Investigating the type VI secretion systems found in diazotrophic, grass endophyte *Azoarcus* sp. strain BH72

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Declaration

I hereby declare that this dissertation was written independently and that all resources and aids are stated. The experiments performed during my study were carried out from July 2009 until June 20013 under the guidance of Prof. Dr. Barbara Reinhold-Hurek in the Department of Microbe-Plant Interactions at the University of Bremen, Fachbereich 2, Bremen, Germany.

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Summary

In this study the predicted T6SS gene clusters found within the genome of *Azoarcus* sp. strain BH72 were examined. It was found that both gene clusters share the characteristics of T6SSs of other organisms described in literature. Additionally, the T6SS encoded by the *sci* cluster (T6SS-2) contained several genes with similarity to the HSI-1 T6SS of *Pseudomonas aeruginosa* PA01, which utilizes a threonine phosphorylation pathway mode of post-translational regulation.

The hallmark of a functioning T6SS is the presence of Hcp in culture supernatants. Although the antibodies used in this study did display cross-reactivity, these antibodies were used to confirm *Azoarcus* sp. strain BH72 expresses and secretes Hcp proteins. Furthermore, deletion of predicted core components manipulated the on and off secretion status, as did deletion of a predicted component of the threonine phosphorylation pathway. However, the putative threonine phosphorylation pathway in *Azoarcus* sp. strain BH72 was not stimulated by surface contact growth in the assays used in this study, like it is in bacterial competition by *P. aeruginosa* PA01.

The up-regulation of *imp* gene cluster (T6SS-1) genes had been confirmed previously. An increase of Hcp secretion was also observed with growth under nitrogen fixing conditions in this study using Western blot analyses. Because of this, secretome data was acquired from cultures grown under nitrogen fixing conditions. From this, the secreted effector protein(s) could not be distinguished, but it was observed that T6SS-2 components were more represented in the supernatant than T6SS-1 components.

Still, without identification of an effector, inoculation of rice seedlings with an *Azoarcus* strain where both T6SSs had been turned off resulted in a plant response not seen in rice seedlings that had been inoculated with wild type *Azoarcus* sp. strain BH72. Additionally, the T6SS deficient strain colonized rice seedlings in higher numbers than wild type.

Finally, in a quest for T6SS transcriptional regulators, several potential regulators were screened. Azo0559 was determined to at least partially activate expression of T6SS-1 under nitrogen fixing conditions.

Zusammenfassung

In dieser Studie wurden die prognostizierten T6SS Gencluster, welche im Genom von *Azoarcus* sp. Stamm BH72 gefunden wurden, untersucht. Es wurde herausgefunden, dass beide Gencluster Eigenschaften mit anderen in der Literatur beschriebenen T6SSen gemein haben. Zusätzlich wurde herausgefunden, dass das T6SS, welches vom *sci* Cluster codiert wird (T6SS-2), mehrere Gene mit Ähnlichkeiten zum HSI-1 T6SS von *Pseudomonas aeruginosa* PA01 besitzt, das eine Threoninphosphorylierungspfad zur posttranslationalen Regulierung nutzt.

Das Kennzeichen eines aktiven T6SS ist das Vorhandensein von Hcp im Kulturüberstand. Obwohl die Antikörper, die in dieser Studie verwendet wurden, eine Kreuzreaktivität besaßen, wurden sie genutzt, um Expression und Sekretion von Hcp Proteinen durch *Azoarcus* sp. Stamm BH72 zu verifizieren. Es konnte gezeigt werden, dass sowohl die Deletion einiger Kernkomponenten die Sekretion durch die T6SSe beeinflussen, als auch die Deletion von prognostizierten Komponenten des Threoninphosphorylierungspfads. Im Gegensatz zur bakteriellen Kompetition durch *P. aeruginosa* PA01 wurde der Threonin-phosphorylierungspfad in *Azoarcus* sp. Stamm BH72 in den durchgeführten Versuchen jedoch nicht durch Oberflächenkontakt-abhängiges Wachstum stimuliert.

Die Induktion der Genexpression des *imp* Genclusters (T6SS-1) wurde bereits im Vorfeld dieser Studie bestätigt. Ein Anstieg in der Hcp Sekretion wurde außerdem unter stickstofffixierenden Bedingungen in dieser Studie durch Western Blot Analysen gezeigt. Um diesen Umstand zu nutzen, wurden Sekretomdaten von Kulturen, die unter stickstofffixierenden Bedingungen wuchsen, erarbeitet. Diese Daten konnten nicht zur Identifikation eines oder mehrerer Effektormoleküle beitragen, es wurde jedoch beobachtet, dass die T6SS-2 Komponenten stärker im Überstand vertreten waren als die des T6SS-1.

Trotz der fehlenden Identifikation eines Effektormoleküls konnte bei der Inokulierung von Reissämlingen mit einem *Azoarcus* Stamm, in welchem beide T6SSs abgeschaltet waren, ein Änderung im Verhalten der Reissämlingen im Vergleich zur Inokulierung mit dem Wildtyp *Azoarcus* sp. Stamm BH72 beobachtet werden. Zusätzlich infizierte der T6SS defiziente Stamm Reissämlinge in größerer Zahl als der Wildtyp.

Schlussendlich wurden in einem Versuch, transkriptionelle Regulatoren der T6SSe zu identifizieren, mehrere potentielle Regulatoren untersucht. Es stellte sich dabei raus, dass Azo0559 zumindest zu einem gewissen Teil an der Aktivierung der Expression des T6SS-1 unter stickstofffixierenden Bedingungen beteiligt ist.

Abbreviations

aa	amino acid
AAA	ATPase associated with diverse activities
AMF	arbuscular mycorrhizal fungi
APS	ammoniumpersulfate
AT	autotransporter
BAK1	BRI1-associated kinase 1
bEBP	bacterial enhancer binding protein
BIK1	botrytis-induced kinase 1
BRI1	brassinosteroid insensitive 1
bp	base pairs
BSA	bovine serum albumin
CCRH	colonized curled root hair
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CFU	colony forming unit
Cm	chloramphenicol
DAMP	damage-associated molecular patterns
dH_2O	deionized water
DIG	digoxigenin
DEPC	diethylpyrocarbonate
DNase	deoxyribonuclease
Dot	defect in organelle trafficking
DTT	dithiothreitol
DUB	deubiquitinase
EAEC	enteroaggregative *Escherichia coli*
ECM	extracellular milieu
EDTA	ethylene diamine tetraacetic acid

EFR	EF-Tu receptor
EMBL-EMI	European Molecular Biology Laboratory- European Bioinformatics Institute
ET	ethylene
ETS	effector triggered susceptibility
FLS2	flagella sensing 2
Flg22	22 amino acid conserved peptide from flagellin
gDNA	genomic DNA
GFP	green fluorescent protein
gusA/uidA	gene encoding β-glucuronidase enzyme
Hcp	Heamolysin co-reulated protein
HR	hypersensitive response
HSI	Hcp secretion islands
IAHP	IcmF-associated homologous protein
IcmF	Intracellular mulitiplication Factor
JA	jasmonic acid
Kb	kilobases
kDa	kilodaltons
Km	kanamycin
LPS	Lipopolysaccharide
LRR	leucine-rich repeat -containing Toll-like receptors
M-MuLV	Moloney Murine Leukemia Virus
MAMPS	microbe-associated molecular patterns
MAPK	mitogen activated protein kinase
MTI	MAMP triggered immunity
na	not applicable
nt	nucleotide

OD_{578}	optical density measured at 578 nm
OM/P	outer membrane/protein
ON	overnight
PBS	Phosphate Buffered Saline
PGIP	polygalacturonase-inhibitor protein
RISC	RNA-induced silencing complex
RLK	LRR-receptor-like kinases
RNAi	RNA interference
RNAP	RNA polymerase
RNI	reactive nitrogen intermediates
ROS	reactive oxygen species
PRR	pattern recognition receptors
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SA	salicylic acid
SAR	systemic acquired resistance
SM	synthetic medium
SSC	sodium chloride/sodium citrate
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	superoxide dismutase
Sp	spectinomycin
St	streptomycin
STK	serine/threonine kinase
STP	serine/threonine phosphatase
T1SS	type I secretion system
T2SS	type II secretion system

T3SS	type IIIsecretion system
T4SS	type IV secretion system
T5SS	type V secretion system
T6S	type VI secretion
T6SS	type VI secretion system
T7SS	type VII secretion system
TAE acid	tris(hydroxymethyl)aminomethane acetate ethylenediaminetetraacetic
tag	type VI secretion system associated genes
Tat	twin-arginine translocation export pathway
TE	tris(hydroxymethyl)aminomethane ethylenediaminetetraacetic acid
TES	tris(hydroxymethyl)aminomethane ethylenediaminetetraacetic acid sodium
TGS	tris(hydroxymethyl)aminomethane glycine sodium dodecyl sulfate
TEMED	Tetramethylethylenediamine
TPS	two-partner secretion pathway
Tris	tris(hydroxymethyl)aminomethane
tss	type VI secretion genes
U	units
VgrG	valine-glycine repeat
VME	voll (full) medium supplemented with ethanol
WT	wild type

I. Introduction

I. A. Bacterial Secretion Systems

Secretory systems in bacteria are responsible for the translocation of proteins from within the bacterial cytoplasm to the periplasmic space, the extracellular milieu (ECM) or directly into a target eukaryotic or prokaryotic cell. Protein secretion is vital; it has been estimated that 20-30% of bacterial proteins are destined to reside outside of the cytoplasm (Kudva et al., 2013). Gram positive bacteria are predominately monoderm and typically use a general secretion (Sec) protein export pathway or the twin-arginine translocation (Tat) export pathway (Freudl, 2013). Both of these systems translocate proteins with an N-terminal signal sequence through the cytoplasmic membrane post-translation or co-translationally. The passage of proteins through the cell envelope of Gram negative organisms is generally considered more complicated due to their didermic nature. To date, six different secretion systems have been identified, types 1-6 (Bingle et al., 2008). A seventh secretion system (T7SS) has also been identified, unique to Gram positive Mycobacterium, for export of proteins across its outer mycomembrane (Abdallah, 2007). Figure 1 provides a schematic of the different secretion systems introduced in this section (adapted from Tseng et al., 2009).

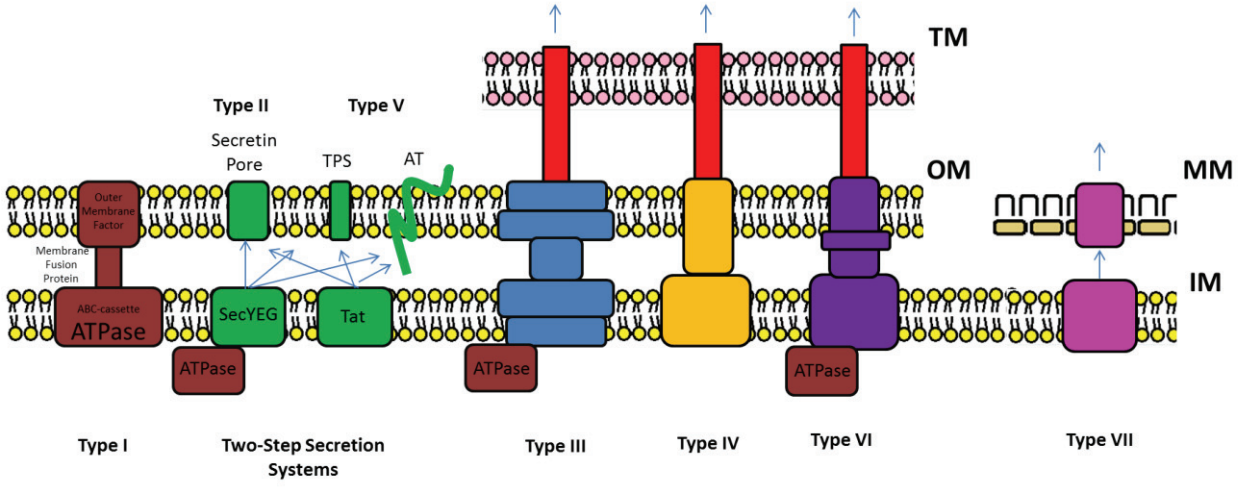


Figure 1. **Secretion systems used by Gram negative bacteria.** Depiction of the one and two step secretion systems used by Gram negative bacteria and Mycobacteria. Secretion systems translocate proteins and or nucleic acids across the inner membrane (IM) and outer membrane (OM) and the mycomembrane (MM) in the case of Mycobacterium in a one or two step process. Type III, IV and VI secrete proteins past the target membrane in a single step (Tseng et al., 2009).

The Sec and Tat pathway are considered universal and can be found in eukaryotes, archaea and eubacteria. These systems pass proteins only into the periplasmic space; an additional

secretion step is required to pass through the outer membrane (OM) (Müller, 2005; Tseng et al., 2009). The type II secretion system (T2SS) is a Sec-dependent system, also known as a secreton. Substrates arrive in the periplasm via the Sec or Tat system, and the N-terminal signal sequence is cleaved. At the OM a 12- to 16-mer of secretins form a pore large enough for folded proteins to pass (Filloux, 2004). Similarly, in type V secretion (T5SS), proteins arrive in the periplasmic space via the Sec or Tat system. The T5SS is a general classification for two-partner secretion (TPS) and autotransporter (AT) pathways across the OM. Two proteins are involved in the TPS pathway: the secreted protein and the transporter. The AT pathway consists of only one protein with a C-terminal beta-barrel that integrates into the OM and allows passage of the N-terminal passenger domain (Jacob-Dubuisson et al., 2004). Still, the Gram negative bacteria have the capacity to and do utilize one step secretion systems.

The type I secretion system (T1SS) is used for the export of usually acidic proteins ranging from 20-900 kDa. A C-terminal signal that is not cleaved triggers assembly of the secretion apparatus that is composed of three primary subunits: an ABC-binding cassette, outer membrane factor and membrane fusion proteins. T1SSs are classified into three groups based primarily on the presence or absence of an N-terminal peptidase for cutting off a substrates leader sequence (Delepelaire, 2004).

The type III secretion system (T3SS), also referred to as an ‘injectisome’, can translocate proteins from the bacterial cytoplasm directly into the cytoplasm of the target cell (Cornelis, 2006). The T3SS secretes potentially hundreds of different protein effectors that modulate the proteins of their target plant or animal host, usually to suppress host defenses and promote invasion (Yang et al., 2004). Translocated proteins require an N-terminal recognition sequence that can be used to secrete even non-effectors (Wang et al., 2013). About 20 genes encode for T3SS components, and eight are very similar to the flagella, suggesting they share a common evolutionary origin (Yang et al., 2004; Macnab, 2004). Whatever the origin, there are seven distinct families of T3SS. There are indications that these evolved late and were distributed horizontally (Gophna et al., 2003; Tseng et al., 2009). Only two of these families are predominant in plant pathogens (Hrp1 and Hrp2), while rhizobia use a third family, and one bacterial species may utilize more than one family of T3SS (Cornelis, 2006; Tseng et al., 2009).

Like the T3SS, the type IV secretion system (T4SS) is a single-step secretion system that can translocate substrates from the bacterial cytoplasm directly into its target cell. T4SS can be

found in Gram negative, Gram positive and some *Archea* spp. Substrates may or may not have a C-terminal signal peptide (Pukatzki et al., 2009). Unique to the T4SS is its capability to translocate proteins or nucleic acids and its wide range of target cells including plants, animals, yeast or other bacteria (Lawley et al., 2003; Cascales and Christie, 2003; Bhatty et al., 2013). A typical T4SS contains about 12 proteins, and they have evolved to be highly diverse. T4SS can generally by classified into three different subfamilies. The largest subfamily is the conjugation system and includes the T–DNA system found in *Agrobacterium tumefaciens*. *A. tumefaciens* is the causative agent of crown gall disease resulting from tumorigenesis (Records, 2011; Wu et al., 2008). Another subfamily includes effector translocation systems, most notable for their role in pathogenesis. No effector (when functioning for attachment) to as many as 100 effectors are delivered this way (Bhatty et al., 2013). The third subfamily is the only T4SS that functions independent of a target cell. This system is responsible for the import and export of DNA from the ECM as found in the *Helicobacter pylori*'s ComB system for taking up DNA and *Neisseria gonorrhoeae*'s gonococcal genetic island for DNA release (Hofreuter et al., 2001; Dillard and Seifert, 2001; Alvarez-Martinez et al., 2009).

The final secretion system to mention is the type VI secretion system (T6SS). Since its identification in 2006, information on T6SSs has rapidly grown, and while details of its structure and function are quickly unraveling, several unknowns remain. It has thus far been established that, similar to the T3SS and T4SS, the T6SS forms a conduit from the bacterium to its target cell allowing direct passage of effector protein/molecule(s) from the bacterial cytoplasm to the target cell's cytoplasm. The predicted structure of a type VI secretion system is depicted in Figure 2 (adapted from Filloux et al., 2008).

Consistently, T6SSs have been strongly correlated to interactions with eukaryotic hosts, but it has also been found that the T6SS targets other prokaryotes for toxin injection (Fritsch et al., 2013; Hood et al., 2010; Kapitein and Mogk, 2013). There are three T6SSs in *Pseudomonas aeruginosa* PAO1, and each exports an antibacterial toxin, Tse1-3 (type six exported 1-3). Tse1 and Tse3 have peptidoglycan amidase and muramidase activity, and Tse2 is a cytoplasmic toxin. The donor strain's resistance to the toxic effects is dependent on expression of immunity proteins (Tsi1-3) (Hood et al., 2010; Li et al., 2012). However, most T6SS clusters were discovered through screening procedures for loss of virulence and for genes transcriptionally induced during infection of a eukaryotic host (Cascales, 2008;

Pukatzki et al., 2007). T6SS genes are frequently necessary for virulence within a eukaryotic host, but they are also found in non-pathogenic species. There are several examples where the T6SS limits virulence (Parsons and Heffron, 2005; Records, 2011), or in the case of *R. leguminosarum*, it was shown that the T6SS impairs its ability to nodulate peas (Bladergroen et al., 2003). In this study the T6SS encoded by *Azoarcus* sp. strain BH72 was investigated and its role in plant-microbe interactions assessed.

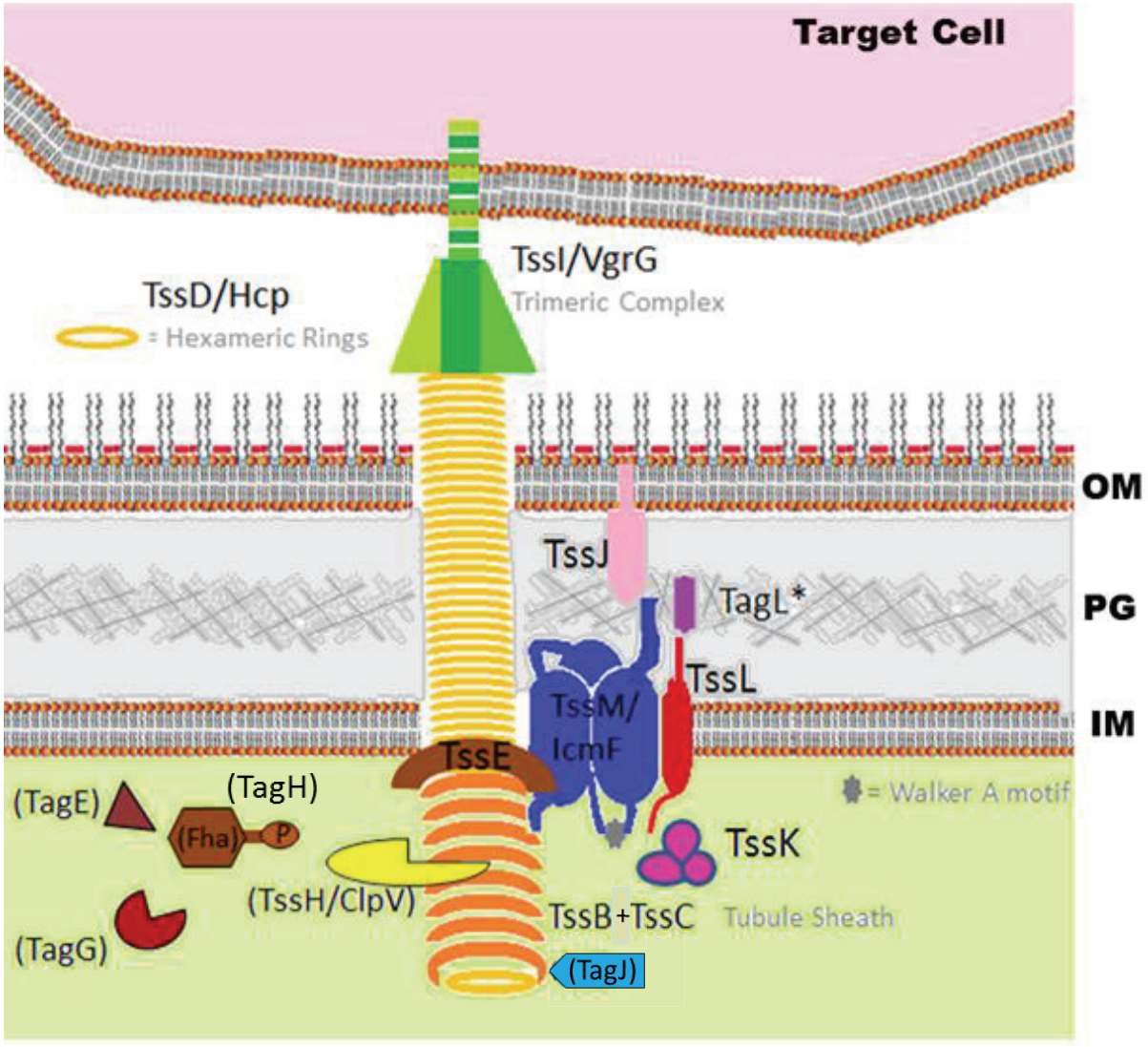


Figure 2. Diagram of the proposed structure of the type VI secretion apparatus and accessory components. Components of a functional T6SS are depicted on the left with their positions relative to the bacterial outer membrane (OM), peptidoglycan (PG), inner membrane (IM) and the target cell. TagL, encoded only by T6SS-1, is marked with an asterisk. Proteins encoded only in or near T6SS-2 are labeled in parenthesis. The figure was adapted from Filloux et al., 2008.

I. B. Microbe-Plant Interactions

Because of their photosynthetic capabilities and sessile lifestyle, plants have the ability and need to be autonomous organisms. However, it has long been known that the vast majority of plants live in close association with microorganisms. Occasionally, plant survival is even contingent upon association with microbes. For example, the orchid Neottia parasitizes fungi to compensate for its lack of photosynthetic genes (Cafasso and Chinali, 2012). Chisholm et al. state in a 2008 review, "Current research suggests that *all* plants in native ecosystems are symbiotic with fungi and other microbes (bacteria, yeast) on their leaf and root surfaces, rhizosphere and internal tissues that influence their performance." It has even been suggested that it was only because of early plants' symbiotic relationship with fungi that they were able to move to land, and since this time microbes have played a large role in shaping the evolution of plants (Chisholm et al., 2006; Barrow et al., 2008). Plant-microbe interactions cover a full spectrum, from being beneficial to being pathogenic to the host plant (Kogel et al., 2006). Investigators continue to be intrigued by the question of which factors determine where on the spectrum the interaction will lie. Other questions include what signals the plant to mount an immune response and what does the microbe do to circumvent these measures, at what point do the organisms find a balance, or do they ever reach equilibrium?

I. B. 1. Plant Defense Responses

Phytopathogens–such as viruses, fungi, bacteria and oomycota–can be classified as biotrophic (requiring a living host), necrotrophic (killing and feeding off their host) or hemibiotrophic (initially requiring a living host, but ultimately killing it) (as reviewed: Pel and Pieterse, 2013; Muthamilarasan and Prasad, 2013). Plants lack a systemic adaptive immunity like that seen in mammals; however, each cell is well-equipped to contribute to the plant's comprehensive innate immunity. Despite the plethora of plant pathogens found in nature, most plant species are resistant to the majority of them. Because of the usually limited host-range of particular microbes, most plants are 'non-hosts'. A suitable host can only be found when the pathogen has the capacity to overcome the several lines of plant defense, which become increasingly species specific as the plant-microbe interaction becomes more intimate, sometimes a 'gene for gene' interaction (Thordal-Christensen, 2003). The first line of defense against pathogens includes preformed barriers that function passively, such as the plant's epidermis that is usually coated aerially by a waxy cuticle. This barrier is disrupted at stomatal pores (where guard cells control the pore size), newly emerging lateral roots, the

root cap or wounds (Reina-Pinto and Yephremov, 2009; Muthamilarasan and Prasad, 2013). Some fungal pathogens produce enzymes that soften or breakdown the cell wall and form an appressorium, a modified hyphae that can directly penetrate the plant's epidermis through an infection peg (Howard et al. 1991; Muthamilarasan and Prasad, 2013). Other modes for direct penetration into plants include the use of vectors such as insects or nematodes. These modes are especially important for plant viruses (Bragard et al., 2013). Additionally, potential pathogens must tolerate antimicrobial enzymes and secondary metabolites that might be found on the plant's surface (Habib and Fazili, 2007; Ahuja et al., 2012; Bednarek, 2012; Muthamilarasan and Prasad, 2013).

Pathogens breeching the preformed barriers reach the apoplast and may have already triggered the plants innate immune system. In the event of a viral attack, the mode of plant defense is gene silencing through RNA interference (RNAi) or histone modifications that make expression of viral genes impossible (Al-Kaff et al., 1998; Jaskiewicz et al., 2011; Muthamilarasan and Prasad, 2013). RNAi can briefly be described as viral dsRNA that is recognized, processed into the ribonucleoprotein RISC (RNA-induced silencing complex) and used to target complimentary viral mRNA for degradation by the Argonaut protein component of RISC. In response, viruses usually encode one or two proteins that interfere with the plant's gene silencing machinery (Berstein et al., 2001; Muthamilarasan and Prasad, 2013; de Faria, et al., 2013).

For detection of other potential pathogens, plant cells have pattern recognition receptors (PRR) found on the plant cell's surface that recognize microbe-associated molecular patterns (MAMPs). These are small molecules, often conserved over an entire group of microorganisms. A list of potential MAMPs that are recognized by some but not all PRR include, but are not limited to, bacterial Lipopolysaccharide (LPS), a conserved polypeptide of flagellin, peptidoglycan (data is not conclusive), the RNA binding motif of cold-shock proteins, elongation factor Tu, fungal-specific glycolsylated proteins, ergosterol, chitin, β-glucan and the well-studied Pep13-domain of oocmycota cell wall transglutaminase (Zipfel and Felix, 2005). These were previously called PAMPs (pathogen-associated molecular patterns); however, MAMPs became the favored term because it was realized the term should encompass molecules of non-pathogenic origins (Staal and Dixelius, 2007; Muthamilarasan and Prasad, 2013). Stimulation of PRR by MAMPs is called MAMP triggered immunity (MTI). In addition to recognition of foreign microbial molecules, some PRR also recognize

endogenous damage-associated molecular patterns (DAMPs) derived from degradation products of plant material by pathogens. Plants release protein inhibitors in response to standard pectin and xylan degrading enzymes of fungal pathogens. Interestingly, polygalacturonase-inhibitor protein (PGIP) does not completely stop degradation. Instead, it aids in generating larger, DAMP-active fragments (Nühse, 2012; Federici et al., 2006).

PRR are homologous to the leucine-rich repeat (LRR)-containing Toll-like receptors found in mammals and include LRR-receptor-like kinases (RLK) and LRR-receptor-like proteins (RLP). Both RLK and RLP span the membrane and have an extracellular LRR recognition domain, but only RLK has a cytoplasmic Serine/Threonine effector domain at its C-terminus (Jones and Takemoto, 2004). RLP requires adaptor proteins for signal transduction (Jones and Takemoto, 2004). The list of RLK proteins has been growing. In a review from 2013, it was stated that *Arabidopsis thaliana* encodes over 600 RLKs (Pel and Pieterse, 2013); in 2004 only 233 were reported (Jones and Takemoto, 2004). It should be noted that the majority of these receptors do not seem to play a role in defense but rather plant development (Lehti-Shiu et al., 2009). Most PRR are in the RLK-LRR family, and one of the best characterized examples of PRR-triggered immunity is when flagella sensing 2 (FLS2) finds the 22 amino acid conserved peptide from flagellin (flg22). Upon association, FLS2 is phosphorylated at the kinase domain and almost instantly heterodimerizes with BRI1-associated kinase 1 (BAK1), a co-receptor of brassinosteroid insensitive 1 (BRI1) (Chinchilla et al., 2006). BRI1 is a hormone receptor for the brassinosteroid hormones, which are involved in controlling growth and development. Botrytis-induced kinase 1 (BIK1) becomes phosphorylated along with FLS2/BAK1, BIK1 is released and MAPK (mitogen activated protein kinase) cascades are stimulated, activating WRKY transcription factors and leading to expression of defense genes (Rushton et al., 1996; Chinchilla et al., 2006; Heese et al., 2007; Schulze et al., 2010; Muthamilarasan and Prasad, 2013). A second well-characterized PRR triggered immunity occurs when translational elongation factor, EF-Tu, is recognized by EF-Tu receptor (EFR), and BAK1 is also used for signal transduction (Jones and Dagl, 2006).

MTI responses include alkalization of the media (as seen in cell cultures), production of reactive nitrogen intermediates (RNI), production of reactive oxygen species (ROS), accumulation of callose between the cell wall and plasma membrane, closure of stomata, and synthesis of phytohormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). ROS are microbiocidal but also strengthen the cell wall through oxidative cross linking of

glycoproteins (Lamb and Dixon, 1997; Muthamilarasan and Prasad, 2013). Microbial attempts at pathogenesis clearly do not stop here. Successful pathogens often use effectors to interfere with MTI. These are injected directly through the plant cell wall and induce effector triggered susceptibility (ETS). Unlike MAMPs, effectors are less conserved and usually species specific. H. H. Flor hypothesized in the 1950s that the interaction is often 'gene-for-gene'. This high degree of specificity likely resulted from plant and pathogen co-evolution. For every new mechanism of attack, there was the selective pressure to evade it, tit for tat. Once an effector is recognized, effector-triggered immunity (ETI) is activated (Jones and Dagl, 2006; Schulze-Lefert, 2010).

To stimulate ETI, plants have nucleotide-binding (NB)-LRR proteins residing in the cytoplasm. NB-LRR make up the largest family of resistance (R)-proteins, and they can bind effectors directly or indirectly via accessory proteins or detection of by-products resulting from the effectors activity on its target (Keen, 1990; Hoorn RAL, Kamoun S 2008; Muthamilarasan and Prasad, 2013). They have a central regulatory domain (NB), a C-terminal LRR domain for recognition and a coiled-coil effector domain at the N-terminus (Jones and Takemoto, 2004). The majority of R-proteins are NB-LRR, but there are also extracellular LRR (eLRR), which include PGIP (De Lorenzo et al., 2001; Muthamilarasan and Prasad, 2013). Several of the cascades of signal transduction overlap with MTI, but ETI is distinct. It is said to be an amplified MTI response (Jones and Dagl, 2006).

A well-studied model of ETI is *Pseudomonas syringae* DC3000 and tomato plants. *P. syringae* DC3000 uses T3SS for delivery of about 30 effector proteins, including AvrPto and AvrPtoB, into tomato plant cells. Immune tomato plants harbor genes encoding for the resistance protein Pto and NB-LRR immune sensor Prf. With the help of Prf, the AvrPto-Pto complex forms, Pto kinase is active and signal transduction proceeds. The function of AvrPto and AvrPtoB is likely to thwart MTI. These effectors have been shown to inhibit PRRs FLS2 and EFR kinase activity, and the effector target appears to be BAK1, as evidenced by studies where BAK1-AvrPto or BAK1-AvrPtoB complexes could be co-immunoprecipiated (Schulze-Lefert, 2010; Lu et al., 2010; Muthamilarasan and Prasad, 2013).

ETI response overlaps the MTI response somewhat (e.g. MAPK cascade and WRKY transcription factors), but it is not redundant. ETI leads to activation of pathogenesis-related (PR) genes for the biosynthesis of SA, JA, ET, cell wall strengthening, lignifications, and the production and secretion of antimicrobials (Tsuda et al., 2003; Muthamilarasan and Prasad,

2013). Accumulation of SA is a part of systemic acquired resistance (SAR), where distal parts of the plant are signaled or primed for defense against an invading pathogen. SA also binds to the receptor NPR3 (nonexpressor of pr genes 3) to mediate degradation of cell-death suppressor NPR1, thus leading to cell death (Fu et al., 2012; and reviews: Pajerowska-Mukhtar et al., 2013; Muthamilarasan and Prasad, 2013). This programmed cell death is known as hypersensitive response (HR), which is intended to sacrifice infected plant tissues to prevent the further spread of the pathogen (Coll et al., 2011). A comparable systemically-induced resistance phenomenon is ISR (induced systemic resistance). ISR is also under the control of transcriptional regulator NPR1 but typically uses JA and ET signaling rather than SA signaling. Additionally, there is less reprogramming, if any, of the transcriptome in ISR, but the plants are primed for defense (Van Wees et al., 2008).

I. B. 2. Non-pathogenic Plant-Microbe Interactions

Both pathogenic and non-pathogenic microbes typically trigger the same initial defense response from plants. The direction of the plant-microbe interaction is dependent upon the arsenal of microbial elicitors and plant R-proteins, an arms race of co-evolutionary events that can even lead to symbiotic interactions (Zamioudis and Pieterse, 2012; Wang and Dong, 2011). Plant-microbe symbiosis–the long-term, sometimes obligatory interaction, between the two species–can be classified in several different ways. Considering the spatial relationship, ectosymbiosis is where one organism lives on the other and endosymbiosis is where one organism resides within the other. The relationships of interacting species cover a spectrum of behaviors that can be facultative or obligatory including: parasitism, where only one partner benefits to the detriment of the other; mutualism, where both partners benefit, or commensalism, where one partner benefits while the other is seemingly unaffected (Ahmadjian and Paracer, 2000). It should not be overlooked that the interactions are not always permanently established. There are examples of small physical or chemical fluctuations in the milieu or mutation to one of the interacting partners, drastically changing the plant-microbe dynamic (Kogel et al., 2006).

The best studied examples of mutualistic microbes include mycorrhizal fungi (or arbuscular mycorrhizal fungi, AMF) and *Rhizobium* bacteria. These endosymbionts are found closely associated with the plant at the rhizosphere and have systemic effects on the plant including IRS. AMF are obligate symbionts found associated with at least 80% of terrestrial plants, providing the plants with water and minerals, most importantly phosphorous, acquired

through an extensive system of hyphae in the soil. (Harrison, 2005). The fungi penetrate the root surface through an appressorium and, once in the cortex, form elaborate hyphae structures that impregnate the plant cell but remain separated by surrounding plasma membrane. It is here that nutrients are delivered to the plant and likely here that the fungus receives its only source of carbon (Harrison, 2005). It is believed that only after signaling between the plant and AMF does the plant not reject the microbe as a pathogen. In 2005 Güimil et. al. performed a whole-transcriptome analysis of *Oryza sativa* during symbiosis with Glomalean fungi and compared it to the transcriptome of rice infected with pathogenic *Magnaporthe grisea* and *Fusarium moniliforme*. Of 224 differentially regulated genes, 95 (43%) responded similarly to infection by symbiotic and pathogenic fungi (Güimil et al., 2005). In 2003 Liu et al. examined *Medicago truncatula* roots during the development of a symbiosis with *Glomus versiforme* using cDNA arrays. In one group they found an increase in transcripts upon initial contact and a subsequent decrease as the symbiosis developed. They were also able to determine a large portion of these genes were those involved in defense- and stress-response (Liu et al., 2003).

Another well-studied mutualism is rhizobial nodulation of legumes. About 100 species of rhizobial bacteria have been defined so far. They are a polyphyletic group of *Proteobacteria* including genera such as *Rhizobium* and *Bradyrhizobium* from the alpha-subgroup and *Burkholderia* from the beta-subgroup. The majority of nodulating rhizobia are found in the alpha-subgroup (α-rhizobia). Betaproteobacteria are typically considered free-living and only closely associated with roots, but after much effort proving they were not nodule contaminants, β-proteobacteria such as *Cupriavidus taiwanensis* and *Burkholderia* strains were identified as true nodulating rhizobia using microscopic analysis of GFP-marked strains (Gyaneshwar et al., 2011; Chen et al., 2005; Chen et al., 2006; Moulin et al., 2001). Of the over 19,000 species in the Leguminosae (Fabaceae) family, the majority form nodules. Among the three subfamilies, the percentage of species that have been examined so far and found to form nodules are Papilionoideae (96%), Mimosoideae (96%), and Caesalpinioideae (22%) (Gyaneshwar et al., 2011).

Nodulation requires communication between the plant and rhizobia. The plant exudes aromatic compounds called flavonoids (2-phenyl-1.4-benzopyrone derivatives), which induce *nod* gene expression (Maj et al., 2010). Flavonoids vary from plant to plant, and one plant may produce different flavonoids at different times (Fisher and Long, 1992). Within the

Rhizobium, NodD (a LysR family transcriptional regulator) complexes with a specific flavonoid and binds a *nod*-box upstream of the *nod* operon, bending the DNA and acting as a transcriptional activator (Maj et al., 2010; Fisher and Long, 1992; Jones et al., 2007). The N-terminus of NodD is more conserved and functions in DNA binding. The C-terminal portion is more variable and likely explains why NodD proteins respond differently to different flavonoids. This is presumed to be an important aspect in determining host specificity. Some rhizobia encode multiple *nodD* alleles, which would account for their ability to sense different hosts (Fisher and Long, 1992). There is also evidence that the transcription of *S. meliloti nod* genes is inhibited by flavonoids from non-host plants (Peck et al., 2006; Jones et al., 2007). Roughly 25 *nod* genes encode for the proteins required for synthesis and export of Nod factor (Gage, 2004). Nodulation genes are classified into three groups: common (essential for nodulation), host specific (modifications to Nod factors to determine the host range and rate of nodule formation), and nodD regulatory genes (Maj et al., 2010).

Activation of Nod genes leads to the synthesis and export of Nod factors, contributing to the beginning of nodulation (Lerouge et al., 1990). As reviewed in 2007 by Jones et al. calcium spiking causes alterations in the cytoskeleton of root hair, leading to root hair curling and Rhizobial bacteria becoming trapped. An infection thread is formed, extending into the cortex, and the bacteria are endocytosed and differentiate into bacteroids. The plant provides bacteroids with the nutrition and microaerobic environment needed for biological nitrogen fixation. In return, the plant receives an ample source of usable nitrogen, which in many soils is a limiting growth factor (Jones et al., 2007).

Lesser studied examples of non-pathogenic microbes interacting with plants are non-nodulating bacterial endophytes. Endophyte is a Greek-based term meaning ‘within plant’. Characteristic traits of bacterial endophytes include living intercellularly (also intracellularly, but not within a living cell) without a surrounding membrane compartment, having the ability to colonize the plant in high numbers without inducing signs of plant disease, and typically providing the plant with an advantage against biotic (such as pathogens) and abiotic (such as poor soils) stresses (Kogel et al., 2006; Reinhold-Hurek and Hurek 2011; Quispel, 1992). The work presented herein focuses on aspects of endophytic interactions of the model grass endophyte *Azoarcus* sp. strain BH72 with Asian rice cultivar *Oryza sativa* cv. IR-36.

I. C. *Azoarcus* sp. BH72 and *Oryza sativa*: An Endophytic Model

I. C. 1. The diazotrophic grass endophyte *Azoarcus* sp. strain BH72

The genus *Azoarcus* belongs to the beta subgroup of *Proteobacteria* (Reinhold-Hurek et al., 1993a). *Proteobacteria* are a diverse lineage of gram negative bacteria (diderms) (Kersters et al., 2006). As mentioned previously, rhizobial bacteria are found in the alpha and beta subgroups (Gyaneshwar et al., 2011). The gamma subgroup is one of the largest and includes several human, animal and plant pathogens such as *Enterobacteriaceae* (*E. coli*, *Yersinia*, *Klebsiella*), *Vibrio* and *Pseudomonas* (Gao et al., 2009). The beta subgroup is known to contain species with the ability to degrade diverse substrates and include non-nodulating diazotrophic bacteria. However, at least two different strains of *Burkholderia*, which are included in this subgroup, were also found to be nodulating bacteria (Moulin et al., 2001). *Azoarcus* sp. strain BH72 was originally isolated from surface sterilized Kallar grass (*Leptochloa fusca* (L.) Kunth) roots from the Punjab region of Pakistan (Reinhold et al., 1986). Plant-associated strains of *Azoarcus* spp. are strict aerobes and are very limited as to the carbon sources on which they can grow–namely organic acids or ethanol. The optimal temperature for growth is 37-42°C, with a doubling time of about 2h (Reinhold-Hurek et al., 1993a; Hurek et al., 2003). *Azoarcus* usually have an arched rod shape and form yellow pigmented colonies. They are capable of biological nitrogen fixation, hence the name *Azoarcus* (azo, nitrogen; arcus, arch) (Reinhold-Hurek et al., 1993a). *Azoarcus* sp. strain BH72 expression of *nif* genes, genes necessary for biological nitrogen fixation, has been demonstrated in rice roots and at the rice rhizosphere (Egener et al., 1998 and 1999; Hurek et al., 2002). *Azoarcus* spp. are motile with a single (rarely bi-) polar flagellum. In the initial studies on isolated strains, cells were found to range from 0.4 to 1.0 μm wide by 1.1 to 4.0 μm long, but they could elongate up to 12 μm under certain growth conditions (Reinhold-Hurek et al., 1993a). Another significant feature of *Azoarcus* sp. strain BH72 is that its 4.3 Mb genome has been sequenced. In the genome, 91.2% is predicted to encode for 3992 protein sequences (67.9% GC). Moreover, there was no extrachromosomal DNA detected in *Azoarcus* sp. strain BH72, and the genome appears to be quite stable with only eight loci containing genes with phage or transposon-related elements (Krause et al., 2006).

I. C. 2. Endophyte associated grass *Leptochloa fusca*

Leptochloa fusca or Kallar grass is a highly salt-tolerant forage plant that grows to be 80-120 cm tall and is found in Pakistan and India (Malik et al., 1986). Kallar grass, along

with the important food crops of the world (such as wheat, rice and corn), is a member of the *Gramineae* (or *Poaceae*) family, one of the largest families of monocot plants. Kallar grass thriving in salt-affected, alkaline, often flooded, low-fertility soil without nitrogenous fertilizers prompted the study of nitrogen fixing bacteria colonizing Kallar grass roots (Reinhold et al., 1986; Malik et al., 1986). In the rhizosphere, soil directly associated with plant roots, *Azospirillum* had regularly been isolated and was also found along the rhizoplane of Kallar grass. Within the endorhizosphere, *Azoarcus* spp. were the predominant culturable diazotroph in a survey of nitrogen fixing bacteria. In Kallar grass, up to10^8 cells per gram root dry weight were found (Reinhold et al., 1986; Reinhold-Hurek et al., 1993a). *Azoarcus* sp. strain BH72 colonized rice in equally high numbers (10^9 cells per gram dry weight) without rice showing any signs of distress (Hurek et al., 1994b). *Azoarcus* sp. strain BH72 does not survive well and cannot be cultured from root free soils, but black sclerotia a basidiomycete has been shown to act as a shuttle vector (Hurek et al., 2002).

I. C. 3. *Azoarcus* sp. strain BH72's interaction with rice

Colonization by endophytes occurs predominately in the outer cell layers (exodermis and sclerenchymatous cells) and root cortex (Hurek et al., 1994b). Light and electron microscopic immunogold studies showed *Azoarcus* sp. strain BH72 colonizes Kallar grass and rice seedlings in gnotobiotic cultures at similar sites without invoking signs of plant defense. Interestingly, *Azoarcus* sp. strain BH72 was one of the first endophytes to be confirmed colonizing the stele, likely the means by which infections spread systemically into the shoot (Egener et al., 1999; Hurek et al., 1994b; Hurek et al., 1997). It had previously been thought that this vascular tissue was sterile unless infected by pathogens. The largest microcolonies occur in the arenchyma (Hurek et al., 1994b). Formation of aerenchyma tissue is an adaptive response to limited oxygen (and sometimes because of other minerals), usually resulting from flooded, waterlogged soils. This tissue contains large air-filled cavities, which allow lower resistance for gas exchange between the plant parts above the water and the submerged tissues (Postma and Lynch, 2011; Sairam et al., 2008).

Azoarcus sp. strain BH72 and other endophytes invade plants in a fashion similar to pathogens at lateral root emergence points and along the zone of elongation and differentiation at the root tip (Egener et al., 1999). Ultimately, *Azoarcus* sp. strain BH72 has a very limited arsenal for plant invasion. A typical phytopathogen produces cellulose degrading enzymes, a major component of plant cell walls of grasses. True cellulolytic bacteria or fungi

would utilize the degradation products as an energy source. However, of almost 50 mono- or disaccharides tested, *Azoarcus* sp. strain BH72 grew on none of them (Reinhold-Hurek et al., 1993a). Despite the fact *Azoarcus* sp. strain BH72 cannot grow on cellulose or its breakdown products, it encodes putative glycosidases (*palZ* (*azo2310*), *spr1* (*azo2059*), *ndvC* (*azo1284*), *eglA* (*azo2236*)) (Reinhold-Hurek et al., 1993b; Krause et al., 2006). This is relatively few compared to cellulytic and phytopathogenic bacteria, which usually have multiple cellulases (Reinhold-Hurek et al., 2006). Of these predicted cellulose degrading enzymes, endoglucanase, *eglA*, in *Azoarcus* sp. strain BH72 has been the most extensively studied through cloning and expression in *E.coli*. The expressed gene cleaved carboxymethyl cellulose and 1,4-β-cellooligosaccharides. The EglA sequence was most similar to endoglucanases of phytopathogens. Contrary to true cellulytic bacteria, in a transcriptional gene fusion study, *eglA::gusA* was neither induced by the presence of celluloses nor repressed by presence of breakdown products such as D-glucose. In *Azoarcus* it was shown to be the most active in the presence of rice roots (three-fold higher) and to some extent when grown microaerobicially on N_2, conditions optimal for biological nitrogen fixation (Reinhold-Hurek et al., 1993b; Reinhold-Hurek et al., 2006). Additionally, these cellulases are not secreted into the culture medium, like in most Gram negative phytopathogens. The exo- and endoglucanases of *Azoarcus* spp. studied were cell surface bound. As a result, the degradation is more localized compared to pathogens and cellulytic bacteria, reducing overall plant damage and subsequent plant defense responses (Reinhold-Hurek et al., 1993b). Although its activity is less effective than an excreted cellulase, EglA is a key factor for colonization of rice roots and for the systemic spread into shoots. It was seen through microscopic detection that an *eglA*- mutant colonized rice roots significantly less than wild-type, but colonization efficiency could be partially restored through complementation. Spreading into the shoots could no longer be detected by the *eglA*- mutant using PCR based methods (Reinhold-Hurek et al., 2006).

Few additional features have been determined to aid in colonization, one being type IV pili. In *Azoarcus* sp. strain BH72, *pilA* (co-transcribed with *pilB*) encodes for an abnormally short prepilin (Dörr et al., 1998). It is regulated by the PilSR two component regulatory system, but there are at least 30 genes for the biogenesis and function of type IV pili (Alm and Mattick, 1997). Pili are typically 6 nm thick and play a role in cell specificity, phage adsorption, twitching motility, transformation competency, and social gliding, and act as virulence factors (Strom and Lory, 1993; Jonsson et al., 1994; Bradley, 1974; Bradley, 1980; Darzins

and Russell, 1997; Fusenegger et al., 1997; Wu and Kaiser, 1995; Dörr et al., 1998). *Azoarcus* sp. strain BH72 endophytic colonization of rice plants was reduced in a PilAB mutant compared to wild type and could be partially restored with complementation of *pilAB* (Dörr et al., 1998). Interestingly, it was also determined that population density and carbon starvation increase *pilAB* expression (Hauberg-Lotte et al., 2012). PilT mutants (that still made pili but were deficient in twitching motility) were slightly impeded in their ability to colonize rice root surfaces, but endophytic colonization was strongly impaired (Böhm et al. 2007).

