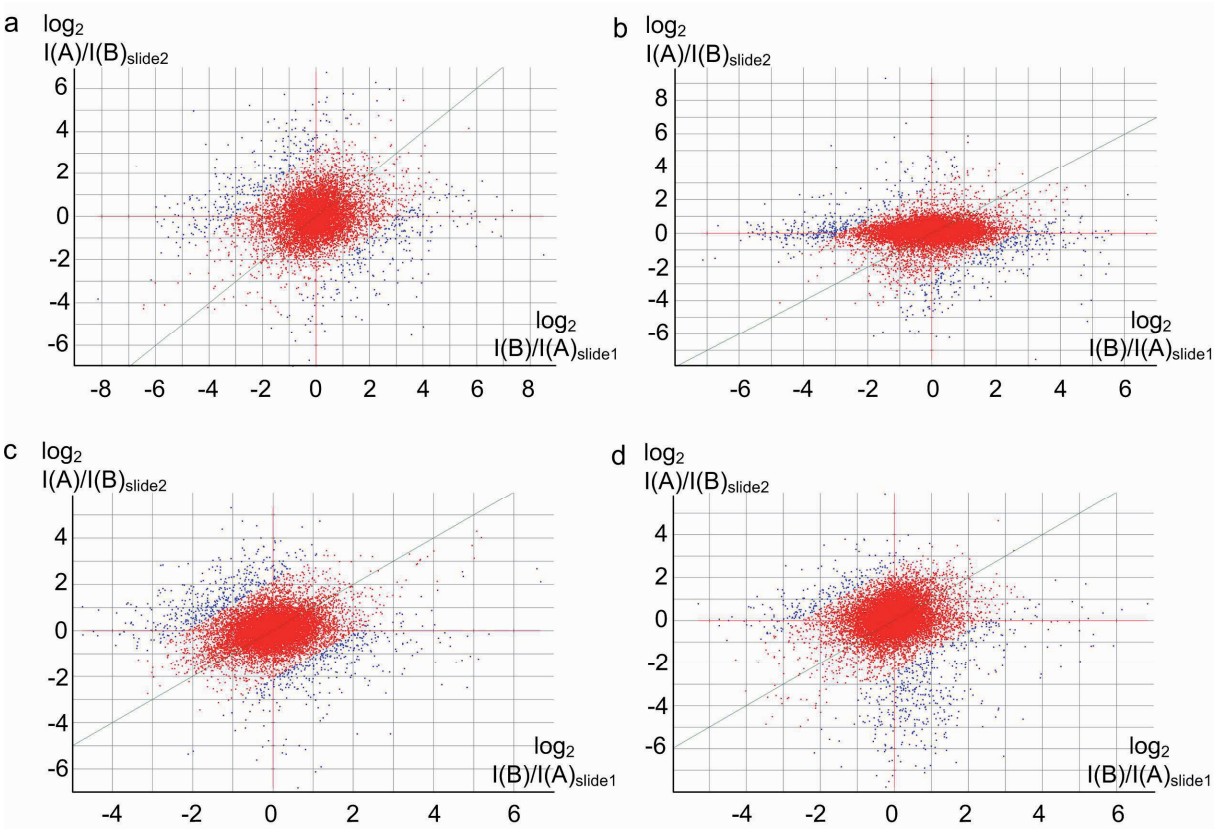

Figure A 3: Schematic representation of flip dye results of the *Azoarcus* sp. BH72 microarray approaches performed under one hour (slide pair 302942 (slide1) and 302943 (slide2)) and four hours (slide pair 301713 (slide1) and 301715 (slide 2)) of quorum sensing conditions. The flip dye diagnostic graph is based on $\log_2$ I(B)/I(A) in slide 1 versus $\log_2$ I(A)/I(B) in slide 2. In slides 302942 and 301715 the cDNA targets from quorum sensing conditions were labeled with Cy3 whereas the cDNA from the early exponential growth phase was Cy5 labelled. In slides 302943 and 301713 the fluorescent dyes used for labeling of the respective cDNA targets were swapped (see Table 7). The pre-filtered data are represented in blue, red dots show post-filtered data of the dye flip analyses.

Table A 2: In *Azoarcus* sp. BH72 under quorum sensing conditions differentially expressed genes detected by microarray analyses.

Acc. No.[a)]	Fold[b)]		Gene	Product Name	P-value[c)]	
	1h	4h			1h	4h
azo0011	1.02	-1.97		short-chain dehydrogenase family protein	0.089	0.012
azo0018	-1.02	2.46		ThiJ/Pfpl family protein	0.445	0.012
azo0038	1.24	-2.18		short-chain dehydrogenase family protein	0.096	0.008
azo0086	-1.90	-1.95	*efp*	translation elongation factor P (EF-P)	0.014	0.041
azo0092	1.54	2.13	*mucD1*	probable serine protease	0.031	0.002
azo0099	1.95	1.15	*def1*	peptide deformylase	0.001	0.053
azo0119	1.56	3.23		amino acid/amide ABC transporter substrate-binding protein, HAAT family	0.006	0.009
azo0152	1.11	-2.11		conserved hypothetical membrane protein	0.010	0.012
azo0153	1.11	-1.92	*atpB*	probable ATP synthase A chain	0.025	0.008
azo0154	-1.39	-2.45	*atpE*	ATP synthase F_0 subcomplex C subunit	0.058	0.007
azo0155	-2.21	-2.45	*atpF*	ATP synthase F_0 subcomplex B subunit	0.010	0.009
azo0156	-2.37	-2.53	*atpH*	ATP synthase F_1 subcomplex delta subunit	0.025	0.012
azo0157	-2.19	-1.84	*atpA*	ATP synthase F_1 subcomplex alpha subunit	0.008	0.003
azo0158	-2.55	-2.08	*atpG*	ATP synthase F_1 subcomplex gamma subunit	0.008	0.009
azo0159	-1.48	-2.77	*atpD*	ATP synthase beta chain	0.023	0.004
azo0160	-1.75	-2.07	*atpC*	ATP synthase F_1 subcomplex epsilon subunit	0.132	0.012
azo0161	1.00	1.96		conserved hypothetical protein	0.030	0.005
azo0163	1.67	2.00		conserved hypothetical protein	0.000	0.000
azo0165	1.58	3.13	*phoR*	PAS/PAC sensor signal transduction histidine kinase	0.385	0.000
azo0223	1.98	1.16		conserved hypothetical secreted protein	0.010	0.029
azo0247	1.89	1.15	*bfr1*	putative bacterioferritin	0.006	0.036
azo0275	3.75	1.61		conserved hypothetical secreted protein	0.017	0.003
azo0291	1.43	-2.75		conserved hypothetical protein	0.137	0.020
azo0294	-1.93	-2.07		putative penicillin-binding protein	0.046	0.005
azo0300	-2.44	1.92	*paaG1*	probable enoyl-CoA hydratase	0.002	0.014
azo0301	-2.00	1.22	*paaH1*	3-hydroxyacyl-CoA dehydrogenase	0.028	0.416
azo0302	-2.93	1.40	*paaI*	phenylacetic acid degradation protein	0.016	0.051
azo0303	-2.18	1.86	*paaK*	phenylacetate-CoA ligase	0.006	0.002
azo0304	-1.57	1.89	*paaA*	phenylacetic acid degradation protein	0.010	0.006
azo0305	-1.98	1.65	*paaB*	phenylacetic acid degradation protein	0.010	0.014
azo0307	-1.83	1.84	*paaD*	probable phenylacetic acid degradation protein	0.007	0.000
azo0308	-1.84	1.43	*paaE*	probable phenylacetic acid degradation NADH oxidoreductase	0.009	0.008
azo0321	1.90	1.85	*cutA2*	putative protein disulfide-isomerase	0.002	0.000
azo0347	1.96	2.86		hypothetical secreted protein	0.032	0.003
azo0352	2.12	2.09		conserved hypothetical protein	0.000	0.010
azo0368	-1.20	-1.99	*mmpI*	probable immunodominant 35kDa protein	0.222	0.008
azo0376	2.65	2.82		conserved hypothetical protein	0.036	0.022
azo0386	4.01	1.93		hypothetical membrane protein	0.018	0.045
azo0391	1.22	2.01		putative MerR-family transcriptional regulator	0.057	0.011
azo0427	-1.55	-2.79	*hslR*	heat shock protein Hsp15	0.191	0.011
azo0429	-1.66	-2.22	*cysD*	sulfate adenylyltransferase subunit 2	0.468	0.016
azo0430	1.11	-3.53	*cysH*	phosphoadenylylsulfate reductase (thioredoxin)	0.056	0.013
azo0432	-1.17	-2.99	*cysI*	putative sulfite reductase	0.371	0.009
azo0438	2.15	-1.09	*ansB2*	periplasmic L-asparaginase II	0.011	0.034
azo0440	1.86	-1.09	*glnM*	amino acid ABC transporter membrane protein 2, PAAT family	0.000	0.021
azo0456	13.30	13.06		hypothetical secreted protein	0.000	0.001
azo0457	-1.82	-1.89	*ragA*	two component transcriptional regulator, winged helix family	0.005	0.016
azo0465	2.94	2.42	*fadB1*	probable enoyl-CoA hydratase / 3-hydroxyacyl-CoA dehydrogenase	0.026	0.013
azo0468	1.17	1.89	*fadB2*	3-hydroxyacyl-CoA dehydrogenase	0.055	0.005

azo0477	2.10	1.24	*recG*	ATP-dependent DNA helicase	0.022	0.347
azo0488	2.05	1.84		conserved hypothetical protein	0.002	0.000
azo0499	1.97	1.98	*pheC*	amino acid ABC transporter substrate-binding protein, PAAT family	0.008	0.004
azo0536	-1.44	-3.15		conserved hypothetical protein	0.090	0.002
azo0544	-1.00	-2.13		hypothetical protein	0.202	0.003
azo0574	-1.31	-2.22	*yebC*	conserved hypothetical protein	0.030	0.016
azo0578	-2.67	-1.87	*emrY*	multidrug resistence transmembrane protein	0.006	0.020
azo0586	-2.05	-2.07		Hypothetical protein	0.001	0.014
azo0587	-3.08	-1.81	*acyH*	adenosylhomocysteinase	0.000	0.025
azo0590	-2.31	-2.16	*htrB*	putative lipid A biosynthesis lauroyl acyltransferase	0.057	0.009
azo0592	-1.68	-1.94	*msbBb*	putative lipid A biosynthesis acyltransferase, truncated	0.018	0.013
azo0616	1.88	2.00	*bfr2*	putative bacterioferritin	0.002	0.001
azo0617	1.86	1.84	*bfd*	conserved hypothetical bacterioferritin-associated ferredoxin	0.003	0.049
azo0622	1.87	2.18		diguanylate cyclase/phosphodiesterase	0.003	0.001
azo0644	2.08	1.39		putative regulatory protein	0.010	0.002
azo0647	1.48	2.70	*hylD*	putatice HlyD family secretion protein	0.150	0.005
azo0649	2.33	2.12		conserved hypothetical protein	0.001	0.001
azo0650	1.66	2.01		conserved hypothetical glycosyltransferase	0.013	0.005
azo0659	1.00	2.14	*gor*	NADPH-glutathione reductase	0.111	0.015
azo0669	2.18	2.66	*napC1*	periplasmic nitrate reductase subunit	0.003	0.010
azo0670	1.07	1.83	*napB1*	periplasmic nitrate reductase subunit	0.144	0.000
azo0671	1.62	2.81	*napA1*	periplasmic nitrate reductase subunit apoprotein	0.007	0.014
azo0672	1.07	3.51	*napD1*	putative NapD protein	0.183	0.012
azo0673	2.72	4.60	*napE*	putative periplasmic nitrate reductase accessory protein	0.020	0.000
azo0682	-2.05	-2.74		conserved hypothetical sodium:solute symporter	0.006	0.015
azo0717	1.27	3.28		conserved hypothetical secreted protein	0.055	0.003
azo0718	-1.52	-2.36	*rpsF*	SSU ribosomal protein S6P	0.492	0.005
azo0719	-3.03	-3.23	*priB*	putative primosomal replication protein	0.012	0.017
azo0720	-1.17	-1.91	*rpsR*	SSU ribosomal protein S18P	0.095	0.010
azo0721	-2.02	-2.69	*rplI*	LSU ribosomal protein L9P	0.007	0.013
azo0746	-3.16	-2.25		hypothetical membrane protein	0.023	0.002
azo0752	-1.29	-2.14	*ychF*	conserved hypothetical protein	0.153	0.020
azo0753	-1.43	-2.54	*pth*	peptidyl-tRNA hydrolase	0.002	0.017
azo0754	1.02	-1.97	*rplY*	LSU ribosomal protein L25P	0.363	0.010
azo0755	-1.81	-2.65	*prsA*	Ribose-phosphate diphosphokinase	0.004	0.013
azo0820	1.08	-2.22	*pckG*	probable phosphoenolpyruvate carboxykinase	0.034	0.019
azo0864	-1.10	-2.27	*accB*	biotin carboxyl carrier protein	0.036	0.005
azo0865	-1.17	-1.88	*aroQ*	3-dehydroquinate dehydratase	0.032	0.003
azo0867	1.95	1.75		conserved hypothetical protein	0.000	0.010
azo0875	-1.70	-2.96	*mraZ*	protein MraZ	0.016	0.017
azo0898	1.42	2.35		putative serine/threonine protein kinase	0.222	0.004
azo0899	1.81	2.28		hypothetical membrane protein	0.000	0.001
azo0923	-1.15	-2.51	*ndk*	nucleoside diphosphate kinase	0.101	0.005
azo0973	-1.59	-2.16	*groES1*	chaperonin	0.010	0.013
azo0974	-2.10	-1.82	*groEL1*	chaperonin	0.007	0.000
azo1046	-2.03	-1.46	*trpF*	phosphoribosylanthranilate isomerase	0.030	0.023
azo1062	-1.20	-2.65	*dnaJ1*	chaperone protein	0.092	0.008
azo1063	-1.38	-4.35	*dnaK*	chaperone protein	0.387	0.000
azo1064	-1.24	-2.84	*grpE*	probable heat shock protein	0.019	0.012
azo1072	-2.11	-2.32	*rpsA*	SSU ribosomal protein S1P	0.027	0.016
azo1073	-1.51	-2.52	*ihfB*	probable integration host factor, beta-subunit	0.017	0.012
azo1080	-2.05	-2.55	*infC*	bacterial translation initiation factor 3 (bIF-3)	0.004	0.014
azo1081	-1.30	-2.04	*rpmI*	LSU ribosomal protein L35P	0.133	0.021
azo1082	-2.64	-3.37	*rplT*	LSU ribosomal protein L20P	0.002	0.000
azo1083	-2.01	-2.80	*pheS*	phenylalanyl-tRNA synthetase, alpha subunit	0.009	0.016
azo1090	1.83	2.14	*rpoS*	RNA polymerase, sigma-38 subunit	0.026	0.001