Little is known about additional features that might play a role in endophytic colonization; however, it appears that flagella mediate endophytic competence. As discussed above, flg22 is a well characterized MAMP, sensed by FLS2 in PRR-triggered immunity (Chinchilla et al., 2006). *Azoarcus* sp. strain BH72 encodes for three falgellins (*fliC1*, *fliC2*, *fliC3*), the main structural proteins to produce a single polar flagellum (Krause et al., 2006; Reinhold-Hurek et al., 1993a). In pure culture, FliC3 expression is induced in association with rice roots. Deletion of FliC impairs motility and significantly reduces endophytic colonization (Buschart et al., 2012). Given the defense response commonly stimulated by Flg22, it is easy to assume that without flagella the defense response is decreased and endophytic colonization is improved, which has been observed in other systems (Iniguez et al., 2005). The colonization of a *fliC3*$^{-}$ mutant on the rice surface was not significantly different than wild type, but endophytic colonization was a factor of three less than wild type (Buschart et al., 2012). Additionally, no obvious changes in growth two weeks after inoculating seedlings with any of the *fliC*$^{-}$ mutants in comparison to wild type were seen. Also, rice cell suspensions, OC156, were not stimulated to produce ethylene in the presence of SDS-PAGE purified flagella, unlike the production seen by OC156 in the presence of known elicitors such as chitin or mycelium of *Penicillum chrysogenum*, suggesting that *Azoarcus* sp. strain BH72 flagella do not induce a defense response in rice (Buschart et al., 2012; Felix et al., 1999; Thuerig et al., 2006). Intriguingly, crude *Azoarcus* sp. strain BH72 flagellar extracts did elicit production of ethylene as well as flagella preps from in-frame deletion mutant BHΔfliC3 and polar mutant BHfliC3. This brings into question what other component present in the flagellar preps elicits a response, because in BHfliC3, *fliS*, *fliT* and *fliD* (genes essential for flagella assembly) were also inactivated. Taken together that SDS-PAGE purified flagella did not elicit ethylene production and only crude flagella extracts, even from non-flagellating mutants, did elicit, it is probable that an additional, unknown component is co-purified via

ultracentrifugation with flagella acting as the elicitor (Buschart et al., 2012). It should be borne in mind that although crude extracts elicit ethylene production in rice cell suspensions, *in planta Azoarcus* sp. BH72 ultimately does not elicit visible signs of defense by rice. Nevertheless, identification of this elicitor could shed light on other features involved in the development of *Azoarcus* sp. strain BH72's endophytic lifestyle with rice and other endophytic systems.

In Summary, in *Azoarcus* sp. strain BH72, few factors necessary for colonization have been established but include an endoglucanse, T4P and flagella (Buschart et al., 2012; Reinhold-Hurek et al., 2006; Dörr et al., 1998). These bacterial features provide a physical means to colonize rice, but still lacking is the identification of an effector or component communicating with rice at the molecular level, something that signals or manipulates rice to turn down its defense response(s), allowing endophytic colonization. Regularly, the T3SS or T4SS play an essential role in establishing bacterial interactions with eukaryotic hosts, often by transferring effector proteins or toxins (Bingle et al., 2008; Yang et al., 2004). However, *Azoarcus* sp. strain BH72 has been said to be somewhat 'disarmed' considering there is no genetic evidence for a T3SS, T4SS or toxins (Krause et al., 2006). As discussed above, like the T3SS and T4SS, the T6SS is a one-step secretion system, and its presence is strongly correlated to interactions with eukaryotic hosts. T6SSs are typically necessary for virulence, but there are examples of T6SSs reducing virulence (Records, 2011; Parsons and Heffron, 2005). Via *in silico* analysis, it was found that *Azoarcus* sp. strain BH72 contains two T6SS gene clusters (Julia Herglotz, Diplomarbeit, 2007), prompting further investigations into whether *Azoarcus* sp. strain BH72 encodes a functioning T6SS(s) and if this secretion system plays a role in establishing the endophytic colonization of *Azoarcus* sp. strain BH72 in rice. One aim of this study was to perpetuate this investigation.

I. D. The type VI secretion system (T6SS)

I. D. 1. Discovering a sixth secretion system

In 2003 Das and Chaudhuri identified a 15 gene cluster around *icmF* in *Vibrio cholerae* and other gram negative bacteria commonly found in association with eukaryotes. At the time, IcmF was best characterized in *Legionella pneumophilia* as a component of its Icm/Dot (**I**ntra**c**ellular **m**ulitiplication **F**actor/**D**efect in **o**rganelle **t**rafficking) T4bSS, which is essential for intracellular multiplication in macrophages. Interestingly, in *V. cholera* there

were no other T4SS components present, and so they designated it the **I**cmF-**a**ssociated **h**omologous **p**rotein (IAHP) gene cluster (Das and Chaudhuri, 2003; Nagai and Kubori, 2006). In 2006 Pukatzki *et al.* (John Mekalanos Laboratory) found that the IAHP-like gene cluster in *V. cholerae* encoded a protein secretion system distinct from type III and type IV pathways and proposed it be called the T6SS. The genes were given the name *vas* for **v**irulence **a**ssociated **s**ecretion (Pukatzki et al., 2006). Nearly in parallel, Mougous *et al.* (John Mekalanos Laboratory) identified three IAHP-related loci in *P. aeruginosa* that they termed **H**cp **s**ecretion **i**slands (HSI-1 through HSI-3). They found Hcp1 was exported by HSI-1 and through crystallography learned Hcp formed hexameric rings with an inner diameter of approximately 40 Å and outer diameter of about 85 Å (Mougous et al., 2006; Coulthurst, 2013). Hcp (**h**aemolysin **c**o-regulated **p**rotein) was first identified in 1996 by Williams et al. in *V. cholerae*. They had found its expression was co-regulated along with HlyA by the HlyU (**h**aemo**l**ysin) regulatory system. At that time, it was known that *V. cholerae* secreted Hcp, but it had no cytotoxic effect on HeLa cells, was not required for pathogenesis in infant mice and had no effect on colonization. They knew only that it lacked a secretion signal and could traverse the inner membrane of *E. coli* without expression of *V. cholerae*-specific factors (Williams et al., 1996). It is now understood that secretion of Hcp (TssD, under the newly harmonized nomenclature), or at least its presence in culture supernatants, is the hallmark of T6SS.

In silico analysis revealed T6SS clusters in at least one fourth of sequenced Gram-negative bacterial genomes, and almost all are encoded by Proteobacteria (Bingle et al., 2008; Boyer et al., 2009). Approximately one third of the T6SS encoding genomes have multiple clusters, ranging from two to six, but based on sequence, phenotype and regulations, they do not appear redundant (Leung et al., 2011; Silverman et al., 2012). Four categories based on function have been suggested: (i) bacterial cell targeting, (ii) eukaryotic cell targeting, (iii) bacterial and eukaryotic cell targeting and (iv) other (Silverman et al., 2012). The highest number of T6SSs has been reported in *Burkhoderia pseudomallei* and *Yersinia pestis*, each with six (Coulthurst, 2013). Each gene cluster contains 12 to greater than 20 genes with the function of each gene not yet determined. Additionally, it is known that not all involved genes are necessarily encoded within the gene cluster. Interestingly, unlike most secretion systems, the majority of proteins in T6SS are predicted to be cytoplasmic proteins. Extensive variations occur between species, but there are 13 genes considered essential (Boyer et al., 2009). In an earlier bioinformatic analysis of *Azoarcus* sp. strain BH72, a 15.7 kb *sci*-like

cluster (similar in sequence to a T6SS cluster of ***S**almonella enterica* **c**entisome 7 genomic **i**sland 7) comprised of 13 genes and a 17.4-kb *imp*-like cluster (similar to a T6SS cluster in *Rhizobium leguminosarum,*so named for its **imp**aired nodulation) with 14 genes were identified (fig.IIIA1) (Julia Herglotz, Diplomarbeit, 2007; Blondel et al., 2009). The GC content of the *sci*-(69.22%) and *imp*-cluster (67.58%) was found to have only minor deviations from the average *Azoarcus* sp. strain BH72 genome content (67.92%) (Julia Herglotz, Diplomarbeit, 2007). Boyer *et al.* looked at the relationship between phylogeny and T6SS gene content in a genome wide *in silico* analysis. Based primarily on presence of conserved accessory proteins (in addition to core components), they divided the T6SSs into 5 sub-groups (I-V): in *Azoarcus* sp. strain BH72 the *sci* cluster in sub-group III and the *imp* cluster in sub-group IV (closest to *Chromobacterium violaceum)* (Boyer et al., 2009). As the list of newly discovered components has grown, so has the list of gene names. Genes have been identified by names of the originally discovered homolog or by the names of gene cluster encoded by a particular organism, resulting in several names for the same gene and the same name for multiple genes. To ease confusion, the nomenclature of T6SS components proposed by Shalom et al. will be used here, as that has been the most widely accepted. Core components are named Tss (**t**ype **s**ix **s**ecreted), and accessory proteins are named Tag (**T**ss-**a**ssociated **g**enes) (Aschtgen et al., 2010; Shalom et al., 2007). The core components of a functional T6SS, TssA-M will be briefly reviewed here. Predicted cytoplasmic proteins, TssA, TssF, and TssG, have been determined essential; however, their precise function has not yet been determined.

I. D. 2. The T6SS Membrane Complex

As discussed above, it was the presence of IcmF but the lack of any additional T4bSS components, outside of IcmH/DotU, that provoked a more critical look at IAHP clusters and led to the identification of the T6SS (Das and Chaudhuri, 2003; Nagai and Kubori, 2006). IcmF is a component of the T4bSS, found in several pathogenic bacteria for transport of proteins or nucleic acids (Christie and Vogel, 2000). In *L. pneumophilia*, 19 of the 25 *icm/dot* genes have high sequence similarity to the conjugative transfer system (Sexton and Vogel, 2002). IcmF is required for Icm-Dot complex stability and partially required for intracellular multiplication in macrophages (Van Rheenen et al., 2004). Before the T6SS had been established, expression of IcmF or IAHP gene clusters were found to be induced in infection models, and mutations to these genes influenced virulence in several different studies (Bingle et al., 2008). In 2000, Das et al. discovered expression of an IcmF homolog in *V. cholerae*

was induced in a rabbit illeal loop infection model (Das et al., 2000). In 2004, *evp* genes, including IcmF homolog EvpO, were shown to be vital for *Edwardsiella tarda* pathogenesis in fish (Rao et al., 2004). In 2005 *Salmonella enterica* serovar Typhimurium, *sciS*, an IcmF homolog, was identified after screening a transposon library for the ability to persist in macrophages. It was determined IcmF limited intracellular replication in the late stages of infection, and in a murine host it attenuated virulence (Parsons and Heffron, 2005). Using the standardized nomenclature, IcmF shall hereinafter be referred to as TssM.

The only other remnant of T4bSS and additional component of the membrane structure is IcmH/DotU (TssL). TssM and TssL are among the most highly conserved T6SS components and usually occur as a pair (Robb et al., 2012). It was believed that TssM and TssL are translocated via the Sec pathway, independent of T6SS. However, in the analysis by Boyer et al., 87% of the DotU sequences did not contain a signal peptide (Ma et al., 2009; Boyer et al., 2009). In a structural analysis of *Francisella novicida* TssL, no obvious catalytic domains were found. It was proposed TssL plays purely a structural role (Robb et al., 2012). Like the IcmH/DotU and IcmF pair of the T4bSS in *L. pneumophilia*, TssL has been shown to stabilize TssM. Similarly, TssL has one transmembrane segment with a periplasmic C-terminus and a cytoplasmic N-terminus, where the bulk of the protein resides (Durand et al., 2012). The structure of IcmH/DotU has not been determined, but it is predicted to be similar to the secondary structure of TssL, which has been ascertained. The soluble portion of TssL forms a globular structure comprised of eight α-helices, two three-helix bundles connected by two short helices that all together resemble a hook, which might play a role in protein recruitment (Durand et al., 2012; Aschtgen et al., 2010; Robb et al., 2012). It was shown in *A. tumefaciens* that TssL interacts with TssM in the cytoplasm (Ma et al., 2009). In enteroaggregative *Escherichia coli* (EAEC) it was determined TssL dimerization is necessary for functional a T6SS, which was speculated to trigger recruitment of TssM (Durand et al., 2012). Unlike IcmH/DotU, the periplasmic portion often contains a peptidoglycan binding (PGB) domain similar to the OmpA/MotB/Pal family (Cascales and Cambillau 2012; Filloux et al., 2008).

The third and final core component of the membrane complex reviewed here is TssJ. TssJ is a lipoprotein that fractionates with the OM and is exposed in the periplasm. Proper localization is required for function. Unlike TssM and TssL, TssJ is not a part of the Icm/Dot T4bSS. However, TssJ's interaction with TssM in the periplasm has been demonstrated, and they are

found at a 1:1 ratio in EAEC (Durand et al., 2012). TssJ was originally identified in the *sci*-1 gene cluster, one of two T6SS gene clusters of EAEC, and is encoded by *sciN*. Mutation to *sciN* led to abrogation of Hcp secretion, decreased biofilm formation and 50% less killing of *Caenorhabditis elegans* compared to WT. Mutations to *sci-2* showed no virulence defects (Aschtgen et al., 2008).

Although TssJ does not share homology with the T4bSS as do TssM and TssL, it is interesting to note that structural analysis of EAEC TssJ revealed a β-sandwich fold, which is similar to the T3SS-associated lipoprotein, ExsB, and the T4P lipoprotein, PilP (Cascales and Cambillau 2012). Cascales *et al.* describe T6SS as a 'patchwork' of subunits from different organisms (Cascales and Cambillau 2012). This is further exemplified by the uncanny resemblance of the injection apparatus to a bacteriophage tail spike.

I. D. 3. The T6SS Injection Apparatus

Inarguably, the T6SS apparatus for secreting substrates from the bacterial cytoplasm directly into a target cell is structurally similar to a bacteriophage injection apparatus, only inverted across the bacterial membrane. The actual conduit extending between cells is composed of Hcp. As mentioned earlier, it is well accepted that secretion of Hcp or its presence in culture supernatants is the indicator of a functional T6SS. However, it cannot be assumed that Hcp is the secreted substrate or effector. The most favorable opinion is that its appearance in the supernatant results from the shearing off of these bacterial appendages (Cascales and Cambillau 2012). However, Hcp proteins may play a more active role than a static conduit; electron microscopy revealed effectors bound to the inner surface of *Pseudomonas aeruginosa* Hcp proteins (Silverman et al., 2013). Hcp may or may not be encoded within their given T6SS gene cluster, which is also the case for some other T6SS components. Hcp monomers appear in culture supernatants without a secretion signal and without processing (Pukatzki et al., 2007). Monomers assemble hexameric rings and have been shown to do so *in vitro* (Edward et al., 2008). Rings stack to form tubules up to 100 nm in solution with an internal diameter of about 35-40 Å and external diameter 80-90 Å, as evidenced by electron microscopy and examination of crystal packing (Cascales and Cambillau 2012; Mougous et al., 2006; Jobichen et al., 2010). To date, the existence of this structure *in vivo* has not been documented though (Silverman et al., 2012). Hcp size, tertiary structure and, to some extent, sequence resembles a bacteriophage λ tail tube protein, gene product 19 (gp19). The putative function of Hcp tubules as effector delivering structures is also comparable; the

bacteriophage tube delivers DNA into target bacterial cells (Edward et al., 2008; Records, 2011).

At the tip of the Hcp tubule, extending into the ECM or the point of entry at the target cell, is another bacteriophage-like protein VgrG (**v**aline-**g**lycine **r**epeat protein **G**), newly named TssI (Shalom et al., 2007). The internal diameter of the Hcp tubule is predicted to potentially hold an unfolded protein up to a 50 kDa, but the prism shaped VgrG complex is not large enough for passage of this size of a protein (Silverman et al., 2012). The presence of VgrG in culture supernatants is also considered a hallmark of T6SS. In the T6SS, VgrG is a large protein (529-1027 aa) that forms trimers and is necessary for Hcp secretion (Pukatzki et al., 2007; Zheng and Leung 2007). The VgrG trimer resembles a fusion of gp27 and gp5 of the bacteriophage T4 needle-like protein complex $(gp27)_3$-$(gp5)_3$. The VgrG protein is like a fusion of gp27 and gp5 (Boyer et al., 2009). In bacteriophage, a trimer of gp27 proteins forms a baseplate ring, and the N-terminal end of gp5 proteins associates with it. At the C-terminal end of gp5 is a triple stranded β-helix that forms the spike-like needle, making the total complex length about 190 Å (Kanamaru et al., 2002). The middle domain of gp5 polypeptide includes lysozyme activity, but this is absent in VgrG proteins (Records, 2011). Multiple VgrG proteins are sometimes encoded by a single T6SS containing genome, and in *V. cholerae* it was shown that mulitmeric complexes can form; VgrG1 pulled down VgrG2 and VgrG3 in immunoaffinity assays (Pukatzki et al., 2007).

The discovery of 'evolved VgrGs' has sparked many discussions about their possible role as T6SS effector proteins in early literature. It has become clear that only a small minority of VgrG proteins contain these C-terminal extension effector domains, but the important role of these effector domains in host interactions has been well established (Pukatzki et al., 2009). A list of predicted VgrG C-terminal effector domain functions include ADP-ribosylation, actin cross linking, PG binding or degradation, metalloprotease activity, mannose binding and adhesion to host (Records, 2011; Pukatzki et al., 2009; Pukatzki et al., 2007). It is proposed that, when there is an effector domain, it remains hidden within the VgrG trimer until entering the target cell, at which time it is somehow freed to interact with the host cell (Cascales and Cambillau 2012; Hachani et al., 2011). It is interesting to note that in the genome wide *in silico* analysis, Boyer et al. built 13 trees based on each core components protein families. Extracellular and surface proteins, Hcp (TssD), VgrG (TssI) and SciN

(TssJ), showed the greatest distances to the other trees. They proposed that the distances resulted from a higher rate of mutation leading to greater specificity for their direct interaction with the environment or host cells (Boyer et al., 2009).

At the cytoplasmic end of the Hcp tubule, the two most highly conserved core components are found that also resemble parts of a bacteriophage contractile tail sheath, TssB and TssC (formally most commonly referred to as VipA and VipB). TssB and TssC interact with one another to form tubules up to hundreds of angstroms long. Tubules have a cogwheel shape with 12-fold symmetry (Basler et al., 2012). TssB and TssC protein sequences are so highly conserved that cognate partners from different bacteria have been shown to bind and genes are often co-organized with those encoding for Hcp proteins (Bröms et al., 2013; Boyer et al., 2009). Through time-lapse fluorescence light microscopy, the Mekalanos laboratory has successfully recorded the structure and activity of TssB/TssC tubules. They saw 0-5 highly dynamic tubule formations per cell that could cover the length of the cell up to 1 μm in 20-30 s that would rapidly contract in ≤ 5 ms to half of their extended length (Basler et al., 2012). Tubules have an external diameter of 300 Å and an internal diameter of about 100 Å, which is comparable to sheath tubules of bacteriophage and large enough to encircle Hcp tubules (Cascales and Cambillau 2012; Kanamaru et al., 2002; Bönemann et al., 2009). It is believed that contraction of TssB/TssC tubules provides the energy to push the Hcp syringe with a VgrG needle tip across the membranes and into the target cell (Basler et al., 2012). Essential for this action is an additional core T6SS component with approximately 40% sequence similarity to the bacteriophage gp25 protein, TssE. Like gp25, TssE is believed to be assembled at the cytoplasmic side of the inner membrane forming a baseplate or hub, which is required for secure anchoring of the apparatus to the membrane; mutations led to loss of T6SS function. Unlike gp25, TssE does not have lysozyme activity (Lossi et al., 2011; Cascales and Cambillau 2012).

Once the T6SS sheath has contracted and the Hcp syringe has powered through the membrane, ClpV (TssH) disassembles the sheath over 30-60 s (Basler et al., 2012). This differentiates the T6SS from the bacteriophage injection model; here, the T6SS sheath is recycled whereas the viral one is not (Kapitein and Mogk, 2013). ClpV is a hexamer-forming chaperone of the **h**eat **s**hock **p**rotein, Hsp100/Clp family of AAA^+ (**A**TPase **a**ssociated with diverse **a**ctivities) proteins referred to as TssH. AAA+ proteins have one or two WalkerA and WalkerB motifs for ATP-binding and hydrolysis, energizing the disassembly and unfolding

of proteins (Schlieker et al., 2005). It binds TssC at its substrate specific N-terminal domain and energizes dissociation of TssB and TssC through ATP binding at a C-terminal AAA domain (Cascales and Cambillau 2012; Bönemann et al., 2009, Pietrosiuk et al., 2011). Additionally, TssH is believed to prevent unproductive TssB/TssC tubule formation, as these rapidly assemble in solution (Kapitein et al., 2013).

I. D. 4. Threonine Phosphorylation Pathway (TagE/TagG/Fha)

Phosphorylation of an Fha domain containing protein, TagH, by a serine/threonine kinase (STK/PpkA/TagE) and the antagonistic dephosphorylation by serine/threonine phosphatase (STP/PppA/TagG) is an example of post-translational regulation, well-studied in *P. aeruginosa* (Mougous et al., 2007). It was previously thought that tyrosine and ser/thr kinases were the predominant kinases of eukaryotes and histidine kinases were predominant in prokaryotes, but advances in genomics changed these ideas. In 1999 Mukhopadhyay et al. identified four Ser/Thr kinases in a strain of *P. aeruginosa,* and one was implicated in virulence (*stk1*). Interestingly, at that time they had noted that *stk1* and *stp1* were in close association with an *icmF*-like gene (Mukhopadhyay et al., 1999). H1-T6SS, one of three T6SS gene clusters of *P. aeruginosa* PAO1*,* encodes for a serine/threonine kinase with one transmembrane segment, PpkA (TagE). Binding and dimerization of the extracellular domain, with putative co-receptor TagR, leads to the dimerization and autophosphorylation, hence activation, of PpkA (Hsu et al., 2009). Antagonizing the phosphorylation of Fha (TagH) is phosphatase, PppA (TagG). In *P. aeruginosa*, mutation to *pppA* resulted in T6SS being in a permanently 'on' state*,* essentially a hyper-secreting mutant (Mougous et al., 2007). This form of post-translational regulation was induced by surface contact in *P. aeruginosa* and referred to as the threonine phosphorylation pathway (TPP). However, not all T6SSs utilize such a system. In these examples there may be other means of phosphorylating Fha, but there are also some T6SS that lack Fha all together (Hsu et al., 2009). TPP is one of many modes of T6SS regulation that has been studied so far. Other modes of regulation that have established will be briefly introduced in Section ID6.

I. D. 5. The T6SS Effectors.

The most elusive components of T6SSs are the actual effectors. Hcp is found abundantly in the supernatant of T6SS, but it is not believed to be the effector. However, there is evidence that Hcp plays an additional function in triggering a cell response. Hcp of *E. coli* K1 strain RS218 was shown to rearrange the actin of the cytoskeleton, induce apoptosis, and release

cytokines and chemokines in human brain microvascular endothelial cells (Zhou et al., 2012). The potential also remains that Hcp is 'piggy-backing' cargo out of the cell (Coulthurst, 2013). Another component of the T6SS apparatus that has been shown to play an effector role is VgrG (TssI). Evolved VgrGs with C-terminal extensions are discussed in Section ID3. Few 'true' secreted effectors, those that are not part of the T6SS machinery, have been identified to date but include EvpP (*E. tarda*), TssM (old nomenclature, *B. mallei*), VasX (*V. cholerae*), Tse1/Tse2/Tse3 (*P. aeruginosa*), Ssp1 and Ssp2 (*S. marcescens*), seven T6SS-1 substrates, putative substrates from *B. thailandensis* and a peptidoglycan amidase from *P. fluorescens* (Coulthurst, 2013). EvpP has been shown to be secreted and is required for virulence in blue gourami fish (Zheng and Leung 2007). TssM (different from the core component IcmF) encoded by *B. mallei* is a secreted deubiquitinase (DUB). In eukaryotes, ubiquitination alters a protein's stability or function by addition of ubiquitin, a 76 aa protein. Pathogenic bacteria export DUBs, usually via T3SS, to evade the host immune response and promote survival and replication *in vivo*. In *B. mallei*, the secreted DUB is genetically linked to the T6SS, but its secretion is not T6SS dependent (Shanks et al., 2009). VasX is secreted by *V. cholerae*, and a VasX mutant is attenuated in virulence toward the amoeba, *Dictyostelium discoideum* (Miyata et al., 2011). Tse1, Tse2 and Tse3, toxins secreted by *P. aeruginosa*, are toxic to prokaryotes as introduced in Section IA (Hood et al., 2010). $Tae2^{BT}$ is one of 13 proteins present in the wild type secretome of *B. thailandensis* but absent in the DT6SS-1 (T6SS-1 mutant) secretome. Of these other 12 proteins, two were predicted substrates of alternative secretory pathways because of their N-terminal signal peptides and four were VgrG homologs. Most T6SS-1 substrates were predicted to be involved in interbacterial interactions, such as Tae (type VI amidase effector) proteins and bacteriocin-like proteins. Of these, $Tae2^{BT}$ was predicted to be a cell wall targeting, antibacterial, peptidoglycan amidase (Shanks et al., 2009). Ssp1 and Ssp2 are antibacterial toxins secreted by *S. marcescens* that have been shown to bind cognate immunity proteins (Rap proteins) (Fritsch et al., 2013).

It is evident that the majority of the substrates for T6SSs identified so far are antibacterial. Of course, there still remain several systems that have been identified through effects on eukaryotic hosts. T6SSs were originally identified by mutants with loss of virulence. However, identification of these substrates has been limited mostly to evolved VgrG proteins. Effects of T6SS on eukaryotes are usually seen as whole-organism phenotypes and include actin cross-linking, formation of actin protrusions, limitation of bacterial colonization,

intracellular replication, and adaption to deoxycheolic acid (Miyata et al., 2013; Coulthurst, 2013).

I. D. 6. Regulation of type VI secretion systems.

Soon after the surge of T6SSs findings in several genomes, discovering different modes of regulation followed. The number of different regulatory mechanisms described so far matches the diverse functions and bacteria that utilize the T6SS (Miyata et al., 2013). Environmental cues found to date include but are not limited to cell density, temperature, pH, salinity, osmolarity, and concentrations of iron, phosphate and magnesium (Miyata et al., 2013). Some of the most highlighted mechanisms of transcriptional regulation involve histone-like nucleoid-structuring proteins (H-NS), σ^{54}, quorum sensing, two component systems and various transcriptional activators (Bernard et al., 2010).

Via DNA binding, H-NS and H-NS-like proteins negatively regulate the T6SSs in *S. enterica*, *P. putida* and *P. aeruginosa* (specifically gene clusters HSI-2 and HSI-3) (Lucchini et al., 2006; Renzi et al., 2010; Castang et al., 2006; Bernard et al., 2010). More current studies have focused very little on this aspect of T6SS regulation. However, recently, H-NS was shown to play a role in expression of the T6SS genes of *Acinetobacter baumannii* along with other virulence factors (Eijkelkamp et al., 2013).

The alternative sigma factor σ^{54}, or σ^{N} (RpoN), has reoccurred often in T6SS literature as a positive regulator. RNA polymerase (RNAP) consists of five subunits capable of transcriptional elongation and termination, but the holoenzyme requires a sixth factor, the σ-factor, for initiation. (Helmann and Chamberlin, 1988; Buck et al., 2000). Sigma 54 is recognized for its involvement in regulation of nitrogen metabolism and its unusual spectra of unrelated genes that have been shown to be transcribed, including nitrogen utilization, flagellation, plant pathogenicity, synthesis of several cofactors, and alginate biosynthesis (Cases et al., 2003). Several gene clusters possess a consensus σ^{54}-binding sequence -24/-12 upstream of the +1 transcriptional start. Here, σ^{54} recognizes and binds the sequence and recruits RNAP, forming a closed complex. For transcription to proceed, an ATP-dependent bacterial enhancer binding protein is needed to energize the melting of the DNA to form an open complex (Buck et al., 2000; Bernard et al., 2011). The enhancer binding protein, VasH, of *V. cholerae*, *P. aeruginosa* and *A. hydrophila* is required for expression of their T6SSs (Miyata et al., 2013).

In quorum sensing, cell density is detected via diffusible signals that lead to activation and/or repression of gene expression. T6SS genes are typically induced at high cell densities, but *P. aeruginosa* HSI-1 is an example where the T6SS is repressed (Silverman et al., 2012).

In two component systems an environmental cue (most are unknown) signals a sensor kinase that transduces a signal by transferring a phosphate to a response regulator. The response regulator includes a DNA-binding domain, which binds specific genes and regulates expression. In *E. tarda* (EsrAB) and the Tss1 locus of *B. mallei* (VirAG), a two component system induces a T6SS. In *S. enterica* (SsrAB) the T6SS is negatively regulated (Bernard et al., 2010). Various transcriptional activators have been demonstrated to regulate T6SSs, such as an AraC-like transcription factor inducing expression in EAEC, *E. tarda* and *B. pseudomallei*. A Mar-like transcription factor contributes to expression in *Y. pestis*. A TetR-like transcription factor functions in *V. cholerae*, *B. pseudomallei* and *P.aeruginosa*. There are often multiple T6SSs which can each be induced by multiple transcription factors, increasing complexity (Bernard et al., 2010). Of course, these modes of regulation also play a role in regulation of other bacterial systems, and cross talk often exists amongst these systems. This has been seen with the T3SS and T6SS of *S. enteric*, *E. tarda* and *Aeromonas hydrophila* with an additional connection to the flagella system. Crosstalk among quorum sensing, T6SS, and flagella systems in *V. cholerae* has been demonstrated and among the three T6SSs of *P. aeruginosa* (Leung et al., 2011).

Post-transcriptional regulation has also been documented. GacS/GacA is a two component system that leads to expression of small regulatory RNAs, which sequester mRNA-binding protein, RsmA. By interfering with the ribosome binding site access, RsmA inhibits translation (Silverman et al., 2012). In *P. aeruginosa*, *retS* encodes an activator of GacAS signaling and *ladS* a repressor. In the early discovery of T6SSs, a microarray study of *P. aeruginosa* found T6SS genes were up-regulated in Δ*retS* (Mougous et al., 2006). Post-translational regulation has been studied the most conclusively in *P. aeruginosa*. The HSI-1 gene cluster is regulated by a TPP system (as introduced in Section ID4). Further, regulation upstream of PpkA (TagE) has also been shown. TagR is a periplasmic protein that positively regulates PpkA (Hsu et al., 2009) and is anchored to the OM by OM lipoprotein, TagQ. In *P. aeruginosa*, TagS and TagT, Lol-like lipoproteins, form a membrane complex with ATPase activity. It is believed that TagS and TagT detect an environmental signal and transmit it by modulating TagR (Casabona et al. 2013). A lipoprotein transporter, permease protein, LolE

(*azo1509*), and lipoprotein releasing system ATP-binding protein, LolD (*azo1510*) are also found within the BH72 genome, which may be the homologs of TagS and TagT (Casabona et al., 2013).

Finally, a TPP-independent mode of post-translational regulation is the TagF pathway, which is also found in *P. aeruginosa*. The environmental cue for TagF activity is unknown, but it is known to repress the T6SS similar to PppA (TagG); also known is that Fha is required along with the recruitment of ClpV (Silverman et al., 2011).

Although several examples of regulation have been found, it needs to be noted that there are also examples of constitutively expressed T6SS gene clusters. Examples of T6SSs that are constitutively expressed include those encoded by *Serratia marcescens* strain Db10, *V.cholerae* V52 and T6SS-1 of *Burkholderia thailandensis* (Fritsch et al., 2013).

I. E. Aim and scope of this thesis

Azoarcus spp. were the predominant culturable diazotroph in a survey of nitrogen fixing bacteria within the endorhizosphere of Kallar grass where up to 10^8 cells per gram root dry weight were found (Reinhold et al., 1986; Reinhold-Hurek et al., 1993a). Additionally, *Azoarcus* sp. strain BH72 colonized rice in equally high numbers (10^9 cells per gram dry weight) without rice showing any signs of distress (Hurek et al., 1994b). Understanding how this endophytic lifestyle is established has remained enigmatic, considering there is no genetic evidence for a T3SS, T4SS or toxins encoded within the *Azoarcus* sp. BH72 genome (Krause et al., 2006). Few factors necessary for colonization had been identified, but include an endoglucanse, T4P and flagella (Buschart et al., 2012; Reinhold-Hurek et al., 2006; Dörr et al., 1998). The discovery of a putative T6SS prompted further investigations into its function and whether this secretion system plays a role in establishing the endophytic colonization of *Azoarcus* sp. strain BH72 in rice (Julia Herglotz, Diplomarbeit, 2007; Teja Shidore, Master thesis, 2008). GUS assays determined that transcription of *azo1299* (TssK1, formerly ImpJ) was nearly 20 times higher when cells were grown under nitrogen fixing conditions, than comparable non-fixing conditions. Genes from the *sci* gene cluster, *azo3892* (TssK2, formerly SciO) and *azo3901* (TssF2, formerly SciC), remained at consistent levels of expression regardless of growth conditions tested (Julia Herglotz, Diplomarbeit, 2007). The up-regulation of *azo1302* (TssM1) under nitrogen fixing conditions and constitutive

expression of *sci* genes, *azo3892* and *azo3901*, was also confirmed using semi-quantitative RT PCR (Shidore Teja, Master thesis, 2008). The up-regulation of *imp* genes and constitutive expression of *sci* genes was also demonstrated in microarray studies (Sarkar and Reinhold, 2014). Additionally, *Oryza sativa* cv. IR-36 produced an unusual root hair phenotype when inoculated with a strain where expression of an *imp* encoded protein, TssK1, was inactivated. Endophytic colonization by this mutant was also impaired (Julia Herglotz, Master thesis, 2007; Teja Shidore, Master thesis, 2008). The aim of this study was to perpetuate the investigation of T6SS(s) encoded by the *Azoarcus* sp. strain BH72 genome. This work supports previous findings that *Azoarcus* sp. strain BH72 utilizes both T6SSs and further dissects the roles of yet untested components. A primary focus was given to the *imp* gene cluster because of its up-regulation under nitrogen fixing conditions, and proteomic findings in this study corroborated with transcriptional data that type VI secretion (T6S) is up-regulated under nitrogen fixing conditions. Additionally, this study begins to expose important features of the *sci* encoded T6SS. Finally, potential transcriptional regulators of T6SS genes were screened and the role of RseC-like Azo0559 as a positive partial regulator was demonstrated.

II. Materials and Methods

II. A. Materials

II. A. 1. Media

All media was prepared and sterilized in an autoclave at 121°C at 2 barr for 20 min. Heat labile components were added after autoclaving and sterilized by filtration using a 0.2 µm pore filter (Schleicher and Schuell, Dassel, Germany).

Luria–Bertani medium (LB) for *E. coli* (Sambrook et al., 1989)

10 g/l		Tryptone
5 g/l		Yeast Extract
10 g/l	(171 mM)	NaCl

For agar plates: 15 g/l agar

Synthetic Media (SM) (Reinhold et al., 1986)

5 g/l	(37 mM)	DL–malic acid
0.2 g/l	(0.8 mM)	$MgSO_4 \cdot 7H_20$
0.1 g/l	(17 mM)	NaCl
26 mg/l	(180 µM)	$CaCl_2 \cdot 2H_2O$
2 mg/l	(8.3 µM)	$Na_2MoO_4 \cdot 2H_2O$
10 mg/l	(60 µM)	$MnSO_4 \cdot H_2O$
66 mg/l	(180 µM)	Fe(III)- EDTA
0.87 g/l	(6 mM)	KH_2PO_4
1.12 g/l	(6 mM)	K_2HPO_4

pH 6.8

For semi-solid media: 2 g/l Difco agar (Franklin Lakes, New Jersey, USA)

Synthetic Media + NH_4Cl (SM+N)

1 L		SM Medium
0.1 g		Bacto Yeast Extract
0.5 g	(9.3 mM)	NH_4Cl

For plates: 17 g/l Difco agar

For semi-solid media: 2 g/l Difco agar

Voll Medium-Ethanol (VME) Medium (Reinhold-Hurek et al. 1993a)

5 g/l	(37 mM)	DL–malic acid
0.2 g/l	(0.8 mM)	MgSO4•7H20
1.1 g/l	(19 mM)	NaCl
26 mg/l	(180 µM)	CaCl2•2H2O
2 mg/l	(8.3 µM)	Na2MoO4•2H2O
10 mg/l	(60 µM)	MnSO4•H2O
66 mg/l	(180 µM)	Fe(III)- EDTA
0.87 g/l	(6 mM)	KH2PO4
1.12 g/l	(6 mM)	K2HPO4
0.5 g/l	(9.3 mM)	NH_4Cl
1 g/l		BactoYeast Extract
3 g/l		Bacto Peptone
6 ml/l	(0.6%)	Absolute Ethanol (sterile filtered)

For agar plates: 17 g/l agar

Conjugation agar (KON)

5 g/l	(37 mM)	DL–malic acid
0.2 g/l	(0.8 mM)	MgSO4•7H20
1.1 g/l	(19 mM)	NaCl
26 mg/l	(180 µM)	CaCl2•2H2O
2 mg/l	(8.3 µM)	Na2MoO4•2H2O
10 mg/l	(60 µM)	MnSO4•H2O
66 mg/l	(180 µM)	Fe(III)- EDTA
0.87 g/l	(6 mM)	KH_2PO_4
1.12 g/l	(6 mM)	K_2HPO_4
5 g/l		Bacto Yeast Extract
17 g/l		Difco agar

Germination Agar

1 g/l		Bacto Yeast Extract
3 g/l		Bacto Peptone
1 g/l	(5.6 mM)	α-D-Glucose
10 g/l		Difco Agar

Plant Medium (Egener *et al.*, 1999)

20 mg/l	(170 µM)	DL–malic acid
0.03 mg/l	(48.5 µM)	H_3BO_3
0.2 mg/l	(0.69 µM)	$ZnSO_4$•7 H_2O
0.1 mg/l	(0.40 µM)	$CuSO_4$•5 H_2O
0.2 g/l	(0.91 mM)	$MgCl_2$•6 H_2O
0.1 g/l	(1.71 mM)	NaCl
12 mg/l	(70 µM)	$MnSO_4$•H_2O
4 mg/l	(16 µM)	Na_2MoO_4•2 H_2O
26 mg/l	(0.23 mM)	$CaCl_2$•2 H_2O*
0.87 g/l	(6 mM)	KH_2PO_4
1.12 g/l	(6 mM)	K_2HPO_4
13 mg/l	(49 µM)	$C_6H_5FeO_7$•H_2O

Semi-semi-solid Media (for pour plating)

1 L		SM Medium
0.1 g/l		Bacto Yeast Extract
8 g/l		Difco agar

II. A. 2. Solutions and Buffers

GUS buffer

8.5 g/l	(40 mM)	NaH_2PO_4
5.52 g/l	(60 mM)	Na_2HPO_4 x H_2O
1 mM		EDTA
10 mM		β-mercaptoethanol
pH 7.0		

GUS Assay Lysis Solution

5 mM	Tris (hydroxymethyl) aminomethyane (Trizma™)
0.5 mM	EDTA
0.05%	SDS

Stop Solution

2.5 M	2-amino-2methyl 1,3 propandiol

TE Buffer

10 mM	Tris (hydroxymethyl) aminomethyane (Trizma™)
1 mM	EDTA
pH 8.0 adjusted using HCl	

Colony Lysis Buffer

0.1% (v/v)		Tween20
12.1 mg/l	(10 mM)	Tris (hydroxymethyl) aminomethyane (Trizma™)
1.86 g/l	(1 mM)	EDTA
pH 8.0		

TES Buffer

10 mM	Tris (hydroxymethyl) aminomethyane (Trizma™)
1 mM	EDTA
50 mM	NaCl
pH 8.0 adjusted using HCl	

Solution 2

200 mM	NaOH
1% (w/v)	SDS

Phosphate Buffered Saline (PBS)

135 mM	NaCl
2.7 mM	KCl
20 mM	Na_2HPO_4
1.5 mM	KH_2PO_4

pH 7.0 adjusted using HCl/NaOH

Tris Buffered Saline (TBS)

100 mM	Tris (hydroxymethyl) aminomethyane (Trizma™)
150 mM	NaCl

pH 7.5 adjusted with HCl

Plant Wash Solution

0.2 g/l	(0.8 mM)	$MgSO_4 \cdot 7H_20$
0.1 g/l	(17 mM)	NaCl
26 mg/l	(180 μM)	$CaCl_2 \cdot 2H_2O$
2 mg/l	(8.3 μM)	$Na_2MoO_4 \cdot 2H_2O$
10 mg/l	(60 μM)	$MnSO_4 \cdot H_2O$
66 mg/l	(180 μM)	Fe(III)- EDTA
0.87 g/l	(6 mM)	KH_2PO_4
1.12 g/l	(6 mM)	K_2HPO_4

Plant Sterilization Solution

4% (v/v)		NaClO Solution
1 g/l	(9.3 mM)	Na_2CO_3
33 g/l	(3.3 mM)	NaCl
1.67 g/l	(41.7 mM)	NaOH

Prehybridization Solution (for Southern Blots)

0.9 M	NaCl
90 mM	Natriumcitrate-Dihydrate
0.1% (w/v)	Bovine Serum Albumin (BSA)
0.1% (w/v)	Ficoll 400
0.1% (w/v)	Polyvinyl pyrollidone
0.5% (w/v)	SDS

SDS-Extraction Buffer

7.5 g/l	Tris (hydroxymethyl) aminomethyane (Trizma™)
5% (v/v)	β-mercaptoethanol
10% (v/v)	Glycerol

pH 6.8 adjusted using HCl

SDS-PAGE Running Buffer (TGS)

25 mM	Tris (hydroxymethyl) aminomethyane (Trizma™)
192 mM	Glycine
0.1% (w/v)	SDS

pH 8.4 adjusted using HCl

Transfer Buffer

14.4 g/l	(0.2 M)	Glycine
3 g/l	(25 mM)	Tris (hydroxymethyl) aminomethyane (Trizma™)
0.0075% (w/v)		SDS
20% (v/v)		Methanol

Colloidal Coomassie Brilliant Blue Stain (Candiano et al. 2004)

2% (w/v)	H_3PO_4
10% (w/v)	$(NH_4)_2SO_4$
0.1% (w/v)	Coomassie Brilliant Blue G-250
20% (v/v)	Ethanol Absolute

Buffer W (for strep-tagged protein purification)

100 mM	Tris (hydroxymethyl) aminomethyane (Trizma™)
450 mM	NaCl
1 mM	EDTA

pH 8.0 adjusted using HCl

Buffer E (for strep-tagged protein purification)

100 mM	Tris •HCl
150 mM	NaCl
1 mM	EDTA
2.5 mM	Desthiobiotin,

pH 8

20X SSC (saline-sodium citrate)

3 M	NaCl
0.3 M	Natriumcitrate-Dihydrate

pH 7.0 adjusted using HCl/NaOH

10X DNA Loading Buffer

20% (w/v)	Ficoll 400
0.1% (w/v)	Bromophenol Blue
0.1% (w/v)	Xylenxyanol
0.1% (w/v)	OrangeG
10 µl/ml	RNaseA

Southern Blot Neutralization Solution

1.5 M	NaCl
0.5 M	Tris (hydroxymethyl) aminomethyane (Trizma™)
1 mM	Ethylendiamintetraacetat

pH 7.0 adjusted with HCl

Southern Blot Denaturation Solution

1.5 M	NaCl
0.5 M	NaOH

DIG-P1 (Washing Buffer)

0.1 M	Malic Acid
0.15 M	NaCl

pH 7.5 adjusted using NaOH

DIG-P3

0.1 M	Tris (hydroxymethyl) aminomethyane (Trizma™)
0.1 M	NaCl
0.05 M	$MgCl_2 \bullet 6H_2O$

pH 9.5 adjusted using HCl

TAE Buffer

40 mM	Tris (hydroxymethyl) aminomethyane (Trizma™)
2 mM	EDTA

pH 8.5 adjusted with Glacial Acetic Acid

Protein Sample Buffer for SDS-PAGE

15% (v/v)	Glycerol
2.3%	SDS
62.25 mM	Tris (pH 6.8)
0.003% (w/v)	Bromophenol Blue
1.25% (v/v)	β-Mercaptoethanol

2D-Gel Sample Buffer

9.5 M	Urea
2% (v/v)	Bio-Lyte 3/10 Ampholyte (BioRad, München, Germany)
100 mM	DTT

2D-Gel Overlay Buffer

6 M	Urea
1% (v/v)	Bio-Lyte 3/10 Ampholyte (BioRad, München, Germany)
100 mM	DTT
2% (w/v)	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)

2D-Gel Equilibration Buffer

60 mM	Tris (hydroxymethyl) aminomethyane (Trizma™)
1% (w/v)	SDS
20% (v/v)	Glycine
50 mM	DTT
pH 6.8	

NAES Solution

50 mM	Na-acetate (pH 5.1)
10 mM	EDTA
1%	SDS

Protein Suspension Buffer$^{(+)}$

5M	Urea
2 M	Thiourea
0.65 M	Dithiothreitol (DTT)
0.3 M	CHAPS
0.65 M	Caprylyl Sulfobetaine SB3-10

$^{(+)}$EDTA-free protease inhibitor cocktail tablet (cOmplete ULTRA Tablets, Mini, Roche, Mannheim, Germany)

II. A. 3. Antibiotics

All antibiotics were prepared in stock solutions and then sterile filtered through a 0.2 µm pore filter (Schleicher and Schuell, Dassel, Germany) before adding to media.

Table 1. Final Concentrations of Antibiotics used in this study.

Antibiotic	*E. coli*	*Azoarcus* sp. strain BH72
Ampicillin (Ap)	150 µg/ml	30.0 µg/ml
Chloramphenicol (Cm)	not applicable	12.5 µg/ml
Kanamycin (Km)	30.0 µg/ml	30.0 µg/ml
Spectinomycin (Sp)	80 µg/ml	40 µg/ml
Streptomycin (St)	20 µg/ml	20 µg/ml

II. A. 4. Bacterial Strains

Bacterial strains and their descriptions used in this study are listed in Table 2.

Table 2. Bacterial strains used in this study.

Bacterial Strain	Description	Source
Azoarcus sp. strains		
Azoarcus sp. BH72 (WT)	wild type (WT)	Reinhold et al. 1986
BHimpL::pK18GGST (BHazo1302)	Km^R, pK18TimpL (with a 719 bp PCRFragment 67-786 bp into *azo1302*, *Hind*III/*Xba*I) integrated into the chromosome of BH72 through a single recombinational event	Herglotz, Julia 2007
BH1301	Km^R, Transcriptional fusion of *gusA* 82 bp after *azo1301* through a single recombination of pK18GGST1301pro without disruption of BH72 genes	This study
BHLAO	Sp^R/Sm^R, BH72 *nifL* :: Ω	Egener et al., 2002

BHΔimpLsciO	Km^R, In-frame deletion (408 bp-3423 bp) of *azo1302* through double recombination of pK18msBazoUpDown1302 and a polar mutation to *azo3890* through the integration of pK18TsciO (Herglotz, 2007) in *azo3892* at bp 754 bp in a single recombination event	This study
BHΔ3331	In-frame deletion (180-576 bp) of *azo3331* through double recombination of pK18msBazo3331 into BH72	This study
BHΔ3885	In-frame deletion (207-687 bp) of *azo3885* constructed through double recombination of pK18msBH3885 in BH72	This study
BHazo3903	Km^R, single recombination of pK18Gazo3903 in gene *azo3903* after 593 bp	This study
BHazo3888	Km^R, pK18GGSTazo3888 into *azo3888* after 642 bp through a single recombinational event. Last gene of operon, no polar effects in BH72	Disch, Eva 2010
BHΔgacA	In-frame deletion of *gene azo2980* (71-632 bp) through the double recombination of pK18mobsacBΔgacA into the chromosome of strain BH72	This study
BHazo0559	Km^R, *azo0559* with insertion of pK18GGSTazo0559 through a single recombinational event, polar mutation to BH72	Laboratory course
BHΔ0559	In-frame deletion of *azo0559* (108-384 bp) through the double recombination of pK18mobsacBΔ0559 into the chromosome of strain BH72	This study
BHΔ0559;1301pro::gusA	Km^R, Transcriptional fusion of *gusA* with gene *azo1301* after 82 bp through a single recombination of pK18GGST1301pro into the chromosome of strain BHΔ0559	This study

BHLAO;1301::gusA	Km^R, Transcriptional fusion of *gusA* with *azo1301* after 82 bp through a single recombination of pK18GGST1301pro without disruption of genes into the chromosome of strain BHLAO	This study
BHΔRnf1	In-frame deletion of Rnf1 through double recombination of pJRnf1UD-MSB into the BH72 chromosome.	Sarkar et al., 2012
Escherichia coli		
DH5αF'	F′/ φ80*lac*ZM15 (*lacZYA-argF*)$_{U169}$ *deoRrecA1endA1 hsdR17* (rk^-, mk+) *phoA supE 44λ¯ thi*-1 *gyrA96relA*	Invitrogen, Karlsruhe, Germany
S17-1	Sp^R, MM294, RP4-2-Tc::Mu-Km::Tn7 chromosomally integrated	Simon et al., 1983
pRK2013	Km^R, DH5αF′ containing the helper plasmid pRK2013 with *tra*+, ColE1 replicon	Figurski et al., 1979
pASK-IBA-azo1305	Ap^R, *E. coli* DH5αF' containing pASK-IBA3 plasmid with PCR fragments from BH72 *azo1305* (527 bp *Bsa*I/*Bsa*I)	Öhrlein, Johannes 2009
pASK-IBA-azo3898	Ap^R, *E. coli* DH5αF' containing pASK-IBA3 plasmid with PCR fragments from *Azoarcus* sp.BH72 *azo3897* (518 bp *Bsa*I/*Bsa*I)	Öhrlein, Johannes 2009
pASK-IBA-azo3897	Ap^R, *E. coli* DH5αF' containing pASK-IBA3 plasmid with PCR fragments from BH72 *azo3898*(538 bp *Bsa*I/*Bsa*I)	Öhrlein, Johannes 2009

II. A. 5. Plasmids

The plasmids used in this study are listed and their features described in Table 3.

Table 3. Plasmids used in this study.