azo1117	-1.82	-2.20	*aceA*	isocitrate lyase	0.001	0.009
azo1133	-1.51	2.01	*clpS*	ClpS protein	0.036	0.016
azo1134	-2.19	-1.81	*cspE*	cold-shock DNA-binding protein family	0.001	0.014
azo1135	-1.87	-2.99	*rpmG*	LSU ribosomal protein L33P	0.000	0.043
azo1159	-2.36	1.23	*aceB*	AceB protein	0.009	0.015
azo1167	2.22	2.19		conserved hypothetical iron-sulfur protein	0.037	0.004
azo1193	1.57	2.07	*hppD*	probable 4-hydroxyphenylpyruvate dioxygenase	0.466	0.012
azo1198	-1.88	-2.18	*dxs*	1-deoxy-D-xylulose-5-phosphate synthase	0.013	0.015
azo1212	-1.86	-2.59		conserved hypothetical protein	0.006	0.004
azo1222	1.92	1.16	*dmpM*	phenol 2-monooxygenase	0.000	0.027
azo1226	-2.09	-2.47	*lguL*	lactoylglutathione lyase	0.013	0.039
azo1262	1.83	2.32	*lemA1*	conserved hypothetical LemA family protein	0.018	0.018
azo1272	2.77	2.12		conserved hypothetical secreted protein	0.452	0.019
azo1277	1.87	2.30		conserved hypothetical glutathione peroxidase	0.025	0.003
azo1280	-1.47	-5.39	*fpr1*	ferredoxin-$NADP^+$ reductase	0.001	0.004
azo1285	-2.03	1.13	*gcvP*	glycine dehydrogenase (decarboxylating) alpha subunit/beta subunit	0.003	0.036
azo1286	-2.39	1.18	*gcvH*	glycine cleavage system H protein	0.004	0.106
azo1287	-2.64	-1.06	*gcvT*	aminomethyltransferase	0.050	0.064
azo1317	2.45	2.08		conserved hypothetical protein	0.003	0.021
azo1345	1.29	1.83	*ccoG*	putative iron-sulfur 4Fe-4S ferredoxin transmembrane protein	0.311	0.003
azo1349	2.29	2.10		putative universal stress protein f	0.000	0.014
azo1350	2.04	3.13	*phbC2*	probable poly-beta-hydroxybutyrate synthase	0.018	0.005
azo1368	2.57	7.54		conserved hypothetical protein	0.044	0.018
azo1377	-1.47	-2.04	*folD*	5,10-methylenetetrahydrofolate dehydrogenase ($NADP^+$)/methenyltetrahydrofolate cyclohydrolase	0.125	0.014
azo1396	-1.23	-3.33	*nuoA*	NADH dehydrogenase subunit A	0.047	0.007
azo1397	-1.82	-2.49	*nuoB2*	NADH dehydrogenase subunit B	0.005	0.015
azo1398	-2.03	-2.06	*nuoC*	NADH dehydrogenase subunit C	0.010	0.021
azo1399	-1.38	-2.21	*nuoD*	NADH dehydrogenase subunit D	0.013	0.000
azo1400	-1.59	-2.26	*nuoE*	NADH dehydrogenase subunit E	0.003	0.031
azo1401	-2.12	-2.21	*nuoF*	NADH dehydrogenase subunit F	0.017	0.017
azo1402	-1.91	-3.52	*nuoG*	NADH dehydrogenase subunit G	0.027	0.011
azo1403	-2.28	-2.11	*nuoH*	NADH dehydrogenase subunit H	0.009	0.009
azo1404	-2.19	-2.45	*nuoI*	NADH dehydrogenase subunit I	0.027	0.007
azo1406	-2.72	-2.80	*nuoK*	NADH dehydrogenase subunit K	0.007	0.006
azo1407	-1.53	-2.22	*nuoL*	NADH dehydrogenase subunit L	0.000	0.002
azo1408	-1.10	-2.26	*nuoM*	NADH dehydrogenase subunit M	0.215	0.008
azo1409	-1.82	-1.90	*nuoN*	NADH dehydrogenase subunit N	0.010	0.022
azo1425	-2.13	-1.87	*htpG*	probable chaperon protein	0.008	0.019
azo1431	1.19	1.98		conserved hypothetical protein	0.085	0.013
azo1442	1.51	2.04		putative nuclease	0.070	0.016
azo1448	-1.66	-1.98	*motA2*	flagellar motor protein MotA	0.192	0.008
azo1468	-1.12	-2.17	*exbB3*	conserved hypothetical biopolymer transport protein	0.054	0.015
azo1497	-1.16	-2.14	*prfB*	bacterial peptide chain release factor 2 (bRF-2)	0.052	0.013
azo1521	-1.13	-2.43	*rpmE*	LSU ribosomal protein L31P	0.473	0.000
azo1544	1.83	1.98		diguanylate cyclase/ phosphodiesterase with PAS/PAC sensor(s)	0.000	0.000
azo1586	2.14	1.29		conserved hypothetical secreted protein	0.001	0.005
azo1595	-1.05	2.07	*tmk*	thymidylate kinase	0.239	0.006
azo1608	3.37	3.08		hypothetical protein	0.004	0.002
azo1620	-2.22	-2.30		conserved hypothetical protein	0.020	0.011
azo1621	1.16	-2.57	*rpmF*	LSU ribosomal protein L32P	0.106	0.005
azo1622	-1.57	-2.32	*plsX*	phosphate:acyl-[acyl carrier protein] acyltransferase	0.153	0.005
azo1623	-1.33	-2.87	*fabH*	3-oxoacyl-(acyl-carrier-protein) synthase III	0.087	0.014
azo1625	-1.99	-2.12	*fabG1*	3-oxoacyl-(acyl-carrier-protein) reductase	0.009	0.017
azo1630	1.15	1.96	*algU*	RNA polymerase, sigma-24 subunit	0.032	0.014
azo1652	1.29	1.88		conserved hypothetical protein	0.079	0.008