Plasmid Name	Plasmid Features	Source
pUC19	Ap^R, ColE1 replicon	Yanisch-Perron et al., 1985
pJET1.2/blunt	Ap^R, rep (pMB1), *eco47IR*, P_{lacUV5}, T7 promoter	Fermentas, ThermoScientific, Waltham, MA, USA
pK18GGST	Km^R, derivative of the mobilizable cloning vector pK18mob2, promoterless *gfp* and *gusA*, T4 transcription terminator	Krause et al., 2011
pK18*mobsac*B	Km^R, RP4 mob region, containing *sac*B gene	Schaefer et al., 1994
pRK2013	Km^R, RK2 Transfer genes cloned into the ColE1 Replicon	Figurski et al. 1979
pK18TsciO	Km^R, PCR-Fragment (44-757 bp of gene *azo3892*) with *Hin*dIII and *Xba*I restriction sites in the pK18GGST vector	Herglotz, Julia 2007
pK18GGSTazo3888	Km^R, PCR fragment (91-642 bp of gene *azo3888*) cloned at *Xba*I-*Hind*III site in pK18GGST	Disch, Eva 2010
pK18Gazo3903	Km^R, PCR fragment (53-593 bp of gene *azo3903*) with *Xba*I and *Hind*III restriction sites in the pK18GGST vector	Disch, Eva 2010
pK18GGST1301pro	Km^R, PCR amplified fragment (495 bp upstream – 82 bp into *azo1301*) in pK18GGST with *XbaI* and *Hind*III	This study
pUC18Δ0559	Ap^R, Up and down fragments (with 276 bp deletion of *azo0559*) from pK18mobsac18Δ0559 into pUC18 using *Sma*I/*Xba*I sites	This study
pK18mobsac18Δ0559	Km^R, 906 bp up fragment (798 bp upstream-108 bp into gene *azo0559* with *Xba*I-*Bam*HI) and 817 down fragment (from 384 bp into *azo0559*-797 bp downstream with *Bam*HI/*Sma*I) cloned into a pK18*mobsac*B vector	This study
pUC19updown Ω	Ap^R, Sp^R, Sm^R, up (801 bp with *Eco*RI/*Bam*HI sites) and down (823 bp with *Bam*HI and *Hind*III sites) fragments of *azo1302* with an omega cassette positioned in the 2994 bp deleted region between, in pUC19	Öhrlein, Johannes 2007

pUC-UpDown1302	Ap^R, 801 bp up fragment (393 bp upstream-408 bp into gene *azo1302*) cut from pUC19updown Ω with *Eco*RI/*Bam*HI sites and 823 bp PCR amplified down fragment (3423 bp in-532 bp downstream of gene *azo1302*) in pUC19 with *Bam*HI and *Hind*III sites	This study
pK18msBazoUpDown1302	Km^R, up and down fragments from pUC-UpDown1302 using *Eco*RI/ *Hind*III sites into pK18*mobsac*B	This study
pUCΔ3885	Ap^R, 782 bp up fragment (575 bp upstream-207 bp into gene *azo3885* with *Eco*RI and *Sac*I sites) and 983 bp down fragment (687 bp in-899 bp downstream of *azo3885* with *Sac*I and *Xba*I sites) in pUC19	This study
pK18msBH3885	Km^R, *azo3885* up and down (207-687 bp deleted) from pUCΔ3885 inserted into pK18*mobsac*B using *Eco*RI and *Xba*I sites	This study
pK19azo3331	Ap^R, 247 bp up fragment (67 bp upstream-180 bp into gene *azo3331* with *Eco*RI and *Bam*HI sites) and 243 bp down fragment (576 bp in-63 bp downstream of azo3331 with *Bam*HI and *Hind*III sites) in pUC19	This study
pK18msBazo3331	Km^R, Up and down fragments from pK19azo3331 (with 396 bp deletion) in pK18*mobsac*B using *Eco*RI/*Hind*III sites	This study
pUC18ΔgacA	Ap^R, 985 bp PCR amplified up fragment (913 bp upstream-71 bp into *azo2980*) with *Bam*HI/*Xba*I sites and 772 bp PCR amplified down fragment (632 bp in-736 bp downstream of *azo2980*) with *Xba*I/*Hind*III sites cloned into pUC18	This study
pK18mobsacBΔgacA	Km^R, Up and down fragments from pUC18ΔgacA with 540 bp deletion cloned into pK18*mobsac*B using *Bam*HI/*Hind*III sites	This study

II. A. 6. Primers

Primers were designed using sequences of interest entered into PrimerBlast (National Center for Biotechnology Information (NCBI), Bethesda, MD, USA) or Primer3 (v. 0.4.0) (http://sourceforge.net/projects/primer3) (Untergrasser et al., 2012; Koressaar and Remm, 2007) programs. The primers used were ordered from Eurofins MWG GmbH (Ebersberg,

Germany). Primers were dissolved to 500 µM with nuclease-free TE buffer. Working solutions were prepared by making a 50 µM dilution with RNase free water.

Table 4. List of Primers used in this study.

Product Name: **Forward Primer Name** **Reverse Primer Name**	**Sequence (5´-3´)**	**Product length (bp)**	**Annl. temp. (°C)**
16S:* TH3fw TH5rv	 GATTGGAGCGGCCGATGTC CTGGTTCCCGAAGGCACCC	800	70
1301 fusion: 1301fsnF 1301fsnR	 AAAtctagaATATCTCCGTGTCCGACCTG (*Xba*I) AGGaagcttAGCCAGCCATCCAGAAAGC (*Hind*III)	578	55
3331UP: Aazo3331-F Aazo3331-R	 TTTgaattcTTTTGTCTACCCGCGAAATC (*Eco*RI) TTTggatccGACGACGTCTACTGCGA (*Bam*HI)	247	57
3331Down: Bazo3331-F Bazo3331-R	 TTTggatccGAAACCGTGCTTCTGTG (*Bam*HI) TTTaagcttTATCAACTTCGGCATCACCA (*Hind*III)	243	57
S/T Kinase: 3888(551)F 3888(551)R	 GGGtctagaTAAGCTGGGCGAATTCGAGAT (*Xba*I) GGGaagcttGGCTTGAGGATGACCGTAAG(*Hind*III)	551	60-68
gacAUP: UPgacA-F UPgacA-R	 ATggatccAGAACGCCGCATCATCGCCC(*Bam*HI) ACtctagaAGGCGGAAACCCATGCGGAC (*Xba*I)	985	65
GacADown: DNgacA-F DNgacA-R	 GGtctagaGATTGCGGTACGGGCGGGTT (*Xba*I) AGGaagcttTGCTGGCGTCGAAGCACTGG (*Hind*III)	772	65
0559UP: 0559F(906) 0559UpR(809)	 AAAtctagaATCAAGACCGGCAGCCTT (*Xba*I) AAAggatccGCTGCCGCAGACCTTGTT (*Bam*HI)	906	60.8
0559Down: 0559DwnF(817) 0559DwnR(817)	 AAAggatccGCATCCGAATTCAACGACTGA(*Bam*HI AAAcccgggCGGGTCAACAGCACACAG (*Sma*I)	817	55
3885UP: 3885upR(780) 3885upR(780)	 AAAgaattcCCGAACTGATGGAGCACAT (*Eco*RI) AAAgagctcGGCTTCGCTCACTTCGGGATG (*Sac*I)	780	55
3885Down: 3885dnF(983) 3885dnR(983)	 AAAgagctcGACAACCACCACAGCGATAA (*Sac*I) AAAtctgagCAGCACCAGGCTGAAGTTCT (*Xba*I)	983	60.8
3890 (SciS):* 3890F349bp 3890R349bp	 GTACAGCTTCGACGGCT GTCGATCACCGACTTGTCCT	349	50
ClpV: 3903(560)F 3903(560)R	 GGGtctagaTAACAAACTCAACAGCCTCGCCTA (*Xba*I) GGGaagcttGTGAGGTCGACGGTGAATTT(*Hind*III)	564	60-68
impL: downforC downrev4real	 GTTggatccCAGACCTGGGCGAGTTTCAGT(*Bam*HI) GCGaagcttCTTCGTTGAGCAGGGCTTC (*Hind*III)	823	65

Gfp:*** gfppK18GGSTfor gfppK18GGSTrev	 AGTGGAGAGGGZGAAGGTGA AAAGGGCAGATTGTGTGGAC	535	58
Universal Primers:** M13F M13R	 GTAAAACGACGGCCAGT AACAGCTATGACCATG	--	55
pJET Primers:** pJET1.2 F (23mer) pJET1.2 R (24mer)	 CGACTCACTATAGGGAGAGCGGC AAGAACATCGATTTTCCATGGCAG	--	--

Application: *RT-PCR, **sequencing, ***DIG-labeled probe. Underlined sequences do not compliment the template sequence. Small case letters indicate restriction sites and are listed in parentheses after the sequence.

II. B. Methods

II. B. 1. Conditions used for growing cultures

II. B. 1a. Standard growth conditions of *E. coli* and *Azoarcus* strains for generation of precultures and propagation of plasmids

Standard growth conditions were generally used to generate precultures and propagate plasmids in *E. coli* strains. *E. coli* strains were grown in LB broth, and strains of *Azoarcus* were grown in VME or SM+N broth containing the appropriate antibiotic at 37°C, overnight, with shaking at 200 rpm.

II. B. 1b. Growing cultures in the bioreactor to establish conditions promoting biological nitrogen fixation by *Azoarcus* strains

Conditions promoting biological nitrogen fixation were established using a bioreactor, BIOSTAT B (Braun Biotech International GmbH, Melsungen, Germany). Cultures used for inoculation were grown overnight in SM+N media and washed two times before inoculating the bioreactor. For washing, cells were pelleted at 3220 *x g* at room temperature for 10 min and then resuspended in 10 ml of SM medium. The bioreactor was filled with SM media or supplemented with 20 mM glutamate or 9.3 mM NH_4Cl when indicated. In this study, the bioreactor was set to maintain a constant temperature of 37°C, an oxygen concentration of 0.6% and a pH of 7.0, which was controlled by the addition of 0.5 M H_3PO_4 by the. Constant stirring was set at 600 rpm. The bioreactor was inoculated with a beginning OD_{578} of 0.009, cells were harvested once an OD_{578} of 0.8 was reached unless otherwise specified. *Azoarcus* sp. strain BH72 reached an OD_{578} of 0.8 after roughly 15 h. Some strains required multiple days to reach that cell density.

II. B. 1c. Growth of batch cultures in microaerobic conditions

To provide *Azoarcus* strains used in this study with microaerobic conditions, the bioreactor was used or conditions were set in 1 L flasks. Flasks were sealed with rubber stoppers and flushed for 30 min with N_2. Air was injected into the sealed flasks so that the O_2 concentration was 1.8% for overnight cultures and 1.2% for 6 h induction assays. These cultures were incubated at 37°C with 200 rpm shaking.

II. B. 1d. Surface Growth on agar plates to assay for stimulation of Hcp expression and secretion

Precultures were grown overnight at 37°C with shaking in VME. Cultures were set to an OD_{578} of 0.5 and 1 mL was pipetted onto a VME agar plate. Five plates were incubated overnight at 37°C upright in a sealed bag with wet paper to keep conditions moist. Cells were scraped off the agar plates, and a minimal amount of liquid media (600–1000 µl) was used to aid in removing all cells from the plate. Cells were pelleted out of this suspension at 4°C for 20 min at 10,400 *x g* before further processing of the supernatant proteins (Section IIB4d) and acquiring the total cell fractions (Section IIB4e).

II. B. 1e. Surface Growth on Erlenmeyer flask glass bottoms to assay for the stimulation of expression and secretion of Hcp

Precultures were grown overnight at 37°C with shaking in VME. Cultures were set to an OD_{578} of 0.5 using VME media, and 2 mL was pipetted onto each bottom of seven Erlenmeyer flasks. Following overnight growth without shaking, the liquid cultures were pooled and cells were pelleted out via centrifugation at 4°C for 20 min at 10,400 *x g*. Total protein was extracted from the pelleted cells as described in Section IIB4e, and supernatant proteins were precipitated as described in Section IIB4d.

II. B. 2. Methods used for construction of plasmids

II. B. 2a. Amplification of DNA fragments using Polymerase Chain Reaction (PCR)

PCR amplification of DNA was performed in 50 µl reactions (60-150 ng of template DNA, 50 µM dNTPs, 2.5 U polymerase, 0.5 µM reverse primer, 0.5 µM forward primer, and the supplied buffer at 1X concentration). Pfu polymerase (ThermoFischer Scientific, St. Leon-Rot, Germany), with 1X PCR reaction buffer (20 mM Tris-HCl (pH 8.8 at 25°C), 10 mM $(NH_4)2SO_4$, 10 M KCl, 1% (v/v) Triton X-100, 0.1 mg/mL BSA) was used for DNA

fragments that would be used for cloning (1% DMSO was also added for these reactions). DreamTaq DNA polymerase (Thermo Scientific, Waltham, MA, USA) was used for colony PCR (Section IIB3g), PCR of cDNA and other sequence analyses. Cycling times and annealing temperature were optimized and varied for each reaction. The standard cycling used was preceded with a 5 min denaturation at 95°C and then 25-35 cycles of 1 min denaturation at 95°C, 1 min annealing at 5°C less than the mean melting temperature of primers used (annealing temperatures used are indicated in Table 4), and a 2 min elongation at 72°C. A final elongation step was done after the cycling for 7 min at 72°C (Primus, MWG-Biotech, Ebersberg, Germany).

II. B. 2b. Ligation of insert and vector DNA using T4 DNA ligase

Five units of T4 DNA ligase (Thermo Fischer Scientific, Waltham, MA) was used for the ligation of insert and vector with a ratio of 2 to 10 molecules of insert DNA to 1 molecule of vector DNA. Ligation reactions were set up in 10 µl reactions with 1X T4 ligase buffer (400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP, pH 7.8) and incubated for 2 h at room temperature or overnight at 15°C. Each ligation was heat deactivated at 65°C for 10 min and iced briefly before proceeding with transformation of *E. coli*.

II. B. 2c. Blunt-End Cloning Protocol

Blunting reactions were set up as needed prior to ligations. These were set up as recommended by the protocol provided with the blunting enzyme from the CloneJET PCR Cloning Kit (Fermentas, ThermoScientific, Waltham, MA, USA). Reactions were set up on ice with 1X Reaction Buffer (as supplied by company), DNA (0.15 pmol ends insert DNA of 0.05 pmol ends of vector DNA) and 1 µl DNA Blunting Enzyme (as supplied by company). This was vortexed briefly and then centrifuged for 3-5 s to collect the entire volume. The reaction was incubated at 70°C for 5 min and then placed on ice. For ligation into the pJET1.2/blunt cloning vector provided in the kit, 0.05 pmol ends of the kit provided vector and 5 U T4 DNA ligase were added to the entire blunting reaction on ice. The ligation mixture was then incubated for 5 min at 22°C before directly transforming *E. coli* DH5α cells.

II. B. 2d. Isolation of plasmid DNA from *E. coli* (Birnboim and Doly, 1979; Birnboim, 1983)

Plasmid harboring *E. coli* was grown overnight in 2.0 ml of LB broth with the appropriate antibiotic at 37°C, rolling in reagent tubes. Cells were pelleted for 2 min at 16,100 *x g* at room temperature and resuspended in 100 µl TE. Cells were lysed for 5 min on ice with the addition of 200 µl of Solution 2 and then neutralized on ice for 5 min with the addition of 150 µl of 3 M K-Acetate (pH 5.2). Cell debris was pelleted via centrifugation at 16,100 *x g* for 10 minutes at room temperature. DNA was precipitated from the supernatant with 500 µl of isopropanol on ice for 10 min. DNA was pelleted for 15 minutes, spinning at 16,100 *x g* at room temperature and then resuspended in 500 µl of 70% ethanol and pelleted again. After drying, DNA was resuspended in 35 µl of TE.

II. B. 2e. Preparing plasmid DNA for sequencing using QIAprep (Qiagen, Hilden, Germany)

Plasmid harboring *E. coli* was grown overnight in 2.0 ml of LB broth containing the appropriate antibiotic at 37°C, rolling in reagent tubes. Cells were pelleted for 2 min at 16,100 *x g* at room temperature and resuspended in 250 µl P1 buffer (provided by the kit, with RNase added) before adding 250 µl of P2 buffer. After mixing by inverting the tube 4-6 times, 350 µl of buffer N3 was added. Cell debris was pelleted out via centrifugation at 16,100 *x g* for 10 minutes at room temperature and the supernatant was applied to a QIAprep spin column. The column was spun for 60 s at 16,100 *x g* and the flow through discarded. The column was washed one time by applying 750 µl of PE buffer. Excess liquid was removed by spinning a second time for 1 min. Plasmid DNA was eluted from the column with 35 µl of nuclease free H_2O.

II. B. 2f. Digestion of DNA using restriction endonucleases

DNA from plasmid preparations were digested in restriction endonuclease reactions. The recommended buffers were added to a 1X final concentration in a 20 µl reaction with approximately 2-3 µg DNA and 5 U of Fermentas (St. Leon-Rot, Germany) and/or New England Biolabs (Ipswich, MA, USA) restriction enzymes for 2 h at 37°C (except digestions with *Sma*I were incubated at room temperature). For chromosomal DNA, 3 µg of DNA was digested with 20 U of enzyme and the recommended commercial 1X buffer, overnight at 37°C (unless otherwise recommended) in 30 µl reactions.

II. B. 2g. Agarose Gel Electrophoresis for separation of DNA fragments

Agarose gels were cast with 0.8-1.2% agarose melted in TAE buffer for size separation of DNA. Higher concentrations of agarose were used for separation of smaller DNA fragments. DNA samples were mixed with DNA loading buffer to a final concentration of 1X. Gels were run from 60-100 V for 1-5 h in TAE buffer. Digested chromosomal DNA was separated by running the gel from 30-60 V for 3-5 h. *Pst*I digested lambda DNA was loaded as a size marker in parallel. After sufficient separation, gels were stained for 20 min in an ethidium bromide solution (0.5 µg/ml). Bands of DNA fragmants were visualized using an Image Master VDS (GE Healthcare, Freiburg, Germany) with UV light (312 nm) or a Typhoon 8600 Variable Mode Imager (GE Healthcare, Freiburg, Germany).

II. B. 2h. Purification of DNA fragments from agarose gel

PCR products and digested DNA fragments were run on agarose gels using electrophoresis to verify size and separate from other DNA fragments. Purification of DNA from agarose gels was performed using one of two kits available.

***i.* GeneClean kit (MP Biomedicals, Santa Ana, CA, USA)**

Ethidium bromide (0.5 µg/ml) stained DNA fragments were visualized with an Image Master VDS (GE Healthcare, Freiburg, Germany) using 312 nm UV light. DNA fragments of the expected sizes were excised from the gel. The gel mass was determined, and 300 µl of the NaI solution provided by the kit (pH 7-7.4) was added for every 100 mg. The gel piece was dissolved in the NaI solution at 55°C before adding 5 µl of glass bead slurry. The suspension was incubated at room temperature for 5 min before pelleting the glass beads for 30 s at 16,100 *x g* at room temperature. The pellet was resuspended in 500 µl of New Wash buffer and pelleted again, twice. Excess liquid was removed by briefly allowing the pellet to dry at room temperature. DNA was eluted with 5-10 µl of nuclease free water for 3 minutes at 52°C. Glass beads were pelleted out by centrifugation at 16,100 *x g* for 30 s at room temperature.

***ii.* NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany)**

Ethidium bromide (0.5 µg/ml) stained DNA fragments were visualized using 312 nm UV light using an Image Master VDS (GE Healthcare, Freiburg, Germany) and excised from the gel. The gel piece mass was determined, and 200 µl of NT buffer was added to every 100 mg.

After dissolving the gel piece at 50°C for 5-10 min, the solution was transferred to a NucleoSpin column with a silica membrane. The column was spun for 1 min at 11,000 *x g* at room temperature. The membrane was washed with 600 µl NT3 buffer and then dried with an additional 2 min spin at 16,100 *x g* at room temperature. DNA was eluted with 35 µl of NE buffer (5 mM Tris/HCl, pH 8.5) pre-warmed to 70°C.

II. B. 2i. Preparing DNA for sequencing by LGC genomics

All sequences were obtained from LGC genomics. Samples were prepared following guidelines given by LGC genomics. Samples contained either 1 µg of plasmid DNA or 100–400 ng of PCR product, depending on the product size, and 20 pmol of primer. Primers used are specified in the results of the construction of each mutant. Primer sequences are listed in Table 4.

II. B. 3. Methods used for construction of mutants

II. B. 3a. Transformation of *E. coli* DH5αF´ and S17-1

Chemically competent strains of *E. coli* were transformed by incubating 50 µl of cells with 5µl of ligation mix on ice for 15 min. Cells were then heat shocked for 2 min at 42°C followed by icing for 1 min before adding 1 ml of room temperature LB broth. Cells were allowed 45 min of outgrowth at 37°C with shaking. On LB plates containing the appropriate antibiotic(s), 200 µl and the concentrated rest of the cells were plated. After overnight growth transformants were picked based on their antibiotic resistance.

II. B. 3b. Transfer of plasmid DNA into *Azoarcus* sp. BH72 through a biparental conjugation

Cultures to be conjugated were grown overnight at 37°C with shaking (200 rpm). Carrying the plasmid to be transferred, *E. coli* S17-1 was grown in 10 ml LB media containing the appropriate antibiotic. *Azoarcus* sp. strain BH72 was grown in 10 ml SM+N media. Cells were pelleted (3220 *x g*, 10 min, RT) and resuspended in LB for *E. coli* and SM for *Azoarcus* so that they each had an OD_{578} of 1. A mix of 250 µl *E. coli* and 750 µl of *Azoarcus* was pelleted and resuspended in 100 µl of SM media. The entire conjugation mix was then spotted on a KON agar plate and sealed shut with parafilm to avoid drying out of the plate

and incubated upright overnight at 37°C. Cells were scraped from the plate with a Drigalsky spatula and resuspended in 600 µl SM medium and pelleted. Pelleted cells were resuspended in 500 µl SM media and from this, 200 µl was spread plated on a VME agar media containing the 60 mg/l Km and 12.5 mg/l Cm. The remaining volume of cells was spread plated on a second plate. After 2-3 d colonies were screened via colony PCR (Section IIB3g) for the mutant genotype, and mutants were confirmed using Southern blot analysis (Section IIB3e).

II. B. 3c. Transfer of plasmid DNA into *Azoarcus* sp. strain BH72 through a triparental conjugation

Cultures to be conjugated were grown overnight at 37°C with shaking (200 rpm). *E. coli* DH5α donor and helper strains were grown in 10 ml LB media containing the appropriate antibiotic, and *Azoarcus* sp. strain BH72 was grown in 10 ml SM+N media. The donor strain contained the *mob* region containing plasmid to be transferred, and the helper strain contained *tra* genes on the plasmid pRK2013 to allow DNA transfer. Cells were pelleted (3220 *x g*, 10min, RT) and resuspended in LB for *E. coli* strains and SM for *Azoarcus* so that they each had an OD_{578} of 1. A mix of 20 µl helper strain, 20 µl donor strain and 1 ml of *Azoarcus* was pelleted and resuspended in 600 µl of SM medium. The entire conjugation mix was then spotted on a KON agar plate and processed the same as in the biparental conjugation (Section IIB3b)

II. B. 3d. Extraction of genomic DNA

Strains of *Azoarcus* were grown in 3 ml of VME media in reagent tubes rolling overnight at 37°C. Cells were pelleted (16,100 *x g*, 2 min, RT) and resuspended in 1 ml of TES and pelleted again. Cells were resuspended in 300 µl of TE before adding 100 µl of 5% (w/v) Laurylsarcosin and 23 µl of 5% (w/v) Pronase E. Cell suspensions were incubated for 1 h at 37°C. Genomic DNA was further disrupted by vigorous vortexing and pipetting up and down at least ten times. An equal volume of phenol:chloroform:isoamylalcohol (24:24:1, pH 7.0-7.5) was added, and the mixture was vortexed for 15 s. After centrifugation (16,110 *x g*, 15 min, RT) the upper aqueous phase was removed and the phenol extraction was performed two more times. A final chloroform extraction was done by adding an equal volume of TE-saturated chloroform, vortexing and centrifuging as done before. Genomic DNA was precipitated by adding of 1/9 volume 3 M Na-acetate (pH 5.2) and 1 volume of isopropanol and incubating on ice for 15 min. DNA was pelleted (16,100 *x g*, 15 min, RT) and then resuspended in 500 µl of 70% (v/v) ethanol before repelleting. The pellet was allowed to dry

before resuspending in 30 µl of TE. DNA concentrations were measured using a NanoDrop UV/Vis spectrophotometer (ThermoScientific, Waltham, MA, USA) before storing at -20°C.

II. B. 3e. Southern blot analysis of genomic DNA (Southern, 1975)

Genomic DNA was digested in a 30 µl reaction (3 ng genomic DNA, 1X restriction endonuclease buffer, 20 units restriction endonuclease) incubated overnight at the temperature recommended by the enzyme (25 or 37°C). Digest DNA were separated on 1% agarose gels at 65-75 V until the size marker dye suggested sufficient migration for size determination of separated DNA fragments. Gels were stained with ethidium bromide solution (500 mg/l) for at least 15 min, and the image was visualized using an Image Master VDS with 312 nm UV light (GE Healthcare, Freiburg, Germany). Distances migrated by DNA fragments in the marker were recorded. Fragmented DNA within the gel was depurinated for 15 min in 0.78% (v/v) HCl and then briefly rinsed in dH_2O. Gels were then incubated in Denaturation Soultion with shaking. After 20 minutes the Denaturation Solution was replaced with fresh solution and the gels were rinsed twice in dH2O. Agarose gels were then incubated in Neutralization Solution two times for 20 min with shaking. DNA from the gel was then transferred to an Amersham Hybond-N membrane (GE Healthcare, Buckinghamshire, England) via capillary action overnight. The transfer was set up so that 20X SSC was absorbed by Whatmann paper (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) under the gel were it passed through another layer of Whatmann paper, the gel, the membrane, another layer of Whatmann paper and then about a 15 cm stack of green paper towel.

DNA transferred to the nylon membrane (Hybond™ N, GE Healthcare, Freiburg, Germany) was UV cross-linked with 0.4 J/cm^2 of energy (FluoLink, Biometra, Göttingen, Germany). The membrane was then blocked with Prehybridization Solution for four hours at 65°C with shaking. The Prehybridization Solution was replaced with a hybridization solution, Prehybridization Solution with DIG-labeled probe (Section IIB3f) that had been boiled for 20 min before adding. Blotted membranes were incubated overnight in the hybridization solution at 65°C with shaking. The hybridized membrane was then rinsed in successively stringent solutions: 2X SSC for 10 min, 2X SSC with 0.1% SDS for 20 min, and finally 0.1X SSC with 0.1% SDS for 10 min. The membrane was then briefly rinsed in DIG-P1 before blocking in DIG-P2 solution (5% (w/v) skim milk powder in DIG-P1). After 30-60 min of blocking, alkaline phosphatase conjugated anti-DIG Fab fragments (Roche, Mannheim,

Germany) were added (1:25,000) and the membrane was incubated for 30 min with shaking at room temperature. The membrane was then washed by shaking in DIG-P1 and replacing the DIG-P1 wash solution every 10 min, three times. The membrane was then incubated in DIG-P3 (0.1 M Tris; 0.1 M NaCl; 0.05 M $MgCl_2$ x 6 H_2O; pH 9.5) for 3 minutes before adding CDP-star Chemiluminescent Substrate (Sigma, Aldrich, St. Louis, MO, USA) (1:500). After 3 min incubation, excess substrate was wiped away and bands were detected using the FUJI imager LAS-3000 mini (FUJI Photo Film Co, Tokyo, Japan).

II. B. 3f. Labeling of probes used for Southern blot analysis with Digoxigenin-11-dUTP (DIG labeling)

The hybridization solution used for Southern blot analysis contained DIG labeled probes. Probes were prepared using DIG DNA 10X Labeling Mix (Roche, Mannheim, Germany), which includes DIG-dUTP for incorporation into newly synthesized DNA during PCR amplification. PCR reactions were set up in 50 µl reactions (1 µl plasmid or PCR amplified DNA template (60-150 ng); 10 µM dNTPs; 0.6X DIG DNA Labeling Mix; 2.5 U DreamTaq DNA polymerase; 0.5 µM reverse primer; 0.5 µM forward primer; and the DreamTaq buffer at a final 1X concentration). The cycling conditions used were optimized for the primer pair used (Section IIB2a). To control that the oligo had been labeled, a shift in DNA fragment size was seen using agarose gel electrophoresis (Section IIB2g). Additionally, dilutions of the probe were spotted and cross linked onto a membrane to determine the amount of probe necessary for detection using anti-DIG Fab fragments and CDP-star Chemiluminescent Substrate (Section IIB3e).

II. B. 3g. Preparation of DNA for colony PCR

Using a sterile toothpick one bacterial colony was picked up and resuspended in Colony Lysis Buffer. This cell suspension was incubated at 95°C for 10 min and then centrifuged for 2 min at 16,100 *x g* at room temperature. The supernatant was stored at -20°C until use for PCR. For PCR reactions, 1 µl of plasmid DNA was used and 5 µl of chromosomal DNA was used (Section IIB2a).

II. B. 3h. Isolation of total RNA from bacteria using the hot phenol method (Bielefeld Method)

Cells were grown under given growth conditions (e.g. promoting nitrogen fixation) to the desired OD_{578}. Cells were pelleted out of the media for 15 min at 3220 *x g* at 4°C. Cells were stored at -80°C until further use, or RNA was extracted immediately. Cells were resuspended

in a 1:1 mix of phenol:chloroform: isoamylacohol (25:24:1, pH 5) and NAES prewarmed to 65°C and incubated at 65°C for 5 min. Suspensions were then placed on ice for 5-10 minutes before centrifugation at 10,000 *x g* for 15 min at 12°C. The upper aqueous phase was removed, and phenol extractions were repeated by adding an equal volume of phenol:chloroform:isoamylacohol (25:24:1, pH 5). Mixtures were vortexed for 30 sec before centrifugation (15 min at 3220 *x g* at 4°C). Phenol extractions were repeated until all visible protein was removed. A final chloroform extraction was performed with 1 volume of chloroform:isoamylalcohol (24:1) and using the same vortexing an cenrifugation steps used above. One volume of isopropanol was added to precipitate the RNA for 30-60 min on ice. RNA was pelleted for 5 min at 10-12,000 *x g* at 4°C and resuspended in one volume of 70% ethanol prepared using DEPC water and then pelleted for 5 min at 10-12,000 *x g* at 4°C. The pellet was dried at 65°C and dissolved into 1X RNAsecure™ water heat deactivated at 65°C for 5 min (Life Technologies (Ambion), Carlsbad, CA). The RNA concentration was measured using a NanoDrop UV/Vis spectrophotometer (ThermoScientific, Waltham, MA, USA).

II. B. 3i. DNase I recombinant enzyme treatment of total RNA extracted from bacteria (Roche, Mannheim, Germany)

Each reaction mixture contained a final volume of 200 µl with 1X DNase buffer (40 mM Tris-HCl, 10 mM NaCl, 6 mM $MgCl_2$, 1 mM $CaCl_2$; pH 7.9), 8 µg of RNA, 1X RNAsecure (Ambion, Life Technologies, Carlsbad, CA). This mixture was incubated at 60°C for 10 min and then cooled on ice for 5 min before adding 40 U of SUPERase (Ambion, Life Technologies, Carlsbad, CA) and 80 U of DNase I recombinant enzyme (Roche, Mannheim, Germany). This mixture was incubated at 37°C for 30 min before performing a phenol extraction with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1, pH 4). After vigorous mixing, this was centrifuged at 16,110 *x g* for 10 min at room temperature. The upper phase was removed and the RNA precipitated overnight at -80°C with the addition of Na-acetate (pH 5.2) and three volumes of ethanol. The next day the RNA was pelleted (16,110 *x g*, 4°C, 15 min). The pellet was resuspended in 70% ethanol and pelleted again. The pellet was air dried and then resuspended in 20-40 µl 1X heat deactivated RNAsecure. The concentration was measured using a NanoDrop UV/Vis spectrophotometer (ThermoScientific, Waltham, MA, USA). RNA was stored at -80°C.

II. B. 3j. Generartion of cDNA by reverse transcription with RNA dependent DNA polymerase Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) for PCR amplification

First-strand cDNA synthesis was done following the protocol provided with the enzyme M-MLV (Promega, Madison, WI). An initial annealing step was done by adding 2 pmol of a gene specific primer to 1 ng-1 µg of RNA. This mixture was heated to 70°C for 5 min before quickly cooling on ice. The following components were then added in this order 1X M-MLV reaction buffer (50mM Tris-HCl (pH 8.3 @ 25°C), 75mM KCl, 3mM $MgCl_2$ and 10mM DTT), 1.5 mM dNTP mix, 50 U M-MLV room temperature and nuclease free water to a final volume of 25 µl. The reaction was mixed well and then incubated at 47.5°C for 1 h. The reaction was inactivated at 70°C for 15 min. Directly from this reaction, 5-10 µl was used for PCR reactions (Section IIB2a).

II. B. 4. Methods used for protein expression and purification.

II. B. 4a. Overexpression of strep-tagged recombinant proteins by *E. coli.*

Proteins were overexpressed and purified using the pASK-IBA3 expression vector (IBA, Göttingen, Germany), which contains an 8 amino acid strep-tag. *E. coli* DH5αF' was transformed with the specified plasmid DNA (Table 3). A 2 ml preculture was grown overnight in LB media with 150 µg/ml ampicillin at 37°C. Main cultures of 100 ml LB media with 150 µg/ml ampicillin were inoculated to an OD_{578} of 0.1 and grown at 37°C with 200 rpm shaking. Expression was induced by adding 0.02 ng/ml anhydroustetracycline after the main cultures reached an OD_{578} of 0.5. At 3 h post induction cells were harvested (4500 *x g*, 12 min, 4°C). Cells were either stored at -20°C or further processed to obtain clear lysates.

II. B. 4b. Preparation of cleared lysates

Cell pellets containing overexpressed protein were resuspended in chilled Buffer W. Cell suspensions were sonicated multiple times with 50 watt outputs for 30 s, with 30 s of icing between each sonication, until cleared lysates were obtained. Cellular debris was pelleted out via centrifugation (16,110 *x g*, 15 min, 4°C).

II. B. 4c. Purification of recombinant proteins containing a strep tag using Strep-Tactin sepharose columns (IBA, Göttingen, Germany)

Strep-tagged proteins were purified using Strep-Tactin sepharose columns (IBA, Göttingen, Germany). All steps were performed at 4°C. A column bed volume (CV) of 500 µl was established by loading 1 ml of the 50% sepharose slurry into a 1 ml polypropylene column (Qiagen, Hilden, Germany) and allowing the liquid to flow through. The column was equilibrated with 2 CV (1 ml) of BufferW before loading the cleared lysates and collecting the flow through fraction. The column was washed five times by running 2 CV of Buffer W thought the column. Each of the wash fractions were collected before eluting the column bound proteins in four fractions of 1 CV with Buffer E. Purified proteins were stored at -20°C until used. Protein content was verified using SDS-PAGE gels and Western blot analysis.

II. B. 4d. Isolation of culture supernatant proteins

Cultures of strains of interest were grown in the designated media to a desired OD_{578}. Cells were pelleted by centrifuging for 20 min at 4°C at 10,400 *x* g using a GSA rotor (Sorvall, Waltham MA, USA). A second centrifugation of the supernatant with the same conditions was performed to be sure all cells were removed. Proteins from the supernatant were then precipitated overnight at 4°C with the addition of trichloroacetic acid to a final concentration of 10% (v/v). Precipitated proteins were pelleted via centrifugation at 4°C for 1 h at 10,400 *x* g. Pellets were resuspended in 70% ethanol and then pelleted by centrifuging at 16,110 *x g* for 7.5 min at room temperature. Protein pellets were then washed three times by resuspending protein pellets in 70% ethanol centrifuging at 16,110 *x g* for 7.5 min at room temperature. A final wash step was performed in 100% ethanol. The pellets were briefly dried at room temperature and then resuspended in Protein Suspension Buffer$^{(+)}$. Supernatnat proteins were stored at -20°C. Samples of supernatant proteins to be analyzed using LC-MS/MS were resuspended in a solution of 8 M urea and 2 M thiourea.

II. B. 4e. Extraction of SDS-soluble proteins (total cell protein extracts)

Cells were pelleted from cultures grown to the desired OD_{578} in the appropriate media. A 150 mg fresh weight cell pellet was transferred into a 1.5 mL cup and resuspended in 1 ml SDS-extraction buffer before addition of 100 µl 20% SDS. Cell suspensions were incubated for 15 min at 95°C and then cooled on ice. Cellular debris was removed via centrifugation for 5 min at 16,100 *x g* at room temperature. The supernatant was retained and stored at -20°C until further use.

II. B. 4f. Preparation of flagella from extracellular fractions (Buschart et al., 2012)

Cultures were grown overnight at 37°C with shaking in VME. Cultures were set to an OD_{578} of 0.5, and 1 mL was pipetted onto a VME agar plate. Plates were incubated overnight at 37°C upright in a sealed bag with wet paper to keep conditions moist. Cells were removed by scraping them from the plates with a Drigalsky spatula and using a minimal amount of 50 mM Tris HCl (pH 8.0) to resuspend the cells. Cell suspensions were vortexed vigorously for 2 min and then passed through a needle (0.6 mm x 25 mm) three times. Cellular debris was removed from the flagella filaments by centrifugation at 3220 *x g* at 4°C for 10 min. This separation was then repeated with the supernatant. Flagella filaments were pelleted via ultracentrifugation at 67,400 *x g* for 30 min at 4°C in a Beckman T145 rotor (Beckman Coulter, Pasadena, California, USA). Pellets were resuspended in 10 mM Tris HCl (pH 7.2) and briefly spun for 1 min at 16,100 x g. Crude flagella extracts were stored at -20°C until further use.

II. B. 4g. Bradford Assay for Protein Quantification (Bradford, 1976).

Bradford Assays were performed to determine the protein concentration. From protein samples, 5 µL was added to 795 µl PBS and 200 µL Roti® Quant (Carl-Roth, Karlsruhe, Germany) was added to this. Solutions were mixed well and incubated for 5 min in the dark. The absorption at 595 nm wavelength was determined. The blank used was a mixture of 800 µl PBS and 200 µl Roti® Quant. For use as a standard, preparations of BSA (2, 4, 6, 8, 10, 20 µg/ml) were also prepared in 800 µl TBS. Protein standards were treated in parallel to protein samples with the addition of Roti® Quant and absorption measured. A linear equation was determined from the protein standards and used for calculating the concentration of proteins in the samples.

II. B. 5. Protein detection using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

II. B. 5a. Preparation of SDS-PAGE gels

SDS-PAGE gels were prepared by first pouring a 12% polyacrylamide separating gel and allowing it to polymerize between glass plates sealed along each side and bottom. A layer of isopropanol was applied across the top to minimize the presence of oxygen. After polymerization of the 12% gel for 30 min or more, a 5% polyacrylamide stacking gel was

poured and a comb inserted for formation of loading wells. The stacking gel was allowed to polymerize for 30 min or more before loading protein samples. Protein samples were prepared for loading by adding a 4X loading buffer to a final concentration of 1X. Samples were then boiled for 5-10 min at 95°C before loading the SDS-PAGE gels. A commercially prepared molecular weight marker, PageRuler™ Prestained Protein Ladder, was also loaded (Fermentas, ThermoFisher, Rockford, IL, USA). Gels were run at 100-130V for 1-2 h.

Table 5. Composition SDS-PAGE gels

	12.5% Separation gel	5% Stacking gel
Tris (pH 8.8)	375 mM	---
Tris (pH 6.8)	---	125 mM
SDS	0.1% (w/v)	0.1% (w/v)
Acryl/bisacrylamide (24:1)	12.5% (v/v)	5% (v/v)
Ammonium persulfate	0.3% (w/v)	0.3% (w/v)
Tetramethylethylenediamine	0.2% (v/v)	0.3% (v/v)

II. B. 5b. Using two-dimensional (2D) gels for protein separation

Isoelectric focusing gels were polymerized in 8.5 cm long capillary tubes with a 1.3 cm diameter (13.5 mM Urea; 5.7% Acrylamide/Bisacrylamide (24:1); 3% (w/v) CHAPS; 12% (v/v) Bio-Lyte 3/10 Ampholyte (BioRad, München, Germany); 3% (v/v) Bio-Lyte 4/6 Ampholyte; 3% Bio-Lyte 5/8 Ampholyte; 0.1% (w/v) Ammonium persulfate; 0.1% (v/v) Tetramethylethylenediamine). Samples prepared in 2D-Gel Sample Buffer were loaded. Samples were overlayed with 20-40 µl Overlay Buffer (6 M urea; 1% (v/v) Bio-Lyte 5/8 Ampholyte; 100 mM DTT; 2% (w/v) CHAPS), and any area remaining at the top of tube was filled with Cathode Buffer (0.02 mM NaOH). The upper chamber was filled with Cathode Buffer and the lower chamber with Anode Buffer (0.01 M H_3PO_4). The gels were run at 500 V for 0.16 h and then 750 V for 3.5 h. Gel tubes were stored at 20°C until second dimension gels were run. First dimension gels were ejected between the glass plates and laid across the top of the second dimension gel (prepared as described previously for a 12.5% (w/v) polyacrylamide separating gel) The first dimension gel was covered with Equilibration Buffer

for 10-20 min. The Equilibration Buffer was then removed, and the first dimension gel was pushed flush against the SDS-PAGE gel and run as described previously (Section IIB5a). After incubating the gels in water with shaking for 5 min, the second dimension gels were further Western blotted (Section IIBb) or stained with Colloidal Coomassie Brialliant Blue (Section IIB5c) or Silver Stain (Section IIB5d).

II. B. 5c. Protein detection via Western blot analysis

SDS-PAGE gels with separated protein bands were incubated in water with shaking for one minute at room temperature before incubating with shaking in Transfer Buffer for 5 min at room temperature. Proteins were next blotted onto a Nitrocellulose membrane (Whatmann, GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) for 45 min at 8 V using a Trans-Blot SD Semi-Dry Transfer Cell (BioRad, München, Germany). Alternatively, when indicated, proteins were transferred to a PVDF membrane (polyvinylidene fluoride) that was first dipped in methanol before setting up the semi-dry transfer. For the protein transfer, the gel and membrane were sandwiched between two pieces of Watmann paper saturated with Transfer Buffer. Membranes were incubated with agitation for one hour in 5% (w/v) milk powder in PBS before incubating overnight at 4°C with shaking in an antibody solution comprised of 1% (w/v) BSA in TBS and the specified antibody. Anti-Hcp contained a mixture of each anti-1305, anti-3897 and anti-3898 with a 1:3000 dilution of each of the anti-Hcp antibodies (Öhrlein, Johannes, 2007). Anti-NifH was prepared as a 1:5000 dilution. Anti-GlnB was prepared as a 1:1000 dilution. After washing the membranes three times for 10 min in TBS, membranes were incubated with shaking at 4°C for 2-4 h in a secondary antibody solution comprised of 1% BSA prepared in TBS and a 1:10,000 dilution of swine anti-Rb HRP conjugated antibody (Dako, Agilent Technologies, Eching, Germany). Membranes were washed three times. In each wash step membranes were incubated in TBS with shaking for 10 min. A mixture of equal volumes of Luminol/Enhancer and Stable Peroxide Buffer from the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher, Rockford, IL) was added to the membrane and incubated for 1 minute. Excess liquid was removed, and protein bands were detected using a LAS-3000 imager (FUJI, Tokyo, Japan). To detect weaker signals, Lumi-Light^{PLUS} western blotting substrate (Roche, Rockford, IL, USA) was used. Equal volumes of Luminol/Enhancer and Stable Peroxide

Buffer were added to the membrane. After a 1 min incubation, protein bands were detected using the LAS-3000 imager (FUJI, Tokyo, Japan).

II. B. 5d. Colloidal Coomassie Brilliant Blue staining of SDS-PAGE resolved proteins.

One- and two-dimensional SDS-PAGE gels (Section IIB5a-b) were incubated overnight in Colloidal Coomassie Blue Stain with shaking at room temperature. Gels with a high degree of background staining were destained by incubating in water or a 50% ethanol solution until distinct bands appeared.

II. B. 5e. Silver Staining of SDS-PAGE resolved proteins using PageSilver Silver Staining Kit (Fermentas, ThermoScientific, Waltham, MA, USA)

For silver staining of proteins in SDS-PAGE gels, PageSilver Silver Staining Kit (Fermentas, ThermoScientific, Waltham, MA, USA) was used. The staining protocol provided by the kit was used. Proteins were fixed in the gels with a solution of 50% (v/v) ethanol and 10% (v/v) Glacial Acetic Acid overnight. Following the overnight incubation, proteins were fixed in the gel with a 30% (v/v) ethanol solution. The 30% ethanol solution was replaced with fresh solution after 20 min and again after another 20 min. Gels were briefly rinsed twice for 20 s in 100 ml of deionized water (dH_2O) and then incubated for 1 min in 100 ml of Sensitizing Solution (0.4% sensitizing concentrate prepared in dH_2O). After rinsing twice for 20 s in 100 ml of dH_2O, the gels were stained in 100 ml Staining Solution (4 ml Staining Reagent; 54 µl Formaldehyde; prepared in dH_2O) for 20 min and then rinsed again twice for 20 s in dH_2O. The Developing Solution (10 µl sensitizing concentrate; 10 ml developing reagent; 27 µl Formaldehyde; prepared in 100 ml of dH_2O) was then added until bands appeared. To stop the developing reaction, the Developing Solution was replaced by 100 ml of Stop Solution (8% (v/v) Stop Reagent; prepared in dH_2O). Gels were then stored in plastic foil kept moist with water until images were scanned or bands of interest excised for in gel trypsin digestion.

II. B. 6. Methods used for matrix-assisted laser desorption/ionization (MALDI) analysis

II. B. 6a. Digestion of in Gel proteins with trypsin (Shevchenko, 1996)

Protein containing gel pieces were excised from SDS-PAGE gels and diced into 1 mm x 1 mm cubes. Gel pieces were incubated in a 1:1 mixture of 100 mM NH_4NCO_3 and acetonitrile (ACN) for 15 min at room temperature, the solution was removed and the gel pieces were again incubated in a 1:1 mixture (NH_4NCO_3:ACN) for 15 min at room temperature. Gel pieces were then incubated in ACN until gel pieces became white and sticky. The ACN was removed and gel pieces were then rehydrated by adding 50 mM NH_4HCO_3 for 5 min before adding an equal volume of ACN. This mixture was removed, and gel pieces were dried with the addition ACN. The ACN was removed, and pieces were thoroughly dried by using a Concentrator 5301 (Eppendorf, Hamburg, Germany) for 5 min at room temperature. To reduce the in gel proteins, gel pieces were rehydrated in 10 mM DTT and incubated at 56°C for 60 min. Gel pieces were then cooled to room temperature, and the DTT was replaced with 55 mM Iodoacetamide and incubated at room temperature for 45 min in the dark for alkylation of the in gel proteins. All of the wash steps preceding reduction and alkylation were then repeated before drying the gel pieces in an Eppendorf Concentrator 5301 (Hamburg, Germany). Sequencing-grade modified Trypsin (Roche, Mannheim, Germany) was prepared in a 1 mM HCl solution (100 ng/µl). Gel pieces were then rehydrated with a minimal volume of trypsin (5 ng/µl in 25 mM NH_4HCO_3) and incubated overnight at 37°C.

A volume of ACN equal to the volume of trypsin solution was added to the gel pieces. The pieces were then incubated for 10 min in a Transonic T420 (Elma GmbH, Singen, Germany) sonicating water bath at room temperature to extract peptides from the gel pieces. The peptide containing supernatant was removed, and 100 µl of 30% (v/v) ACN and 0.1% (v/v) TFA was added to the gel pieces. After sonicating for 15 min at room temperature, this supernatant was pooled with the first extraction. A final extraction was done by sonicating the samples in 50% (v/v) ACN and 0.1% (v/v) TFA for 15 min. The pooled supernatants were concentrated using an Eppendorf Concentrator 5301 (Hamburg, Germany) at 240 *x g* at 30°C until samples were dry. These were sealed to avoid any moisture and stored at -20°C until further processing.

II. B. 6b. Matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS) analysis of peptides from trypsin digested proteins

The concentrated peptides were resuspended in 0.1% (v/v) TFA. Two different matrices were used: α-cyano-4-hydroxycinnamic acid (CHCA) saturated in 50% (v/v) ACN and 2,5-dihydroxybenzoic acid (DHB) suspended in 70% (v/v) ACN and 0.3% (v/v) TFA. Peptide and matrix were mixed 1:1 and 1:10. One microliter of these dilutions were spotted on a 96 × 2-position, hydrophobic plastic surface plate (Applied Biosystems, Foster City, CA, USA) and dried. A Voyager DE-Pro MALDI-TOF mass spectrometer (Applied Biosystems, Darmstadt, Germany) was used. The settings used were with the accelerating voltage at 20,000 V, the guide wire at 0.01% of accelerating voltage, the grid at 70% and a delay time of 150 nsec. The laser intensity ranged from 8000-15,000 V. Most spectra were accumulated over 200 shots. Peptides were identified using Mascot Wizard (Matrix Science, Boston, MA), or peaks were manually picked using Data Explorer (Applied Biosystems, Foster City, CA).