azo1654	-1.19	-1.95		cell division topological specificity factor MinE	0.476	0.010
azo1659	1.07	-2.05	*tex*	transcription accessory protein	0.202	0.012
azo1674	-2.05	-1.26		conserved hypothetical protein	0.014	0.003
azo1675	2.22	2.10		putative phosphoribosyltransferase	0.000	0.006
azo1684	1.86	10.85		conserved hypothetical protein	0.018	0.010
azo1694	1.81	1.83		conserved hypothetical protein	0.001	0.005
azo1699	-2.48	-1.39	*etfB1*	electron transfer flavoprotein, beta subunit	0.018	0.032
azo1700	-2.58	-1.29	*etfA1*	probable electron transfer flavoprotein, alpha subunit	0.016	0.026
azo1701	-2.14	-1.86		conserved hypothetical membrane protein	0.012	0.023
azo1715	2.22	1.10	*rbcR*	transcriptional regulator	0.009	0.053
azo1727	1.97	1.82	*treS*	trehalose synthase	0.012	0.009
azo1743	1.00	2.59		conserved hypothetical protein	0.169	0.015
azo1790	1.35	1.91	*rpoN2*	RNA polymerase, sigma-54 subunit	0.206	0.013
azo1851	2.17	1.82		conserved hypothetical protein	0.003	0.010
azo1864	1.88	2.14		hypothetical protein	0.031	0.014
azo1874	1.86	1.28	*rmlC*	dTDP-4-dehydrorhamnose 3,5-epimerase	0.002	0.004
azo1875	1.88	1.17	*rmlA*	glucose-1-phosphate thymidylyltransferase	0.014	0.065
azo1877	1.86	1.33	*rmlB*	dTDP-glucose 4,6-dehydratase	0.000	0.018
azo1887	1.94	1.28		conserved hypothetical protein	0.000	0.002
azo1896	1.33	-1.90	*lpxB*	lipid-A-disaccharide synthase	0.044	0.008
azo1908	-2.19	-2.47	*tsf*	translation elongation factor Ts (EF-Ts)	0.017	0.007
azo1909	-1.26	-3.47		SSU ribosomal protein S2P	0.103	0.002
azo1912	-1.05	-2.04		conserved hypothetical membrane protein	0.096	0.016
azo1918	2.23	2.48		conserved hypothetical secreted protein	0.020	0.012
azo1922	-2.15	-1.29	*etfB2*	electron transfer flavoprotein, beta-subunit	0.020	0.059
azo1948	-1.40	-1.89	*padD*	phenylacetyl-CoA:acceptor oxidoreductase PadD subunit	0.099	0.002
azo1965	1.19	1.89		transcriptional regulator, AraC family	0.121	0.010
azo1978	1.98	1.93		conserved hypothetical protein	0.032	0.013
azo1985	2.58	2.09		putative AMP-binding protein	0.003	0.014
azo1995	3.18	2.23		conserved hypothetical protein	0.033	0.019
azo2008	1.89	1.44		(LSU ribosomal protein L3P)-glutamine N5-methyltransferase	0.000	0.017
azo2062	1.24	2.32		conserved hypothetical peptidyl-prolyl cis-trans isomerase	0.283	0.001
azo2063	1.20	2.06	*bcp1*	putative bacterioferritin comigratory protein	0.017	0.004
azo2070	1.82	-1.12	*clpX*	ATP-dependent Clp protease, ATP-binding subunit	0.012	0.031
azo2072	-1.03	-2.93	*tig*	trigger factor	0.025	0.015
azo2073	1.99	4.33	*prkA*	putative serine protein kinase	0.006	0.011
azo2074	2.64	4.49	*yeaH*	conserved hypothetical protein	0.001	0.006
azo2075	2.05	2.42	*ycgB*	putative cytoplasmic protein	0.000	0.001
azo2080	1.07	-2.13	*thrC*	L-threonine synthase	0.026	0.019
azo2088	-2.00	-1.38		conserved hypothetical secreted protein	0.017	0.020
azo2103	-1.47	-2.31	*pnp*	polyribonucleotide nucleotidyltransferase	0.099	0.016
azo2104	-1.42	-2.38	*rpsO*	SSU ribosomal protein S15P	0.008	0.049
azo2106	-1.29	-1.96		ribosome-binding factor A	0.004	0.023
azo2108	-1.04	-2.75	*nusA*	NusA antitermination factor	0.077	0.018
azo2109	-1.24	-2.31		conserved hypothetical protein	0.003	0.016
azo2120	1.00	-2.12	*ylqF*	probable GTPase	0.228	0.005
azo2121	-2.03	-2.85	*cspA*	cold-shock DNA-binding protein family	0.000	0.009
azo2130	2.58	-1.10	*fabI2*	Enoyl-(acyl-carrier-protein) reductase (NADH)	0.026	0.022
azo2142	1.58	4.14		putative inosine-5'-monophosphate dehydrogenase related protein	0.030	0.013
azo2148	1.25	1.95	*sndH*	putative L-sorbosone dehydrogenase	0.130	0.000
azo2151	1.09	-1.97	*etf1*	probable electron transfer flavoprotein-ubiquinone oxidoreductase	0.082	0.018
azo2156	2.42	-1.32		probable TonB-dependent receptor	0.020	0.017
azo2175	1.79	2.43	*pilY1A*	putative type IV pilus biogenesis protein	0.378	0.004
azo2177	1.28	2.06	*pilW*	putative type IV pilus biogenesis protein	0.048	0.008

azo2179	-1.02	2.13		conserved hypothetical protein	0.056	0.000
azo2180	1.10	3.00		conserved hypothetical prepilin like protein	0.166	0.008
azo2186	-1.93	-1.94	*argG*	argininosuccinate synthase	0.006	0.015
azo2187	1.00	-1.84	*argF*	ornithine carbamoyltransferase	0.273	0.007
azo2190	-1.28	-2.27	*rpsT*	SSU ribosomal protein S20P	0.178	0.006
azo2192	-1.81	-2.22		hypothetical protein	0.000	0.014
azo2197	2.24	1.84	*bcp2*	putative bacterioferritin comigratory protein	0.006	0.001
azo2220	2.97	1.95	*cbiM*	putative cobalt transport system, permease protein	0.032	0.005
azo2224	-1.11	-2.38	*yaiI*	YaiI/YqxD family protein	0.060	0.003
azo2257	-1.11	-2.32	*mauA*	probable methylamine dehydrogenase, L chain	0.491	0.005
azo2290	2.06	1.51		conserved hypothetical protein	0.019	0.008
azo2296	2.55	2.61	*phhB*	pterin-4-alpha-carbinolamine dehydratase	0.016	0.031
azo2314	1.35	2.67		glycosyltransferase	0.067	0.005
azo2315	1.27	1.89		conserved hypothetical tyrosine/serine phosphatase	0.168	0.004
azo2324	2.43	2.47		putative polysaccharide deacetylase	0.004	0.004
azo2338	2.02	2.49	*prc*	C-terminal processing peptidase-1, serine peptidase, MEROPS family S41A	0.003	0.010
azo2396	-1.73	-2.00		putative TonB-dependent receptor	0.007	0.023
azo2405	-1.75	-2.18	*ohr*	probable organic hydroperoxide resistance protein	0.019	0.013
azo2408	9.75	2.24		hypothetical sensor protein	0.000	0.009
azo2442	2.02	2.00	*poxC*	phenol 2-monooxygenase P2 subunit	0.009	0.002
azo2469	1.00	2.63		conserved hypothetical protein	0.353	0.009
azo2484	-1.22	2.01		response regulator receiver modulated metal dependent phosphohydrolase	0.061	0.001
azo2491	1.27	-1.92	*etfA3*	probable electron transfer flavoprotein, alpha subunit	0.002	0.019
azo2492	-1.81	-1.87	*etfB3*	electron transfer flavoprotein, beta subunit	0.005	0.023
azo2498	2.00	1.09	*paaG3*	putative enoyl-CoA hydratase	0.003	0.134
azo2515	1.00	2.81	*nagH*	putative ring hydroxylating beta subunit	0.205	0.008
azo2552	1.10	2.41	*pilU1*	twitching motility protein	0.323	0.000
azo2561	1.96	3.91		conserved hypothetical protein	0.055	0.006
azo2562	3.08	1.91	*ccp*	putative cytochrome c peroxidase	0.005	0.011
azo2563	1.90	2.27		conserved hypothetical secreted protein	0.001	0.000
azo2573	3.51	1.62	*fsr*	putative fosmidomycin resistance protein	0.008	0.110
azo2588	-2.38	1.15	*ompA1*	outer membrane protein A precursor	0.002	0.047
azo2640	-1.37	2.71	*mdcH*	putative transcriptional factor	0.190	0.008
azo2645	1.86	1.84		hypothetical protein	0.023	0.010
azo2646	1.71	2.48		conserved hypothetical protein	0.006	0.011
azo2651	2.66	2.53		conserved hypothetical protein	0.010	0.019
azo2656	1.53	2.50		conserved hypothetical membrane protein	0.074	0.004
azo2664	-1.28	2.41		conserved hypothetical protein	0.243	0.011
azo2672	1.50	1.84		conserved hypothetical sensor histidine kinase	0.022	0.001
azo2690	1.08	2.35		conserved hypothetical protein	0.019	0.001
azo2694	1.89	2.00		nucleotide sugar aminotransferase	0.003	0.012
azo2696	1.57	1.80		conserved hypothetical protein	0.002	0.008
azo2698	2.71	1.93	*pepM*	putative phosphoenolpyruvate phosphomutase	0.040	0.004
azo2740	1.97	1.64	*flgB*	flagellar basal-body rod protein	0.007	0.001
azo2758	2.16	2.72		conserved hypothetical protein	0.001	0.008
azo2759	-2.03	-3.16	*rpsI*	SSU ribosomal protein S9P	0.012	0.002
azo2760	-1.35	-2.99	*rplM*	LSU ribosomal protein L13P	0.384	0.002
azo2763	-1.09	-2.08		conserved hypothetical secreted protein	0.116	0.003
azo2790	2.24	2.82		conserved hypothetical protein	0.026	0.012
azo2813	-1.95	-1.65		conserved hypothetical secreted protein	0.046	0.006
azo2827	2.03	1.76	*phoU*	phosphate uptake regulator	0.002	0.025
azo2835	1.91	1.39	*prkB*	probable phosphoribulokinase	0.004	0.008
azo2844	1.00	2.32	*exaA1*	putative quinoprotein ethanol dehydrogenase	0.064	0.002
azo2845	1.00	2.03	*qbdB2*	conserved hypothetical secreted protein	0.023	0.011
azo2859	2.50	1.84	*estB*	probable carboxylesterase	0.037	0.009
azo2871	2.14	5.46		conserved hypothetical YceI like protein	0.004	0.005

azo2876	3.33	6.82		conserved hypothetical membrane protein	0.006	0.015
azo2883	1.93	2.02		conserved hypothetical secreted protein	0.012	0.008
azo2898	-1.09	-3.06	*rpsP*	SSU ribosomal protein S16P	0.084	0.007
azo2900	-2.08	-2.10	*trmD*	tRNA (guanine37-N(1)-) methyltransferase	0.023	0.017
azo2901	-2.04	-2.07	*rplS*	LSU ribosomal protein L19P	0.034	0.000
azo2914	1.88	1.41	*pilV*	putative prepilin-like protein	0.021	0.002
azo2916	3.41	2.16	*pilX*	putative type IV pilus assembly protein	0.002	0.001
azo2931	1.48	2.00		conserved hypothetical protein	0.020	0.003
azo2956	-1.16	2.55		putative cooper-transporting ATPase protein	0.055	0.000
azo2961	1.05	2.16		hypothetical secreted protein	0.231	0.013
azo2962	1.31	2.10	*sigZ*	putative RNA polymerase sigma factor	0.432	0.021
azo2969	1.95	2.21		conserved hypothetical secreted protein	0.024	0.015
azo2975	1.50	3.11	*exaA3*	putative quinoprotein ethanol dehydrogenase	0.007	0.021
azo2977	1.10	1.92	*cphA*	putative beta lactamase precursor	0.018	0.007
azo2987	1.57	2.32		dihydrofolate reductase, putative	0.151	0.013
azo3023	-5.24	-1.15		probable TonB-dependent receptor	0.001	0.457
azo3041	2.21	2.41	*rubA*	probable rubredoxin	0.008	0.012
azo3042	-5.02	-1.28		NADPH quinone oxidoreductase, putative	0.008	0.102
azo3047	1.98	1.12	*livG1*	amino acid/amide ABC transporter ATP-binding protein 1, HAAT family	0.003	0.024
azo3050	1.00	4.26	*livJ*	amino acid/amide ABC transporter substrate-binding protein, HAAT family	0.344	0.013
azo3051	1.33	3.20		conserved hypothetical protein	0.049	0.024
azo3057	1.41	2.39	*boxB*	benzoyl-CoA oxygenase, component B	0.002	0.021
azo3060	3.79	2.42		conserved hypothetical protein	0.004	0.006
azo3073	2.47	1.49		hypothetical protein	0.005	0.004
azo3077	2.47	2.18		conserved hypothetical protein	0.021	0.013
azo3128	2.15	2.13	*nikR*	transcriptional regulator, CopG family	0.001	0.012
azo3146	1.00	3.08		conserved hypothetical protein	0.051	0.013
azo3154	1.04	-2.11	*ilvI*	acetolactate synthase, large subunit	0.097	0.020
azo3157	1.32	1.81	*pssA*	CDP-diacylglycerol-serine O-phosphatidyltransferase	0.004	0.003
azo3160	3.09	3.62		conserved hypothetical protein	0.043	0.013
azo3167	-1.08	-2.30	*rplU*	LSU ribosomal protein L21P	0.454	0.049
azo3168	-1.23	-3.25	*rpmA*	LSU ribosomal protein L27P	0.007	0.030
azo3170	-1.26	-2.16	*proB*	glutamate 5-kinase	0.049	0.003
azo3174	1.93	1.99		putative methyltransferase	0.035	0.011
azo3194	1.41	2.54		conserved hypothetical protein	0.016	0.019
azo3212	1.60	1.93	*parA3*	ParA family protein	0.000	0.012
azo3225	-1.11	-1.82	*rpsU*	SSU ribosomal protein S21P	0.139	0.000
azo3287	1.14	2.01		conserved hypothetical secreted protein	0.057	0.007
azo3293	2.42	3.42	*senC*	SCO1/SenC family protein	0.000	0.001
azo3294	5.07	11.34	*coxD*	4-hydroxybenzoate octaprenyltransferase	0.001	0.003
azo3300	-1.87	-2.61		conserved hypothetical membrane protein	0.017	0.014
azo3303	1.00	1.82	*coxA*	probable cytochrome c oxidase, subunit I	0.095	0.012
azo3304	1.50	2.10	*coxB*	conserved hypothetical cytochrome c oxidase, subunit II	0.068	0.006
azo3319	1.93	1.25	*fkbP*	peptidyl-prolyl cis-trans isomerase	0.004	0.037
azo3320	1.88	2.14	*msrA*	putative peptide methionine sulfoxide reductase	0.009	0.012
azo3325	-1.98	-2.17	*trpE*	anthranilate synthase, component I	0.014	0.006
azo3327	1.28	-1.81	*rpe*	ribulose-5-phosphate 3-epimerase	0.084	0.010
azo3330	2.10	1.83		FHA-domain containing protein	0.004	0.005
azo3354	1.58	1.74	*pilB*	type IV pilus assembly protein	0.006	0.006
azo3355	1.38	2.98	*pilA*	type IV pilus structural protein	0.028	0.021
azo3356	1.97	1.38	*pilR*	two-component response regulator	0.001	0.024
azo3364	-1.57	-1.92		glycosyltransferase	0.310	0.009
azo3388	2.03	3.16		hypothetical protein	0.000	0.000
azo3390	-2.12	-2.27	*rplQ*	LSU ribosomal protein L17P	0.024	0.003
azo3391	-3.38	-2.22	*rpoA*	DNA-directed RNA polymerase subunit alpha	0.010	0.011
azo3393	-2.75	-2.02	*rpsK*	SSU ribosomal protein S11P	0.003	0.036
azo3394	-1.55	-2.20	*rpsM*	SSU ribosomal protein S13P	0.023	0.000