II. B. 6c. LC-MS/MS (Liquid chromatography-tandem mass spectrometry)

Supernatant protein samples were obtained from strains grown under nitrogen fixing conditions in the bioreactor (Section IIB1b) and isolated as described above (Section IIB4d). These samples were then sent to the Functional Genome Research Group directed by Prof. Dr. Uwe Völker at the Ernst Moritz Arndt University of Greifswald for LC-MS/MS analysis by PhD student Praveen Kumar. The student there prepared and analyzed samples containing 5 µg of protein. Proteins for analysis were prepared by first reducing and alkylating them followed by a 16 h trypsin digest. Peptides were desalted and purified using C18 columns. Peptides were reconstituted in a solution of 2% (v/v) ACN and 0.1% (v/v) Acetic Acid. For MS analysis, 400 ng of peptides were injected to online Proxeon-LC coupled with Thermo LTQ Velos-Orbitrap instrument (Thermo Fischer Scientific, Waltham, MA). An 80 min LC gradient was used. The MS parameters were in collision-induced dissociation (CID) mode. Thermo raw files were converted to universal MzXml files, and these were searched against NCBI forward and reverse database of *Azoarcus* using a Sequest search engine. These results were returned to this lab as Scaffold files.

II. B. 7. Determination of β-glucoronidase activity of strains with *gusA* fusions (Jefferson et al., 1987)

Precultures of *Azoarcus* strains with *gusA* fusions in the genome were grown in 20 ml of SM+N media containing the appropriate antibiotic aerobically, overnight at 37°C with

shaking at 200 rpm. Cells were then washed (pelleted by centrifugation and resuspended in 10 ml of SM media) two times before inoculating the main cultures, 20 ml of media with kanamycin in 1 L flasks containing 1.2% O_2. SM media was inoculated so that the starting OD_{578} was 0.3 and SM+N and SM+Glu media were inoculated with 0.15 as a starting OD_{578}. Cells were incubated for 6 h at 37°C with shaking at 200 rpm.

After 6 h, 2 ml of the main culture was pelleted for 20 min at room temperature at 16,100 *x g*. Cell pellets were resuspended in 2 ml GUS buffer. Half of the cell suspension was used for measuring the OD_{600}; the other half was used for assaying GUS activity. Fifty microliters of the GUS Assay Lysis Solution was added to 500 µl of the cell suspension (done in duplicate) and vortexed well. Cells were lysed for 10 min at 37°C, and then 100 µl of substrate was added (4 mg p-nitrophenyl-β-D-Glucoronide/1 ml GUS Buffer). Cell lysates were incubated further at 37°C until the solution of cellular contents turned yellowish. The color change occurred after 5-15 min and was stopped by the addition of 240 µl Stop Solution (2.5 M 2-amino-2methyl 1,3 propandiol). Cell debris was removed with a 2 min spin at room temperature (16,100 *x g*). The extinction was measured at 420 nm. Miller Units (U) were determined as $U = 1000(E_{420}/(t_{min} \bullet Vol \bullet OD_{600}))$

II. B. 8. Plant infection and colonization experiments

II. B. 8a. Infection of Asian rice cultivar, *Oryza sativa* IR-36 seedlings

Oryza sativa cv. IR-36 caryopses were husked and sterilized in Plant Sterilization Solution for 45 min with continuous shaking at room temperature. Grains were then washed 3 times. For each washing step, fresh sterile dH_2O was added to the grains and flasks were left shaking for 1 h or more at room temperature. The rice grains were then shaken in fresh sterile dH_2O overnight before placing on germination plates (maximum 10 grains/plate). Plates were sealed with perforated parafilm and incubated in the dark at 37°C. After 3 d, the germinating seedlings were transferred to a phytotron (30°C, 80% humidity, 15 kLux light intensity, 14 h day cycle) for one day of growth. In sterile glass reagent tubes, seedlings were planted in sterile quartz and inoculated with bacteria. Bacterial strains used for inoculation were grown in SM+N media shaking at 200 rpm at 30°C until they reached an OD_{578} of 1.0-1.2. Cells were then pelleted at 3220 *x g* in a swing bucket rotor at room temperature for 10 min and resuspended with Plant Medium. Cells were again pelleted and resuspended. A master mix of

inoculum was prepared by setting the culture to OD_{578} = 1.0 and adding 100 µl of this to every 3 ml of Plant Medium used for planting the seedlings. An additional 100 µl of bacterial suspension with an OD_{578} = 1.0 was added to the planted seedling. The reagent tubes were stopped with sterile paper plugs and placed in a black ink water bath in the phytotron (30°C, 80% humidity, 15 kLux light intensity, 14 h day cycle) for 7 d. After 7 d the seedlings were watered with sterile dH_2O and incubated for an additional 7 d.

II. B. 8b. Rice Seedling CFU Counts of Endophytic Bacteria (Böhm et al. 2007)

Seedlings were loosened from the quartz in their reagent tubes with vortexing and then vigorously dipped in sterile dH_2O twice to remove quartz and some bacteria adhering to the rice roots. In a 250 ml beaker of sterile dH_2O, seedlings were ultrasonicated in a Transonic T420 water bath (Elma GmbH, Singen, Germany) for 15 min to further remove bacteria adhering to the root surface. Roots were dried by blotting dry on sterile filter paper. All root material was removed and the fresh weight was recorded. The root mass was then ground with a sterile mortar and pestle in Plant Wash Solution and some quartz. Serial dilutions of the homogenized roots were pour plated in semi-semi-solid SM medium. Root suspensions were also streaked on a VME plate to control for any microorganisms contaminating the plants. Plates were incubated for 3 d at 37°C and then colony forming units (CFU) were counted on countable plates (15-150 CFU). The CFU/mg fresh root weight was then determined, and an average was calculated for each plant that had at least two countable plates. Three independent trials (infection of plants on different days from different precultures) inoculating rice seedlings with mutant strains were compared to three independent trials inoculating seedlings with the wild-type strain. A p-value was determined using a two-tailed paired t-test

II. B. 8c. Assaying for ethylene production by rice cell cultures (Thuerig et al., 2006)

Ethylene production by rice cell culture suspensions when exposed to proteins and buffer solutions was measured. Rice cell cultures OC156 were maintained at 23ºC with non-stop shaking under continuous light for seven or eight days. Each assay was performed with five replicas: 500 µl of cell culture suspension was pipetted into 4 ml reagent tubes and elicitor was added to this. An amount of 5 ng, 500 ng or 5 µg of an overexpressed Hcp protein isolated from *E. coli* and purified using Strep-Tactin sepharose columns (Section IIB4a-c) was added. The volume added never exceeded 5 µl. As positive controls Chitin (5 ng) and Pen1 (5 ng and 5 µg) were added. As a negative control ethylene production by cells alone,

without addition of an elicitor or buffer, was measured. Ethylene production by cells exposed to the protein suspension solutions used was also measured as a negative control. Cells were incubated with shaking at 200 rpm at 23°C. The amount of ethylene produced (nmoles) after 4 h was measured by injecting 200 µl of the headspace from the sealed reagent tubes containing the cell cultures into the Chromatograph HRGC-4000A (Konic, Barcelona, Spain). Hydrogen was the carrier gas and a flame ionization detector was used. The nmole of ethylene produced was calculated by dividing the product of the peak area of total gas measured (GP) and the total gas volume (GV) by the product of the peak area of calibration gas measured (CGP) and volume of calibration gas (CGV), this quotient multiplied by 0.44, a factor determined by the given vpm (volumes per million) and volume of the calibration gas used.

$$\text{Ethylene production (nmole)} = [(GP \bullet GV)/(CGP \bullet CGV)] \times 0.44$$

Five replicas were measured for each elicitor and each control tested, in each trial. The middle three values were taken as an average. Three trials were done with eight day old cell cultures and three trials were done with seven day old cell cultures and averaged.

III. Results

In literature, other T6SSs have been seen to promote or inhibit bacterial interactions with a eukaryotic host. T6SS components of *Azoarcus* sp. strain BH72 were found to be similar to well-studied T6SSs used by other organisms. It was proposed that one or both of the T6SS(s) of *Azoarcus* sp. strain BH72 was necessary for the establishment of an endophytic lifestyle in rice. The hallmark of a functioning T6SS is the presence of Hcp in culture supernatants. In this study, the detection of Hcp proteins in the supernatant using Western blot analysis was used to assess the functionality of the system under different growth conditions and after mutation to core components.

Although secretion of Hcp is the Hallmark of T6S, it is not believed to be the secreted protein. Identification of the secreted T6SS effector protein was attempted using LC-MS/MS analysis (performed in the Laboratory of Uwe Völker at the Ernst-Moritz-Arndt-Universität Greifswald). However, the effector protein(s) could not be deciphered.

Before identification of a secreted effector protein, the impact of type VI secretion on rice was still assessed. The response of rice cell suspension cultures to purified Hcp proteins was monitored, and rice seedlings inoculated with T6SS deficient strains were compared to those inoculated with wild type strain BH72. The plant response was observed, and the colonization efficiencies of the bacteria were also determined.

Finally, a screening method was developed to search for a T6SS transcriptional regulator. Because growth under nitrogen fixing conditions had already been seen to up-regulate expression of *imp* cluster genes (T6SS-1), conditions seen at the rhizosphere, primary focus was given to regulation of that secretion system.

III. A. Bioinformatic Analysis of T6SS gene clusters encoded by the genome of *Azoarcus* sp. strain BH72 indicated that both systems share several features with T6SSs of other organisms.

A selective bioinformatic analysis of *Azoarcus* sp. strain BH72 had been performed prior to the experimental work in this thesis. A 15.7 kb *sci*-like gene cluster (similar in sequence to a T6SS cluster of ***S****almonella enterica* **c**entisome 7 genomic **i**sland 7), or T6SS-2, comprised of 13 genes and a 17.4-kb *imp*-like cluster (similar to a T6SS cluster in *Rhizobium leguminosarum,* so named for its **imp**aired nodulation), or T6SS-1, with 14 genes were identified (Figure 3) (Julia Herglotz, Diplomarbeit, 2007; Blondel et al., 2009). The GC

content of T6SS-2 (69.22%) and T6SS-1 (67.58%) were found to have only minor deviations from the average *Azoarcus* sp. strain BH72 genome content (67.92%) (Julia Herglotz, Diplomarbeit, 2007). Boyer et al. looked at the relationship between phylogeny and T6SS gene content in a genome-wide *in silico* analysis. Based primarily on presence of conserved accessory proteins (in addition to core components), they divided the T6SSs into 5 sub-groups (I-V). The T6SS-2 was categorized as belonging to sub-group III and the T6SS-1 to sub-group IV (closest to *Chromobacterium violaceum)* (Boyer et al., 2009). In Figure 3, genes identified in the study by a former student and additional genes since then determined to be involved in T6S are illustrated (Julia Herglotz, Diplomarbeit, 2007). Since the earlier study, a better understanding of proteins involved and their functions has been determined along with a number of genes near T6SS-2 gene cluster that have since been recognized as T6SS associated genes. The harmonized nomenclature proposed by Shalom et al. was used, with core components named Tss (**t**ype **s**ix **s**ecreted), and accessory proteins named Tag (**T**ss-**a**ssociated **g**enes) (Aschtgen et al., 2010; Shalom et al., 2007).

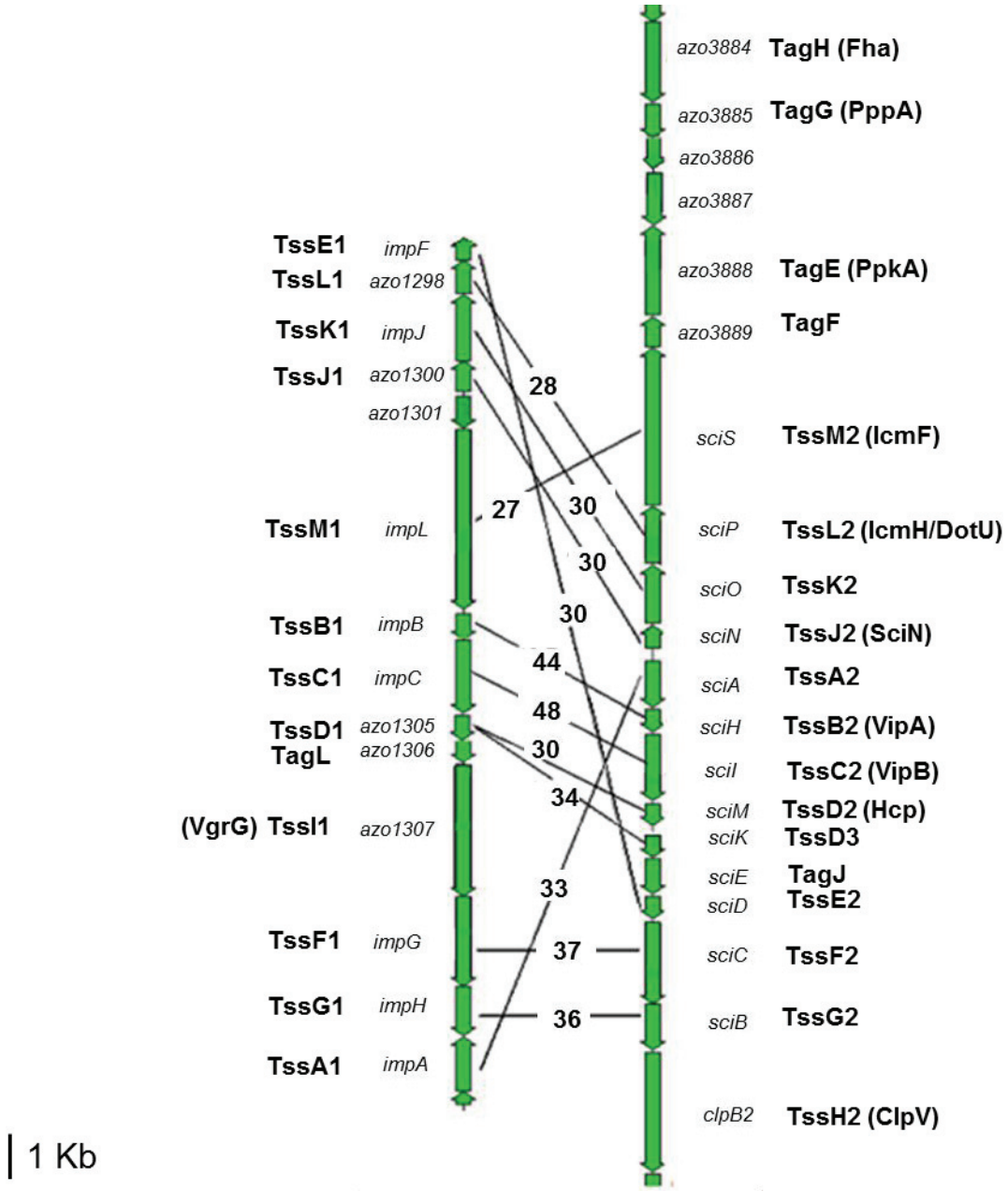


Figure 3. **T6SS Gene Clusters Identified in the genome of *Azoarcus* sp. strain BH72.** *In silico* analysis of the *Azoarcus* sp. strain BH72 genome revealed the presence of two type VI secretion system gene clusters, T6SS-1 (formerly the *imp* cluster) and T6SS-2 (formerly the *sci* cluster). Percent protein sequence identity between similar genes is indicated (revised figure from Julia Herglotz, Diplomarbeit, 2007). Gene names are written in italics nearest the gene cluster schematic, and proteins names using the harmonized nomenclature are written in bold. Components encoded by T6SS-1 (*imp* cluster) are designated by following the protein name with "1", and T6SS-2 (*sci* cluster) enocoded proteins are followed by a "2". A 1 kb scale bar is at the lower left corner of the figure.

TssM. TssM1 and TssM2 homologs (previously named ImpL and SciS) were found within the *Azoarcus* sp. strain BH72 genome. A short, less than 9 or 11 amino acids, N-terminus (probably cytoplasmic) followed by two 22 residue transmembrane segment with an 11 or

5 amino acid loop (likely periplasmic) are encoded by *tssM2* and *tssM1*, respectively. Between the second and third 22 amino acid transmembrane segments, there was a 394 (TssM2) or 365 (TssM1) amino acid region containing a Walker A motif at residue 146 and 120, respectively. The 711 or 782 residues at the C-terminal end would then likely reside in the periplasm. Predictions concerning the IcmF-like proteins encoded by both T6SS gene clusters were in line with typical TssM structure (Cascales and Cambillau, 2012).

TssL and Azo1306. In T6SS-2 gene cluster, *tssL2* and *tssM2* were found consecutively (*azo3891* and *azo3890*), and in the T6SS-1 gene cluster, *tssL1* and *tssM1* were in opposing ORFs (*azo1298* and *azo1302*). In the *Azoarcus* sp. strain BH72 genome, *azo3891* (TssL2, previously named SciP) was predicted to encode 178 amino acids after a transmembrane segment at the C-terminal end with an OmpA/MotB-like peptidoglycan binding domain. At the N-terminal side, 233 amino acids were encoded before the transmembrane segment with 28% sequence identity to the N-terminal region of TssL1 (*azo1298*). TssL1 had only 10 amino acids after the transmembrane segment and did not contain a peptidoglycan binding domain. However, Azo1306, also found in T6SS-1, was predicted by the online tool for classification and secondary structure prediction of membrane proteins, SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui), to have a transmembrane segment at residues 15-37 of this 158 amino acid protein. C-terminal residues 73-142 were predicted to be OmpA/MotB-like by InterPro: protein sequence analysis & classification (European Molecular Biology Laboratory- European Bioinformatics Institute, EMBL-EMI). It is probable that Azo1306 anchors the T6SS apparatus to the peptidoglycan.

TssJ. TssJ homologs were encoded by both T6SS gene clusters, *azo3893* and *azo1300*, respectively. TssJ2 was previously referred to as SciN. A predicted lipobox (LAAC) was found in TssJ2 and TssJ1, 18 amino acids and 46 amino acids from the N-terminus, respectively, without an aspartate in the +2 position (a requirement for localization, Aschtgen et al., 2008; Cascales and Cambillau, 2012). Additionally, the genome of *Azoarcus* sp. strain BH72 encoded for proteins involved in proper localization of TssJ, a requirement for function; signal peptidase II (LspA, *azo1204*), LolA (*azo1366*) and LolB (*azo0757*).

TssB – TssE. TssB and TssC cognates were encoded within both T6SS gene clusters of the *Azoarcus* sp. strain BH72 genome, *azo3895/azo3896* and *azo1303/azo1304,* respectively.

They were co-organized with their respective Hcp proteins (TssD). *Azoarcus* sp. strain BH72 expresses three Hcp proteins. TssD1 (*azo1305*) is encoded by the T6SS-1 gene cluster. TssD2 and TssD3 (*azo3897* and *azo3898*), by the T6SS-2 gene cluster. The two genes encoding for Hcp proteins in the T6SS-2 gene cluster are predicted to contain a hairpin loop structure between them (cgggcgacgc ctttccaggt gtcgcccg). TssE (phage gp25-like) homologs were found encoded by *azo3900* (TssE2) and *azo1297* (TssE1).

TssH. Of the 13 proteins considered core components of a T6SS, the T6SS-1 lacked only a gene encoding for a TssH (ClpV) homolog. The Hsp100/Clp family of proteins are part of the AAA+ superfamily and energize the disassembling of proteins through ATP binding and hydrolysis (Schlieker et al., 2005). The *Azoarcus* sp. strain BH72 genome encodes for eight Clp proteins (Clp, ClpA, ClpB1, ClpB2, ClpS, ClpP, ClpX, and ClpXP). Most Clp proteins are involved in protein quality control; however, that does not appear to be the case of ClpV (Schlieker et al., 2005). Variations between the different Clp types occur at Walker A and Walker B motif containing AAA domains and at the N-terminal domain, impacting primarily their substrate specificity. ClpA differs the most from ClpV and ClpB in that it has the capacity to directly interact with peptidase ClpP at a conserved IGF/L tripeptide at its AAA-2 domain. Neither ClpB nor ClpV are involved in proteolysis. It is believed to be unlikely that ClpA plays a role in T6SS (Schlieker et al., 2005). Both ClpB and ClpV have two AAA domains with an M-domain (middle domain) between the AAA domains, which is a domain for solubilization of aggregated proteins. The ClpB M-domain is predicted to from a coiled-coil structure; in contrast, ClpV is predicted to form an α-helical structure, which might be why ClpV is not able to solubilize aggregated proteins (Schlieker et al., 2005). Schlieker et al. looked at the protein sequence conservation between ClpA of *E. coli* and ClpB and ClpV of *E. coli*, *Salmonella typhimurium*, *Photorhabdus luminescens*, *V. cholerae* and *Mesorhizobium loti* (Schlieker et al., 2005). Their published results are in Figure 4. They found over 100 residues conserved in ClpA, ClpB and ClpV and about 10 that differentiate ClpA from ClpB from ClpV.

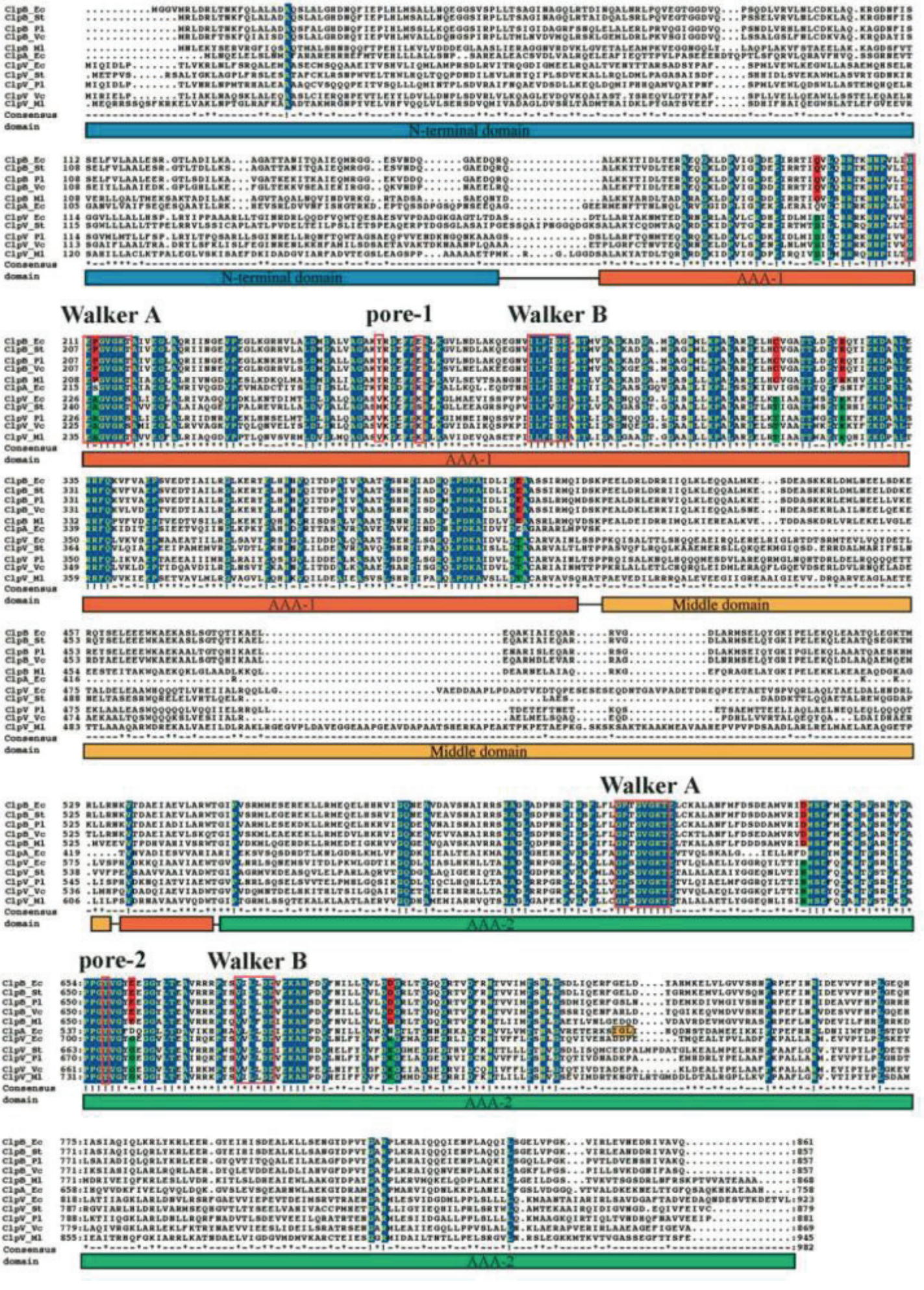

N-terminal domain
N-terminal domain
AAA-1
Walker A
pore-1
Walker B
AAA-1
AAA-1
Middle domain
Middle domain
Walker A
AAA-2
pore-2
Walker B
AAA-2
AAA-2

Figure 4. Sequence alignment performed by Schlieker et al. 2005.

ClpB and ClpV sequences from *Escherichia coli* O157:H7 EDL933 (Ec), *Salmonella typhimurium* LT2 (St), *Photorhabdus luminescens* (Pl), *Vibrio cholerae* (Vc) and *Mesorhizobium loti* (Ml), were aligned along with the ClpA sequence from the *E. coli* strain using ClustalW. Conserved residues were highlighted in blue. Walker A and B motifs and pore sites were boxed in red lines. Domain organization was indicated below the alignment. Residues that were invariant for ClpB and ClpV, but different from each other, were highlighted in red and green, respectively. The ClpP-interaction motif (IGL) of ClpA was highlighted in yellow. (Schlieker et al., 2005)

In Figure 5 a sequence alignment of the ClpA, ClpB and ClpV (TssH2) proteins of *Azoarcus* sp. strain BH72 with reference strain *E. coli* O157:H7 strain EC4042 is shown. The red residues are those conserved amongst all three Clp types. Residues found consistently to only one type of Clp, but not other types, are boxed in black. Residues not conforming to the reference Clp sequences are highlighted in yellow. The IGF/L sequence of ClpA is highlighted in green. Considering the findings of Schlieker et al, Azo1132 was closest to ClpA, Azo1566 was closest to ClpB and Azo3903 matched ClpV (TssH) sequence criteria.

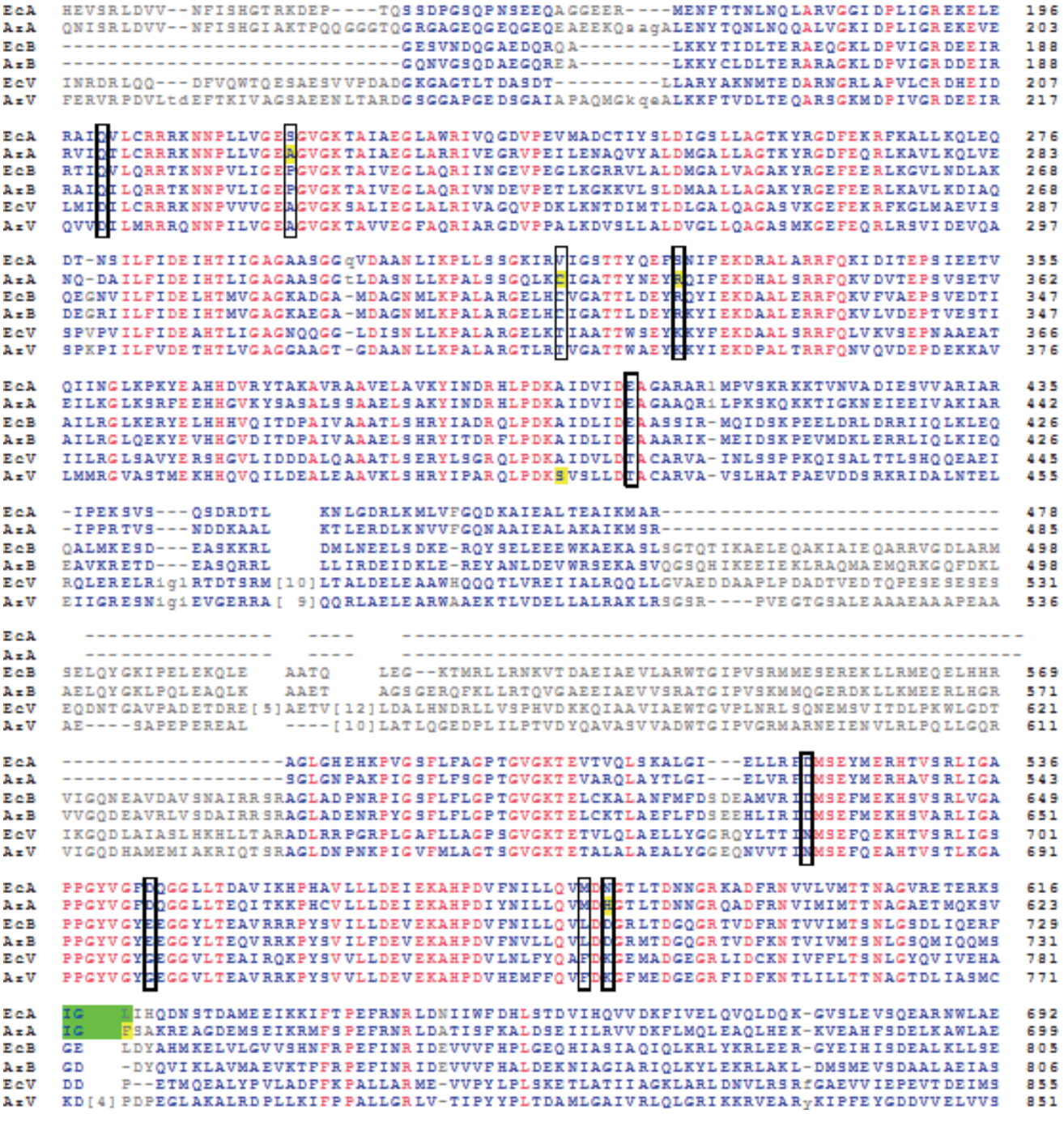


Figure 5. Sequence Alignment of ClpA, ClpB and ClpV protein sequences from an *E. coli* strain and *Azoarcus* sp. strain BH72. ClpA, ClpB and ClpV protein sequences of *E. coli* O157:H7 strain EC4042 (EcA, EcB, EcV, respectively) were compared to Azo1132, Azo1566 and Azo3903 protein sequences of *Azoarcus* sp. strain BH72 (AzA. AzB, AzV, respectively). Red residues are conserved between all Clp types. Residues found consistently to only one type of Clp, but not other types, are boxed in black. Residues not conforming to the reference Clp sequences are highlighted in yellow. The IGF/L sequence of ClpA is highlighted in green.

An interesting feature stood out (highlighted in yellow and not boxed in): One residue encoded within the AAA-1 domain, between the pore and Walker B motif of *azo3903* (ClpV-type), was a polar serine instead of the nonpolar, aliphatic alanine. Using blastp, the NCBI standard protein BLAST® (Basic Local Alignment Search Tool) search of Azo3903, genomes from a lengthy list of organisms also encoded for a serine at this position. The list included several *Pseudomonas* species and several strains of *P. aeruginosa*. Azo1132 had a high identity to ClpA; however, there were residues that instead matched the ClpB sequence and in one instance matched the sequence of ClpV. Still, Azo1132 had the conserved IGF/L motif (highlighted in green) which is necessary for interaction with proteolytic ClpP (Schlieker et al., 2005).

TssI. Three VgrG-related proteins (TssI) were found encoded by the *Azoarcus* sp. strain BH72 genome. One was found encoded within the T6SS-1 gene cluster, TssI1 (*azo1307*), with a length of 905 amino acids. One was encoded not within but near the T6SS-2 gene cluster, TssI2 (*azo3876*), with a length of 690 amino acids. The last was found encoded distant from both T6SS gene clusters, Azo3470, with a length of 905 amino acids.

TssK. TssK is a cytoplasmic protein believed to establish the contact between the trans-envelope complex (TssJ, TssL, TssM) and the phage-like complex (TssB-D) to the membrane through interactions with TssL, TssD (Hcp) and TssC (Zoued et al., 2008). TssK homologs were found encoded by both T6SS gene clusters of *Azoarcus* sp. strain BH72, *azo3892* (*tssK2*, previously *sciO*) and *azo1299* (*tssK1*, previously *impJ*).

TssA, TssF and TssG. Three T6SS proteins that have been determined as core components remain uncharacterized but were predicted to be cytoplasmic proteins. They too were found encoded by each T6SS gene cluster. The gene products of *azo1310* (*tssA1*, previously *impA*) and *azo3894* (*tssA2*, previously *sciA*) were TssA homologs. TssF proteins were found encoded by *azo1308* (*tssF1*, previously *impG*) and *azo3901* (*tssF2*, previously *sciC*). In both clusters genes encoding for TssG, proteins were found in tandem with genes encoding for TssF proteins, *azo1309* (*tssG1*, previously *impH*) and *azo3902* (*tssG2*, previously *sciB*).

Accessory Proteins., TagE-, TagF-, TagG- and TagH-like proteins were found encoded near the T6SS-2 gene cluster (*azo3888*, *azo3889*, *azo3885* and *azo3884*, respectively) and were believed to function in a mode of T6SS utilizing a threonine phosphorylation pathway. Also encoded by the T6SS-2 gene cluster, between gens for core components, was Azo3899 (*sciE*). A similar protein found encoded by the HSI-1 T6SS gene cluster of *P. aeruginosa* and the genome of *S. marcescens* was identified as TagJ, a non-essential protein for T6S that interacts with TssB, modulating its incorporation into the T6SS apparatus (Lossi et al., 2012). Nothing encoded by or in the vicinity of the T6SS-1 gene cluster was found to share homology with these proteins.

The T6SS-1 gene cluster encoded for Azo1301 and Azo1306, which are not core components of T6SSs and were not found in T6SS-2. InterPro: protein sequence analysis & classification (EMBL-EMI) predicted the C-terminal half of Azo1301, a 223 amino acid protein, to be a metallopeptidase belonging to MEROPS peptidase family M15 (clan MD), subfamily M15B (VanY, D-Ala-D-Ala carboxypeptidase) or subfamily M15C (Ply, L-alanyl-D-glutamate peptidase). As discussed above (Section IIIA - TssL and Azo1306), Azo1306 is potentially the peptidoglycan binding domain needed for anchoring TssL and the T6SS apparatus to the peptidoglycan.

III. B. Hcp antibodies used in this study detected Hcp (TssD) proteins in the supernatant and total cell fractions of *Azoarcus* sp. strain BH72 cultures, but not in the culture supernatant of a T6SS deficient mutant.

It has been well established that the presence of Hcp (TssD) in the supernatant is the hallmark of a functioning T6SS. Comparing amino acid sequences of known T6SS proteins from other organisms in the NCBI databank to *Azoarcus* sp. strain BH72 sequences, two Hcp proteins were predicted to be encoded in T6SS-2 gene cluster (*azo*3897 and *azo*3898) and one in the T6SS-1 gene cluster (*azo*1305) (Julia Herglotz, Diplomarbeit, 2007). According to the findings of a former diplom student of this laboratory, the percentage identity to known Hcp protein sequences from other organisms varied, but very similar tertiary structures were predicted. This was sufficient evidence to substantiate generating antibodies against each Hcp (Johannes Öhrlein, Diplomarbeit, 2009).

III. B. 1. Each of the three Hcp antibodies used in this study cross-reacted with each Hcp protein.

Each of the three Hcp proteins encoded by the *Azoarcus* sp. strain BH72 genome were overexpressed and purified. The pASK-IBA3 expression vector (IBA, Göttingen, Germany), encodes for an 8 amino acid C-terminal Strep-Tactin® affinity tag, and was used for the expression of each Hcp protein (TssD1 (*azo1305*), TssD2 (*azo3897*) and TssD3 (*azo3898*)). Recombinant proteins were overexpressed by *E. coli* and purified (Johannes Öhrlein, Diplomarbeit, 2009). Polyclonal antibodies were generated by rabbits, and third bleed sera were used for Hcp detection (Johannes Öhrlein, Diplomarbeit, 2009). Figure 6 shows that each rabbit was truly naïve to each Hcp protein by the lack of reactivity of preimmune sera to the overexpressed Hcp. Additionally, each of the three Hcp were detected by the corresponding antibody that was generated against it. However, each antibody showed cross-reactivity, to some extent, with each of the other Hcp proteins (Figure 6).

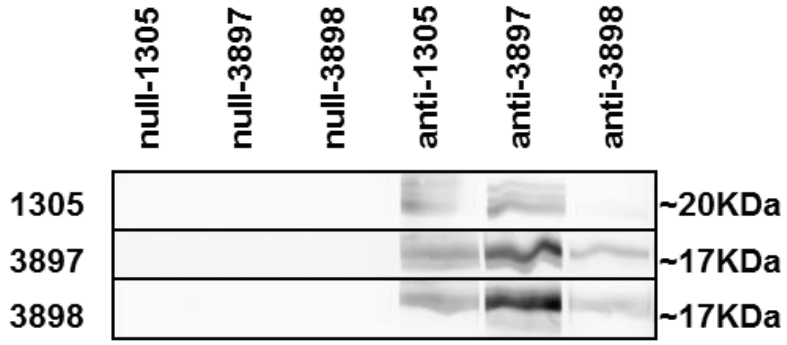


Figure 6. **Cross-reactivity of antibodies used against each of the overexpressed Hcps.** Each Hcp (Azo1305, Azo3897 and Azo3898) was overexpressed by *E. coli* DH5α grown in LB media at 37°C with shaking at 200 rpm upon induction with 0.02 ng/ml anhydroustetracycline for 3 h. Proteins were extracted and then purified using Strep-Tactin sepharose columns. For each Hcp protein (1305, 3897, 3898) about 200 µg was run through a 10 cm wide 12% SDS-PAGE gel and then blotted onto nitrocellulose membranes. Blots were cut into 1 cm wide strips. Strips were probed with one of six different sera, preimmune sera from each naïve rabbit (null-) (1:3000) and anti-sera from the third bleed of each rabbit used (anti-) (1:3000). Protein bands were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher, Rockford, IL, USA).

To overcome this, several attempts were made at purifying each antibody by selectively removing unspecific antibodies from the polyclonal serum. In what appeared to be the most successful strategy, sepharose columns were prepared with two of the three overexpressed Hcp bound to the column. Serum containing antibodies generated for the Hcp protein that was not bound in the column was passed through this column. After multiple rounds eluting

antibodies, the serum no longer cross-reacted with Hcp proteins that were not specific to that antibody. However, the antibody became so dilute that standard quantities of Hcp found in wild type culture supernatants were no longer detected (data not shown). It was next tested whether the affect was merely a result of serum dilution. Figure 7 shows that a 1:10,000 dilution of the anti-3897 serum effectively eliminated visible cross-reactivity against Azo1305 and Azo3898.

Figure 7. Assessment of cross-reactivity of anti-3897 dilutions. Each Hcp was overexpressed by *E. coli* DH5α grown in LB media at 37°C with shaking at 200 rpm upon induction with anhydroustetracycline for 3 h. Proteins were extracted and purified using Strep-Tactin sepharose columns. About 540 µg of each Hcp (Azo1305, Azo3897 and Azo3898) were run through 10 cm wide 12% SDS-PAGE gels. Dilutions of anti-3897 were prepared as 1:1000, 1:5000 and 1:10,000 and used to probe Western blots for each Hcp on 1 cm wide membrane strips of each blot. SuperSignal West Pico Chemiluminescent Substrate was used for detection (Thermo Fisher, Rockford, IL, USA).

Once again, this degree of dilution was ineffective at detecting physiological levels of Hcp found in the supernatant of *Azoarcus* sp. strain BH72 cultures. Using 'purified' and diluted anti-1305 and anti-3898 produced similar results (data not shown). It was determined that use of the undiluted, cross-reacting polyclonal antibodies was unavoidable for effective Hcp detection. In this study different Hcp proteins expressed were not differentiated in Western blot analysis. To minimize any sort of bias, a mixture containing equal parts of anti-1305, anti-3897 and anti-3898 was always used for Hcp detection in further experiments.

III. B. 2. Hcp was detected in the supernatant and total cell fractions of *Azoarcus* sp. strain BH72 cultures.

Methods for harvesting supernatant proteins were optimized to eliminate cellular proteins and maintain detection of secreted Hcp by pelleting the cells out of the culture via centrifugation at 10,400 *x* g at 4°C for 20 min. From this supernatant a second centrifugation step was performed with the same conditions to remove residual cells. Initial methods for harvesting cell supernatant proteins involved a filtration step with 0.2 µm pore membranes. It was clear that a substantial amount (and often all detectable amounts) of Hcp were lost in this step

(Figure 16). The formation of TssB/TssC tubules up to 1 µm has been observed through time-lapse fluorescence light microscopy (Basler et al., 2012). Potentially, tubules of Hcp hexamers maintain structure lengths greater than 200 nm in the supernatant, and so filtration was avoided. Instead, centrifugation steps and handling were optimized during the harvesting to avoid contamination.

Supernatant proteins were precipitated from this second supernatant with the addition of Trichloroacetic acid to a final concentration of 10% (v/v), pelleted and then washed as described (Section IIB4d). Using the antibodies described above, detection of Hcp in the supernatant of wild type cultures was possible under standard growth conditions at 37°C with shaking (200 rpm) in SM+N medium. Figure 8 is representative of several trials where Hcp was detected via Western blot analysis in the supernatant and total cell protein fractions obtained from wild type strain BH72 grown in SM+N media.

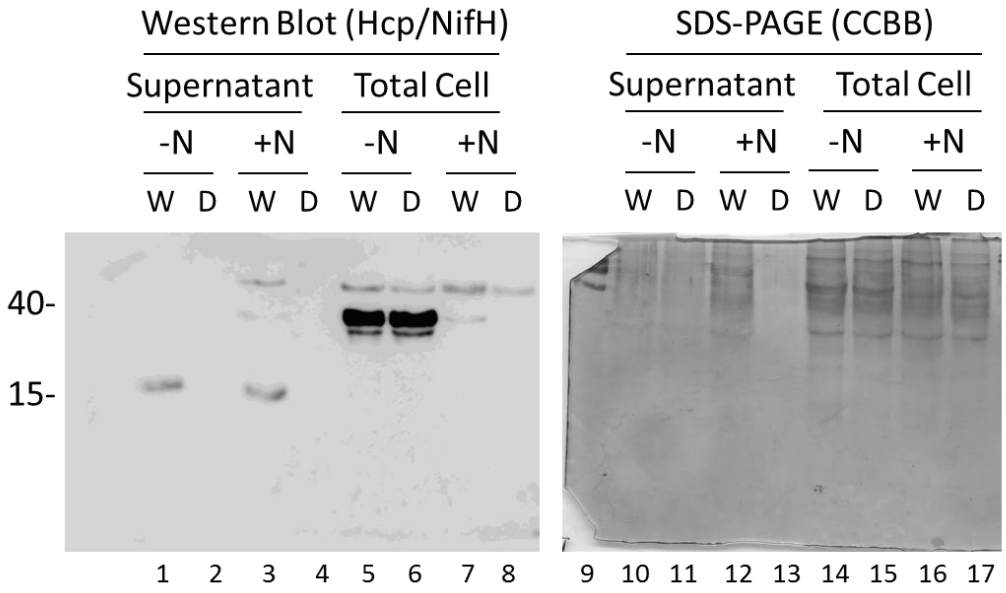
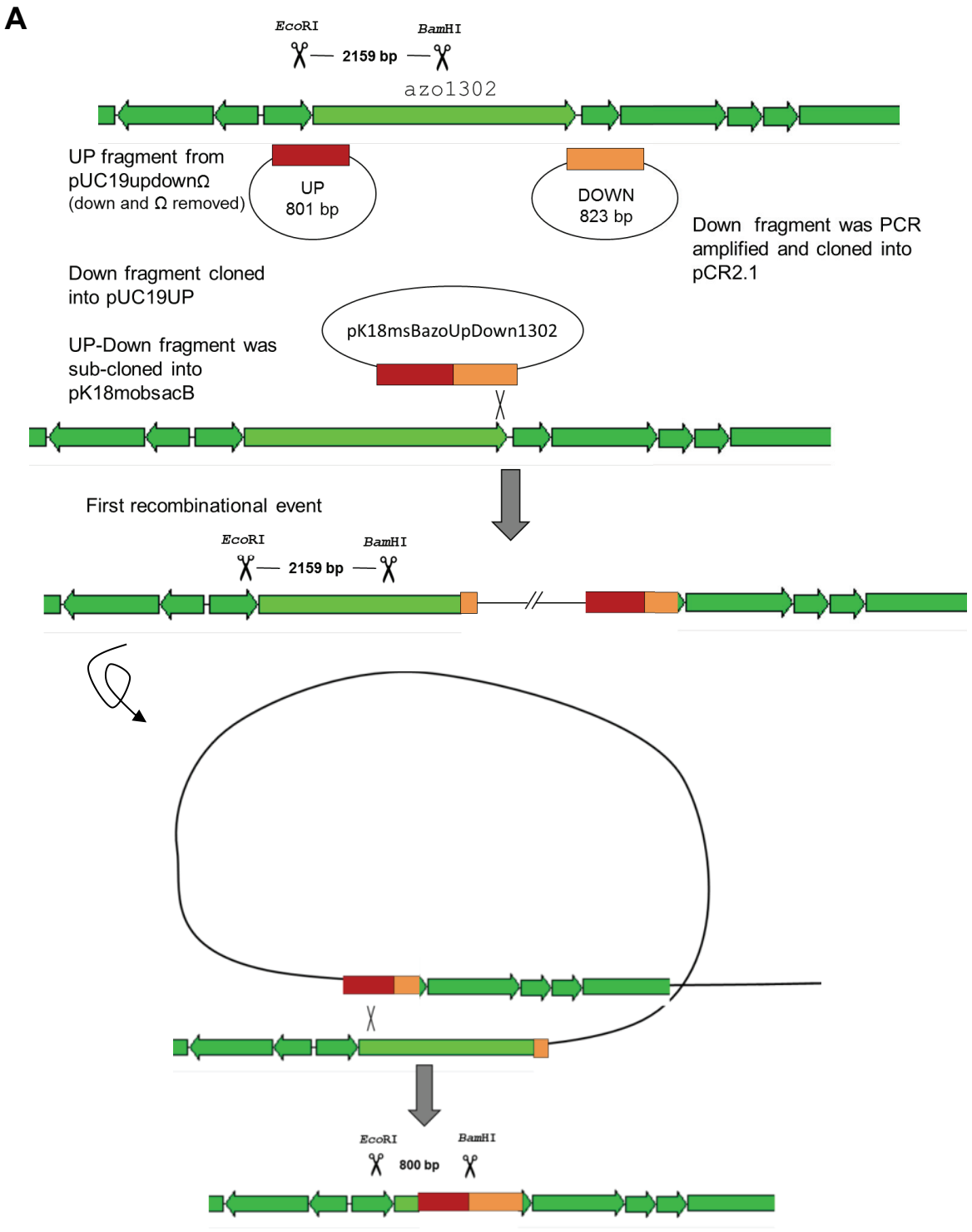


Figure 8. Detection of Hcp in supernatant and total cell protein fractions of *Azoarcus* sp. strain BH72. Wild type cells (W; lanes 1, 3, 5, 7, 10, 12, 14, 16) and mutant type, BHΔimpLsciO, (D; lanes 2, 4, 6, 8, 11, 13, 15, 17) were grown in a bioreactor (37°C, pH 7.0, 600 rpm, 0.6% O_2) in SM medium to an OD_{578} of 0.8 (-N; lanes 1, 2, 5, 6, 10, 11, 14, 15) or overnight in SM medium supplemented with 9.3 mM NH_4Cl (+N; lanes 3, 4, 7, 8, at 37°C with shaking at 200 rpm. Proteins from about 5 ml of culture supernatant and extracts from about 70 µg total cells (fresh pellet weight) were resolved in a 12% polyacrylamide SDS-PAGE. The Western blot was probed with anti-Hcp. SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher, Rockford, IL, USA) was used for detection. The blot was then incubated with Anti-NifH and further developed as before (lanes 1-8). Protein band sizes were estimated in kDa (left) using PageRuler™ Prestained Protein Ladder (Fermentas, ThermoFisher, Rockford, IL, USA) (lane 9). Protein load was controlled by an SDS-PAGE gel stained with colloidal coomassie blue (CCBB; lanes 9-17).

III. B. 3. The double TssM mutant, BHΔimpLsciO, was constructed with an in-frame deletion of *azo1302* (*tssM1*) and disruption of *azo3890* (*tssM2*) by integration of plasmid DNA into the chromosome.