azo3395	-1.24	-2.05	*rpmJ*	LSU ribosomal protein L36P	0.111	0.001
azo3398	-1.73	-2.16	*rplO*	LSU ribosomal protein L15P	0.032	0.008
azo3399	-2.10	-2.07	*rpmD*	LSU ribosomal protein L30P	0.179	0.008
azo3400	-2.34	-2.03	*rpsE*	SSU ribosomal protein S5P	0.017	0.012
azo3401	-1.49	-2.59	*rplR*	LSU ribosomal protein L18P	0.223	0.011
azo3402	-2.38	-2.45	*rplF*	LSU ribosomal protein L6P	0.039	0.017
azo3403	-3.11	-2.57	*rpsH*	SSU ribosomal protein S8P	0.034	0.019
azo3404	-2.32	-2.66	*rpsN*	SSU ribosomal protein S14P	0.010	0.010
azo3405	-2.95	-2.51	*rplE*	LSU ribosomal protein L5P	0.014	0.014
azo3406	-2.54	-1.83	*rplX*	LSU ribosomal protein L24P	0.016	0.019
azo3407	-1.37	-2.80	*rplN*	LSU ribosomal protein L14P	0.050	0.003
azo3408	-1.99	-2.30	*rpsQ*	SSU ribosomal protein S17P	0.008	0.000
azo3409	-1.51	-2.33	*rpmC*	LSU ribosomal protein L29P	0.017	0.008
azo3410	-1.34	-2.07	*rplP*	LSU ribosomal protein L16P	0.057	0.009
azo3411	-1.93	-2.89	*rpsC*	SSU ribosomal protein S3P	0.002	0.013
azo3412	-2.62	-3.50	*rplV*	LSU ribosomal protein L22P	0.011	0.004
azo3413	-2.49	-2.67	*rpsS*	SSU ribosomal protein S19P	0.014	0.002
azo3414	-1.87	-3.73	*rplB*	LSU ribosomal protein L2P	0.036	0.000
azo3415	-3.01	-2.86	*rplW*	LSU ribosomal protein L23P	0.004	0.005
azo3416	-2.56	-3.18	*rplD*	LSU ribosomal protein L4P	0.012	0.016
azo3417	-2.30	-2.98	*rplC*	LSU ribosomal protein L3P	0.010	0.000
azo3418	-1.55	-2.14	*rpsJ*	SSU ribosomal protein S10P	0.085	0.016
azo3419	-2.05	-1.69	*tufA*	elongation factor Tu	0.013	0.073
azo3420	-1.93	-2.09	*fusA2*	translation elongation factor 2 (EF-2/EF-G)	0.006	0.014
azo3422	-1.65	-3.66	*rpsL*	SSU ribosomal protein S12P	0.398	0.013
azo3425	-1.71	-3.24	*rplL*	LSU ribosomal protein L12P	0.009	0.002
azo3426	-2.29	-2.36	*rplJ*	LSU ribosomal protein L10P	0.020	0.015
azo3427	-2.77	-2.19	*rplA*	LSU ribosomal protein L1P	0.020	0.021
azo3428	-2.71	-3.45	*rplK*	LSU ribosomal protein L11P	0.009	0.000
azo3429	-1.52	-2.07	*nusG*	transcription antitermination protein nusG	0.019	0.022
azo3430	-1.75	-2.23	*secE*	protein translocase subunit secE/sec61 gamma	0.410	0.017
azo3431	-1.84	-1.92	*tufB*	translation elongation factor 1A (EF-1A/EF-Tu)	0.021	0.000
azo3436	1.90	-1.54	*flcA*	two component transcriptional regulator, LuxR family	0.013	0.005
azo3442	1.88	2.39	*livF*	amino acid/amide ABC transporter ATP-binding protein 2, HAAT family	0.016	0.008
azo3461	1.92	1.27	*pyrR*	phosphoribosyl transferase	0.001	0.003
azo3462	1.91	1.19	*pyrB*	aspartate carbamoyltransferase	0.015	0.078
azo3478	1.00	4.08		conserved hypothetical secreted protein	0.160	0.017
azo3479	2.18	2.53	*fdhC*	formate dehydrogenase gamma subunit	0.024	0.010
azo3481	4.53	3.99	*fdhB*	probable formate dehydrogenase iron-sulfur subunit	0.018	0.013
azo3485	4.37	15.48		conserved hypothetical iron-sulfur cluster-binding protein	0.020	0.005
azo3486	-2.61	-1.13		conserved hypothetical protein	0.014	0.051
azo3498	1.96	2.09		histidine kinase	0.012	0.008
azo3507	2.85	2.13	*ureA*	urease gamma subunit	0.007	0.004
azo3522	-1.73	-3.09	*rhlE3*	putative ATP-dependent RNA helicase	0.417	0.010
azo3523	-1.02	-2.38	*leuA2*	2-isopropyl malate synthase	0.243	0.011
azo3528	-1.56	-2.16		conserved hypothetical membrane protein	0.002	0.014
azo3529	-1.68	-2.34		precorrin-4 C11-methyltransferase	0.159	0.010
azo3530	-1.49	-2.65	*cbiX*	conserved hypothetical protein CbiX	0.113	0.019
azo3570	1.85	1.51	*waaP3*	lipopolysaccharide core biosynthesis protein	0.003	0.000
azo3623	1.90	1.03	*adhC*	alcohol dehydrogenase class III	0.003	0.248
azo3628	1.64	3.08		hypothetical protein	0.207	0.011
azo3642	-1.23	-2.45	*gltB*	glutamate synthase (NADH) large subunit	0.113	0.019
azo3657	1.47	2.35		conserved hypothetical membrane protein	0.295	0.011
azo3668	2.15	2.12		conserved hypothetical membrane protein	0.018	0.004
azo3674	4.95	4.27	*mucD4*	probable serine protease MucD	0.000	0.005
azo3677	1.00	3.36	*dctP6*	putative C_4-dicarboxylate-binding periplasmic protein	0.203	0.004

azo3679	1.14	2.15		glyoxalase-family protein	0.140	0.015
azo3680	1.31	2.73		conserved hypothetical protein	0.292	0.007
azo3700	1.49	2.64	*trxC2*	probable thioredoxin-disulfide reductase	0.019	0.000
azo3711	8.40	2.95		conserved hypothetical protein	0.025	0.020
azo3727	1.97	2.38		conserved hypothetical secreted protein	0.014	0.013
azo3732	2.26	1.88	*livM5*	amino acid/amide ABC transporter membrane protein 2, HAAT family	0.011	0.014
azo3758	-1.28	-2.19	*dcrH3*	putative hemerythrin-like protein	0.023	0.020
azo3764	1.15	2.00		conserved hypothetical protein	0.021	0.005
azo3770	1.15	3.72		conserved hypothetical protein	0.208	0.007
azo3772	1.16	2.20		conserved hypothetical protein	0.418	0.001
azo3784	1.08	1.86		hypothetical secreted protein	0.011	0.007
azo3790	1.33	2.14		conserved hypothetical glutathione peroxidase	0.070	0.010
azo3793	1.88	2.95	*hupF*	putative hydrogenase expression/ formation protein	0.029	0.005
azo3815	-1.02	-2.32		probable phasin	0.046	0.010
azo3862	1.88	1.22		conserved hypothetical membrane protein	0.017	0.010
azo3868	10.45	2.92	*acoB2*	probable acetoin dehydrogenase, beta subunit	0.020	0.009
azo3870	1.82	1.19		hypothetical protein	0.021	0.016
azo3872	3.19	1.72		conserved hypothetical secreted protein	0.017	0.005
azo3873	4.11	2.21		putative glucase dehydrogenase alpha subunit	0.020	0.017
azo3874	5.08	2.00		conserved hypothetical secreted protein	0.021	0.022
azo3896	1.90	1.32	*sciI*	putative cytoplasmic protein	0.017	0.076
azo3898	2.11	1.26	*sciK*	putative cytoplasmic protein	0.016	0.028
azo3906	1.92	1.64		hypothetical protein	0.000	0.018
azo3911	1.94	1.27	*fabF2*	3-oxoacyl-(acyl-carrier-protein) synthase	0.000	0.083
azo3966	1.42	2.21		hypothetical protein	0.011	0.002
azo3989	-1.01	-2.01		protein translocase subunit yidC	0.213	0.012
azo3990	-1.95	-2.99		conserved hypothetical protein	0.048	0.016
azo3991	-1.49	-2.09		ribonuclease P protein component	0.012	0.011
azo3992	-1.95	-2.35	*rpmH*	LSU ribosomal protein L34P	0.017	0.000

[a)] Acc. No. = accession number/locus tag of *Azoarcus* sp. BH72 genome

[b)] expression fold: expression ratios for each gene were calculated by dividing the corresponding intensity values from one condition by the intensity values from the other condition, to obtain an average expression fold the mean of the twelve replicate expression ratios was calculated

[c)] P-value: one-tailed paired t-test

Table A 3: C_T-values and melting temperatures of PCR-products obtained from real-time PCR experiments.

Gene	Sample[a)]	C(T)	Tm (-dI/dT max)	Gene	Sample[a)]	C(T)	Tm (-dI/dT max)
16 S	1 EE I a	2.040	86.0	16 S	1 EE IV a	3.476	86.5
16 S	2 EE I b	3.016	86.5	16 S	2 EE IV b	4.766	86.5
16 S	3 EE II a	5.090	86.5	16 S	3 EE V a	4.278	86.5
16 S	4 EE II b	5.397	86.5	16 S	4 EE V b	3.090	86.5
16 S	5 QS I a	2.324	86.5	16 S	5 QS IV a	4.757	86.5
16 S	6 QS I b	5.914	86.5	16 S	6 QS IV b	2.567	86.5
16 S	7 QS II a	5.746	86.5	16 S	7 QS V a	3.826	86.5
16 S	8 QS II b	2.621	86.0	16 S	8 QS V b	5.988	86.5
azo3874	1 EE I a	22.581	89.5	*azo3412*	1 EE IV a	20.131	87.5
azo3874	2 EE I b	24.086	89.5	*azo3412*	2 EE IV b	21.010	87.0
azo3874	3 EE II a	24.128	90.0	*azo3412*	3 EE V a	19.579	87.5
azo3874	4 EE II b	24.186	90.0	*azo3412*	4 EE V b	20.856	87.0
azo3874	5 QS I a	18.186	90.0	*azo3412*	5 QS IV a	22.197	87.0
azo3874	6 QS I b	20.881	90.0	*azo3412*	6 QS IV b	21.495	86.5
azo3874	7 QS II a	17.363	89.5	*azo3412*	7 QS V a	21.071	87.0
azo3874	8 QS II b	18.302	89.5	*azo3412*	8 QS V b	23.025	86.5
azo3868	1 EE I a	22.928	88.5	*azo3294*	1 EE IV a	26.203	74.0
azo3868	2 EE I b	23.780	88.5	*azo3294*	2 EE IV b	24.264	74.0
azo3868	3 EE II a	24.071	88.5	*azo3294*	3 EE V a	24.931	74.0
azo3868	4 EE II b	24.285	88.5	*azo3294*	4 EE V b	26.259	74.0
azo3868	5 QS I a	19.325	88.5	*azo3294*	5 QS IV a	25.845	74.0
azo3868	6 QS I b	19.891	88.5	*azo3294*	6 QS IV b	23.608	73.0
azo3868	7 QS II a	15.178	88.5	*azo3294*	7 QS V a	26.138	73.5
azo3868	8 QS II b	19.816	88.0	*azo3294*	8 QS V b	27.749	74.5
azo3674	1 EE I a	24.736	90.5	*azo0673*	1 EE IV a	25.548	88.5
azo3674	2 EE I b	26.135	91.0	*azo0673*	2 EE IV b	26.469	89.0
azo3674	3 EE II a	24.365	91.0	*azo0673*	3 EE V a	24.307	89.0
azo3674	4 EE II b	26.340	91.0	*azo0673*	4 EE V b	25.251	88.5
azo3674	5 QS I a	17.137	91.0	*azo0673*	5 QS IV a	22.351	88.5
azo3674	6 QS I b	24.061	91.0	*azo0673*	6 QS IV b	20.984	88.5
azo3674	7 QS II a	14.663	91.0	*azo0673*	7 QS V a	22.752	89.0
azo3674	8 QS II b	23.374	90.5	*azo0673*	8 QS V b	23.183	88.5
azo0156	1 EE I a	7.595	87.0				
azo0156	2 EE I b	14.063	87.5				
azo0156	3 EE II a	16.273	90.5				
azo0156	4 EE II b	15.558	87.0				
azo0156	5 QS I a	10.541	90.0				
azo0156	6 QS I b	16.621	90.0				
azo0156	7 QS II a	17.749	87.0				
azo0156	8 QS II b	15.490	90.0				