It was believed that construction of a T6SS deficient mutant would allow for a direct comparison of culture supernatants from wild type strain BH72 and a T6SS deficient strain. One could assume proteins found in the wild type secretome, and lacking in the T6SS deficient secretome, were T6SS dependent. Additionally, manipulating the on and off status of secretion through mutations to core components would help confirm that putative Hcp secretion was not an artifact but rather dependent on an active T6SS. It had been reported repeatedly that TssM (IcmF) is required for T6S (Ma et al., 2009; Rao et al., 2004; Parsons and Heffron, 2005), and so a double TssM mutant, BHΔimpLsciO, was constructed by making an in-frame deletion to TssM1 (*azo1302*) and a polar mutation disrupting TssM2 (*azo3890*). A mutant with an in-frame deletion of *azo1302,* BHΔ1302, was first constructed. The upstream fragment was already cloned into pUC19updownΩ in previous work, an 801 bp up fragment (393 bp upstream-408 bp into gene azo1302) (Johannes Öhrlein, Diplomarbeit, 2009). The omega cassette and downstream fragment were cut out via restriction digestion with *Bam*HI and *Hind*III, so that only the upstream fragment remained in pUC19 vector (Johannes Öhrlein, Diplomarbeit, 2009). The 823 bp downstream fragment (3423 bp in-532 bp downstream *azo1302*) was amplified and cloned into a pCR^{TM}2.1TOPO$^{®}$TA cloning vector and subcloned into the pUC19 vector already containing the up fragment, utilizing the *Bam*HI and *Hind*III restriction sites (pUC-UpDown1302). The combined up and down fragments with 2994 bp in-frame deletion (pUC-UpDown1302) were further subcloned into the pK18*mobsac*B (pK18msBazoUpDown1302) utilizing the *Eco*RI and *Hind*III restriction sites and transformed into *E. coli* DH5α. The gene fragments in pK18*mobsac*B were PCR amplified for sequencing using universal primers M13F and M13R. The pK18*mobsac*B was transferable via conjugation with the help of a pRK2013 plasmid containing helper strain of *E. coli* Dh5α. After a tri-parental conjugation (Section IIB3c), transconjugants having undergone one recombinational event were selected for their kanamycin resistance (Km^{R}), encoded by the pK18*mobsac*B vector. The vector could not replicate in wild type strain BH72 if not integrated in the chromosome. A period of growth without antibiotic selection allowed for a second recombinational event. Clones were selected for their lack of sucrose sensitivity encoded by the *sacB* gene of the vector used. Proper construction of the mutant was confirmed via Southern blot analysis. Genomic DNA digested

with *Eco*RI and *Bam*HI was separated on a 1% agarose gel and blotted onto a membrane as described (Section IIB3e). The membrane was probed with DIG-labeled up fragment DNA. Figure 9 provides a schematic of the cloning strategy along with the Southern blot confirming the genomic content of the mutant.



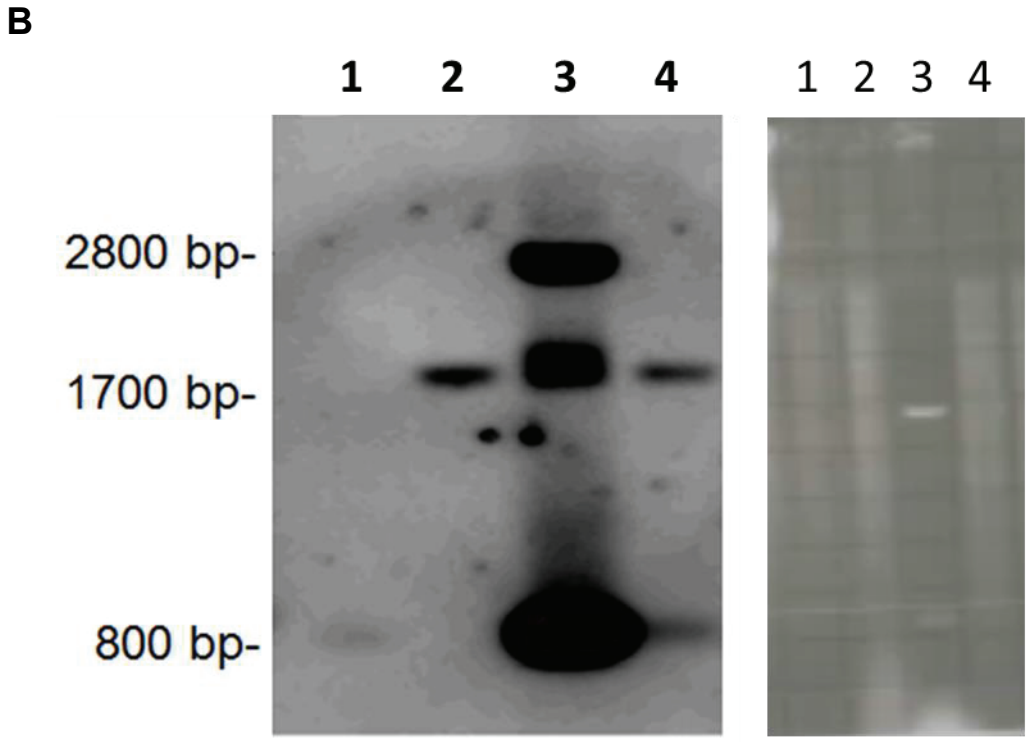
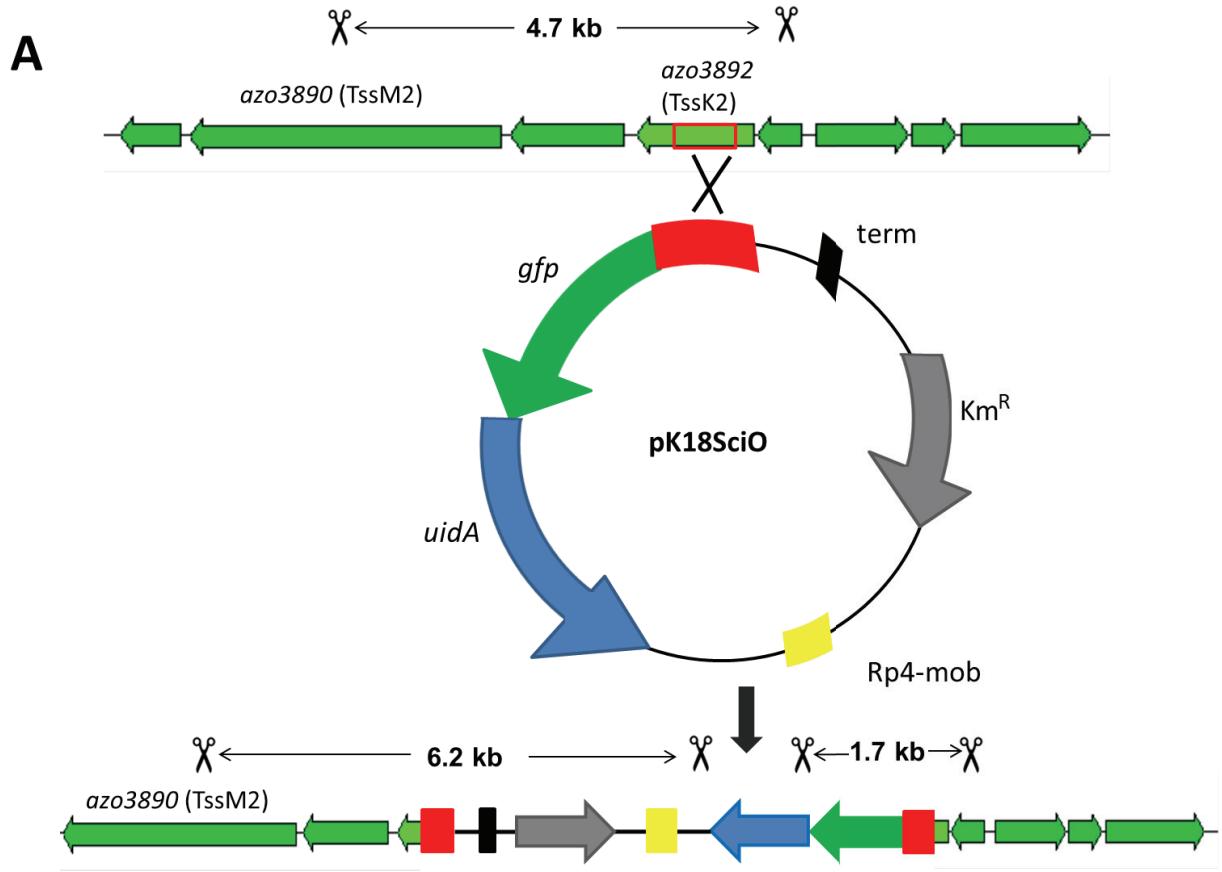


Figure 9. **Construction of in-frame deletion mutant BHΔ1302.** (**A**) Strategy used for construction of the in-frame deletion of *azo1302*. Wild type DNA is depicted in green and the light and dark brown are PCR amplified DNA fragments. Restriction sites are indicated by scissors above the genomic DNA. (**B**) 3 µg of genomic DNA extracted from BHΔ1302 (lane 1), wild type (lane 2) and from the first recombinational event (lane 4) were digested with *Eco*RI and *Bam*HI overnight at 37°C. The positive control, 2 µg of plasmid DNA containing the joined up and down fragments, was loaded in lane 3. DNA fragments and molecular size marker, *Pst*I digested lambda phage DNA (200 ng/µl), were separated by agarose gel electrophoresis (right of Panel B) then blotted and cross linked onto a membrane that was probed with the DIG-labeled up fragment (left side of Panel B) and detected using a 1:25,000 dilution CDP-star Chemiluminescent Substrate (Sigma, Aldrich, St. Louis, MO, USA).

The second mutation in BHΔimpLsciO was the disruption of TssM2 expression. A plasmid constructed in previous work, pK18TsciO (Julia Herglotz, Diplomarbeit, 2007), contained a 731 bp portion of *azo3892* within the pK18GGST vector. With the help of a pRK2013 containing helper strain, the plasmid was conjugated from the *E. coli* Dh5α donor strain into *Azoarcus* sp. strain BH72 through triparental conjugation (Section IIB3c). The plasmid was integrated into the chromosome through a single recombinational event, and mutants were selected for their Km^R encoded by the vector. This mutant was passaged multiple times to ensure purity before confirming the correct integration via Southern blot analysis (Figure 10,

Panel B). To confirm the proper integration of the plasmid, a Southern blot analysis was performed by digesting DNA with *Nru*I.



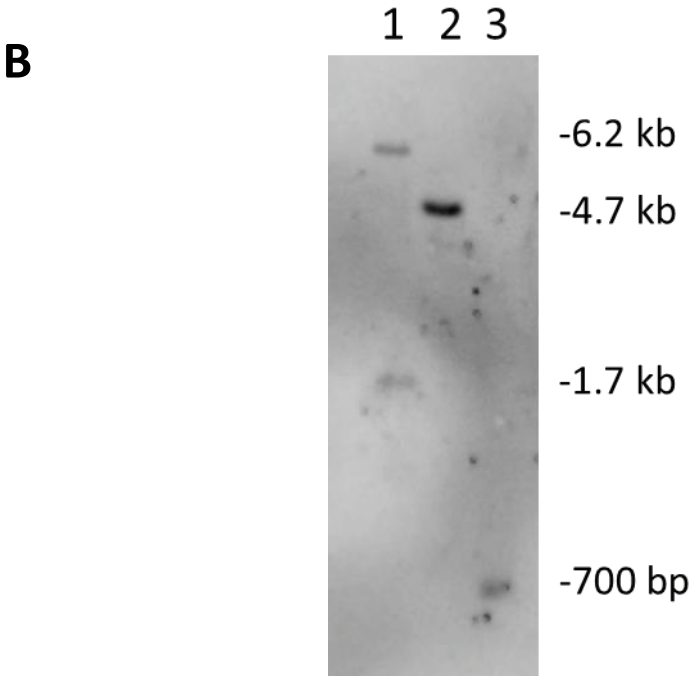


Figure 10. **Integration of pK18SciO into BHΔ1302. (A)** The scheme used for integration of the pK18SciO plasmid into BHΔ1302 is illustrated. Wild type chromosomal DNA is depicted in green and the pK18GGST plasmid is depicted with various colors representing the genes present (red=PCR fragment of *azo3892*; dark green=*gfp*; blue=*uidA(gusA)*; gray=Km^R; black=transcriptional terminator; yellow=Rp4 genes for mobilization). The plasmid contained a 731 bp PCR amplification product from the *sciO* (*tssK2*) gene (596 bp from the 5' end) (Julia Herglotz, Diplomarbeit, 2007) and was transferred into BHΔ1302 via triparental conjugation. (**B**) 3 µg of genomic DNA extracted from BHΔimpLsciO (lane 1) and wild type BH72 (lane 2) was digested with *Nru*I. DNA fragments, PCR amplified *sciO* fragment (lane 3) and molecular size marker, *Pst*I digested lambda phage DNA (200 ng/µl), were separated in a 1% agarose gel via electrophoresis. DNA fragments were blotted onto a membrane and probed with a DIG-labeled *sciO* PCR fragment and detected using CDP-star chemiluminescent substrate (Sigma, Aldrich, St. Louis, MO, USA).

To control that integration of the plasmid at the gene *azo3892* (TssK2, previously SciO) effectively eliminated transcription of *azo3890* (TssM2), an RT-PCR analysis was performed. Amplification of 16S RNA using primers TH3fw and TH5rv confirmed that an equal amount of RNA was present in the RNA extracts used, and PCR amplification of the RNA extract without a reverse transcriptase step confirmed that DNA was not present. The primer 3890R349bp was used to generate cDNA from the total RNA extracted from wild type and BHΔimpLsciO. A 329 bp PCR product was produced after amplification of wild type cDNA using primers 3890F349bp and 3890R349bp (Table 4), confirming PCR conditions used were appropriate. There was no amplification using the same primers and cDNA generated from total RNA extracted from BHΔimpLsciO (Figure 11), confirming transcription of *azo3890* (TssM2) was disrupted in BHΔimpLsciO.

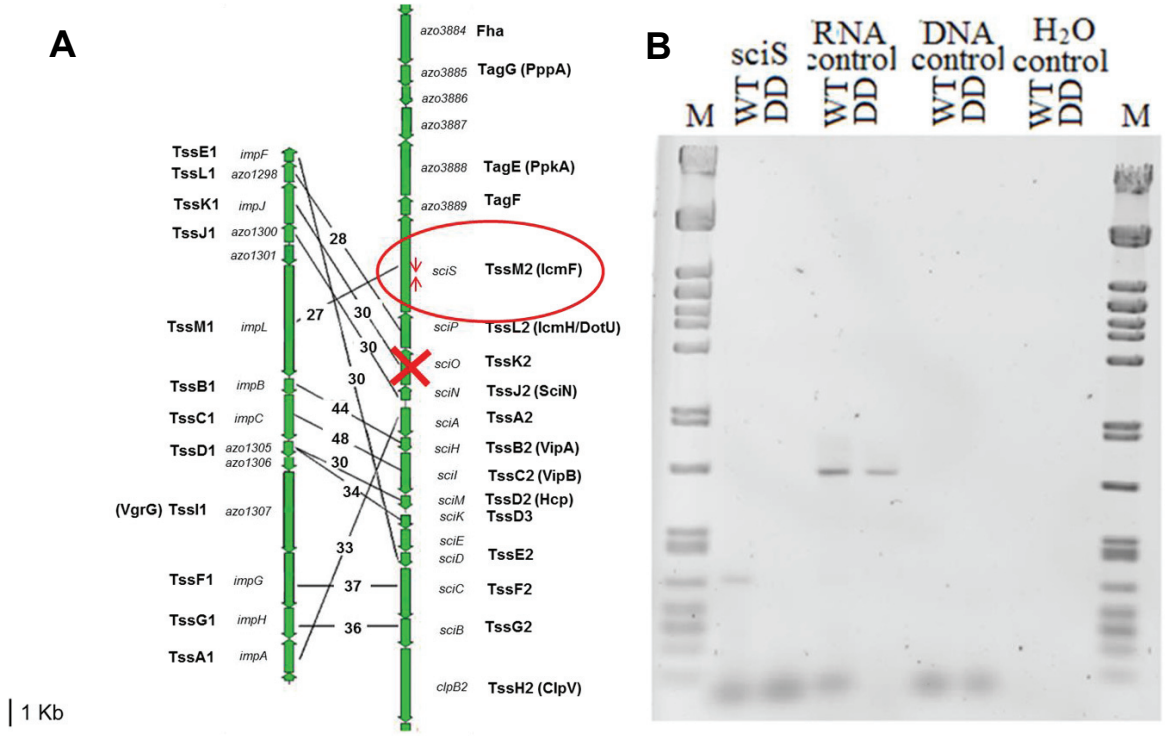


Figure 11. RT-PCR analysis confirmed *tssM2* (sciS) was not transcribed in the BHΔimpLsciO mutant. (A) Schematic of the T6SS-2 gene cluster depicting the organization of *tssK2* (*azo3892*) and *tssM2* (*azo3890*). The disrupted gene *tssK2* is marked with a red X, and the position of *tssM2* is circled in red. (**B**) RT-PCR products and size marker (*Pst*I digested lambda phage DNA (200 ng/µl)) were visualized in an Ethidium Bromide stained 1.2% agarose gel. The 3890R349bp primer was used to generate cDNA from 2 ng of total RNA extracted from wild type (WT) and BHΔimpLsciO (DD). Further amplification of a 329 bp product from cDNA was done with the 3890 (sciS) primers (binding 2162-2508 bp downstream of 5' end; marked with red arrows in Panel A). In the RNA control, 16SRNA was amplified (primers TH3fw and TH5rv) from cDNA generated from 2 ng of total RNA (WT and DD) using primer TH5rv. In the DNA control, RNA extracted from WT and DD were used for amplification using 3890 (sciS) primers without a reverse transcription step. In the water controls, no primers were added to the RT-PCR reactions.

III. B. 4. Secretion of Hcp was eliminated in a double TssM mutant (BHΔimpLsciO).

In an effort to turn off the T6SS, a double TssM mutant (BHΔimpLsciO) was constructed with an in-frame deletion of *azo1302* and an insertional mutation to *azo3890*, so that neither *tssM1* nor *tssM2* were expressed. Wild type and BHΔimpLsciO strains were grown in liquid cultures, and supernatant proteins were harvested and analyzed in a Western blot analysis. A representative Western blot of several trials (Figure 8 and Figure 12) demonstrates that disruption of both TssM proteins, encoded by both T6SS gene clusters, in the BHΔimpLsciO mutant successfully abrogated detection of Hcp proteins in the supernatant.

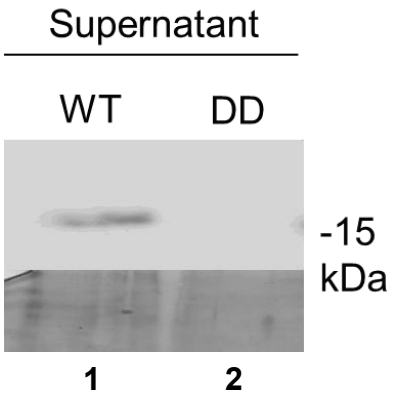


Figure 12. Western blot analysis of Hcp secretion by wild type and BHΔimpLsciO. Wild type (WT, lane 1) and BHΔimpLsciO (DD, lane 2) strains were grown in 2 L of SM media to an OD_{578} of 0.8 in a bioreactor (37°C, pH 7.0, 600 rpm, 0.6% O_2). Supernatant proteins from about 20 ml of culture were loaded and separated on a 12% polyacrylamide SDS-PAGE gel and transferred onto nitrocellulose. The Western blot was probed with anti-Hcp. Protein bands were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher, Rockford, IL, USA). Protein loaded was further controlled by staining the SDS-PAGE gel with Colloidal Coomassie Brilliant Blue Stain (below). Approximate sizes are indicated in kDa, estimated using PageRuler™ Prestained Protein Ladder (Fermentas, ThermoFisher, Rockford, IL, USA).

III. B. 5. Hcp Antibodies used in this study showed unspecific binding.

Using GenDB (Bielefeld, Deutschland, https://gendb.cebitec.uni-bielefeld.de), Hcp proteins of about 17 kDa encoded by the T6SS-2 gene cluster (TssD2/Azo3897 and TssD3/Azo3898) and a 20 kDa Hcp protein (TssD1/Azo1305) encoded by the T6SS-1 gene cluster were predicted. Proteins of this size were detected in the supernatant and total cell protein fractions. Throughout this study, only one protein band was routinely detected in the supernatant fractions, but multiple bands were detected in the total cell fractions using Hcp antibodies in Western blots analysis. Several protein bands detected were too large to be considered Hcp monomers. The larger protein bands detected in Western blots might be Hcp multimers or the antibodies cross reacting with cellular proteins.

Table 6. GenDB predicted sizes of Hcp (TssD) proteins encoded by the *Azoarcus* sp. strain BH72 genome (Meyer et al., 2003).

Hcp Protein	nucleotides (bp)	Amino acids	kDa	pI
TssD1/Azo1305	498	165	20.7	6.23
TssD2/Azo3897	486	162	17.3	5.42
TssD3/Azo3898	504	168	17.6	6.14

The Hcp antibodies used also putatively cross reacted with flagella protein. In a single trial, proteins from a crude extract of flagella from *Azoarcus* sp. strain BH72 (prepared as described, Section IIB4f) were separated using 12% polyacrylamide SDS-PAGE gel. In a Western blot using Hcp anti-serum, a protein band the same size as flagella (~50 kDa), but not a band similar to the predicted monomeric sizes of Hcp (Table 6), was detected. A protein band about of about 50 kDa was also detected in the extracellular extract from the BHΔimpLsciO mutant, which does not secrete Hcp. It was only in the FliC3 mutant, where flagella were not produced, that detection of this 50 kDa protein band was eliminated (Figure 13).

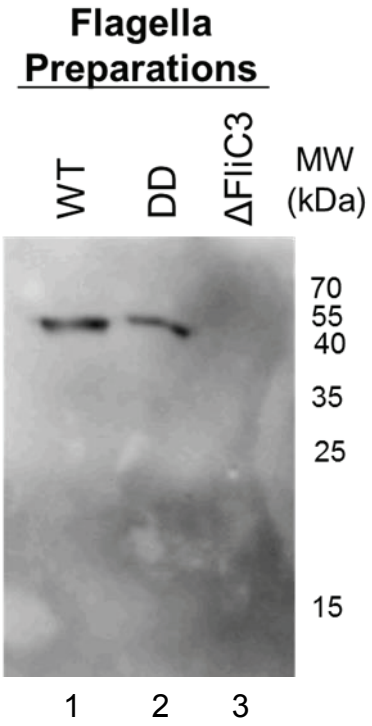


Figure 13. Hcp detection in a Western blot analysis of flagella preparations. Crude flagella extracts from wild type (WT, lane 1), BHΔimpLsciO (DD, lane 2), and BHΔFliC3 (ΔFliC3, lane 3) cultures grown overnight on VME agar plates at 37°C were prepared. Proteins from each sample were separated in a 12% polyacrylamide SDS-PAGE gel and blotted onto a nitrocellulose membrane. The Western blot was probed with anti-Hcp. SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher, Rockford, IL, USA) was used for detection. A parallel gel stained with Colloidal Coomassie Blue controlled the relative amount of total protein. Molecular weights are indicated in kDa, estimated using PageRuler™ Prestained Protein Ladder (Fermentas, ThermoFisher, Rockford, IL, USA).

Additionally, from the same crude flagella extract, a flagellin-sized protein band (about 50 kDa) was detected by the anti-Hcp serum in Western blots of flagella preparations and not

the remaining supernatant fractions after the flagella had been pelleted out (Figure 14). A protein band the size of an Hcp monomer was detected in the supernatant of the wild type flagella preparation, where the flagella had been removed by centrifugation, but not detected in the same fraction prepared from BHΔimpLsciO (Figure 14). Taken together, this was strong evidence that flagella was detected by the Hcp anti-serum

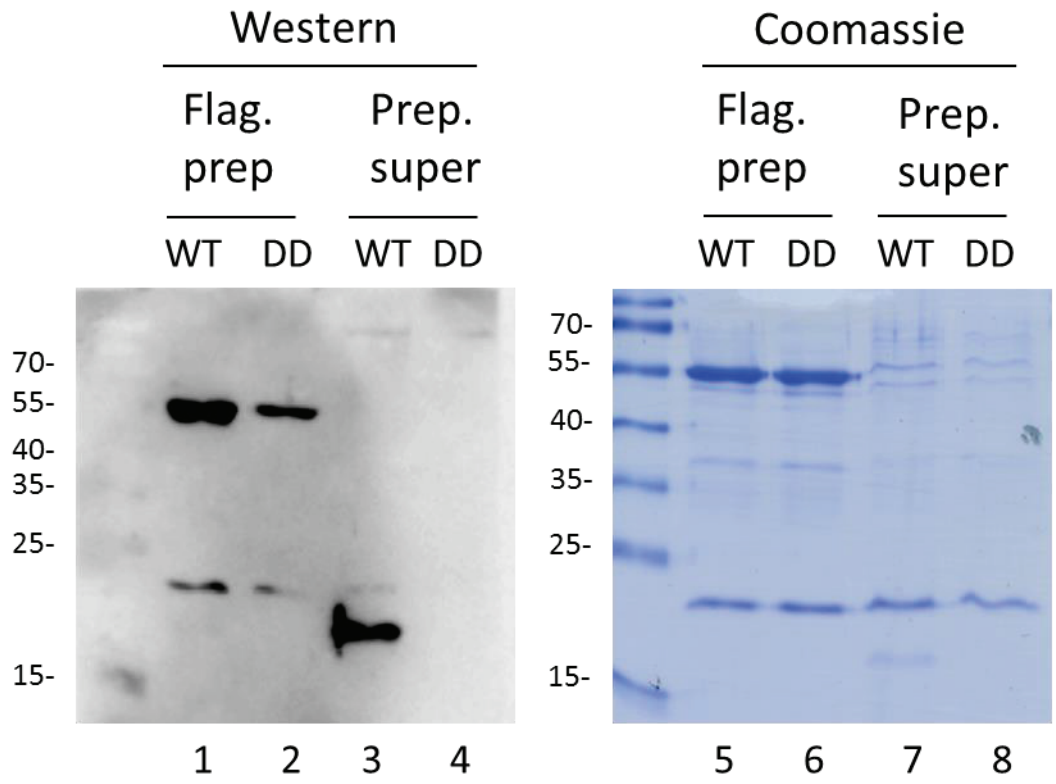


Figure 14. Western blot analysis of flagella preparations. Crude flagella extracts (Flag. prep) were prepared from wild type (lanes 1 and 5) and BHΔimpLsciO (lane 2 and 6). The remaining supernatant fraction (Prep. super) from the same flagella preparations of wild type (lanes 3 and 7) and BHΔimpLsciO (lanes 4 and 8) were also loaded. About 30 µg of protein loaded from each sample were separated on 12% polyacrylamide SDS-PAGE gels and blotted onto a nitrocellulose membrane. The Western blot was probed with anti-Hcp. SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher, Rockford, IL, USA) was used for detection of protein bands (lanes 1-4). A parallel gel stained with Colloidal Coomassie Blue (lanes 5-8) controlled the relative amount of total protein loaded. Approximate protein band sizes were estimated using PageRuler™ Prestained Protein Ladder (Fermentas, ThermoFisher, Rockford, IL, USA) and indicated in kDa.

III. C. The Function of T6SSs in *Azoarcus* sp. strain BH72

III. C. 1. Growing *Azoarcus* sp. strain BH72 under nitrogen fixing conditions induced T6SS-1 gene expression and increased the amount of Hcp (TssD) detected in total cell protein extracts.

In a microarray study, gene transcription by *Azoarcus* sp. strain BH72 grown under conditions promoting nitrogen fixation in a bioreactor (Section IIB1b) was compared to the same growth conditions with 10 mM NH_4Cl added to the medium. The majority of T6SS-1 genes were up-regulated at least 2-fold under nitrogen fixing conditions: *azo1297* (+3.2-fold), *azo1298* (+22), *azo1299* (+10), *azo1300* (+20), *azo1302* (+10), *azo1305* (+16), azo1306 (+5.5), azo1308 (+4.4), *azo1310* (+2.8) (Sarkar and Reinhold, 2014). None of the T6SS-2 cluster genes were up or down-regulated with any statistical significance when grown under nitrogen fixing conditions, but the neighboring *azo3872* was up-regulated 10-fold and *azo3875* was down-regulated 2.6-fold (Sarkar and Reinhold, 2014). Additionally, transcriptional fusions of *azo3901* (TssF), *azo3892* (TssK), *azo1302* (TssM) and *azo1299* (TssK) with a GUS reporter gene were constructed (Julia Herglotz, Diplomarbeit, 2007). Using GUS assays it was determined that transcription of *azo1299* was nearly 20 times higher when cells were grown under nitrogen fixing conditions, than comparable non-fixing conditions. Each of the other genes tested, *azo1302*, *azo3892* and *azo3900*, remained at consistent levels of expression regardless of growth conditions tested (Julia Herglotz, Diplomarbeit, 2007). Further, using semi-quantitative reverse RT-PCR analysis, *azo1299*, *azo1302* and *azo1305* were up-regulated approximately 14-, 12- and 9-fold, respectively (Teja Shidore, PhD thesis, 2012). To corroborate this, it was demonstrated that a greater amount of Hcp was detected in the total cell protein extracts of *Azoarcus* sp. strain BH72 grown under nitrogen fixing conditions compared to growth under non-fixing conditions. This was seen using either a semi-solid or liquid media over several trials throughout the course of this work (Figure 15).

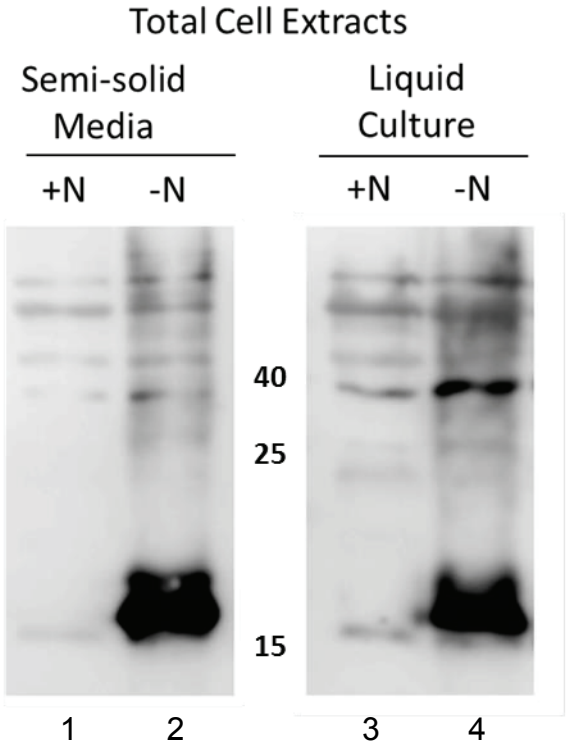


Figure 15. Western blot detection of Hcp in the total cell proteins extracted from *Azoarcus* sp. strain BH72. *Azoarcus* sp. strain BH72 was grown in semi-solid media for 2 d (lanes 1 and 2) or aerobically in liquid culture to an OD_{578} of 0.8 (lanes 3 and 4) in SM media (-N, lanes 2 and 4) or SM media supplemented with 9.3 mM NH_4Cl (+N, lanes 1 and 3). Total protein from about 70 µg of cells (fresh pellet weight) was extracted and resolved on a 12% polyacrylamide SDS-PAGE gels and blotted onto a membrane. Western blots were probed with anti-Hcp. SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher, Rockford, IL, USA) was used to detect protein bands. Protein band sizes are indicated in kDa, estimated using PageRuler™ Prestained Protein Ladder (Fermentas, ThermoFisher, Rockford, IL, USA).

Understanding the independent roles of each T6SS gene cluster, or their possible interdependence, under given growth conditions, was of interest. It was assumed that the high abundance of Hcp detected when cells were grown under nitrogen fixing conditions was expressed from the T6SS-1 gene cluster. Because the different Hcp antibodies cross reacted, it was not possible to discern the Hcp proteins from one another via Western blot analysis. Regrettably, attempts at identifying putative Hcp proteins in 1D and 2D PAGE gels using MALDI-TOF also failed. Despite the inability to distinguish the different Hcp proteins, there were at least some indicators of T6SS interdependence, which are discussed below.

III. C. 2. *Azoarcus* sp. strain BH72 secreted Hcp independent of TssM1.

BHimpL::pK18GGST was constructed by the integration of pK18GGST plasmid into *azo1302*, encoding for TssM1 (Julia Herglotz, Diplomarbeit, 2007). In previous studies the

up-regulation of T6SS-1 genes had been demonstrated under nitrogen fixing conditions. Therefore, it was believed that under nitrogen fixing conditions, turning off T6SS-1 (via deletion of *tssM1*) would be sufficient to eliminate detection of Hcp in culture supernatants. In Figure 16, Western blot analysis was used to assess the presence of Hcp in filtered (+) and non-filtered (–) supernatants of wild type and a TssM1 deletion mutant, BHimpL::pK18GGST, when grown under nitrogen fixing conditions. Blots were later probed with anti-NiH to control for the presence of cellular proteins in the supernatant. To completely eliminate the secretion of Hcp, or its detection in the supernatant, it was necessary to knockout both TssM1 and TssM2 (Figure 12). With T6SS-1 disrupted, in strain BHimpL::pK18GGST, the Hcp detected in the supernatant likely originated from the still functioning T6SS-2.

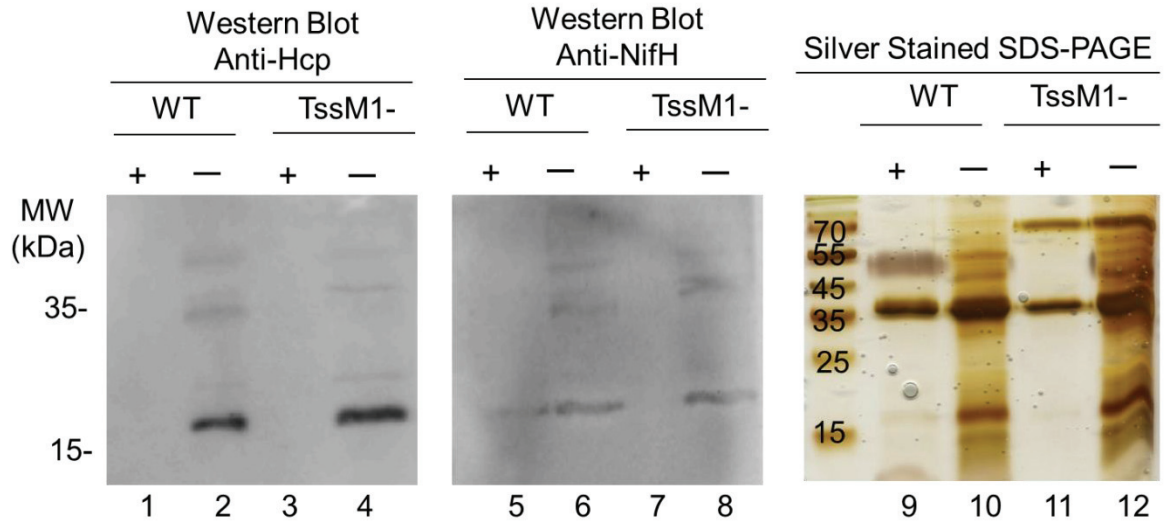


Figure 16. Detection of Hcp in *Azoarcus* sp. strain BH72 and TssM1 mutant culture supernatants. Supernatant proteins from wild type strain BH72 (WT) (lanes 1, 2, 5, 6, 9 and 10) and TssM1 mutant (BHimpL::pK18GGST) (lanes 3, 4, 7, 8, 11 and 12) strains grown under nitrogen fixing conditions (0.6% O_2, pH 7.0, 37°C, 600 rpm) to an OD_{578} of 0.8 in a bioreactor were harvested. Supernatant fractions were loaded directly (-) (lanes 2, 4, 6, 8, 10 and 12) or after filtered through a 0.2 µl pore membrane (+) (lanes 1, 3, 5, 7, 9 and 11), onto 12% polyacrylamide SDS-PAGE gels, resolved and transferred to a PVDF membrane. The Western blot was probed with anti-Hcp (left) and then anti-NifH (middle). Protein bands were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher, Rockford, IL, USA) (lanes 1-8). Amount of protein loaded was controlled by a parallel gel, silver stained (lanes 9-12). Protein band sizes were estimated using PageRuler™ Prestained Protein Ladder (Fermentas, ThermoFisher, Rockford, IL, USA).

The presence of Hcp in the total cell protein extracts was also assessed to provide an insight into the expression rather than the secretion of Hcp (Figure 17). A single trial with limited

exposure of an image allowed for distinction of two protein bands near the size of monomeric Hcp in a Western blot analysis. The absence of a single protein band in the TssM1 mutant, BHimpL::pK18GGST, sample was intriguing (indicated by the arrow in Figure 17). It should be noted that the BHimpL::pK18GGST mutant was constructed by insertion of a plasmid resulting in disruption of all genes downstream of *azo1302* on that operon, potentially including *azo1305*, the Hcp protein TssD1 (Julia Herglotz, Diplomarbeit, 2007). Taken together with the predicted size of TssD1 being slightly larger than other Hcp proteins (Table 6), this was strong evidence that the band in question was TssD1.

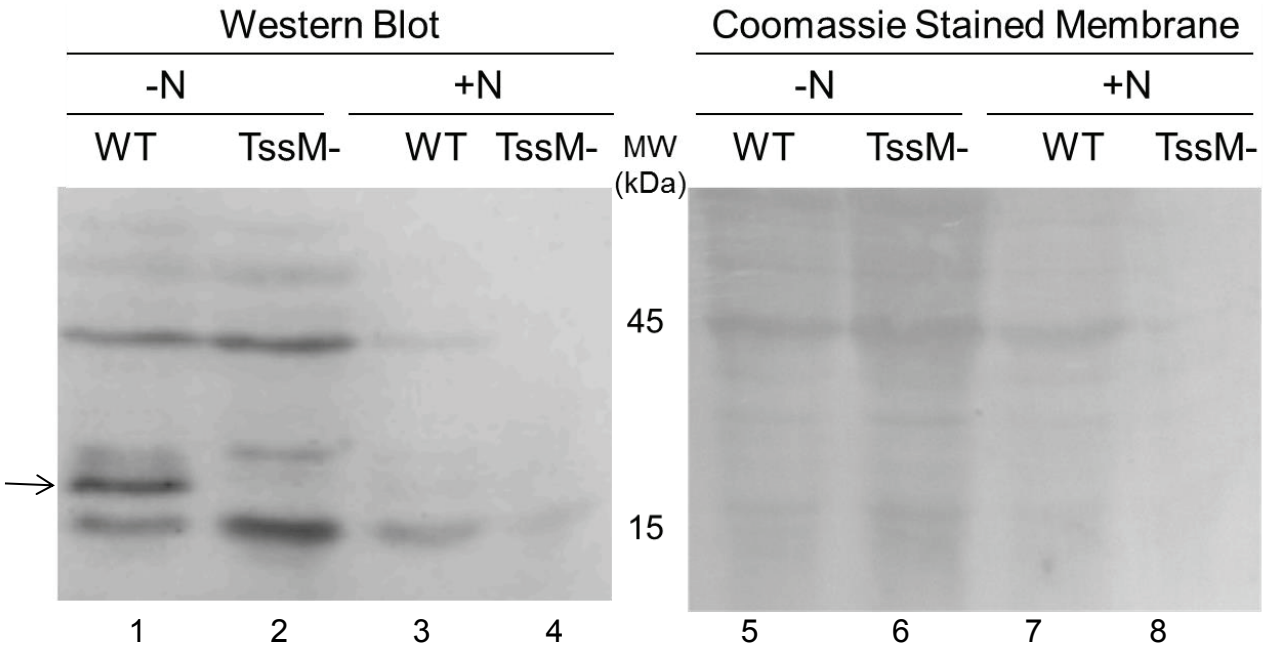


Figure 17. **Detection of Hcp in total cell protein fractions of *Azoarcus* sp. strain BH72 and BHimpL::pK18GGST**. Wild type strain BH72 cells (WT, lanes 1, 3, 5 and 7) or BHimpL::pK18GGST cells (TssM-, lanes 2, 4, 6 and 8) were grown in a bioreactor under nitrogen fixing conditions (0.6% O_2, pH 7.0, 37°C, 600 rpm) (-N, lanes 1, 2, 5 and 6) or grown aerobically and supplemented with 9.3 mM NH_4Cl to an OD_{578} of 0.8 (+N, lanes 3, 4, 7 and 8). Total cellular proteins was extracted from about 12 µg of cells (fresh pellet weight) resolved with a 12% polyacrylamide SDS-PAGE gel and blotted onto a nitrocellulose membrane. The Western blot was probed with a 1:3000 dilution of each anti-Hcp. Proteins bands were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher, Rockford, IL, USA). Protein amount loaded was controlled by staining the PVDF membrane with Coomassie Brilliant Blue (right). Protein band sizes were estimated using PageRuler™ Prestained Protein Ladder (Fermentas, ThermoFisher, Rockford, IL, USA) and indicated in kDa.

Confirming this band's identity with MALDI-TOF would have strengthened this argument, but this was not achieved. This would have been especially useful in determining which

Hcp(s) were detected in the supernatant. Although it was anticipated that the TssD1 band was the predominant protein band detected in Western blots of supernatant proteins from wild type cells grown under nitrogen fixing conditions, Western blots in Figure 16 and Figure 17 advocate otherwise. Expression of Azo1305 was likely interrupted in BHimpL::pK18GGST through a polar mutation and Figure 17 suggests that TssD1 is not expressed by BHimpL::pK18GGST. It is therefore unlikely that the single monomeric Hcp protein band secreted by BHimpL::pK18GGST in Figure 16 originated from T6SS-1. Interestingly, BHimpL::pK18GGST also secreted levels of Hcp similar to wild type in Figure 16. Throughout Western blot analyses in this study, only one protein band was typically detected in the supernatant, near in size to an Hcp monomer. This limited number of trials suggested that TssD1 (*azo1305*) was not typically the predominant Hcp protein detected in the supernatant.

III. C. 3. The TagE mutant, BHazo3888, was constructed by integration of plasmid DNA into the chromosome of Azoarcus sp. strain BH72.

Found encoded near the T6SS-2 gene cluster was the TagE (PpkA) homolog, Azo3888. It is believed that TagE is responsible for the phosphorylation of TagH (Fha), which in its phosphorylated form recruits ClpV. ClpV is believed to energize the T6SS secretion. This post-translational level of regulation is part of the threonine phosphorylation pathway and is well documented in *P. aeruginosa* PA01, but it is lacking in the majority of T6SSs (Mougous et al., 2007; Boyer et al., 2009). To determine if this TagE homolog functioned in the T6SS of *Azoarcus* sp. strain BH72, pK18GGST was inserted into *azo3888* for the construction of insertional mutant BHazo3888. This particular mutant did not lead to polar effects, since *azo3888* lies at the end of an operon. Technical work for the construction of the mutant BHazo3888 and its verification through Southern blot analysis was performed during a lab rotation by a former Master student (E. Disch, 2010). In this strategy a 551 bp portion of the *azo3888* (from bp 91-642) gene was amplified via PCR and cloned into pK18GGST using the added restriction sites *Xba*I and *Hind*III. After construction of the plasmid, pK18GGSTazo3888, it was transformed into *E. coli* S17-1 cells for biparental conjugation with *Azoarcus* sp. strain BH72. Transconjugants were picked based on their Km^R and verification of proper mutant construction was done through Southern blot analysis (Figure 18, Panel C).

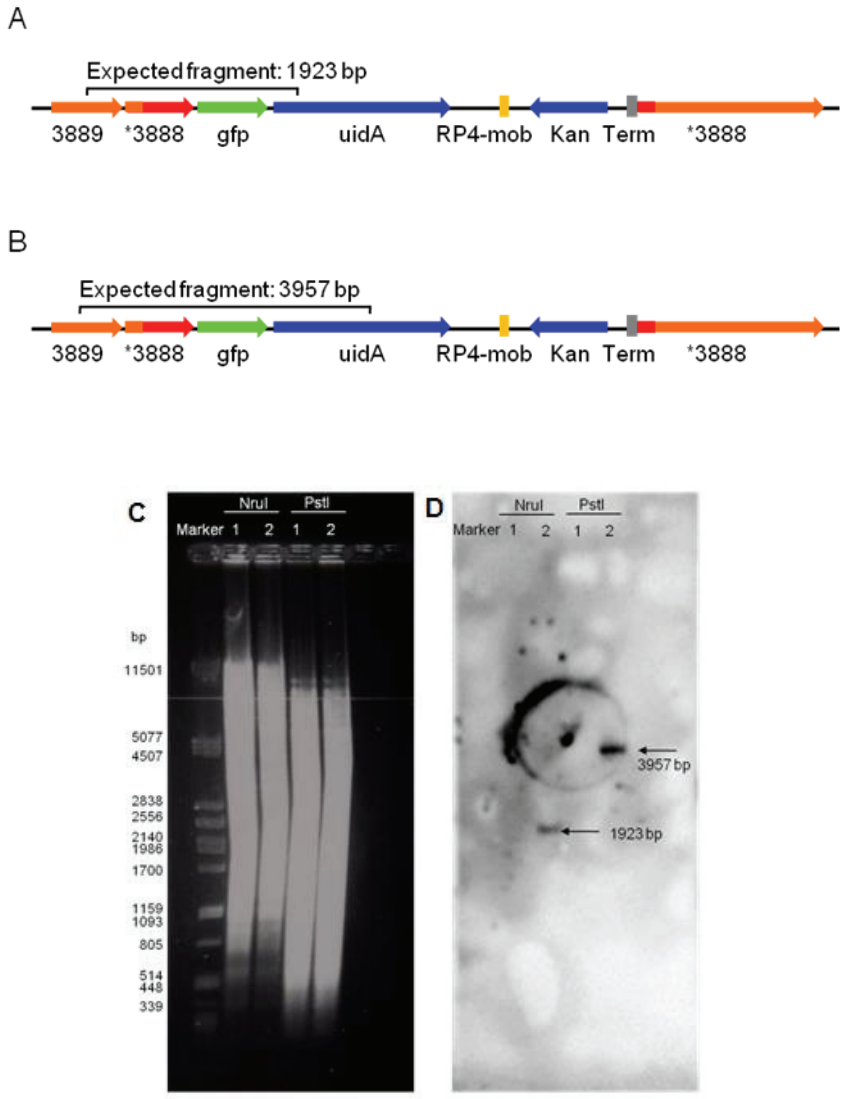
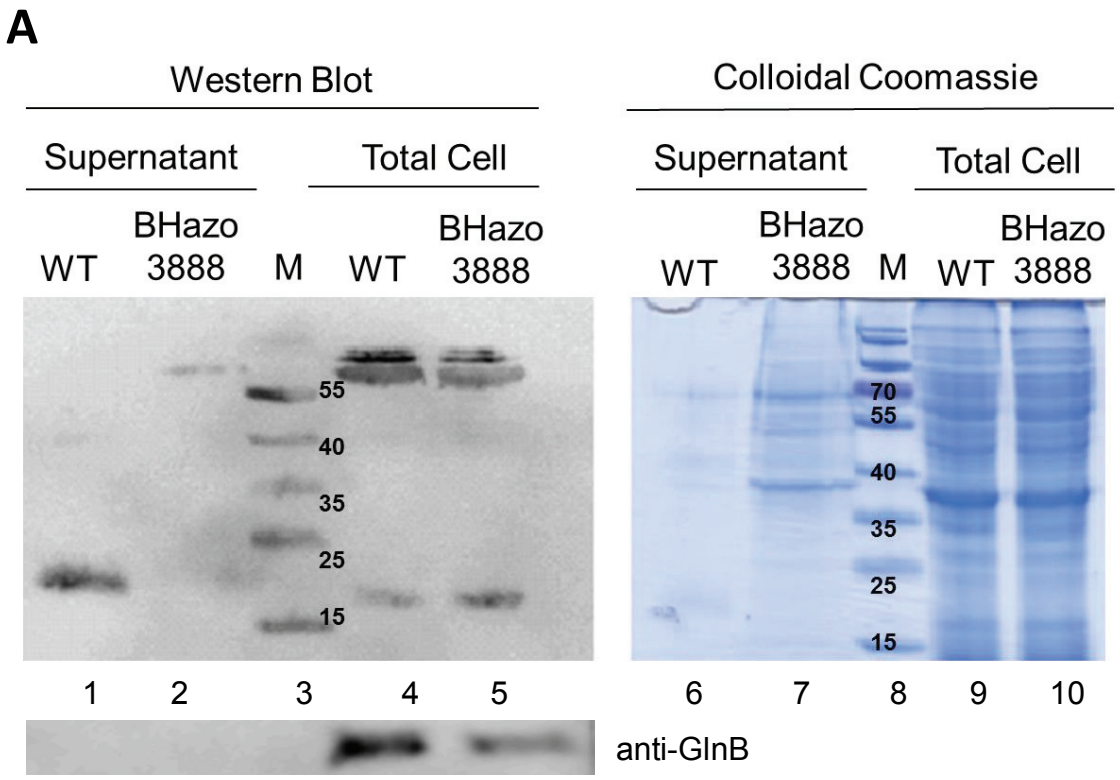


Figure 18. Southern blot analysis of mutant BHazo3888 (Figures from MSc student Eva Disch Lab Report, 2011) Depicted DNA fragment sizes expected after digestion of genomic DNA with restriction enzymes *Nru*I (A) and *Pst*I (B). (C) Ethidium Bromide stained agarose gel of digested genomic DNA extracted from wild type (1) and BHazo3888 (2) and a molecular size marker, *Pst*I digested lambda phage DNA (200 ng/µl) (D) Southern blot probed with DIG-labeled gfp-probe. Hybridized probe was detected by a 1:25,000 dilution of CDP-star Chemiluminescent Substrate (Sigma, Aldrich, St. Louis, MO, USA). Arrows indicate fragments of expected sizes.

III. C. 4. Detection of Hcp proteins in the supernatant of *Azoarcus* sp. strain BH72 cultures was dependent on T6SS accessory protein, TagE.

The expression and secretion of Hcp by BHazo3888 and wild type grown under non-fixing conditions (aerobically in SM+N media) were compared in a Western blot analysis over four independent trials. In Figure 19 a representative gel demonstrates that disrupting the TagE protein (BHazo3888) eliminated detection of Hcp in the supernatant, but expression was

evident from detection of Hcp in the total cell protein extracts (Figure 19, Panel A). Blots were later tested for GlnB (~12kDa) to assess the presence of cellular proteins in the supernatant. Similar results were seen when strains were grown in SM media using a bioreactor to maintain conditions for nitrogen fixation (Section IIB1b). In a single trial Hcp secretion by BHazo3888 was compared to secretion by BHΔRnf1, a strain that secretes Hcp at wild type levels. Also under nitrogen fixing conditions, Hcp was not detected in the supernatant of this BHazo3888 culture. Blots were later tested for NifH (~33kDa) to assess the presence of cellular proteins in the supernatant.



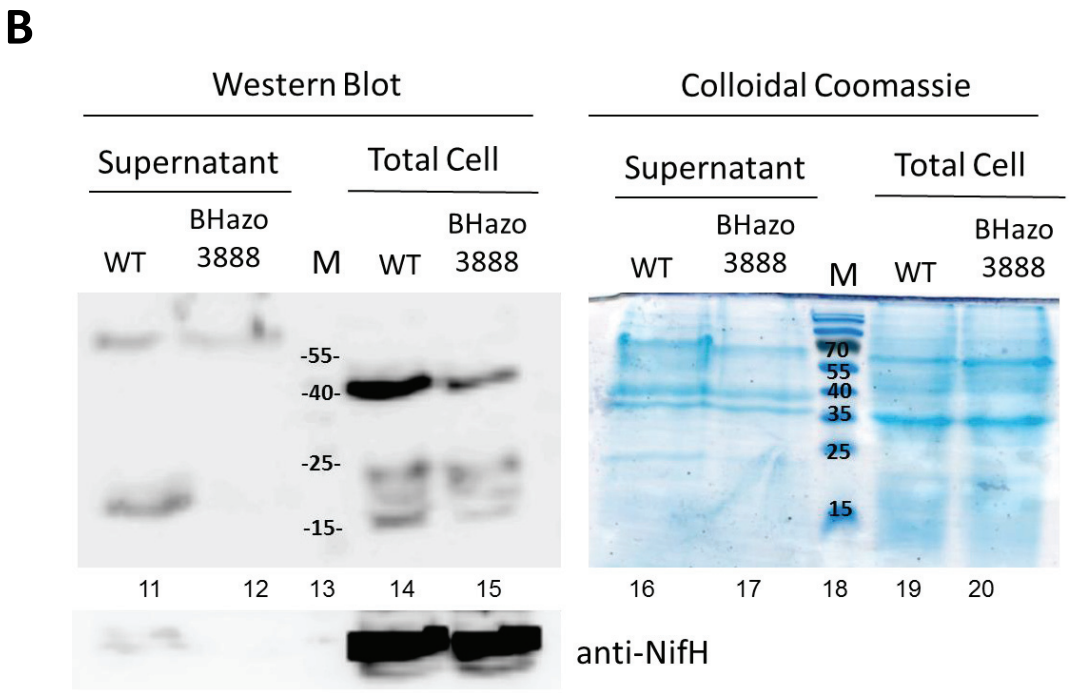


Figure 19. Western blot of supernatant and total cell protein fractions from *Azoarcus* sp. strain BH72 and BHazo3888 strains. **(A)** Wild type strain BH72 (WT, lanes 1, 4, 6 and 9) and BHazo3888 (lanes 2, 5, 7 and 10) strains were grown aerobically, overnight in SM+N media (SM medium supplemented with 9.3 mM NH_4Cl). Supernatant (lanes 1, 2, 6 and 7) and total cell (lanes 4, 5, 9 and 10) protein fractions were isolated. Supernatant protein samples were from about 20 ml of culture, and total proteins were from about 60 µg of cells (fresh weight pellet). **(B)** Wild type-like BHΔRnf1 (WT, lanes 11,14,16 and 19) and BHazo3888 (lanes 12, 15, 17 and 20) strains were grown to an OD_{578} of 0.8 in a bioreactor (SM medium, 37°C, pH 7.0, 600 rpm, 0.6% O_2). Proteins were isolated from about 20 ml supernatant (lanes 11, 12, 16 and 17) and about 60 µg of (fresh weight pellet) total cells (lanes 14, 15, 19 and 20). All samples were loaded and separated by 12% polyacrylamide SDS-PAGE gels and transferred onto nitrocellulose. Hcp was detected on Western blots with anti-Hcp serum. Anti-GlnB (Panel A) or anti-NifH (Panel B) controlled for cellular protein. SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher, Rockford, IL, USA) was used to detect protein bands. Quantities loaded were further controlled in parallel gels stained with Colloidal Coomassie Brilliant Blue Stain. Protein band sizes were estimated using PageRuler™ Prestained Protein Ladder (Fermentas, ThermoFisher, Rockford, IL, USA) and indicated in kDa.

III. C. 5. It could not be demonstrated that Hcp expression and secretion are contact dependent in *Azoarcus* sp. strain BH72.

Some organisms that utilize the threonine phosphorylation pathway in T6SS regulation are dependent on cell-cell or cell-surface contact for secretion (Schwarz et al., 2010b; Silverman et al., 2011; Bleumink-Pluym et al., 2013). To test if the growth on surfaces stimulated the T6SS in *Azoarcus* sp. strain BH72, cells were grown on Voll Media with Ethanol (VME) agar plates. To account for the possible loss of Hcp diffusing into the agar, cells were also grown in 2 ml of VME broth incubated on the glass bottom surfaces of stationary Erlenmeyer flasks. The relative amount of Hcp expressed and secreted by the surface grown cultures was compared to Hcp expression and secretion by cultures grown under standard laboratory

conditions in 100 ml liquid VME at 37°C with shaking at 200 rpm. In Figure 20 a representative Western blot of three independent trials demonstrates that no more or even less Hcp was detected in the supernatants of surface grown *Azoarcus* sp. strain BH72. The same was true for amount of Hcp detected in the total cell extracts. If the threonine phosphorylation pathway regulation was stimulated to secrete more Hcp, it was possible that less Hcp would be present in the total cell extracts. These relative amounts of Hcp still appeared to be similar. Components of the threonine phosphorylation pathway were not found linked to T6SS-1. The over expression or hyper-secretion of TssD1 (*azo1305*) by surface grown cultures was not anticipated. Surface stimulation of T6SS was still worth testing while the potential remained for T6SS interdependence, and disruption of TagE alone abrogated secretion of all Hcp. Interestingly, there was no sign of any Hcp protein expression or secretion being stimulated when cells were grown on surfaces compared to liquid grown cultures.

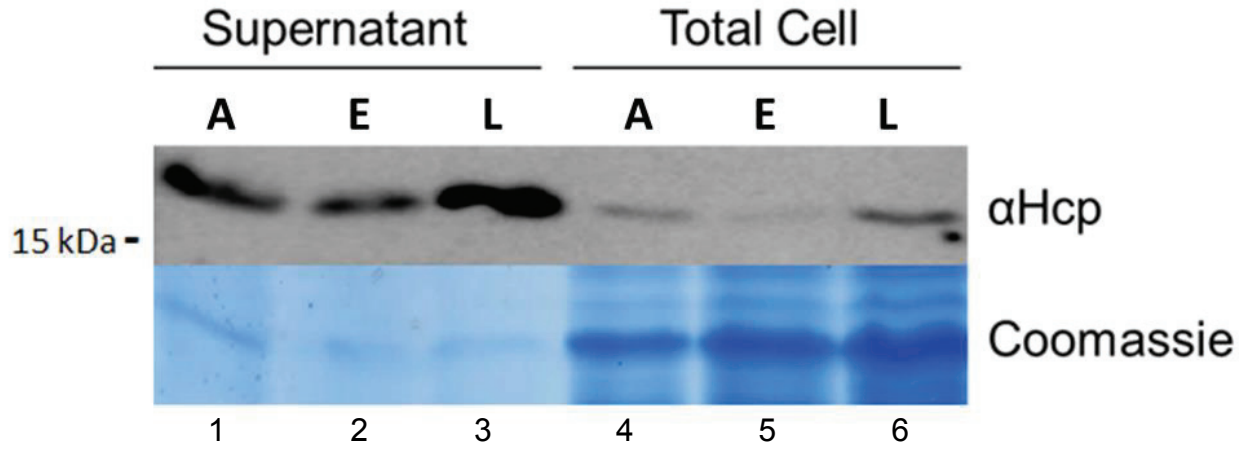
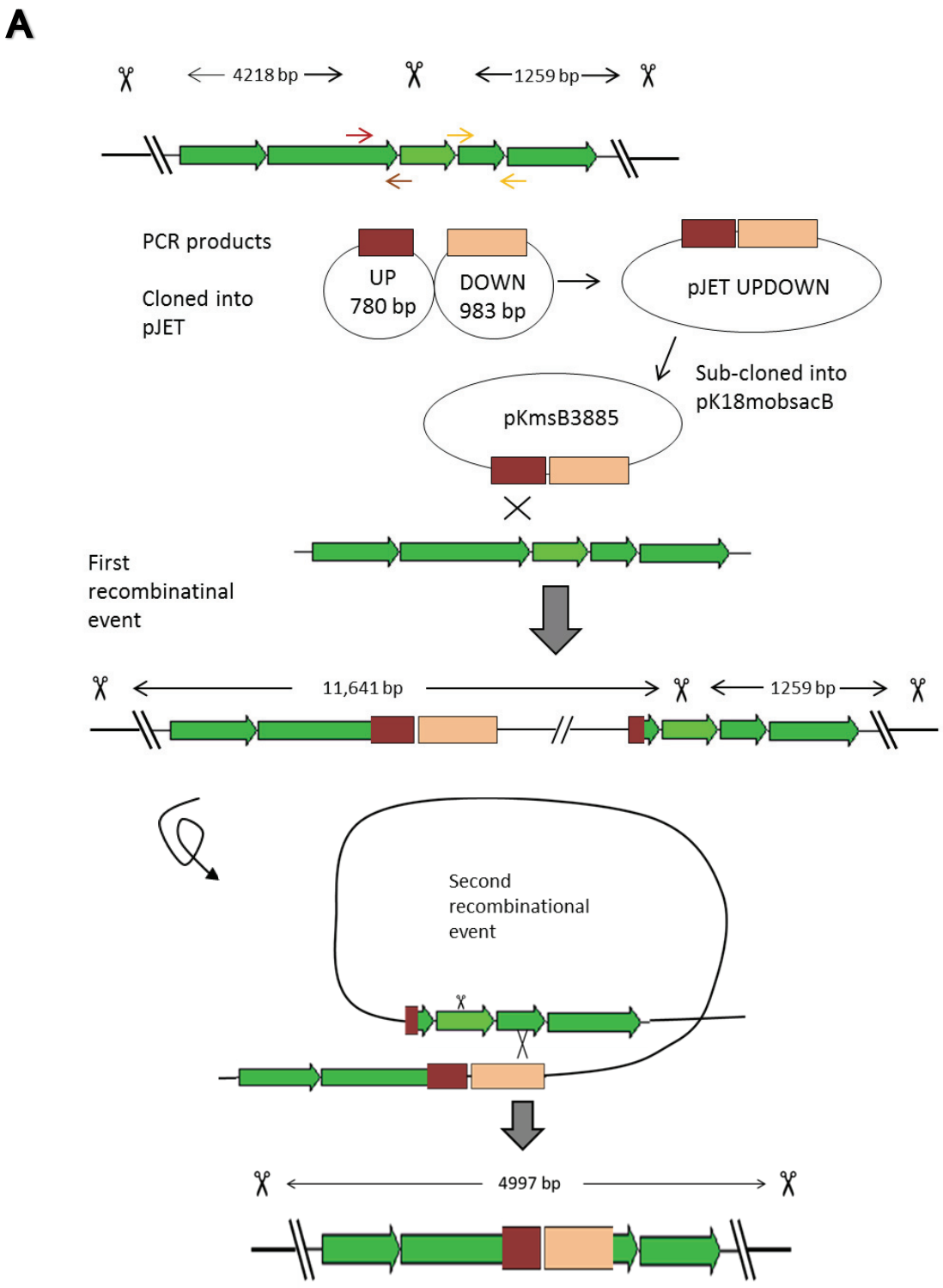


Figure 20. Hcp expression and secretion from surface grown and liquid grown cultures of *Azoarcus* sp. strain BH72. *Azoarcus* sp. strain BH72 cultures grown in VME were set to an OD_{578} of 0.5 and 1 ml was pipetted onto agar plates (A, lanes 1 and 4), 2 ml on the bottom of Erlenmeyer flasks (E, lanes 2 and 5) or 100 ml of liquid cultures shaking at 200 rpm (L, lanes 3 and 6) were incubated overnight at 37°C. Protein isolated from culture supernatants of about 6 mg of cells (pellet fresh weight) (lanes 1-3) and total cell fractions from about 240 µg of cells (pellet fresh weight) (lanes 4-6) were resolved on 12% polyacrylamide SDS-PAGE gels. Western blots were probed with the anti-Hcp. Protein bands were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher, Rockford, IL, USA). Quantities loaded in the SDS-PAGE gel were controlled by a parallel gel stained with Colloidal Coomassie Brilliant Blue Stain. Molecular weight was estimated using PageRuler™ Prestained Protein Ladder (Fermentas, ThermoFisher, Rockford, IL, USA).

III. C. 6. BHΔ3885 was constructed by making an in-frame deletion of *azo3885*.

Because the TagE (PpkA) mutant appeared to effectively eliminate secretion of Hcp, or detection of Hcp in the supernatant (Figure 19), this suggested that a mode of threonine phosphorylation pathway regulation occurred in *Azoarcus* sp. strain BH72. However, unlike the well-studied example of threonine phosphorylation pathway regulation in *P. aeruginosa* PA01, T6SS did not appear to be stimulated by surface contact (Figure 20). Despite this, the TagG (PppA) mutant, BHΔ3885, was constructed. In the threonine phosphorylation pathway, Fha is dephosphorylated by the serine/threonine phosphatase TagG. Ideally, mutation to TagG would keep Fha phosphorylated and T6SS in a constant 'on' state. Construction of BHΔ3885 was done as an in-frame deletion (207-687 bp of *azo3885*). PCR products were generated using Phusion polymerase (New England Biolabs, Ipswich, MA) and *Azoarcus* sp. strain BH72 genomic DNA as a template. The thermo cycler was programmed as follows: 3 min denaturation at 98°C followed by 35 cycles of 98°C for 10 sec, 30 sec at 64°C and 30 sec at 72°C followed by an additional 7 min of elongation at 72°C. A 780 bp upstream region (575 bp upstream-207 bp into gene *azo3885*) was PCR amplified with *Eco*RI and *Sac*I sites added to the 5' and 3' ends, respectively. A 983 bp downstream fragment (687 bp in-899 bp downstream of *azo3885*) was amplified with additional *Sac*I and *Xba*I sites at each end. Each amplified fragment was cloned into the CloneJET PCR cloning kit vector pJET1.2/Blunt (Fermentas, ThermoScientific, Waltham, MA, USA). The down fragment was then removed from its pJET vector and ligated into the pJET plasmid already containing the up fragment using the *Sac*I and *Xba*I restriction sites. The joined *azo3885* fragments, with a 480 bp internal deletion, were then sub-cloned into transfer vector pK18*mobsac*B using *Eco*RI and *Xba*I restriction sites (pK18msBH3885). The pK18msB3885 plasmid was transformed into *E. coli* S17-1 cells for donation of the plasmid to *Azoarcus* sp. strain BH72 in a biparental conjugation. Clones having undergone a single recombinational event were chosen based on their Km^R and picked and restreaked multiple times to ensure purity of the clone. A second recombinational event occurred during a 5 h outgrowth without antibiotics. After a double recombination, mutants were chosen based on their loss of Km^R and lack of sucrose sensitivity encoded by the pK18mobsacB vector. Verification of the mutant construction was done through Southern blot analysis (Figure 21, Panel B). Digestion of wild type DNA with *Sma*I yielded two bands, 4218 bp and 1259 bp. The 480 bp deletion internal to *azo3885* in BHΔ3885 resulted in the loss of a *Sma*I restriction site and yielded a single band of 4997 bp. DNA extracted from a strain carrying the first recombinational event

included cells that had undergone the second cross-over. The resulting 11641 bp band from the first recombinational event was detected in the third lane, but the 1259 bp band of the first event was not detected.



B

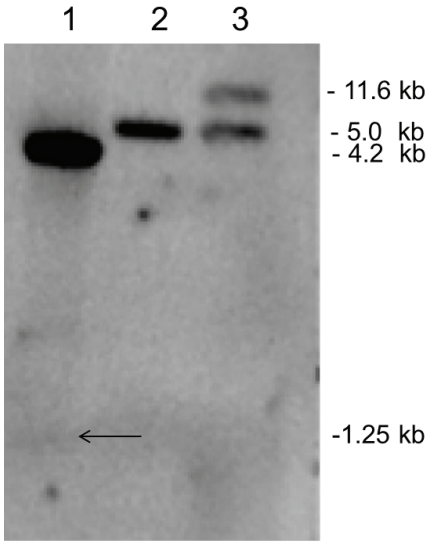


Figure 21. Construction and Southern blot analysis of BHΔ3885. (**A**) Schematic of the construction of the in-frame deletion mutant BHΔ3885. Chromosomal DNA is depicted in green and the light and dark brown are PCR fragments amplified from genomic DNA. Restriction sites are indicated by scissors above the DNA. (**B**) In a Southern blot analysis, 3 µg genomic DNA from *Azoarcus* sp. strain BH72 (lane 1), BHΔ3885 (lane 2) or transconjugant from the first recombinational event (lane 3) was digested with *Sma*I. DNA fragments and molecular size marker, *Pst*I digested lambda phage DNA (200 ng/µl) were separated by agarose gel electrophoresis, transferred to a nylon membrane and probed with DIG-labeled up and down fragments. Hybridized probe was detected using CDP-star Chemiluminescent Substrate (1:25,000) (Sigma, Aldrich, St. Louis, MO, USA). The expected band sizes are indicated in kilobases (kb) on the right.

III. C. 7. Initial findings indicated hyper-secretion of Hcp (TssD) proteins by BHΔ3885.

In agreement with the threonine phosphorylation pathway, disruption of TagG (*azo3885*) appeared to increase secretion of Hcp. Due to time constraints only limited assessment of this mutant could be done. In 1 L flasks, 50 mL of VME was inoculated with wild type or BHΔ3885, reaching an OD_{578} of 0.75 and 0.83, respectively, after 6 h. In Figure 22 the Western blot analysis (Section IIB5) demonstrates a greater amount of Hcp detected in the supernatant of BHΔ3885 (lane 2) cultures than wild type (lane 1). More Hcp was secreted by the TagG mutant when grown in VME, likely due to its inability to dephosphorylate TagH (Fha), keeping TssH (ClpV) continually active, leaving the T6SS in a constant 'on' state (Mougous et al., 2007). It was next decided to test the hyper-secreting activity under nitrogen fixing conditions.

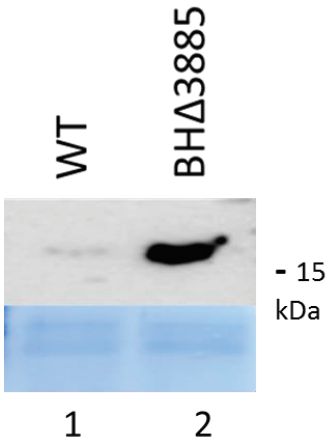


Figure 22. Analysis of Hcp secretion by *Azoarcus* sp. strain BH72 and BHΔ3885 when grown in VME. Wild type strain BH72 (WT) or BHΔ3885 reached an OD_{578} of 0.75 or 0.83 after 6 h growth in VME. Supernatant proteins from about 20 ml cultures of WT (lane 1) or BHΔ3885 (lane 2) were separated on a 12% polyacrylamide SDS-PAGE gel. The Western blot was probed with anti-Hcp. SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher, Rockford, IL, USA) was used for protein band detection. Loading was controlled by a parallel gel stained with Colloidal Coomassie Brilliant Blue (below). Protein band size was estimated using PageRuler™ Prestained Protein Ladder (Fermentas, ThermoFisher, Rockford, IL, USA).

Nitrogen fixing conditions promoted expression of T6SS-1 genes and T6SS activity in *Azoarcus* sp. strain BH72. Therefore, it was worthwhile to test the expression and secretion of Hcp by BHΔ3885 under nitrogen fixing conditions. Time constraints and limited availability of equipment allowed for only one attempt at growing cultures using a bioreactor (Section IIB1b), which was the most reliable method for maintaining constant conditions to promote nitrogen fixing. Wild type strain BH72 and BHΔ3885 were grown to an OD_{578} of 0.8 and 1.0, respectively. Supernatant proteins were harvested (Section IIB4d), and samples from equal volumes of culture supernatant were compared in Western blot analysis (Section IIB5). In this trial, the TagG mutant (BHΔ3885) appeared to secrete more Hcp than wild type (Figure 23). Total cell proteins extracted (Section IIB4e) from cell pellets of equal mass were also examined in a Western blot analysis. More Hcp was detected in the cellular fraction of BHΔ3885, but the Colloidal Coomassie Brilliant Blue stained gel below revealed that there was also a greater amount of total protein loaded in this sample. More trials must be done to confirm these results. Moreover, under nitrogen fixing conditions, additional larger bands were detected in the supernatant by the Hcp antibodies. Possible contamination of the supernatant with cellular proteins was not tested for in this trial.

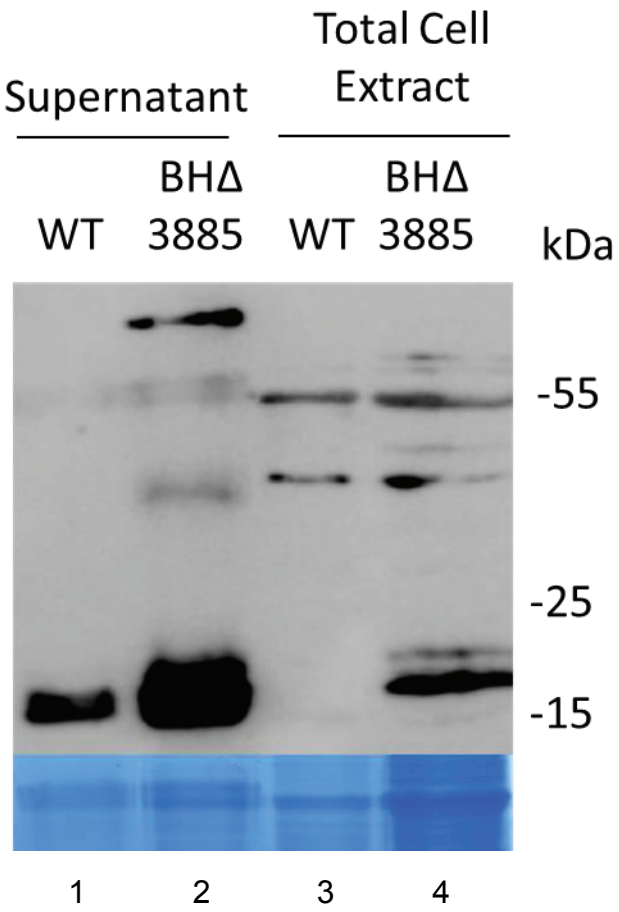
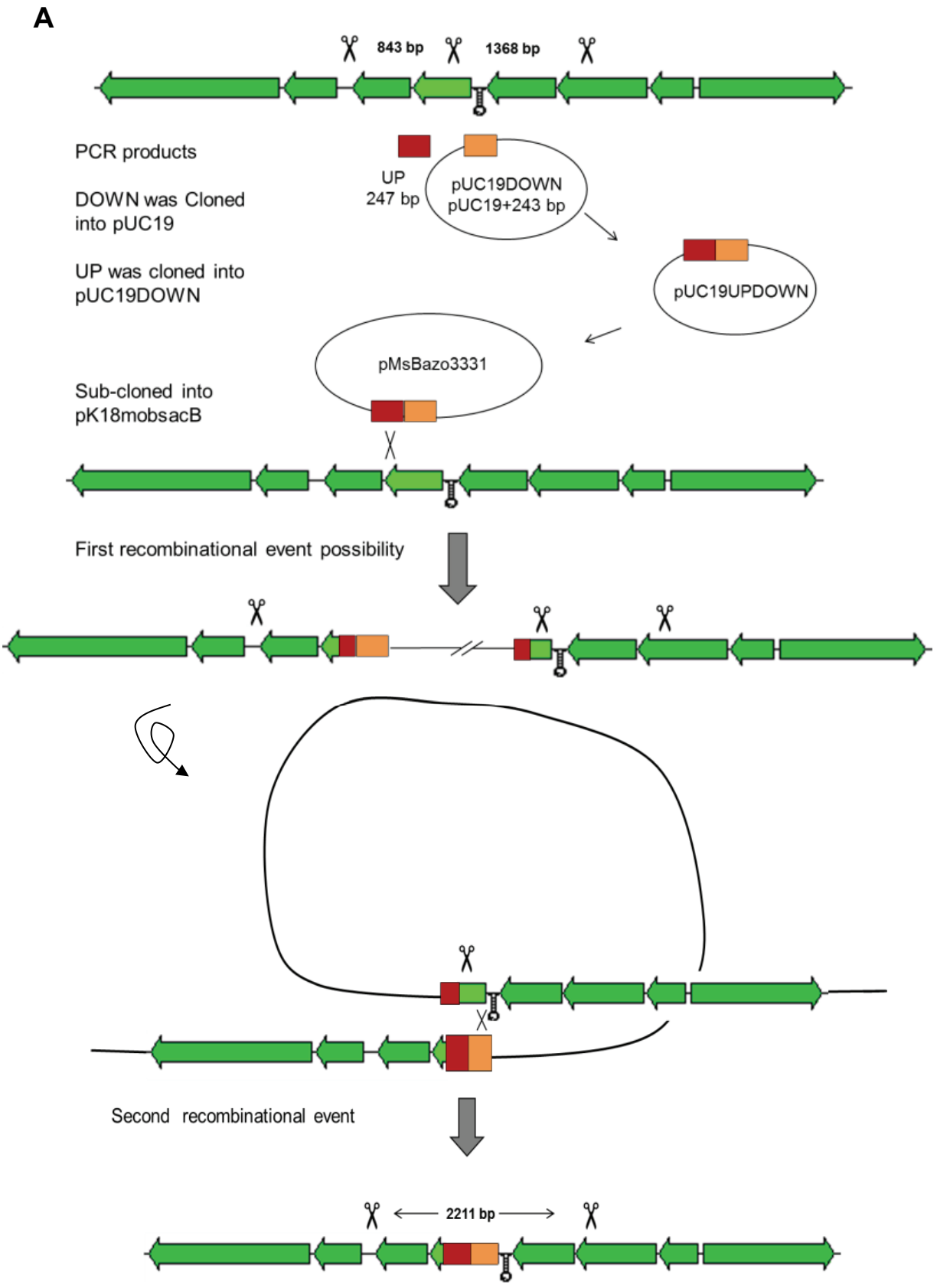


Figure 23. Western blot analysis of Hcp secretion by *Azoarucs* sp. strain BH72 and BHΔ3885 when grown under nitrogen fixing conditions. Wild type strain BH72 (WT, lanes 1 and 3) and BH Δ3885 (lanes 2 and 4) were grown to an OD_{578} of 0.8 and 1.0, respectively, in a bioreactor (37°C, pH 7.0, 600 rpm, 0.6% O_2). Supernatant proteins from 20 ml culture (lanes 1 and 2) and total cell fractions from about 60 µg of cells (pellet fresh weight) (lanes 3 and 4) were prepared, loaded onto 12% polyacrylamide SDS-PAGE gels and separated. The Western was probed with anti-Hcp. Lumi-Light^{PLUS} western blotting substrate (Roche, Rockford, IL, USA) was used to detect protein bands. A parallel gel stained with Colloidal Coomassie Brilliant Blue Stain controlled loading of proteins. Protein band sizes were estimated using PageRuler™ Prestained Protein Ladder (Fermentas, ThermoFisher, Rockford, IL, USA).

III. C. 8. BHΔ3331 was constructed by making an in-frame deletion of *azo3331*.

The putative phosphoprotein phosphatase Azo3331 was also assessed for its role in Hcp secretion. Before completing construction of an in-frame deletion mutant and testing its T6SS phenotype, a bioinformatic approach was taken to look for an alternative phosphatase similar to PppA of *P. aeruginosa*. Using blastp, azo3331 was identified as having greater identity to *pppA* and was linked to an Fha encoding gene. Despite its distance from both T6SSs, it was determined to be worthwhile to test if an deletion of *azo3331* resulted in a hyper-secreting

mutant. BHΔ3331 was constructed as an in-frame deletion. To do this, a downstream fragment was amplified via PCR with additional *Bam*HI and *Hin*dIII restriction sites on the 5' and 3' end, respectively. These restriction sites were used to clone the PCR fragment into the pUC19 vector. The upstream fragment was also amplified via PCR with an additional *Eco*RI site at the 5' end and an additional *Bam*HI site at the 3' end and ligated into the pUC19 vector already containing the downstream fragment, utilizing the *Eco*RI and *Bam*HI restriction sites. The joined up and down fragments with a 396 bp deleted segment were subcloned into the pK18*mobsac*B vector, using *Eco*RI and *Hind*III sites, creating pK18msB3331, which was eventually transformed into *E. coli* S17-1. The S17-1 strain was used to donate the pK18msB3331 vector to strain BH72 in a biparental conjugation. Mutants having undergone a single recombinational event were chosen based on their Km^R and amplification of the correct size of a product in colony PCR. These were then passaged to purify the clone before a 5 h outgrowth without antibiotic to allow for a second recombinational event. These in-frame deletion mutants were selected for their lack of sensitivity to sucrose and loss of Km^R. After picking and replating the cells to guarantee purity, a Southern blot analysis was performed to verify correct integration (Figure 24, Panel B). Digestion of wild type DNA with *Pst*I yielded two bands, 843 bp and 1368 bp. The 396 bp deletion internal to *azo3331* in BHΔ3331 results in the loss of an internal *Pst*I restriction site and yields a single band of 2211 bp.


A
843 bp
1368 bp
PCR products
UP
247 bp
pUC19DOWN
pUC19+243 bp
DOWN was Cloned into pUC19
UP was cloned into pUC19DOWN
pUC19UPDOWN
pMsBazo3331
Sub-cloned into pK18mobsacB
First recombinational event possibility
Second recombinational event
2211 bp

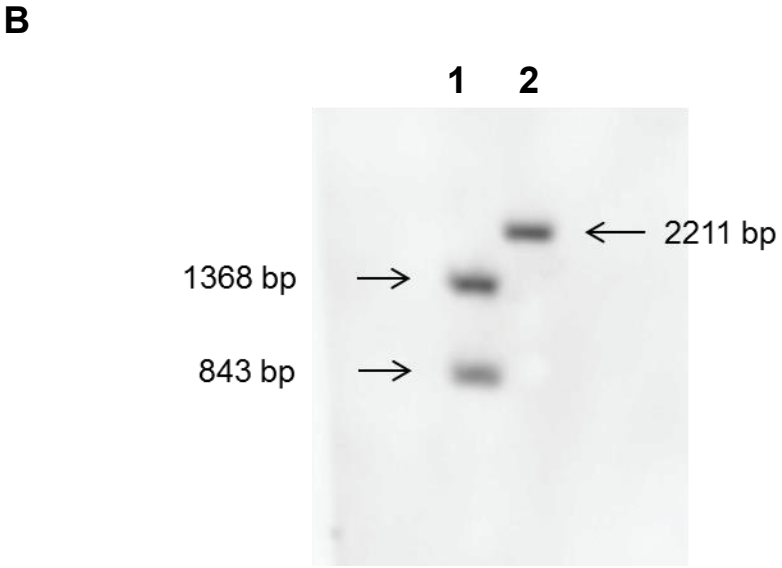


Figure 24. Construction and Southern blot analysis of BHΔ3331. (**A**) Schematic of the construction of BHΔ3331 is illustrated in Panel A. Wild-type DNA is depicted in green and the light and dark brown are Up and Down fragments of PCR amplified from wild-type genomic DNA. *PstI* restriction sites are indicated by scissors above the DNA. (**B**) Chromosomal DNA extracted from wild type strain BH72 (lane 1) and BHΔ3331 (lane 2) was digested with *Pst*I. DNA fragments were separated using electrophoresis of a 1% agarose gel and transferred to a membrane. Southern blots were probed with DIG-labeled probe specific for *azo3331* and detected with CDP-star Chemiluminescent Substrate (1:25,000) (Sigma, Aldrich, St. Louis, MO, USA). The expected band sizes are indicated by arrows.

III. C. 9. BHΔ3331 did not display a hyper-secreting phenotype.

Expression and secretion of Hcp by BHΔ3331 was compared to wild type *Azoarcus* sp. strain BH72 using Western blot analysis. Proteins were extracted from the supernatant, and total cells (Section IIB4d-e) of cultures were grown overnight in 200 ml of SM+N media. Over three independent trials, it was seen that there was no difference in the relative expression or secretion of Hcp. A representative gel can be seen in Figure 25. The phosphatase activity of Azo3331 did not appear to participate in the secretion of Hcp by *Azoarcus* sp. strain BH72.

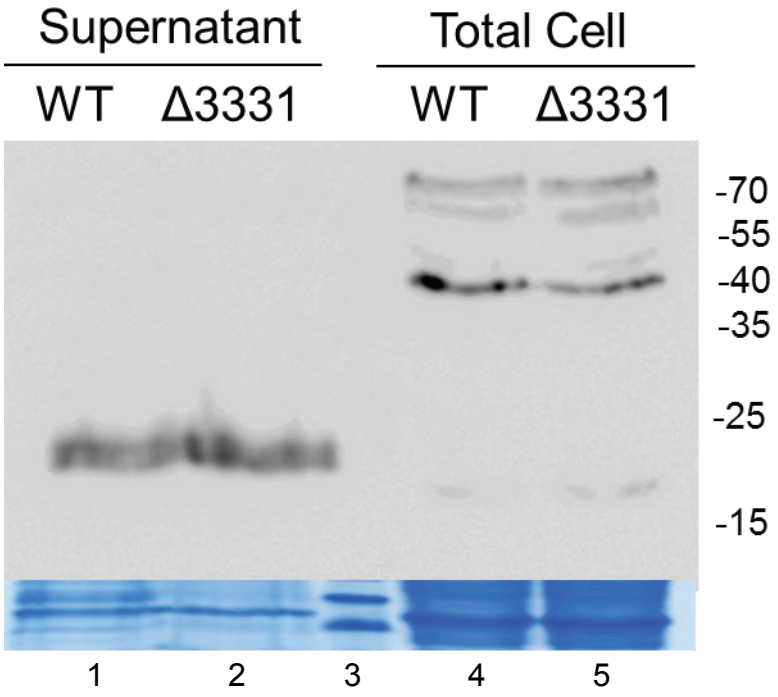


Figure 25. Western blot analysis of Hcp secretion by *Azoarcus* sp. strain BH72 and BHΔ3331 cultures grown under condtions promoting nitrogen fixation. *Azoarcus* sp. strain BH72 (WT, lanes 1 and 4) and BHΔ3331 (Δ3331, lanes 2 and 5) were grown overnight in 200 ml of SM+N medium at 37°C with shaking at 200 rpm to an OD_{578} of 4. Supernatant proteins from 20 ml of culture (lanes 1 and 2) and total cellular proteins from about 60 µg of cell pellet (lanes 4 and 5) were loaded and separated on a 12% polyacrylamide SDS-PAGE. Proteins were transferred onto nitrocellulose. Western blots were probed with anti-Hcp serum. SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher, Rockford, IL, USA) was used to detect protein bands. A parallel gel stained with Colloidal Coomassie Brilliant Blue Stain controlled equal loading. Protein band sizes were estimated using PageRuler™ Prestained Protein Ladder (lane 3) (Fermentas, ThermoFisher, Rockford, IL, USA).

III. C. 10. BHazo3903 was constructed by integration of plasmid DNA into the chromosome of *Azoarcus* sp. strain BH72.

Clp proteins belong to the Hsp100/Clp family of proteins, which are part of the AAA+ superfamily and energize their disassembling of proteins through ATP binding and hydrolysis. However, that does not appear to be the case for ClpV (Schlieker et al., 2005). ClpV (TssH) is considered a core component of T6SSs, and mutation has been shown to eliminate T6S (Bönemann et al., 2009). To test the role of the predicted ClpV protein in *Azoarcus* sp. strain BH72, an Azo3903 mutant was constructed by a former master student via insertion of the pK18GGST plasmid, BHazo3903 (Eva Disch, Lab Report, 2010). The ClpV (TssH) mutant, BHazo3903, was constructed as a polar mutant by the insertion of pK18Gazo3903 into the gene though a single recombinational event. A 564 bp portion of the

gene was amplified via PCR with additional *Xba*I and *Hind*III sites for direct cloning into the multiple cloning site of pK18GGST. This plasmid was amplified in *E. coli* DH5α and then transformed into *E. coli* S17-1. In a biparental conjugation the plasmid was donated to *Azoarcus* sp. strain BH72. After a single recombinational event, clones were chosen based on their Km resistance. These were picked and replated to ensure purity of the clone. Genomic DNA was extracted from BHazo3903 and PCR amplified using a vector specific primer, GFP OUT, and the 3903(560)R primer, and this product was sequenced. In this study the Southern blot analysis was performed to confirm proper integration of the plasmid into the chromosome of *Azoarcus* sp. strain BH72 (Figure 26, Panel B).

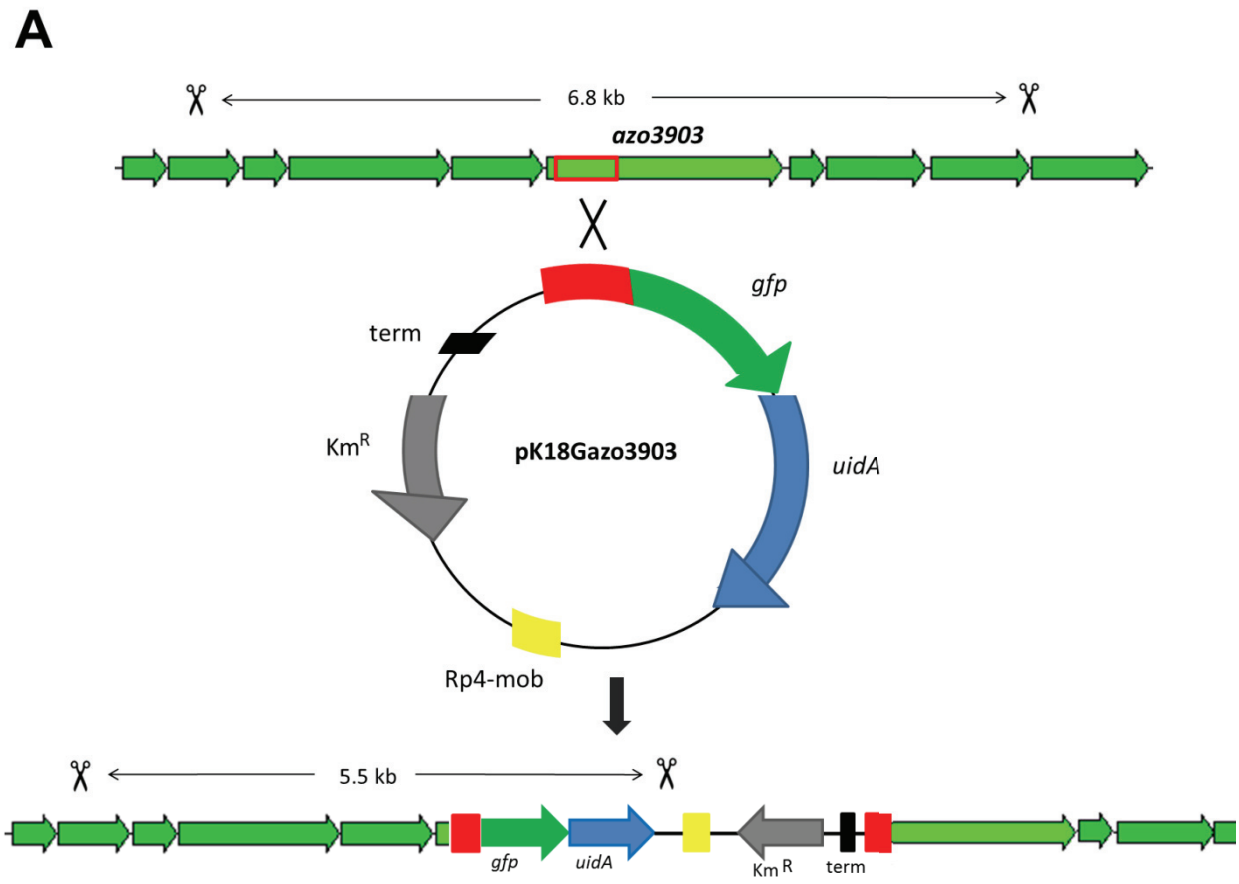


Figure 26. Construction and Southern blot analysis of BHazo3903. (A) Schematic of the methods used to construct BHazo3903. Wild type chromosomal DNA is depicted in green and the pK18GGST plasmid is depicted with various colors representing different genes (red=PCR amplified product from *azo3903*; dark green=*gfp*; blue=*uidA(gusA)*; gray=Km^R; black=transcriptional terminator; yellow=Rp4 mobilization genes). **(B)** Southern blot of genomic DNA extracted from BHazo3903 and digested with *Nru*I (restriction sites marked by scissors). DNA fragments were detected by a DIG-labeled *gfp* probe. Hybridized probe was detected by a 1:25,000 dilution of CDP-star Chemiluminescent Substrate (Sigma, Aldrich, St. Louis, MO, USA).

III. C. 11. Detection of Hcp (TssD) proteins in the supernatant was not eliminated in BHazo3903 cultures.

It was anticipated that disrupting expression of the ClpV protein would eliminate Hcp secretion. To test this, BHazo3903 and wild type cultures were grown in 200 ml of SM+N media, and proteins from supernatant and total cell fractions were compared to those of wild type strain BH72. A representative Western blot of three independent trials can be seen in Figure 27. Unexpectedly, BHazo3903 appeared to secrete more Hcp than wild type *Azoarcus* sp. strain BH72. However, despite practicing the standard methods for Western blot analysis used in this study, detection of Hcp proteins was very poor in every trial. Furthermore, many supernatant proteins identified using MALDI-TOF analysis were non-secreted, cytoplasmic proteins (e.g. elongation factors, chaperonin GroES, a σ^{54} modulation protein, an isomerase and a nucleoside diphosphate kinase). The supernatant of BHazo3903 cultures was littered with non-secreted cellular proteins. The apparent hyper-secreting phenotype was likely attributed to cellular contamination.

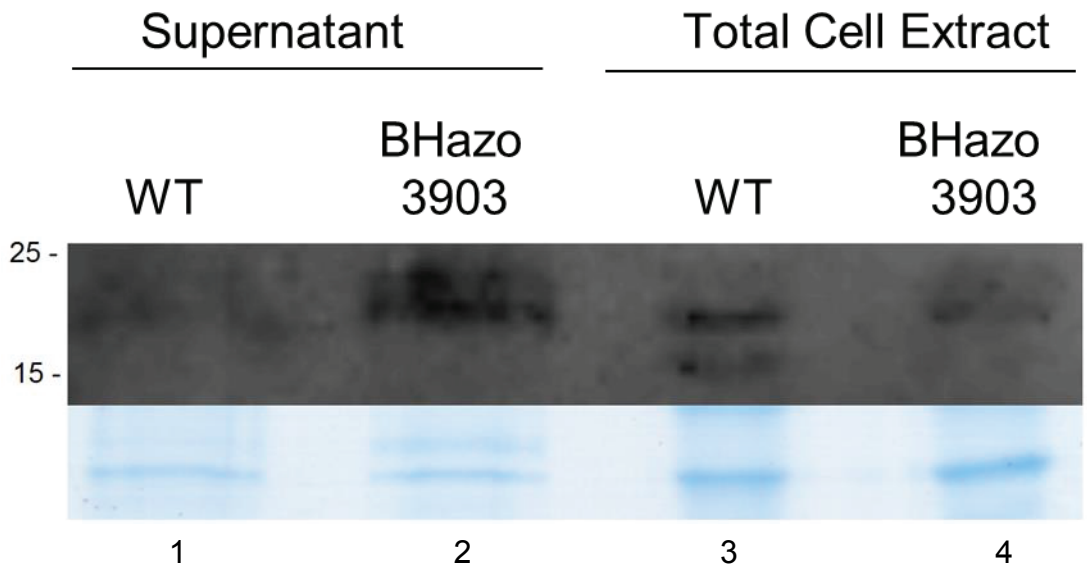


Figure 27. Western blot analysis of Hcp secretion by BHazo3903. Cultures of wild type *Azoarcus* sp. strain BH72 (WT, lanes 1 and 3) andBHazo3903 were grown overnight in 200 ml of SM+N media at 37°C with 200 rpm shaking. Supernatant proteins from 20-40 ml of culture (lanes 1 and 2) and total cell (~120 μg cell pellet) protein extracts (lanes 3 and 4) were separated on a 12% SDS-PAGE gel and transferred onto nitrocellulose. Western blots were probed with anti-Hcp. SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher, Rockford, IL, USA) was used to detect protein bands. A gel was run in parallel and stained with colloidal coomassie blue to control loading. Protein band sizes were estimated using PageRuler™ Prestained Protein Ladder (Fermentas, ThermoFisher, Rockford, IL, USA).

III. D. The *Azoarcus* sp. strain BH72 secretome was compared to the secretome of a T6SS deficient strain, BHΔimpLsciO.

Hcp is not believed to be the secreted effector protein in T6SSs. Its presence in the supernatant was an indicator of a functional T6SS, but only as a consequence of it, along with VgrG (TssI), being the extracellular components of the apparatus. In this study, turning off both T6SSs was done primarily to allow the comparison of wild type supernatant proteins to supernatant proteins from a strain where the T6SSs were turned off. Ideally, comparison of supernatants from an 'off' strain would be compared to a 'hyper-secreting' strain, but construction of a PppA-like, TagG mutant (BHΔ3885) was not completed in time for a secretome analysis. For the secretome analysis, *Azoarcus* sp. strain BH72 was grown under nitrogen fixing conditions and the supernatant proteins harvested. The double TssM mutant, BHΔimpLsciO, was grown under the same conditions and supernatant proteins harvested.

A Western blot analysis done prior to sending samples for LC-MS/MS analysis controlled that supernatants did not contain cytoplasmic proteins. In Figure 28 cytoplasmic NifH was not detected in the supernatant of BHΔimpLsciO or wild type cultures in the Western blot analysis, and Hcp was detected in the wild type culture supernatants.

Figure 28. Supernatant protein samples sent for LC-MS/MS analysis were tested for the presence of cytoplasmic NifH and Hcp. Wild type (lanes 1-4) and BHΔimpLsciO (lanes 5-8) strains were grown in 2 L of SM media to an OD_{578} of 0.8 in a bioreactor (37°C, pH 7.0, 600 rpm, 0.6% O_2). Supernatant proteins were precipitated from supernatants and prepared as described and resuspended in 500 µl of 8 M urea and 2 M thiourea buffer. Protein from 5 µl of supernatant protein suspensions was loaded and separated on 12% polyacrylamide SDS-PAGE gels and blotted onto nitrocelluse. The upper membrane was probed with a 1:3000 dilution (1% BSA; 1X TBS) of anti-NifH. The lower blot was subjected to an additional blocking step of 10 min with biotin (1:1000, 1X PBS, 0.1%(v/v) tween 20) before anti-Hcp (lower membrane). Lumi-Light^{PLUS} western blotting substrate (Roche, Rockford, IL, USA) was used to detect protein bands. Protein band sizes were estimated using PageRuler™ Prestained Protein Ladder (Fermentas, ThermoFisher, Rockford, IL, USA).

Samples were sent to the Laboratory of Uwe Völker at the Ernst-Moritz-Arndt-Universität Greifswald for LC-MS/MS analysis by PhD student, Praveen Kumar. Samples were prepared as described in Section IIB4d for isolation of supernatant proteins but were resuspended in a solution of 8 M urea and 2 M thiourea instead of the normal Protein Suspension Buffer. In Greifswald, protein sample concentrations were estimated using a Bradford Assay, and 5 µg was digested with trypsin for 16 h after being reduced and alkylated. Digested peptides were desalted and purified by C18 columns and reconstituted in 2% ACN and 0.1% acetic acid. From this, 400 ng was injected into Proxeon-LC coupled with Thermo LTQ Velos-Orbitrap. Files obtained were searched against NCBI database of *Azoarcus* using a Sequest search engine and a list of 405 identified proteins was compiled, which is 10% of the 3992 protein sequences predicted to be encoded by the *Azoarcus* sp. strain BH72 genome (Krause et al., 2006). Protein presence in the secretome was quantified by looking at the abundance of peptides identified unique to a given protein. Four independent trials of harvesting secretome

proteins from BHΔimpLsciO and from wild type were analyzed separately. For each protein, the value for abundance listed was determined by taking the average of the four trials.

Some of the most abundant proteins in the supernatant were cellular proteins, such as elongation factor Tu, nitrogenase Mo-Fe protein, chaperone proteins and enzymes involved in metabolism. Therefore, several proteins identified probably arose from contamination of the supernatant fraction with cytoplasmic contents of lysed cells. Presence of cytoplasmic proteins in the supernatant made interpreting the secretome data problematic. It was impossible to identify proteins as truly secreted or cytoplasmic, and the identity of any effector protein(s) was masked. However, the data that was obtained is described below.