[a)] I and II = RNA samples from one hour quorum sensing condition (QS) and the respective control uner early exponential condition (EE), IV and V = RNA samples from four hours quorum sensing condition (QS) and the respective control uner early exponential condition (EE)

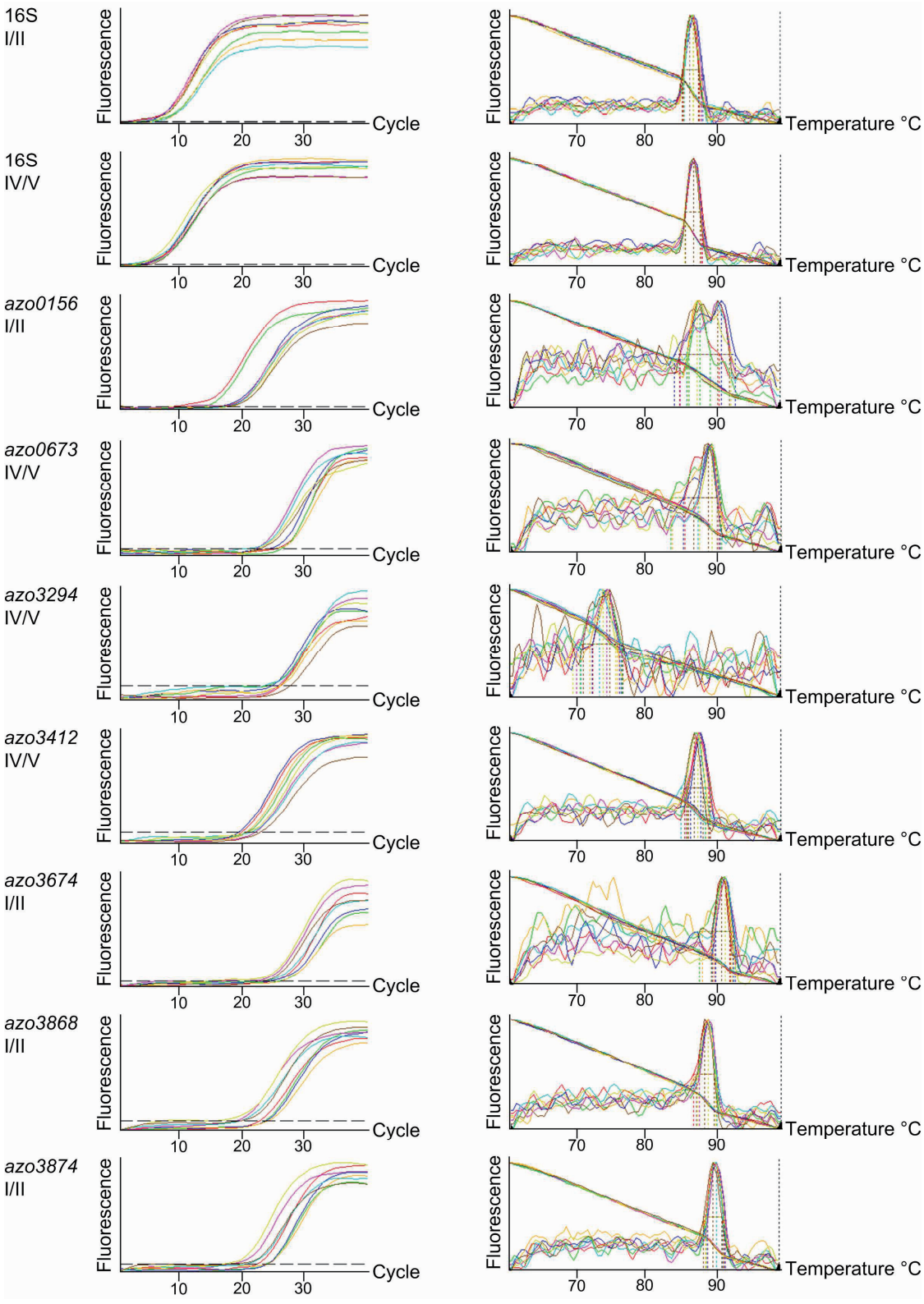

Figure A 4: Diagram of real-time PCR and melting curves for seven *Azoarcus* sp. BH72 genes and the 16S reference gene. Data were obtained from two independent RNA samples and two technical replicates from the early exponential growth phase in comparison to quorum sensing conditions. I and II = RNA samples from one hour quorum sensing condition (QS) and the respective control uner early exponential condition (EE), IV and V = RNA samples from four hours quorum sensing condition (QS) and the respective control uner early exponential condition (EE For colour code of curves see Table A 3 with experimental data: red = 1, green = 2, blue =3, orange = 4, magenta = 5, turquoise = 6, yellow = 7, brown = 8.

VI 2 Appendix B

Table B 1: Quorum sensing systems present in metagenome samples and their corresponding closest BlastP relative.

Table B 2: Complete list of all identified *Azoarcus* sp. BH72 proteins synthesized in the exponential growth phase under standard aerobic growth conditions.

Table B 3: Detailed protein parameters of all identified *Azoarcus* sp. BH72 proteins synthesized in the exponential growth phase under standard aerobic growth conditions.

Table B 4: M-values of all *Azoarcus* sp. BH72 replicate spots obtained by microarray studies after one and four hours quorum sensing conditions in comparison to the control under early exponential growth conditions.

Table B 5: In *Azoarcus* sp. BH72 under quorum sensing conditions differentially expressed genes and the corresponding parameters of gene products.

Table B 6: Protein blast analyses of amino acid sequences of quorum sensing regulated (conserved) hypothetical proteins of *Azoarcus* sp. BH72.

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VI 4 Declaration

I hereby assure that I wrote the dissertation with the title „Molecular characterization of a novel quorum sensing system of the diazotrophic grass endophyte *Azoarcus* sp. BH72“ independently and without any prohibited material. All used resources are stated with the respective reference.

Hiermit versichere ich, dass ich die vorliegende Dissertation mit dem Thema „Molecular characterization of a novel quorum sensing system of the diazotrophic grass endophyte *Azoarcus* sp. strain BH72“ selbstständig und ohne unerlaubte fremde Hilfe verfasst habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Bremen, May 2010

Lena Hauberg