III. D. 1. The majority of T6SS proteins detected in the supernatant were from T6SS-2.

Eight proteins encoded by the T6SSs were present in the secretome and are listed in Table 7. Additionally, two proteins found neighboring the T6SS gene clusters are listed. Proteins from T6SS-2 were the most represented, including TssD2 and TssD3 (*azo3897* and *azo3898*), TssB2 and TssC2 (*azo3895* and *azo3896*) and TssI2 (*azo3876*). This was surprising considering cultures used here were grown under nitrogen fixing conditions. TssD1 was the only core component of T6SS-1 present in the supernatant. Azo1301 was also found in the supernatant and is encoded by the T6SS-1 gene cluster, but it is not considered a core component. As briefly discussed above, it is a putative peptidase. A potential role of Azo1301 in T6S is possibly peptidoglycan degradation.

Table 7. List of proteins detected in an LC-MS/MS analysis of wild type (WT) and BHΔimpLsciO (DD) culture supernatants that are encoded within or near the T6SS gene clusters found in the *Azoarcus* sp. strain BH72 genome.

T6SS-1 Proteins	**DD†**	**WT†**	**Gene Number**
TssD1 (Hcp)	1,81	2,66	azo1305
Peptidase	0,2675	0,725	azo1301
T6SS-2 Proteins			
TssD2 (Hcp)	5,205	42,3925	azo3897
TssD3 (Hcp)	0	0,705	azo3898
TssB2	0	0,9775	azo3895
TssC2	5,1875	6,0275	azo3896
TssI2 (VgrG)	0	5,0675	azo3876
Proteins proximal to T6SSs			
Periplasmic	2,63	1,43	azo3886
Tim44	1,83	2,17	azo1318

†Abundances given are the number of peptides identified unique to a given protein

Most T6SS components are cytoplasmic and not expected to be found in the supernatant. TssB2 and TssC2 were probably identified due to contamination of the supernatant with cytoplasmic proteins and because of the high quantity needed for tubule sheath formation. TssB1 and TssC1 were not identified in the supernatant samples. As an exterior component of the T6SS, TssI (VgrG) proteins were expected to be present in the supernatant. TssI2, encoded near the T6SS-2 gene cluster, was the only VgrG protein that appeared in the supernatant. TssI1, a VgrG homolog (*azo1307*) encoded by the T6SS-1 gene cluster, was missing in the supernatant, as was the third VgrG homolog, Azo3470, encoded outside of both T6SS gene clusters.

Azo3886 is not encoded within a T6SS gene cluster, but *azo3886* likely shares an operon with *azo3884* (TagH) and *azo3885* (TagG) and is one of only two genes between genes encoding for TagE and TagG. Azo3886 was predicted to be a hypothetical protein with an unknown function. Using InterProScan sequence search (EMBL-EBI) a glycine-zipper containing OmpA-like membrane domain (PF13488) was predicted. In a blastp search (NCBI), the Azo3886 sequence showed the highest degree of similarity to mostly other hypothetical proteins, but also with an OmpA family protein in *E. coli*. It was more abundant in BHΔimpLsciO cultures than wild type, which was contrary to effector search criteria. Testing the significance of this difference with a two-tailed paired t-test gave a p-value of 0.09. This was the only T6SS associated protein found to be more abundant in the secretome of the BHΔimpLsciO than the wild type, and because of this, it was doubtful that Azo3886 was the secreted substrate of either T6SSs. Nonetheless, it remains tempting to question if Azo3886 is a yet unnamed accessory component of T6SSs.

Azo1318 is not a component of the T6SS, but it was included on the list because of the close proximity to T6SS-1, just six genes downstream. Azo1318 encodes for a Tim44-related protein with three transmembrane segments with high protein sequence similarity to Tim44 proteins of other *Azoarcus* spp., such as *A. toluclasticus* and *A.* sp. KH32C. Protein sequences from several *Burkholderia* spp. and *Leeia oryzae* were also identified in the database to have a high degree of sequence similarity using the NCBI blastp search, with scores above 200. *L. oryzae* is a strictly aerobic, non-spore-forming, Betaproteobacterium. Interestingly, it was isolated from rice-paddy soil associated with the roots of *Oryza sativa* growing in the Milyang area of Korea (Lim et al., 2007). Using InterPro sequence analysis and classification (EMBL-EBI), the function of bacterial Tim44-like domains (IPR007379) was unknown, but

is likely involved in transport. A crystal structure of yeast mitochondrial peripheral membrane protein Tim44p C-terminal domain was described as having a large hydrophobic pocket for interacting with the acyl chains of lipid molecules in the mitochondrial membrane (Josyula et al., 2006).

Suggesting a role for the aforementioned genes in a T6SS is highly speculative but worthy of mentioning because of their presence in the supernatant and proximity to T6SS gene clusters. The primary aim of the secretome analysis was to identify the effector protein(s), which are not necessarily core components of T6SSs. Still, knowing which Hcp proteins were being detected in Western blot analyses of the supernatant was desired, but was impossible with the cross reactivity of the antibodies used. Data obtained from the secretome analysis did provide some clues as to which Hcp proteins were being secreted. TssD1 was present in the supernatant, but was far less abundant than TssD2. Interestingly, the least abundant Hcp identified was TssD3 (*azo3898*). Despite being encoded adjacent to and in the same transcriptional direction as TssD2 (*azo3897*), the discrepancy in TssD2 and TssD3 abundance could be accounted for. A transcriptional terminator was predicted between *azo3897* and *azo3898*. Overall, the secretome data suggested that each Hcp was expressed and secreted, but TssD2 appeared to have the greatest presence in supernatants.

III. D. 2. Of the twenty most abundant proteins in the supernatant, the majority were not T6SS associated.

The overwhelming amount of proteins identified in the secretome made it impossible to quickly identify the desired effector protein(s). Therefore, several approaches were taken to evaluate the secretome looking largely at overall protein abundance and what was preferentially in the wild type supernatant. The 20 most abundant proteins identified overall are listed in Table 8. The list includes several expected, extracellular proteins such as flagellar proteins, pilus proteins and one of the Hcp proteins, TssD2 (*azo3897*). TssD2 was also identified in the supernatant of BHΔimpLsciO cultures despite the T6SSs being 'off'. This was probably a result of the supernatant fraction being contaminated with cytoplasmic contents of lysed cells. Still, there was considerably more TssD2 in the wild type samples than the BHΔimpLsciO cultures.

Table 8. List of the twenty most abundant proteins detected in an LC-MS/MS analysis of wild type (WT) and BHΔimpLsciO (DD) culture supernatants.

Most abundant supernatant proteins	DD†	WT†	Gene Number	Gene Name	Functional Category‡
flagellin	504	484	azo2704	*fliC3*	Cell motility
c4-dicarboxylate-binding periplasmic protein	157	153	azo0919	*dctP2*	Carbohydrate transport and metabolism
flagellar hook-associated protein	99	84	azo2706	*fliD*	Cell motility
Flagellin	154	156	azo2693	*fliC2*	Cell motility
OMP porin precursor	75	67	azo3290	*omp*	Cell wall and membrane biogenesis
leucine-specific binding protein	52	43	azo3730	*livK4*	Amino acid transport and metabolism
putative glutamine-binding protein	53	49	azo0442	*glnH*	Amino acid transport and metabolism
elongation factor Tu	46	41	azo3419	*tufA*	Translation
type 4 pilus biogenesis protein	46	38	azo2175	*pilY1A*	Cell motility
Iron transport system substrate-binding	37	33	azo2205	*fbp01*	Inorganic ion transport and metabolism
putative superoxide dismutase	28	37*	azo1466		Inorganic ion transport and metabolism
chaperone GroEL	44	29	azo0974	*groEL*	Posttranslational modification /chaperone
chaperone GroES	28	30	azo0973	*groES1*	Posttranslational modification / chaperone
chaperone protein DanK	29	28	azo1063	*dnak*	Posttranslational modification / chaperone
malate dehydrogenase	22	27	azo1547	*mdh*	Energy production and conversion
conserved hypothetical protein	25	31	azo1131	.	Unknown
putative cytoplasmic protein, Hcp (TssD2)	**5**	**42***	**azo3897**	***sciM***	**(T6SS)**
probable flagellar protein	29	29	azo2705	*flaG*	Cell motility
nitrogenase Mo-Fe protein β-chain	24	21	azo0540	*nifK*	Energy production and conversion
putative Tfp pilus assembly protein	29	22	azo2917	*pilY1B*	Cell motility

†Abundances given were the number of peptides identified unique to a given protein
‡Functional categories were assigned by the genome annotation database RhizoBase (genome.microbedb.jp)
*Values had a significant difference with P<0.05 in a two-tailed paired t-test comparing WT and DD samples
Gray colored values highlight WT peptide counts that averaged less than the average in DD samples

In hopes of attaining clues about the secreted effector's identity, proteins that were predominantly in the wild type supernatant became of interest. In Table 8 the majority of proteins listed were found to an equal or greater extent in the BHΔimpLsciO supernatants (gray values indicate counts which were less in wild type samples than mutant samples). Of the 20 most abundant proteins in the supernatant, Azo1466, Azo0973, Azo1547, Azo1131 and Azo3897 (TssD2) were the only ones more abundant in wild type supernatants. Azo1547 is a malate dehydrogenase that reversibly catalyzes the oxidation of malate to oxaloacetate, and Azo0973 is a chaperone protein, both cytoplasmic proteins most likely found in the supernatant as cellular contaminants. Azo1131 is a hypothetical protein with no conserved domains, but in a blastp search against the NCBI database, 29 proteins showed a high degree of similarity. Scores were greater than 100, and e-values ranged from 5e-58 to 1e-28 with almost always greater than 90% query coverage. Sequences were from organisms including, but not limited to, *Pseudomonas stutzeri*, *Tolumonas auensis*, *Azotobacter vinelandii*, *Methylobacter* spp., and other *Azoarcus* spp. Of the 20 most abundant proteins found predominately in the wild type supernatant, only TssD2 (*azo3897*) and Azo1466 showed a significant difference between the average of all supernatant sample trials for each strain with P<0.05 in a two-tailed paired t-test (marked with asterisks). Azo1466 is a putative Fe/Mn-superoxide dismutase (SOD). This could potentially act as an effector protein in interactions with rice, but the data here is inadequate to draw any conclusions.

III. D. 3. Twenty proteins were found in the supernatant of wild type cultures and not BHΔimpLsciO cultures.

In Table 9, the 20 proteins unique to the wild type supernatant are listed. The abundance was very low for each protein. Included in the list were TssI2 (VgrG, *azo3876*), TssB (*azo3895*) and the least abundant Hcp identified in the supernatants, TssD3 (*azo3898*). In Table 9, TssD2 (*azo3897*) and TssD1 (*azo1305*) were not included in this list because a small amount was also found in the supernatant of the BHΔimpLsciO strain. Once again a superoxide dismutase was high on the list, but this time SodC, a Cu/Zn type. Based on protein domains predicted (InterProScan, EMBL-EBI), several proteins in Table 9 were enzymes involved in metabolism and typically found in the cytoplasm: acyl-CoA dehydrogenases (fatty acid β-

oxidation), Frr (ribosome recycling factor), Cytochrome c (electron transport), enoyl-CoA hydratase (metabolizing fatty acids to produce acetyl CoA), diaminopimelate (DAP) decarboxylase (lysine biosynthesis), MurD synthetase (d-glutamine and d-glutamate metabolism and peptidoglycan biosynthesis), lactoylglutathione lyase (detoxification of methylglyoxal), beta-ketoacyl-acyl-carrier-protein synthase I (acyltransferase), Phosphoribosylaminoimidazolesuccinocarbox-amide synthetase (purine nucleotide biosynthesis) and Cys/Met metabolism PLP-dependent enzyme (amino acid biosynthesis). Four identified proteins were hypothetical proteins, two with unknown functions. One of the hypothetical proteins, Azo2154, contained a threodoxin domain (for disulphide bond formation) and another, Azo2168, contained domains for ADP-dependent kinase and inorganic phosphate-dependent pyrophosphorylase activity. There was one transcriptional regulator found in the wild type and not the BHΔimpLsciO supernatant, RseB, a regulator of the anti-sigma E protein RseD. None of these proteins resembled effector proteins so far identified in other organisms (as discussed in Section ID5).

Table 9. List of proteins found in the supernatant of wild type (WT), but not BHΔimpLsciO (DD) cultures in an LC-MS/MS analysis.

Proteins found in the WT supernatant but not the DD supernatant	WT[†]	Gene Number	Gene Name	Functional Category[‡]
conserved hypothetical protein, VgrG (Tssl2)	**5,1***	**azo3876**		**(T6SS)**
superoxide dismutase	1,2*	azo0522	*sodC*	inorganic ion transp and metabolism
probable acyl-CoA dehydrogenase	1,5	azo1702		lipid transport and metabolism
Frr protein	1,7*	azo1906	*frr*	Translation
putative sigma factor regulatory protein	0,96	azo1632	*rseB*	signal transduction
hypothetical protein predicted by Glimmer/Critica	1,9*	azo2635		Unknown
Hypothetical protein	1,4	azo0277	.	Unknown
putative cytoplasmic protein, Hcp (TssD3)	**0,7**	**azo3898**	***sciK***	**(T6SS)**
putative cytochrome c4	0,7	azo3687	*cc43*	energy production and conversion
probable enoyl-CoA hydratase	0,7	azo0790	*paaF1*	lipid transport and metabolism

Prob. UDP-N-acetylmuramoylalanine-D-glut ligase	1,4	azo0882	*murD*	cell wall / membrane biogenesis
conserved hypothetical protein	1,2	azo2183	*yajQ*	nt-binding
probable lactoylglutathione lyase	0,94	azo0692	*gloA1*	aa transport and metabolism
probable 3-oxoacyl-(acyl-carrier-protein) synthase	0,74	azo1623	*fabH*	lipid transport and metabolism
Phosphoribosylaminoimidazole-succinocarboxamide	0,94	azo2873	*purC*	nt transport and metabolism
DapB protein	0,73	azo2576	*dapB*	aa transport and metabolism
conserved hypothetical protein	1,2*	azo2168		Pyrophosphor-ylase activity
unnamed protein product	0,7	azo2154		Thioredoxin-like
putative cytoplasmic protein (TssB2)	**0,98**	**azo3895**	***sciH***	**(T6SS)**
Cys/Met metabol. pyridoxal-PLP-dep. Enzyme	0,71	azo1054	*metZ*	aa transport and metabolism

†Abundance is the number of peptides identified unique to a given protein
‡Functional categories were assigned by the genome annotation database RhizoBase (genome.microbedb.jp)
Bold proteins listed are encoded within or near the T6SS-2 gene cluster
Abbreviations: (aa) amino acid (nt) nucleotide

A common feature of the proteins in Table 9 is that they were all very low in abundance. In such low abundance, some proteins were identified in only two or three of the wild type supernatant samples. Over the four replicates of each sample, using a two-tailed paired t-test, only five proteins present only in the wild type supernatant were significantly different in abundance compared to BHΔimpLsciO supernatants (marked with an asterisk in Table 9); including TssI2/VgrG (*azo3876*), SodC (*azo0522*), Frr (*azo1906*), Azo2635 and Azo2168. Frr is likely a supernatant contaminant from cell lysis. As mentioned above, Azo2168 contained domains for ADP-dependent kinase and inorganic phosphate-dependent pyrophosphorylase activity, and Azo2635 was found to be a hypothetical protein with no known domains. In a blastp search (NCBI) the protein sequence of Azo2635 aligned with a maximum identity of 76% and 49% with hypothetical proteins from two different marine bacteria, *Methylobacter marinus* and *Thalassolituus oleivorans* MIL-1, both of which encode for at least some components of T6SSs in their genomes. This however provides little insight into possible functions of this protein or its involvement in the T6SS. The presence of VgrG in the supernatant of wild type cultures was anticipated, but is not believed to be the effector.

Again, a superoxide dismutase, SodC (*azo0522*), was the most interesting candidate in this list of proteins. However, looking only at proteins present in the wild type cultures (and completely lacking in the mutant strain) was potentially misleading. Although this increased the stringency of the search, being in low abundance also became a prerequisite. In another approach to review the secretome data, only proteins that had a significantly greater presence in the four wild type samples than the four T6SS deficient strain samples were considered.

III. D. 4. Twenty-two proteins were significantly greater in abundance in the supernatant of wild type cultures than BHΔimpLsciO cultures.

Of the 405 proteins identified over the four replicates of wild type and BHΔimpLsciO samples, 36 proteins showed a significant difference between abundance in wild type and BHΔimpLsciO strain supernatants with a p-value <0.05 in a two-tailed paired t-test. Of these, 22 were found more often in wild type supernatants. These proteins are listed in Table 10. Once again, Azo1466 (SOD) and Azo3897 (TssD2/Hcp) were found at the top of the list, as these were also found to be some of the most abundant proteins in the supernatant (Table 10). Azo3876 (TssI2/VgrG), Azo0522 (sodC), Azo1906 (Frr), Azo2635 (unknown) and Azo2168 (phosphotransferase) were also found only in the wild type supernatant and were reviewed to some extent above. Azo2255 was identified as an unknown but conserved protein. In a blastp search (NCBI), the greatest protein sequence similarity was with protein sequences from mostly *Pseudomonas*, *Pseudoalteromonas*, and *Burkholderia* spp., with E-values always less than 4e-103 with greater than 90% sequence coverage.

Table 10. List of proteins found in an LC-MS/MS analysis that had a significantly greater presence in the supernatant of wild type (WT) cultures than BHΔimpLsciO (DD) cultures.

Gene Product	DD[†]	WT[†]	Gene Number	Gene Name	Functional Category[‡]
putative superoxide dismutase	28,042	36,6175	azo1466		Inorganic ion transport and metabolism
putative cytoplasmic protein, Hcp (TssD2)	**5,205**	**42,3925**	**azo3897**	***sciM***	**(T6SS)**
conserved hypothetical secreted protein	7,6625	13,395	azo2255		Unknown
putative flagellar hook-length control protein FliK	5,9925	9,35	azo2721	*fliK*	Cell motility
Probable phosphoenolpyruvate carboxykinase	3,6325	6,22	azo0820	*pckG*	Energy production and conversion

putative phosphoglycerate kinase	2,3425	4,3225	azo2839	*pgk*	Carbohydrate transport and metabolism
NifY protein	3,1775	4,78	azo1248	*nifY*	General
IleS protein	1,8275	3,835	azo1205	*ileS*	Translation
probable dihydroorotase	1,0425	2,895	azo0873	*pyrC*	Nucleotide transport and metabolism
dihydroxy-acid dehydratase	3,1025	4,7875	azo0632	*ilvD*	Amino acid transport and metabolism
Hypothetical protein, VgrG (TssI2)	**0**	**5,0675**	**azo3876**		**(T6SS)**
putative sulfite reductase	0,2675	2,64	azo0432	*cysI*	Inorganic ion transport and metabolism
ArgG protein	1,0225	1,6825	azo2186	*argG*	Amino acid transport and metabolism
superoxide dismutase	0	1,195	azo0522	*sodC*	Inorganic ion transport and metabolism
branched-chain-amino-acid transaminase	1,0425	2,64	azo2775	*ilvE2*	Amino acid transport and metabolism
conserved hypothetical secreted protein	2,5975	3,835	azo1102		Unknown
Frr protein	0	1,6625	azo1906	*frr*	Translation
Digyuanylate cyclase (Predicted GGDEF/EAL domains)	1,315	2,62	azo1476		Signal transduction
putative transaldolase	1,0275	2,8775	azo1464	*tal*	Carbohydrate transport and metabolism
putative oxygen-insensitive NADPH nitroreductase	0,775	1,915	azo0953	*nfsA*	Energy production and conversion
Hypo. protein predicted by Glimmer/Critica	0	1,9175	azo2635		Unknown
conserved hypothetical protein	0	1,195	azo2168		Unknown

†Abundance is the number of peptides identified unique to a given protein
‡Functional categories were assigned by the genome annotation database RhizoBase (genome.microbedb.jp)
Bold proteins listed are encoded within or near the T6SS-2 gene cluster

The remaining proteins on the list were predominantly cytoplasmic proteins including; Azo2721 (flagellar hook-length control), Azo0820 (gluconeogenesis), Azo2839 (glycolysis),

Azo1248 (biosynthesis of the iron-molybdenum cofactor), Azo1205 (attachment of amino acid to its cognate tRNA), Azo0873 (pyrimidine base biosynthesis), Azo0632 (valine and isoleucine biosynthesis), Azo0432 (reduction of sulphite to sulphide), Azo2186 (urea cycle enzyme), Azo2775 (conversion of amino acid and an α-keto acid), Azo1102 (unknown function, but blastp hits showed sequence similarity with some lipoproteins and predicted to have one transmembrane segment), Azo1476 (synthesis of cyclic di-GMP), Azo1464 (link between the glycolytic and pentose-phosphate pathways) and Azo0953 (reduction of nitrogen-containing compounds). It was interesting that Azo1464 was included on this list because *azo1464* was also up-regulated when cells were exposed to plant exudates for 1 h. In total, 10 proteins were identified in the secretome that were also up-regulated in the presence of plant root exudates; Azo1464, Azo2153, Azo2182, Azo3700, Azo0311, Azo2969, Azo3832, Azo2205, Azo3194 and Azo3729 (Teja Shidore, PhD thesis, 2012). Apart from Azo1464, none of these proteins were found with significantly different abundances between wild type and BHΔimpLsciO samples. Azo2205 was one of the most abundant proteins identified in the secretome, but found slightly more often in the BHΔimpLsciO supernatants.

III. E. Rice plants responded differently to T6SS deficient strains compared to wild type *Azoarcus* sp. strain BH72.

III. E. 1. Rice roots inoculated with BHΔimpLsciO were affected.

Several T6SSs were initially identified when screening mutants with loss of virulence and altered interactions with a eukaryotic host (Cascales, 2008; Pukatzki et al., 2007). Therefore, it was of interest to assess the interaction of a T6SS mutant strain with a known *Azoarcus* sp. strain BH72 host, Asian rice cultivar *Oryza sativa* IR-36. Had an effector protein secreted by the T6SS been identified in the secretome, further studies would have been done on its direct interaction with plants. Without a known effector some studies were still possible for determining if the *Azoarcus* sp. strain BH72 T6SS(s) plays a role in plant interactions. In these studies the T6SS deficient strain, BHΔimpLsciO, was used for infecting rice roots, and the effects were compared to infection of rice roots with the wild type strain. As anticipated, the loss of T6S had an effect on the roots of rice seedlings and the colonization efficiency of T6SS mutants. In Figure 29, healthy smooth rice root hairs with no abnormalities two weeks post-inoculation with wild type strain BH72 are seen in Panel A. In panel B, rice seedlings were inoculated with a T6SS mutant, BHΔimpLsciO. Root hair tips were affected.

Irregularities developed along the surface of the tips. Bacteria attached to the exterior surface of the plant had been removed via brief rinses in distilled water and incubation in a sonicating water bath before viewing under the microscope. Live/Dead staining confirmed that these irregularities were not bacterial aggregates (data not shown).

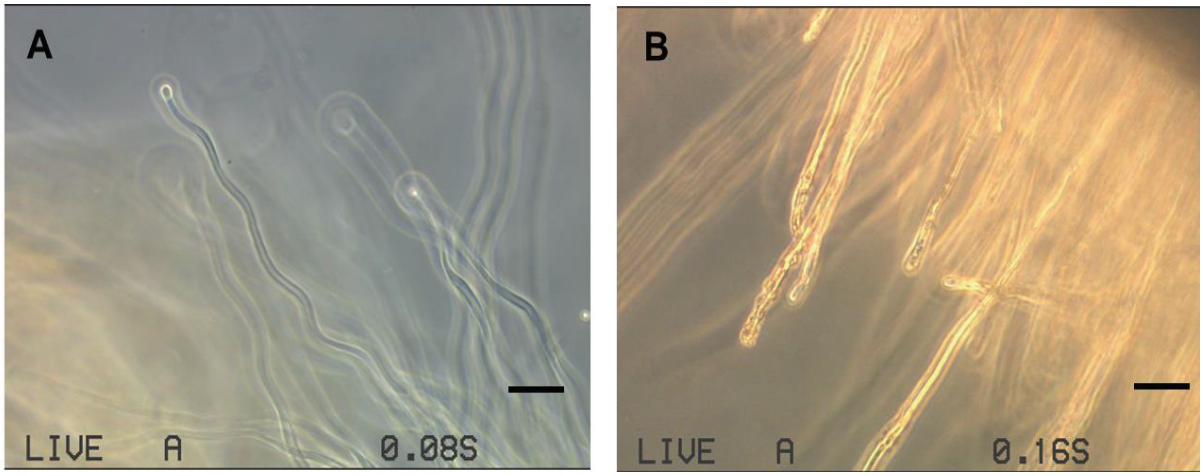


Figure 29. Light microscopic view of root hairs from seedlings 14 d post inoculation with wild type or BHΔimpLsciO strains. *Oryza sativa* cv. IR-36 root hairs 14 d post inoculation with wild type *Azoarcus* sp. strain BH72 (panel A) or BHΔimpLsciO (panel B). Sterilized seeds were germinated on agar plates and then planted in sterile quartz saturated with medium inoculated with bacteria in glass reagent tubes. Reagent tubes were incubated for 14 d in a Phytotron (30°C, 80% humidity, 15 kLux light intensity, and 14/10 hours day/night frequency) in a dark water bath. Roots were rinsed in sterile distilled water and exterior bacteria detached in a sonicating water bath before viewing. The size bars represent 82 μm.

III. E. 2. Turning off both T6SSs increased colonization efficiency.

Surface sterilized rice grains were germinated on plates before planting in sterile quartz and inoculating with wild type or BHΔimpLsciO strains as described (Section IIB8a). After 14 d incubation in a Phytotron (30°C, 80% humidity, 15 kLux light intensity, and 14/10 hours day/night frequency), roots were rinsed in sterile distilled water and then incubated in a sonicating water bath to remove surface associated bacteria before being ground. Serial dilutions of the homogenized roots were pour-plated in semi-semi-solid media, and CFU of endorhizosphere bacteria were counted after 3 d (Section IIB8b). The CFU/mg fresh root weight was then determined, and an average was calculated for each plant that had at least two countable plates (15-150 CFU). The assay is depicted in Figure 30. To control for contamination, homogenates were streaked on a VME plate and incubated in parallel.

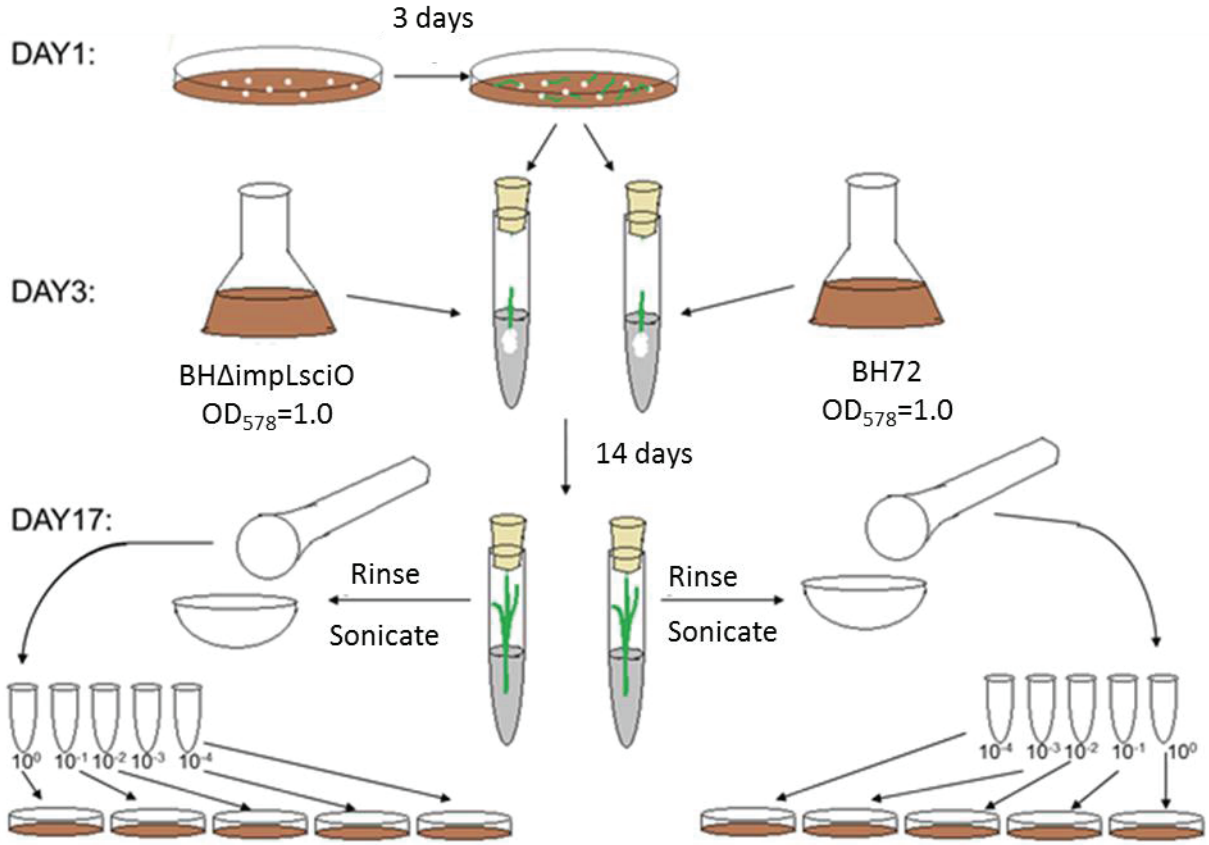


Figure 30. Schematic representation of the rice colonization experiment. Surface sterilized *Oryza sativa* cv. IR-36 rice grains were germinated on plates for 3 d. Seedlings were planted in sterile quartz and inoculated with wild type or BHΔimpLsciO strains and incubated in a Phytotron (30°C, 80% humidity, 15 kLux light intensity, and 14/10 hours day/night frequency). After 14 d, roots were rinsed in sterile distilled water and incubated in a sonicating water bath before grinding. Serial dilutions of the homogenized roots were pour-plated in semi-semi-solid media, and CFU were counted after 3 d. CFU/mg fresh root weight was calculated for all plants with at least three countable plates (15-150 CFU).

For each independent trial, the BHΔimpLsciO mutant colonized the rice roots in higher numbers than wild type. As an average over the four trials, 3297 CFU/mg fresh root weight of wild type and 13,748 CFU/mg fresh root weight of BHΔimpLsciO were counted. The difference was found to be statistically significant with $P<0.05$ in a two-tailed paired t-test (Figure 31). The loss of both T6SSs led to increased colonization of the rice roots.

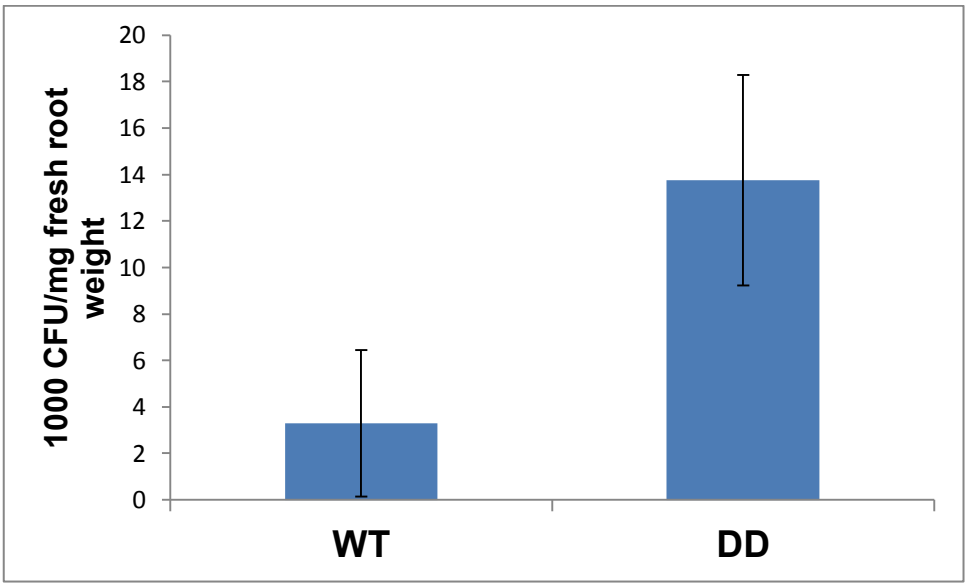


Figure 31. Colonization of *Oryza sativa* cv. IR-36 seedlings by wild type strain BH72 (WT) or BHΔimpLsciO (DD). Rice seedlings were grown under gnotobiotic conditions in quartz saturated with medium inoculated with WT or DD strains for 14 d. Four independent experiments were performed using 3-10 plants per strain of bacteria. The CFU/mg fresh root weight was then determined and an average was calculated for each plant that had at least two countable plates (15-150 CFU). Using a two-tailed paired t-test, P = 0.02499. Error bars depict the standard deviation.

Shidore et al. had similar findings using a competition assay where single rice seedlings (*Oryza sativa cv. Nipponbare*) were inoculated with an equal amount of wild type and BHazo3888 and grown for 3 d in a gel-rite medium (Figure 32). Mutant and wild type colonies were differentiated by using the antibiotic resistance inserted in the mutant strain. Each experiment was performed in triplicate, and the average percentage of colonization by wild type and mutant was calculated in a two-tailed paired t-test $P < 0.05$ (Shidore et al., 2012).

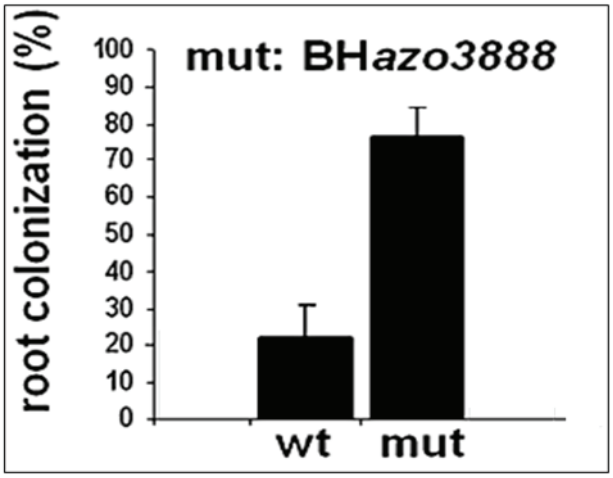


Figure 32. Colonization of *Oryza sativa* cv. IR-36 by *Azoarcus* sp. strain BH72 (wt) and BHazo3888 (mut) in a co-infection. An equal number of wild type and mutant cells were used for inoculation of rice seedlings grown under gnotobiotic conditions in gel-rite containing medium for 3 d. Each experiment was performed in triplicate and average percentage of colonization by wild type and mutant was calculated. Using a two-tailed paired t-test, $P \leq 0.05$ (Shidore et al., 2012).

To reject the possibility that discrepancies in growth rate were a factor in colonization efficiency, doubling times were determined. For each strain the average of four values (two technical replicates for each of two biological replicates) was used to determine the doubling time. Each strain had a doubling time of approximately 2 h (Figure 33).

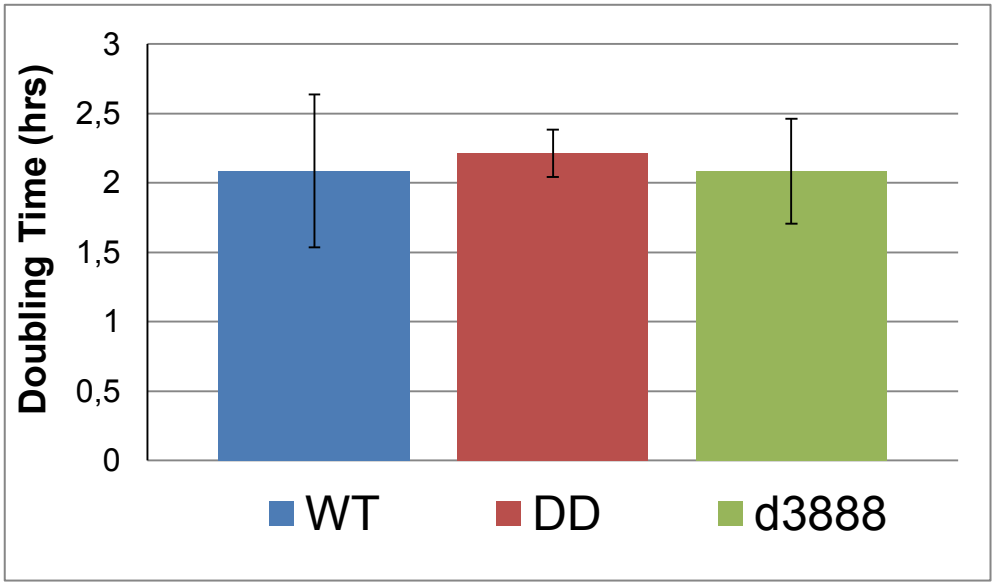


Figure 33. Growth rate of wild type (WT), BHΔimpLsciO (DD), and BHazo3888 (d3888). Strains were grown in VME for 8 h at 37ºC. The increase in optical density was observed every hour and doubling times were calculated for the exponential growth phase. The doubling time was taken as the average of four values (two technical replicates of two biological replicates). The standard deviation is depicted by the error bars.

III. E. 3. Exposure of *O. sativa* OC156 rice cell cultures to overexpressed Azo1305 elicited ethylene production.

An increased production of plant stress hormone, ethylene, can be used to assay for a plant response to putative elicitors (Felix et al., 1991). Suspensions of *O. sativa* OC156 rice cell cultures were exposed to different concentrations of the different Hcp proteins, and the ethylene production was measured. Hcp proteins were overexpressed by *E .coli*, extracted and then purified using Strep-Tactin sepharose columns (Section IIB4a-c). Positive and negative controls were also tested. The last three purple bars on the right were positive controls and represent the ethylene production by known plant elicitors Chitin (Felix et al., 1999) and Pen1, an extract of the mycelium of the ascomycete, *Penicillium chrysogenum* (Thuerig et al., 2006). Ethylene production by cells after the addition of the Hcp protein suspension solutions (BE), 10 mM Tris (pH 7.2), and cells with nothing added were also tested as negative controls (light purple). In Figure 34 the average nmoles of ethylene produced by seven or eight day old rice cell cultures over six trials are displayed with error bars depicting the standard deviation between trials. For each trial, the middle three values of five replicas measuring ethylene were averaged.

When considering the average of all six trials, there was considerable variation. However, when comparing some elicitors to the cells alone, some differences in values were statistically significant. The known elicitors controlled the sensitivity of the assay. Both showed an increase in ethylene production, but only addition of 5 μg of Pen1 yielded a significantly different ethylene production compared to cells not exposed to an elicitor ($p<0.05$ in a two-tailed paired t-test). Of the different Hcp proteins tested, only addition of 5 μg of Azo1305 produced a significantly different amount of ethylene compared to cells not exposed to an elicitor with $p<0.05$ in a two-tailed paired t-test. In a two-tailed paired t-test, ethylene produced by cells exposed to 5 μg of any of the Hcp proteins compared to BE solution alone produced a statistically significant difference in ethylene production ($p<0.05$). Addition of 5 μg of Azo1305 to rice cell cultures resulted in the greatest amount of ethylene production of all elicitors tested including the positive control.

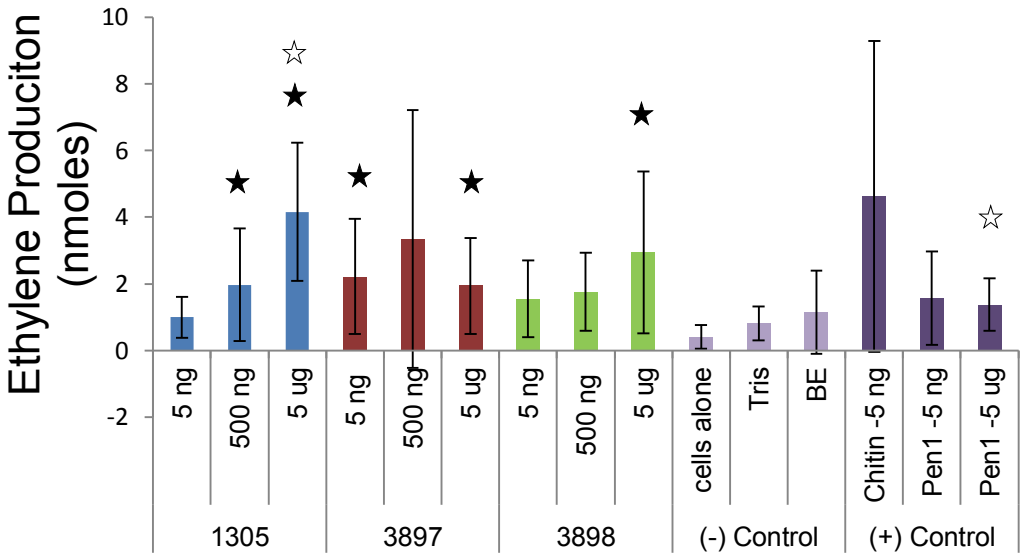


Figure 34. Production of ethylene (nmoles) by seven and eight day old rice cell cultures in the presence of proteins. Ethylene was measured using a Chromatograph HRGC-4000A (Konic, Barcelona, Spain) by taking 200 µl headspace samples from air tight reagent tubes containing 500 µl of seven and eight day old rice cell cultures that had been incubated for 4 h with 5 ng, 500 ng or 5 µg of Hcp (Azo1305, Azo3897 or Azo3898) shaking at 200 rpm at 23°C. In each trial five replicas were measured and the middle three values averaged. The average of six trials, three using eight day old cells and three using seven day old cells, are displayed in the bar chart. As positive controls (+), rice cell cultures were incubated with known elicitors Chitin (5 ng) and Pen1 (5 ng or 5 µg). As negative controls (-), headspace samples were taken from airtight reagent tubes containing cells alone and from tubes containing cells in the presence of 5 µl of protein buffers, Tris and BE (elution buffer for Hcp). The standard deviation between trials for each tested elicitor is displayed by error bars. Open stars indicate elicitation statistically different than ethylene production by cells without addition of elicitors (cells alone) and closed stars indicate ethylene production significantly different than exposure to BE ($p<0.05$ in a two-tailed paired t-test).

III. F. A transcriptional regulator of T6SS-1 in *Azoarcus* sp. strain BH72 was searched for.

In previous work it was speculated that the T6SS-1 might be under the same regulation as genes necessary for nitrogen fixation, since most T6SS-1 genes were up-regulated under nitrogen fixing conditions. Genes necessary for nitrogen fixation, *nifHDK*, are regulated transcriptionally by σ^{54} and enhancer binding proteins. As discussed in Section ID6, σ^{54}, is recognized for its involvement in regulation of nitrogen metabolism and an unusual spectra of unrelated genes (Cases et al., 2003). Transcription from σ^{54}-dependent promoters is tightly controlled by bacterial enhancer binding proteins, which are required to melt the DNA and form an open complex. Without the binding of enhancer proteins to upstream activator sequences and their nucleotide hydrolysis activity, σ^{54}-RNA polymerase would remain bound

to the promoter transcriptionally silent in an unusually stable, closed complex (Buck et al., 2000).

Nitrogen fixation expends a great amount of energy. So, futile production is avoided by the sensing of nitrogen already available and sensing oxygen. One of the key enzymes in nitrogen fixation, nitrogenase, is highly sensitive to oxygen (Egener et al., 2002). Expression of the *nifHDK*, operon requires the σ^{54} activator, NifA (*azo0519*). *nifA* is cotranscribed with *nifL*, which in response to high levels of oxygen and available nitrogen, NifL blocks the function of NifA likely through a direct protein-protein interaction (Merrick and Edwards, 1995). Transcription of the *nifLA* operon requires the σ^{54} activator NtrC (*azo0735*). NtrC is part of the general nitrogen-regulated regulon (Ntr), a global regulatory system (Merrick and Edwards, 1995). When nitrogen is limited, the level of glutamine is low. When glutamine is low, GlnD acts as an uridyltransferase. The *glnB* gene product, the P_{II} protein, is uridylated (with uridine monophosphate, UMP) by GlnD. When nitrogen levels are high, GlnD is stimulated to remove UMP from the P_{II} protein. The free P_{II} protein binds NtrB. Expression of *nifA* requires the two-component phosphorelay system of autophosphorylating sensor kinase NtrB, and response regulator NtrC. NtrC is phosphorylated by NtrB and activates expression of *nifA*. In the Ntr system, only when nitrogen is limited and the P_{II} protein is uridylated, can NtrB autophosphorylation occur. Otherwise, free P_{II} protein binds NtrB and the phosphorelay is inhibited. *nifA* is expressed less and cannot activate *nif* gene expression (Merrick and Edwards, 1995; Egener et al., 2002; Egener et al., 1999).

An *ntrBC*$^{-}$ mutant, BNtrBsp, was constructed in a different study by disrupting *ntrB* and *ntrC* with insertion of an omega cassette (Abhijit Sarkar, 2003). In an earlier study, transcription of T6SS-1 genes (tested using semi-quantitative RT-PCR) was greater in BNtrBsp, suggesting down-regulation by the global regulator NtrC (Teja Shidore, PhD thesis, 2012). Although the differences were not dramatic, they were statistically significant for *azo1299* and *azo1302* (not for *azo1305*), suggesting NtrC was a negative regulator, but not the most essential (Teja Shidore, PhD thesis, 2012). Also in that study, semi-quantitative RT-PCR studies looked at the transcription of *azo1299*, *azo1302* and *azo1305* in a *nifLA*$^{-}$ strain, BHLAO (Teja Shidore, PhD thesis, 2012). There was no statistically significant difference in transcript levels of these genes in BHLAO compared to wild type, suggesting there was a different transcriptional activator (Teja Shidore, PhD thesis, 2012).

Several T6SS gene clusters possess a consensus σ^{54}-binding sequence where RNA polymerase is recruited, and with the help of an equally important enhancer binding protein, transcription occurs (Miyata et al., 2013). The putative importance of σ^{54} in regulation of nitrogen fixation and T6SS made a VasH homolog encoded near the T6SS-2 gene cluster (*azo3875*) worthy of further review. The enhancer binding protein, VasH, of *V. cholerae*, *P. aeruginosa* and *A. hydrophila* is required for expression of their T6SSs (Miyata et al., 2013). In a different study, the VasH-like protein of *Azoarcus* sp. strain BH72 was tested. However, comparing expression of *azo1305* in wild type and BHΔ3875 using semi-quantitative RT-PCR conditions revealed no significant differences in expression levels when cells were grown under conditions promoting nitrogen fixation (SM medium with 20mM glutamate and 1.8% O_2) (Teja Shidore, PhD thesis, 2012). Interestingly, despite being up-regulated during growth under nitrogen fixing conditions, T6S appeared to be regulated in a σ^{54}-independent manner. The search for transcriptional regulators needed to be broadened.

III. F. 1. A screen for transcriptional regulators of the T6SS in *Azoarcus* sp. strain BH72 hinted at involvement of Azo0559.

Upstream of *azo1300* and *azo1301*, a LysR binding box, a Lux box and an Ara box were predicted (unpublished data A. Sarkar). LysR-, LuxR- and AraC- type transcriptional regulators each have a helix-turn-helix (HTH) motif that binds DNA in the major groove (Maddocks and Oyston, 2008; Gallegos et al., 1997; Chen and Xie, 2011). The vast majority of prokaryotic DNA-binding proteins contain a HTH motif (Maddocks and Oyston, 2008) with a C-terminal HTH in transcriptional activators and transcriptional repressors with an N-terminal HTH (Pérez-Rueda and Collado-Vides, 2001; Pérez-Rueda and Collado-Vides, 2000; Gallegos et al., 1997). The HTH is 20–90 amino acids from the N terminus in LysR-type transcriptional regulators regardless of whether activating or repressing. They are often referred to as dual regulators, but the majority are transcriptional activators that negatively regulate their own expression (Maddocks and Oyston, 2008). LuxR-type transcriptional regulators contain a four-helical bundle structure with the second and third helices forming the C-terminal HTH motif for DNA binding (Chen and Xie, 2011). AraC-type transcriptional regulators are almost all positive transcription factors, and they have two C-terminal HTH sub-domains (Gallegos et al., 1997).

Since the *Azoarcus* sp. strain BH72 genome encodes for a great number of transcriptional regulators, a method was developed for quick screening of relative Hcp expression by

transcriptional regulator mutants compared to wild type. The mutant strains tested were picked because of their differential regulation under nitrogen fixing conditions and expression independent of NifA. The list included mostly LysR-, LuxR- and AraC- mutants, a transcription factor for alternate sigma factor E (σ^{24}), and a putative RNA ploymerase sigma factor 70 (Table 11). Most mutants had been constructed in previous work and were polar mutants, disrupting all genes downstream of the shared operon.

Table 11. Potential transcriptional regulators of the T6SS system tested in this study.

Gene number	Gene Name	Definition	Mutant Name
azo0559		Sigma-E factor regulatory protein	BHazo0559
azo0679	*nodD*	LysR-type TR	BHazo0679
azo0830		AraC family TR	BHazo0830
azo1247		LysR family TR	BHazo1247
azo1281		LysR family TR	BHazo1281
azo1376/azo1375	*fixJ2*	LuxR-type TR/putative TCS	BHazo3875
azo2546	*nac*	LysR-type TR	BHazo2546
azo2564		putative RNAP sigma factor	BHazo2564
azo2671/azo2672	*fixJ1*	TCS response regulator/putative sensor histidine kinase	BHazo2672
azo2979	*elmS*	TCS histidine kinase	BHΔelmS
azo2980	*gacA*	LuxR-type TR	BHΔgacA

TR=transcriptional regulator TCS=two component system RNAP=RNA polymerase

In the screening procedure, strains were grown in sealed reagent tubes of semi-solid SM medium without a source of combined nitrogen, promoting nitrogen fixation. After 3 d of growth at 37°C, cells were harvested from the agar medium, and total cell protein extracts were obtained. Proteins were separated by SDS-PAGE, and the relative abundance of Hcp expressed by each mutant strain was compared to wild type. Each of the strains screened were tested in a single trial. None of the strains tested revealed an increased expression of Hcp, suggesting that none of them act as negative regulators of the T6SS. BHazo0559 was further analyzed because of the different levels of Hcp detected in the total protein (Figure 35).

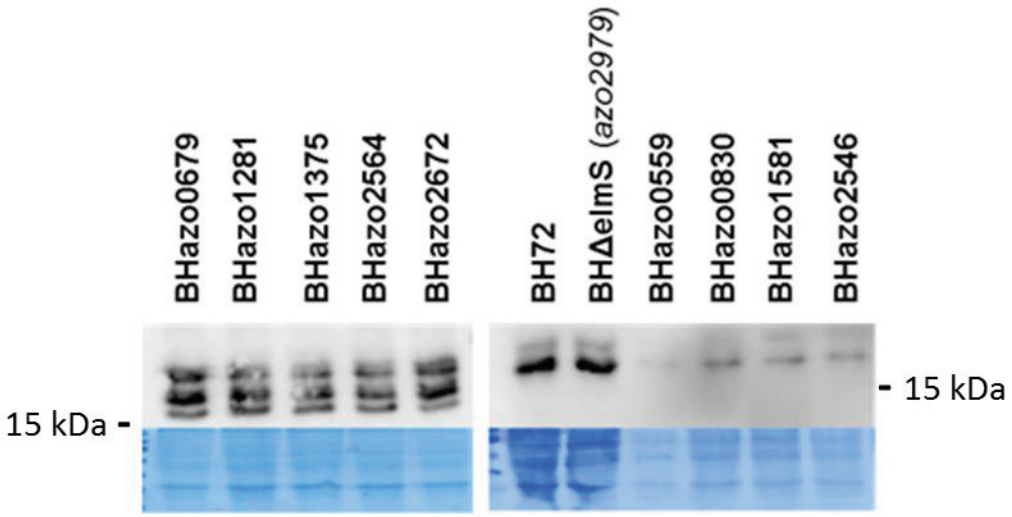


Figure 35. Detection of Hcp in total cell protein extracts to screen mutant strains for potential T6SS transcriptional regulators. Total cell protein extracts were obtained from cells grown in semi-solid media under nitrogen fixing conditions after 3 d. Proteins were seperated on 12% polyacrylamide SDS-PAGE gels. Western blots were probed with anti-Hcp. SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher, Rockford, IL, USA) was used to detect protein bands. A gel run in parallel (left) or the gel post-Western transfer (right) was stained with Colloidal Coomassie Brilliant Blue Stain to control protein quantity loaded. In the right gel wild type BH72 and BHΔelmS samples were 10x more concentrated than the other samples. Protein band sizes were estimated using PageRuler™ Prestained Protein Ladder (Fermentas, ThermoFisher, Rockford, IL, USA).

III. F. 2. Azo0559 played a role in regulating the expression of T6SS(s) in *Azoarcus* sp. strain BH72.

Azo0559 was predicted to be a homolog of RseC (**r**egulator of **s**igma **E**). RseC/MucC is an inner membrane protein with two transmembrane segments that acts as a positive regulator of sigma E (Koo et al., 2003). There are two classes of sigma factors, σ^{54} and σ^{70}. Despite different amino acid sequences and transcription mechanisms, they both produce holoenzymes with RNA polymerase (Buck et al., 2000). Sigma E (σ^{E} or σ^{24}) is an alternative sigma factor and part of the σ^{70} family. Sigma E is activated upon extra cellular stress (such as misfolding of proteins at the cell envelope) by release from its anti-sigma factor, RseA (Missiakas et al., 1997). In the *Azoarcus* sp. strain BH72 genome, two genes encode for RpoE, *azo0075* and *azo1630* (or *algU*). AlgU was found in the *rpoE-rse* gene cluster along with another RseC/MucC homolog, Azo1633 (Figure 36).

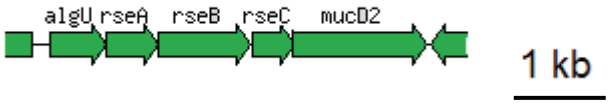


Figure 36. Schematic of the rse gene cluster organization. Gene organization of the *rpoE-rse* operon comprised of genes *azo1630-azo1634* (adapted from GenDB). The scale bar represents 1 kb.

Azo0559 and Azo1633 were respectively predicted to be 135 and 146 amino acid, inner membrane proteins, each with two transmembrane segments that act as anti-anti-sigma factors (SOSUI, http://bp.nuap.nagoya-u.ac.jp/sosui). Both were classified by InterProScan (EMBL-EBI) to be RseC/MucC proteins. In the *Azoarcus* sp. strain BH72 genome, *azo0559* is not a part of the *rpoE-rse* gene cluster and is distant from both T6SS gene clusters. It lies amidst a cluster of *nif* genes necessary for nitrogen fixation (Figure 37).

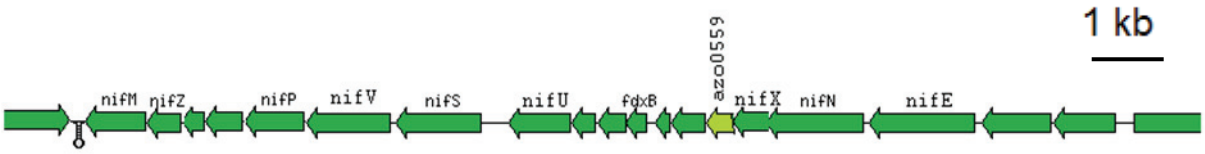


Figure 37. Gene organization of *azo0559* and neighboring *nif* genes. The gene *azo0559* encoded within an operon potentially 14.7 kb long, amidst *nif* genes commonly necessary for nitrogen fixation (adapted from GenDB). The scale bar represents 1 kb.

Azo0559 was examined more closely because less Hcp was detected in the screen for transcriptional regulators (Figure 35). It was also a promising candidate because it was up-regulated 32-fold in a microarray study comparing gene expression by *Azoarcus* sp. strain BH72 under nitrogen fixing conditions compared to non-fixing conditions (Sarkar and Reinhold, 2014). Additionally, a σ^{24} binding site upstream of *azo1301* had been predicted using the program BPROM (http: http://linux1.softberry.com) (Teja Shidore, PhD thesis, 2012). In Figure 38 a representative gel of three independent trials demonstrates that disrupting *azo0559* resulted in a reduction in Hcp expression when cells were grown under nitrogen fixing conditions, but Hcp expression was not completely abrogated in BHazo0559. When assessing the expression of Hcp by cultures grown under non-fixing conditions, there was little or no difference in the small amount of Hcp detected.

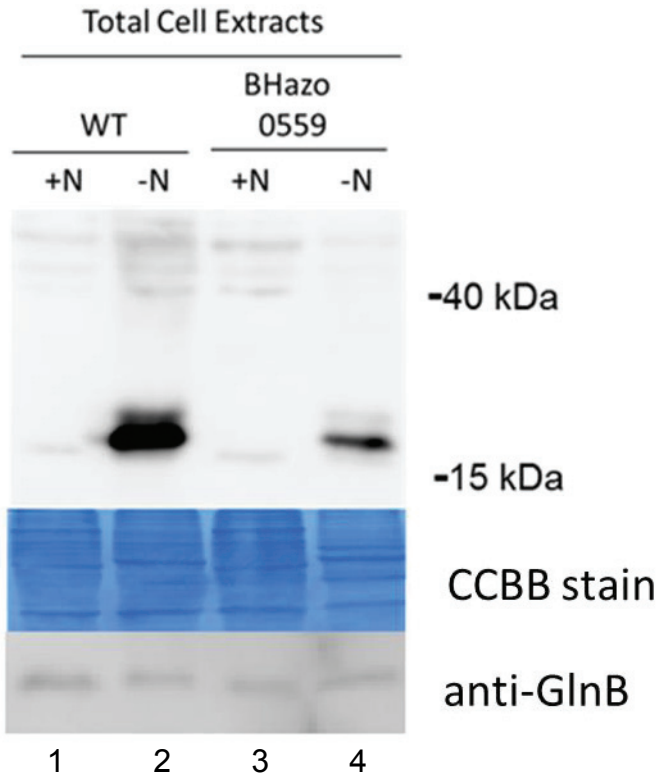


Figure 38. Hcp expression of wild type compared to potential transcriptional regulator mutant, BH0559. Wild type (lanes 1and 2) and BHazo0559 (lanes 3 and 4) were grown for 2 d in semi-solid SM media alone (-N, lanes 2 and 4) or supplemented with 9.3 mM NH_4Cl (+N, lanes 1 and 3). Total cell protein was seperated on a 12% polyacrylamide SDS-PAGE gel and transferred to a nitrocellulose membrane. The Western blot was probed with anti-Hcp. Total protein loaded was controlled in a parallel gel stained with Colloidal Coomassie Brilliant Blue Stained (CCBB) and by detection of cellular protein GlnB (anti-GlnB). Lumi-Light^{PLUS} western blotting substrate (Roche, Rockford, IL, USA) was used to detect proteins. Protein band sizes were estimated using PageRuler™ Prestained Protein Ladder (Fermentas, ThermoFisher, Rockford, IL, USA).

III. F. 3. Hcp expression was stimulated in BHazo0559 cultures grown in medium supplemented with glutamate.

Repeatedly, in Western blot analyses normalizing the total protein loaded based on BHazo0559 cell pellet mass was insufficient. The total cell proteins extracted from BHazo0559 under nitrogen fixing conditions regularly appeared to be less than wild type in SDS-PAGE gels stained with Colloidal Coomassie Brilliant Blue. The mutant BHazo0559 was a polar mutant constructed by insertion of pK18GGST into *azo0560*, disrupting expression of all downstream genes, which potentially includes a great number of *nif* genes typically necessary for nitrogen fixation (Figure 37). The weak growth under nitrogen fixing conditions was thought to be caused by the lack of available nitrogen and a reduced ability to

fix nitrogen. In an effort to overcome this, different approaches were tried. First, the amount of sample loaded for SDS-PAGE was manipulated based on what was seen in stained gels to normalize protein amounts, as was done in Figure 38. Another method was the addition of a poor source of assimilated nitrogen, glutamate, to satisfy growth requirements without ceasing nitrogen fixation. In previous work, *nifHDK* genes were still up-regulated to levels similar to nitrogen fixing conditions when cultures were grown under microaerobic conditions with 20 mM glutamate added (Egener et al., 1999).

The relative expression of Hcp by wild type and BHazo0559 strains grown in sealed reagent tubes of semi-solid SM media was compared using media supplemented with 9.3 mM NH_4Cl, supplemented with 20 mM glutamate or with no additions. In Figure 39 the colloidal coomassie stained SDS-PAGE gel indicates that the total cell proteins extracted from BHazo0559 grown under nitrogen fixing conditions (-N, lane 4) was considerably less than the other samples, and supplementing the medium with glutamate (+G, lane 6) successfully improved the quantity of protein extracted. Addition of glutamate to the medium of wild type cultures stimulated Hcp expression to levels similar to those seen with growth under nitrogen fixing conditions (Figure 39, lanes 3 and 5). It was presumed that addition of glutamate to the media of BHazo0559 cultures would, like wild type, enhance culture growth and that the levels of Hcp expression would remain similar to those with growth under nitrogen fixing conditions. Surprisingly, in a single trial growing BHazo0559 in semi-solid media supplemented with glutamate, there was considerably more Hcp detected in the total protein. Hcp expression by BHazo0559 appeared to be greater than wild type expression in this medium (Figure 39, lanes 5 and 6). Although the addition of glutamate to the media successfully improved growth, it was decided to construct an in-frame deletion mutant of *azo0559*. It was hoped that an in-frame deletion would improve growth under nitrogen fixing conditions without additional glutamate, which appeared to lead to the overexpression of Hcp.

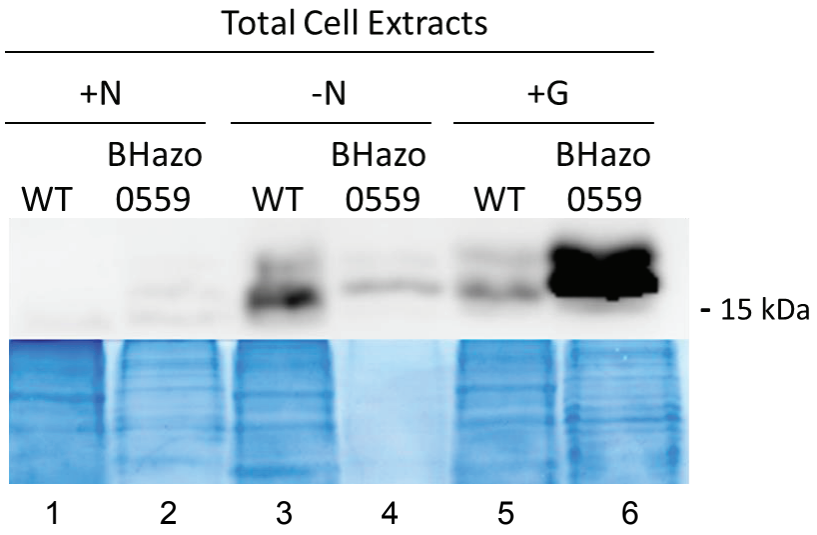
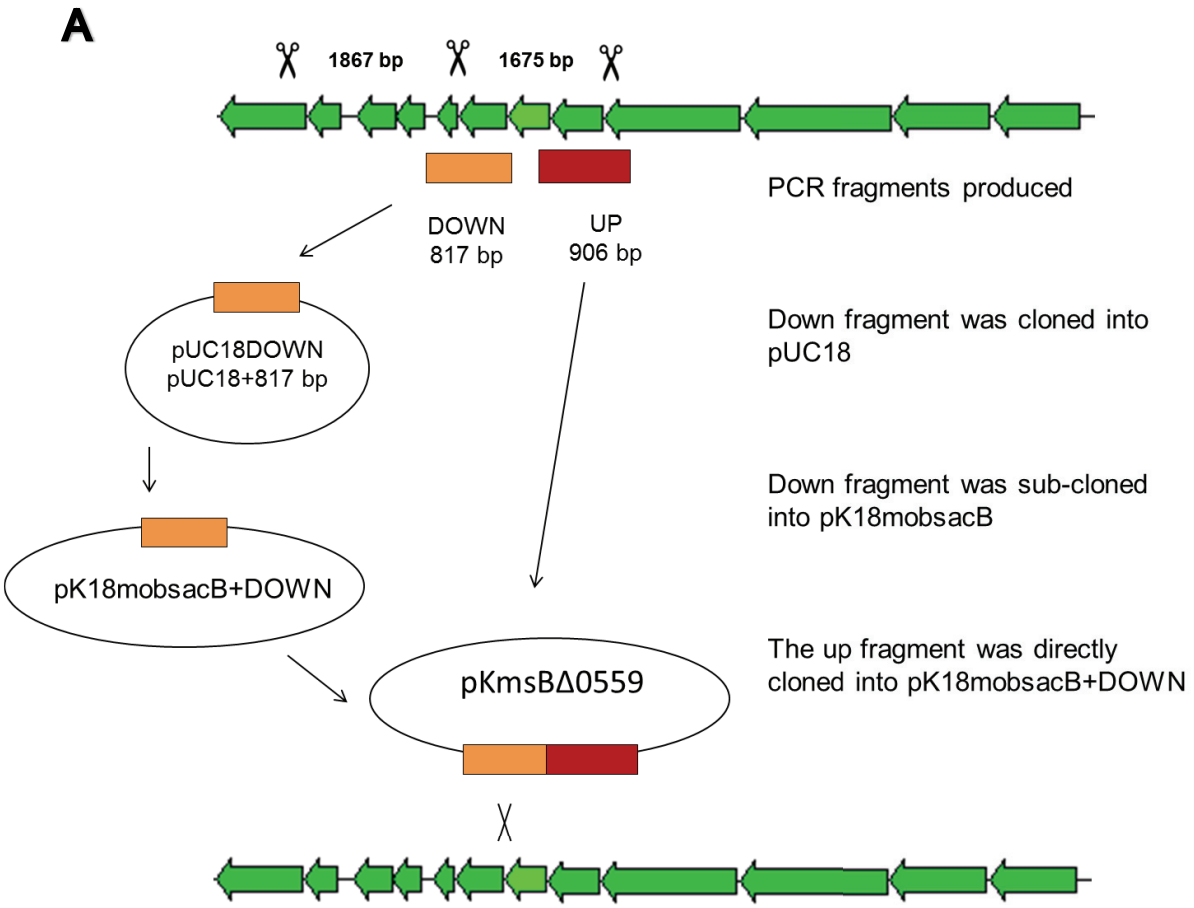


Figure 39. Western blot analysis of Hcp expression by wild type and BHazo0559 grown in semi-solid media with different sources of nitrogen. Wild type (WT, lanes 1, 3 and 5) and BHazo0559 (lanes 2, 4, and 6) cultures were grown in semi-solid media with SM medium supplemented with 9.3 mM NH_4Cl (+N, lanes 1 and 2), with no nitrogen source (-N, lanes 3 and 4) and with 20 mM glutamate (+G, lanes 5 and 6) at 37°C for 2 d. Total cell proteins were extracted, separated through 12% polyacrylamide SDS-PAGE gels and blotted onto a nitrocellulose membrane. Western blots were probed with anti-Hcp. Lumi-Light^{PLUS} western blotting substrate (Roche, Rockford, IL, USA) was used to detect protein bands. Protein loaded was controlled by a parallel gel stained with Colloidal Coomassie Brilliant Blue. Protein band sizes were estimated using PageRuler™ Prestained Protein Ladder (Fermentas, ThermoFisher, Rockford, IL, USA).

III. F. 4. BHΔ0559 was constructed by making an in-frame deletion to *azo0559*.

BHΔ0559 was constructed as an in-frame deletion mutant. An 817 bp downstream region (384 bp-797 bp downstream *azo0559*) was amplified via PCR with additional restriction sites *Bam*HI and *Sma*I added to the 5' and 3' ends, respectively. This down fragment was cloned into the pUC18 vector and then sub-cloned into pK18*mobsac*B, utilizing the added restriction sites each time. The up fragment was a 906 bp piece (798 bp upstream-108 bp into gene *azo0559*) amplified using a Phusion taq polymerase (New England Biolabs, Ipswich, MA) in a 50 µl reaction (60 ng template DNA, 1X HF buffer, 200 µM dNTPs, 3% DMSO, 0.5 µM forward primer, 0.5 µM reverse primer, 1.0 U polymerase). The thermo cycler was programmed as follows: 3 min denaturation at 98°C followed by 35 cycles of 98°C for 10 sec, 30 sec at 60.8°C and 30 sec at 72°C followed by an additional 7 min of elongation at 72°C. Additional *Xba*I and *Bam*HI restriction sites were added to the 5' and 3' ends, respectively. This PCR product was digested and directly cloned into the pK18*mobsac*B vector already containing the down fragment using the *Xba*I and *Bam*HI restriction sites

creating pKmsBΔ0559. The up and down fragments joined with an internal 276 bp deletion of *azo0559* were sub-cloned into pUC18 utilizing the *Xba*I and *Sma*I sites and then sequenced. The plasmid, pKmsBΔ0559, was transformed into *E. coli* S17-1 cells and used for biparental conjugation. Clones having undergone a single recombinational event were picked based on their Km^R. Single colonies were passaged to ensure purity, and then a period of outgrowth without antibiotics for 5 h allowed for the second recombinational event to occur. The BHΔ0559 mutant was selected for its loss of Km^R and lack of sucrose sensitivity encoded by the pK18*mobsac*B vector. After multiple rounds of passaging, the correct mutant construction was confirmed via Southern blot analysis (Figure 40). Digestion of wild type DNA with *Xho*I yielded 1675 bp and 1867 bp bands (PanelB, lane 2). A *Xho*I restriction site was lost in the 376 bp deletion internal to *azo0559* and digestion with *Xho*I yielded bands of 1867 bp and 1360 bp (PanelB, lane 1).



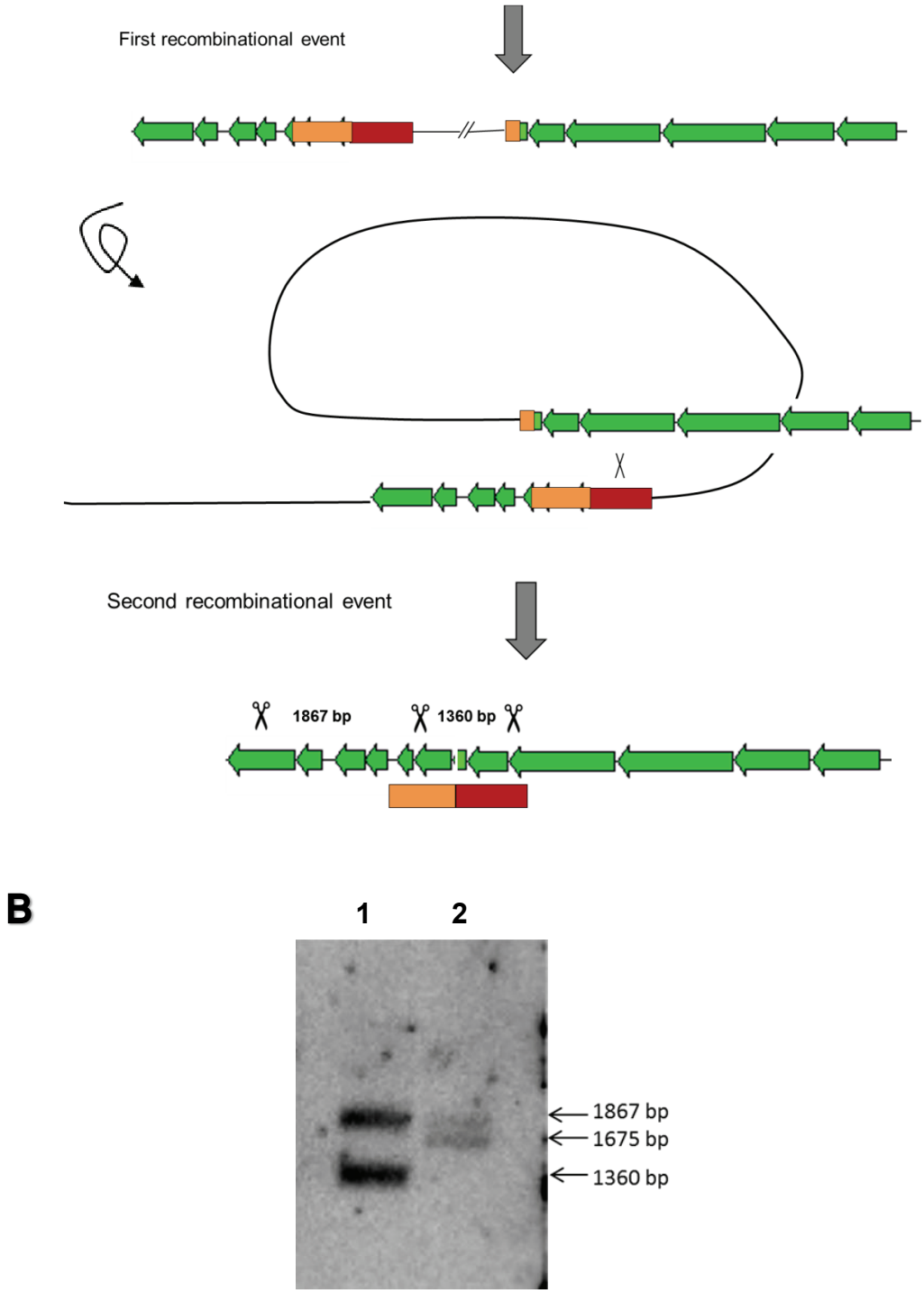


Figure 40. Construction and Southern blot analysis of BHΔ0559. (**A**) Schematic of the construction of in-frame deletion mutant BHΔ0559. Wild type DNA is depicted in green and the light and dark brown are PCR fragments amplified from genomic DNA. *Xho*I restriction sites are indicated by scissors above the DNA. (**B**) In a Southern blot analysis, 3 µg of genomic DNA extracted from wild type (lane 2) or BHΔ0559 (lane 1) was digested with *Xho*I. DNA fragments and molecular size marker, *Pst*I digested lambda phage DNA (200 ng/µl) were separated using agarose gel electrophoresis, transferred to a nylon membrane and detected using a DIG-labeled probe of the down fragment. Hybridized probe was detected using CDP-star Chemiluminescent Substrate (1:25,000) (Sigma, Aldrich, St. Louis, MO, USA). The expected band sizes are indicated in kilobases (kb) on the right.

III. F. 5. BHΔ0559 maintained a phenotype similar to the polar mutant BHazo0559.

It was believed that disruption of genes necessary for nitrogen fixation lead to the poor growth of BHazo0559 cultures under nitrogen fixing conditions. In an effort to normalize total protein production, an in-frame deletion mutant was the constructed. BHΔ0559. To quantify the growth more accurately, BHΔ0559 cultures were grown in liquid media with nitrogen fixing conditions held constant using a bioreactor (Section IIB1b). Unfortunately, the growth of BHΔ0559 under nitrogen fixing conditions was much more stagnant than wild type (Figure 41). A doubling time of 9.4 h in the first trial (diamonds) and 7 h in the second trial (squares) was calculated from the last three time points measured. Doubling times were far greater than the 2 h needed for wild type when grown under identical conditions.

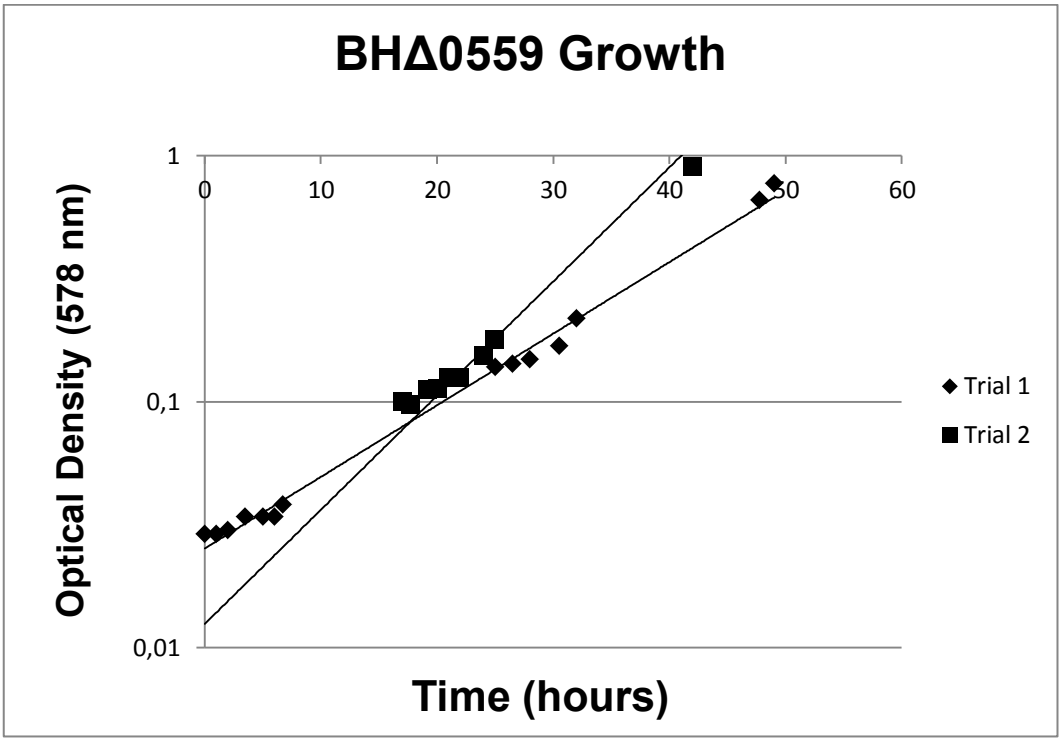


Figure 41. Growth of BHΔ0559 in a bioreactor under nitrogen fixing conditions. The growth of mutant strain, BHΔ0559, was monitored in two independent trials by measuring the optical density (578 nm) of 1 ml samples at different time points of cultures. Nitrogen fixing conditions were maintained by a 2 L bioreactor (SM medium, 37°C, pH 7.0, 0.6% O_2, 600 rpm).

Once again, the addition of glutamate was used to improve growth while promoting nitrogen fixation. Cells were grown in a bioreactor to maintain conditions promoting nitrogen fixation with an SM+G medium. The addition of glutamate to the medium successfully improved

growth of BHΔ0559 cultures under nitrogen fixing conditions. In a single trial, Hcp expression and secretion by BHΔ0559 when grown in a bioreactor with glutamate was also assessed via Western blot analysis (Figure 42). The results were comparable to the expression of Hcp by the polar mutant, BHazo0559, grown on SM+Glutamate media (Figure 39). Using liquid cultures allowed for the assessment of secretion via presence of Hcp in the supernatant. In addition to the increased detection of Hcp in the total cell fraction, a greater amount of Hcp was detected in the BHΔ0559 culture supernatant than wild type (Figure 42, lanes 1 and 2). Hcp expression, and possibly secretion, was induced in Azo0559 mutants with the addition of glutamate.

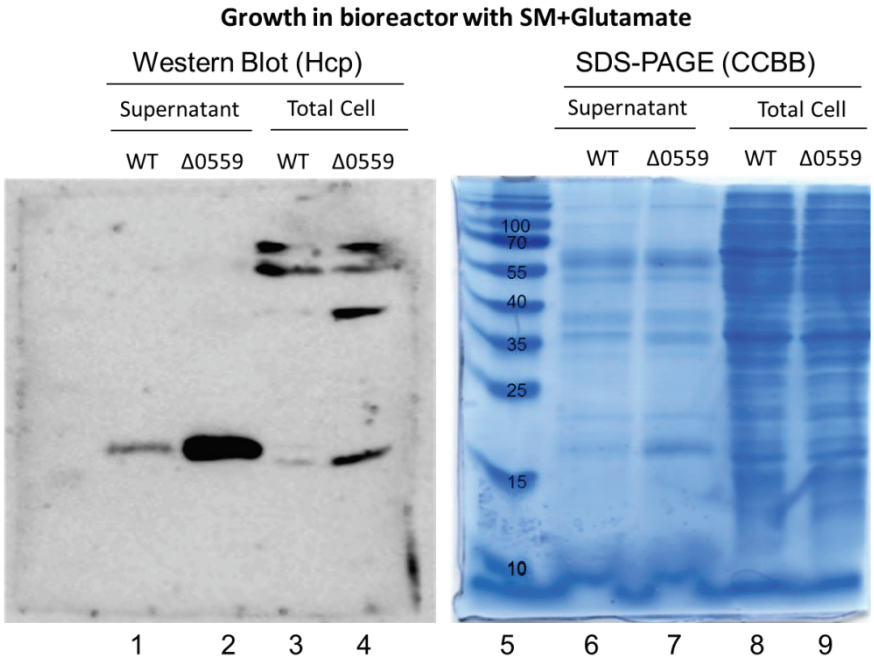


Figure 42. Western blot analysis of Hcp expression and secretion by wild type and BHΔ0559. Proteins from the supernatant (lanes 1, 2, 6 and 7) and total cell protein extracts (lanes 3, 4, 8 and 9) of wild type (WT, lanes 1, 3, 6 and 8) and in-frame deletion mutant, BHΔ0559 (lanes 2, 4, 7 and 9) grown under nitrogen fixing conditions maintained in a bioreactor (37°C, pH 7.0, 600 rpm, 0.6% O_2) in SM media supplemented with 20 mM glutamate to an OD_{578} of 1.4. Proteins were separated on 12% polyacrylamide SDS-PAGE gels and transferred onto a nitrocellulose membrane. The Western blot was probed with anti-Hcp. Lumi-Light^{PLUS} western blotting substrate (Roche, Rockford, IL, USA) was used for detection of protein bands. A parallel gel stained with Colloidal Coomassie Brilliant Blue controlled protein loaded. Protein band sizes were estimated using PageRuler™ Prestained Protein Ladder (Fermentas, ThermoFisher, Rockford, IL, USA).

Western blot analysis limited assessment of Hcp expression to its detection in the total cell fraction or the supernatant, but if levels of secretion were altered independent of expression

levels, incorrect conclusions would be made. For example, if the expression of Hcp was induced in the tested mutant, but secretion was also induced, the amount of Hcp detected in the total cell fraction of the mutant compared to wild type may not be truly indicative of overall relative expression. To investigate the role of a potential transcriptional regulator, overall expression needed to be tested by using a reporter gene system. Because of its up-regulation under nitrogen fixing conditions, changes in T6SS-1 gene expression were monitored.

III. F. 6. The pK18GGST::1301pro plasmid was constructed and incorporated into the chromosomes of *Azoarcus* sp. strain BH72, BHΔ0559 and BHLAO.

Construction of BH1301 began with PCR amplification of 578 bp from wild type chromosomal DNA including the upstream promoter region through 82 bp into *azo1301* with additional *Xba*I and *Hind*III restriction sites added to the 3' and 5' end, respectively. After primer design and optimization of PCR conditions, plasmid construction was performed by students in a lab practical course by incorporation of the DNA fragment into the pK18GGST plasmid. The plasmid was sequenced using the 1301fsnF and 1301fsnR primers, in each direction. The plasmid was then transformed into *E. coli* S17-1, which was used to donate the plasmid to *Azoarcus* sp. strain BH72 in a biparental conjugation. After a single recombinational event (Figure 43, Panel A) the mutants were selected for their Km^R and screened through colony PCR. BH1301 was passaged multiple times to ensure clone purity before doing Southern blot analysis using a *gfp* specific probe to confirm proper integration (Figure. 43, Panel B).

The pK18GGST::1301pro plasmid was also incorporated into BHΔ0559 (from this study) and BHLAO (from a previous study) using the same method as incorporation into *Azoarcus* sp. strain BH72. Proper integration of the plasmid was confirmed via Southern blot analysis (Figure 43, Panel B). Cutting with *Sma*I produced a band of 3072 bp, which was detected by the *gfp*-probe after integration into each strain (wild type (WT), BHLAO and BHΔ0559).

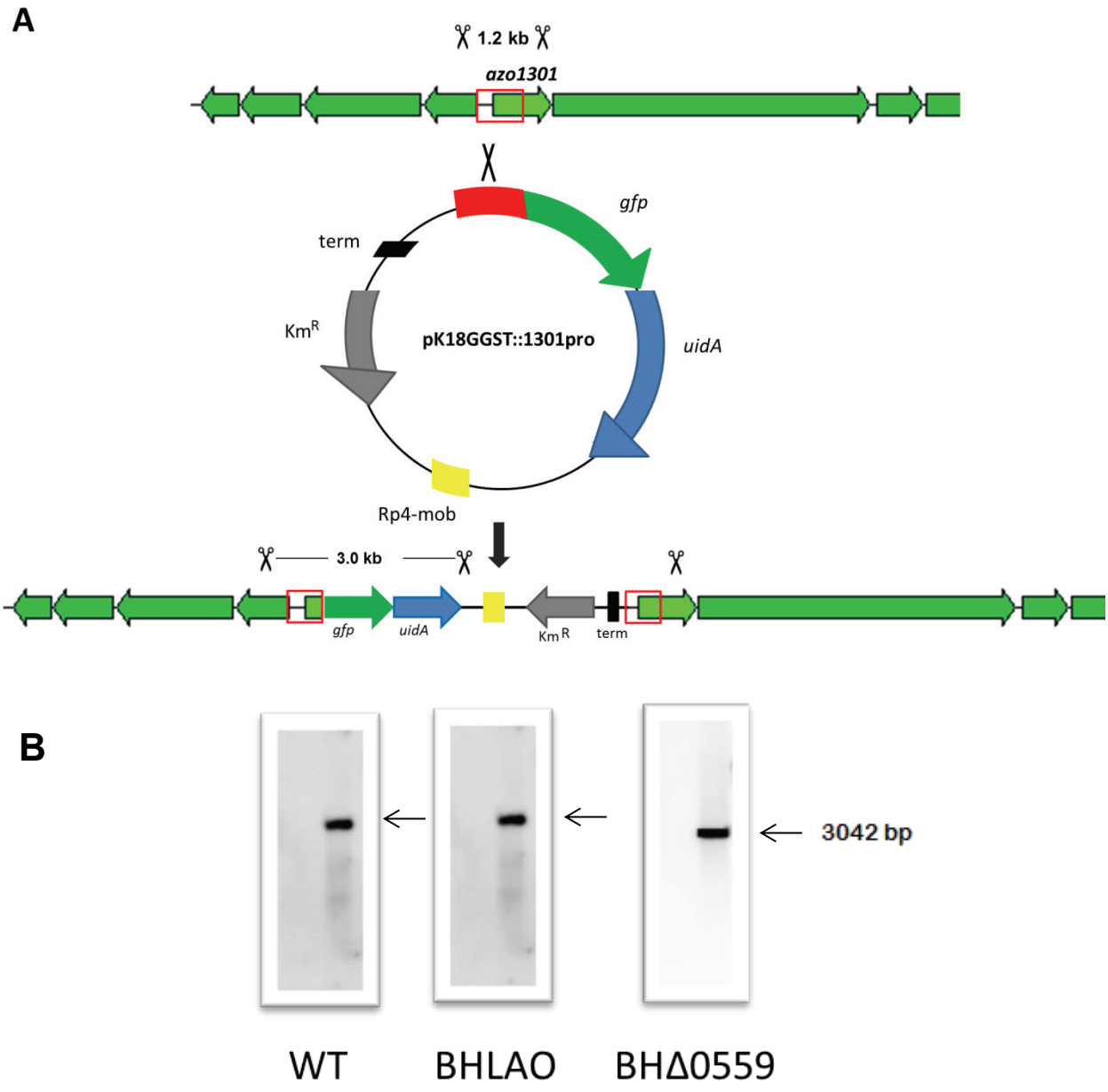


Figure 43.Construction of BH1301 and integration of pK18GGST1301pro into BHLAO and BHΔ0559. (A) Schematic of the methods used to obtain a transcriptional fusion of the *azo1301* promoter region with the β-glucuronidase gene *gusA* in wild type, BHLAO and BHΔ0559 backgrounds. Wild type chromosomal DNA is depicted in green and the pK18GGST plasmid is depicted with various colors representing different genes (red=PCR amplified product from *tssK2*; dark green=*gfp*; blue=*uidA(gusA)*; gray=Km^R; black=transcriptional terminator; yellow=Rp4 mobilization genes). **(B)** Southern blots of genomic DNA extracted from wild type (WT), BHLAO and BHΔ0559 strains with the integrated plasmid and digested with *Sma*I (restriction sites marked by scissors). DNA fragments and molecular size marker, *Pst*I digested lambda phage DNA (200 ng/µl), were separated using agarose gel electrophoresis, transferred to a nylon membrane and probed with DIG-labeled *gfp*. Hybridized probe was detected using CDP-star Chemiluminescent Substrate (1:25,000) (Sigma, Aldrich, St. Louis, MO, USA). The expected band sizes are indicated in basepairs (bp) on the right.

III. F. 7. GUS assays revealed transcription of the *azo1301* operon was altered in transcriptional regulator mutants and in response to different growth media when compared to transcription in the wild type strain.

Transcriptional studies were done using the promoter region upstream of *azo1301* (refer to Figure 3) in a transcriptional fusion with a *gusA* gene encoding for β-glucuronidase on a plasmid as depicted in Figure 43. Testing this promoter region was ideal because multiple genes encoding for core components of the T6SS are likely transcribed from here, a σ^{24} (RpoE) binding site had been predicted upstream of *azo1301* (Teja Shidore, PhD thesis, 2012) and Azo1301 was one of only two T6SS-1 proteins found in the supernatant in the secretome analysis. Incorporation of pK18GGST::1301pro into the chromosomes of wild type and mutant strains allowed for a comparison of overall expression between different strains and different growth conditions. Transcription from the promoter was quantified through GUS activity. GUS assays were performed as described (Section IIB7) on cells grown in sealed 1L flasks set with 1.2% O_2 for six hours with shaking at 200 rpm at 37°C (Section IIB1c). Batch cultures were grown under nitrogen fixing conditions without a nitrogen source (SM-N), with 9.3 mM NH_4Cl (SM+N) or with 20 mM glutamate as a source of nitrogen (SM+Glu). Three independent experiments were done in replica on each strain in each media.

III. F. 7a. GUS assays revealed increased transcription of the *azo1301* operon under nitrogen fixing conditions in the wild type strain.

In the wild type strain, expression of *azo1301* was the greatest when grown in SM-N medium (without a source of combined nitrogen). A 4-fold induction of this T6SS-1 promoter region was seen. An induction of transcription was also observed when cultures were grown in media supplemented with glutamate to a slightly less extent, about 3-fold (Blue Bars, Figure 44). The different inductions observed by the wild type strain in each media were statistically significant differences with $P \leq 0.005$ in two-tailed paired t-tests.

III. F. 7. b. GUS assays revealed transcription from the promoter region of *azo1301* was reduced in the strain BHLAO compared to wild type.

A *nifLA*$^-$ strain, BHLAO, was constructed by insertion of an omega cassette into *nifL*, disrupting both genes of the *nifLA* operon (Egener et al., 2002). The pK18GGST::1301pro plasmid was also incorporated into the BHLAO genome, and transcription from the *azo1301* promoter was assessed by cells grown in batch cultures with microaerobic conditions as described in Section IIB1c. Of the media used for growth, BHLAO cells exhibited the highest

expression from the *azo1301* promoter when grown in SM+G medium (1.5-fold greater than SM+N and 1.75-fold greater than SM-N). Still, expression of *azo1301* in BHLAO was significantly less than wild type when cells were grown in SM+Glu (about 1.5-fold less) and when cells were grown in SM-N media (4.5-fold less). When grown in SM+N medium, transcription from the *azo1301* promoter was 1.3-fold greater by the strain BHLAO than wild type (Red Bars, Figure 44).

A former PhD student had assessed the expression of *azo1299, azo1302* and *azo1305* using semi-quantitative RT-PCR and RNA extracted from BHLAO and wild type strains grown in a microaerobic environment, in media that had been supplemented with glutamate. It appeared induction of these genes was independent of *nifA* since transcriptional levels were not significantly different between BHLAO and wild type strains (Teja Shidore, PhD thesis, 2012).

Comparing the transcription from the *azo1301* promoter of cultures grown under nitrogen fixing conditions with and without media supplemented with glutamate, expression was significantly lower in the BHLAO strain than wild type in both media. Contrary to previous studies, these observations suggested that NifA is indeed a positive regulator of this T6SS-1 gene.

III. F. 7. c. GUS assays revealed transcription of the *azo1301* operon was reduced in BHΔ0559 compared to wild type.

The pK18GGST::1301pro plasmid was also incorporated into BHΔ0559. Cells were grown in flasks set with 1.2% O_2 as described in Section IIB1c. In these assays the expression of *azo1301* by BHΔ0559 was not significantly different between growth under nitrogen fixing conditions in SM-N and growth in SM+N ($P=0.7$ in a two-tailed paired t-test). The expression was highest when grown in SM+Glu. However, the increase of transcription measured in cells grown in SM+Glu was only 1.5-fold higher than growth in SM+N media and 1.7-fold higher than cells grown in SM-N (Green Bars, Figure 44).

The induction of *azo1301* transcription by BHΔ0559 and wild type strains was not significantly different when strains were grown in SM+N. There was a significant difference in *azo1301* expression by BHΔ0559 and wild type strains when cells were grown in batch cultures under nitrogen fixing conditions (SM-N) and when cells were grown in SM+Glu. In

both growth conditions, the expression of *azo1301* was less by the RseC mutant, BHΔ0559 (Figure 44). These observations suggested that Azo0559 is positive regulator of T6SS-1.

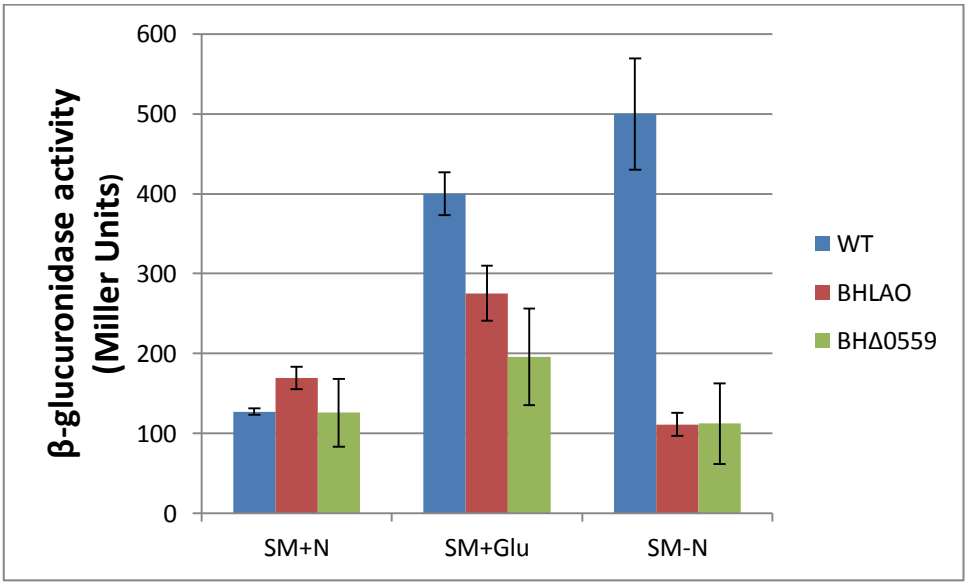


Figure 44. GUS activity of *azo1301* in *Azoarcus* strains grown after six hours of induction. The promoter region upstream of *azo1301* was transcriptionally fused with a *gusA* gene by incorporation of pK18GGST::1301pro into the chromosomes of wild type (WT), BHLAO and BHΔ0559 mutant strains. Airtight 1 L flasks containing SM medium without a nitrogen source (SM), with 9.3 mM NH_4Cl (SM+N) or with 20 mM glutamate (SM+Glu) were flushed with N_2 and then injected with air until 1.2% O_2. Media was inoculated with BH1301 (WT, blue), BHLAO (red) or BHΔ0559 (green) and expression from the promoter was quantified in GUS assays by β-glucuronidase activity after 6 h of shaking at 200 rpm at 37°C. Three independent experiments were done in replica on each strain in each media. Values reported are the average and error bars represent the standard deviation of measurements for the given bacterial strain and growth medium.

III. F. 8. BHΔgacA was constructed by making an in-frame deletion of *azo2980*.

GacS/GacA is a two component system where, in the transcriptome profile of *P. aeruginosa* M18, 15% of the annotated genome was influenced by mutation to GacA. In *P. aeruginosa*, the GacA regulon was shown to include all three of the T6SS gene clusters (Wei et al., 2013). Furthermore, in an earlier microarray study by another group, it was shown that *retS* encodes an activator of GacAS signaling and T6SS genes were up-regulated in ΔretS (Mougous et al., 2006). GacA is activated through a phosphorelay system and leads to expression of small regulatory RNAs that sequesters mRNA-binding protein, RsmA, which inhibits translation by interfering with the ribosome binding site access (Silverman et al., 2012). *Azoarcus* sp. strain BH72 encodes for a GacA protein (*azo2980*), and because of its potential role in T6SS

regulation, Hcp expression an in-frame deletion mutant, BHΔgacA, was assessed. A 985 bp upstream and 5' region of the *gacA* gene (913 bp upstream-71 bp into *azo2980*) was amplified via PCR with additional restriction sites *Bam*HI and *Xba*I at the 5' and 3'ends, respectively. A 772 bp region at the 3' end and downstream (632 bp in-736 bp downstream of *azo2980*) was PCR amplified with additional *Xba*I and *Hind*III sites at the 5' and 3' ends, respectively. These up and down fragments were each cloned into pJET1.2/blunt cloning vectors. The downstream fragment was then subcloned into pUC18 using the *Bam*HI and *Xba*I sites. The up fragment was then cut out of its pJET vector and cloned in the down fragment containing pUC18 vector. The resulting pUC18ΔgacA plasmid was sequenced using universal primers, M13F and M13R. The combined sequences containing the 540 bp deletion were then further subcloned into the pK18*mobsac*B vector. The newly constructed pK18mobsacBΔgacA was next transformed into *E. coli* S17-1 cells for plasmid donation to strain BH72 in a bi-parental conjugation (Section IIB3b). Transconjugants having undergone a single recombinational event were selected for Km^R encoded by the vector. Selected clones were picked and replated to ensure purity before undergoing a period of growth without antibiotic selection. In this time a second recombinational event occurred, and clones were selected for their loss of Km^R and sucrose sensitivity encoded by the *sacB* gene on the pK18*mobsac*B vector and screened via colony PCR. Confirmation of proper genomic mutation was confirmed by Southern blot analysis (Figure 45). Digestion of wild type DNA with *Pst*I yielded two bands, 793 bp and 1007 bp but the probe was made of UP fragment DNA only. The larger downstream band was not probed for. The 540 bp deletion internal to *azo2980* in BHΔgacA resulted in the loss of a *PstI* restriction site and yielded a single band of 1146 bp.

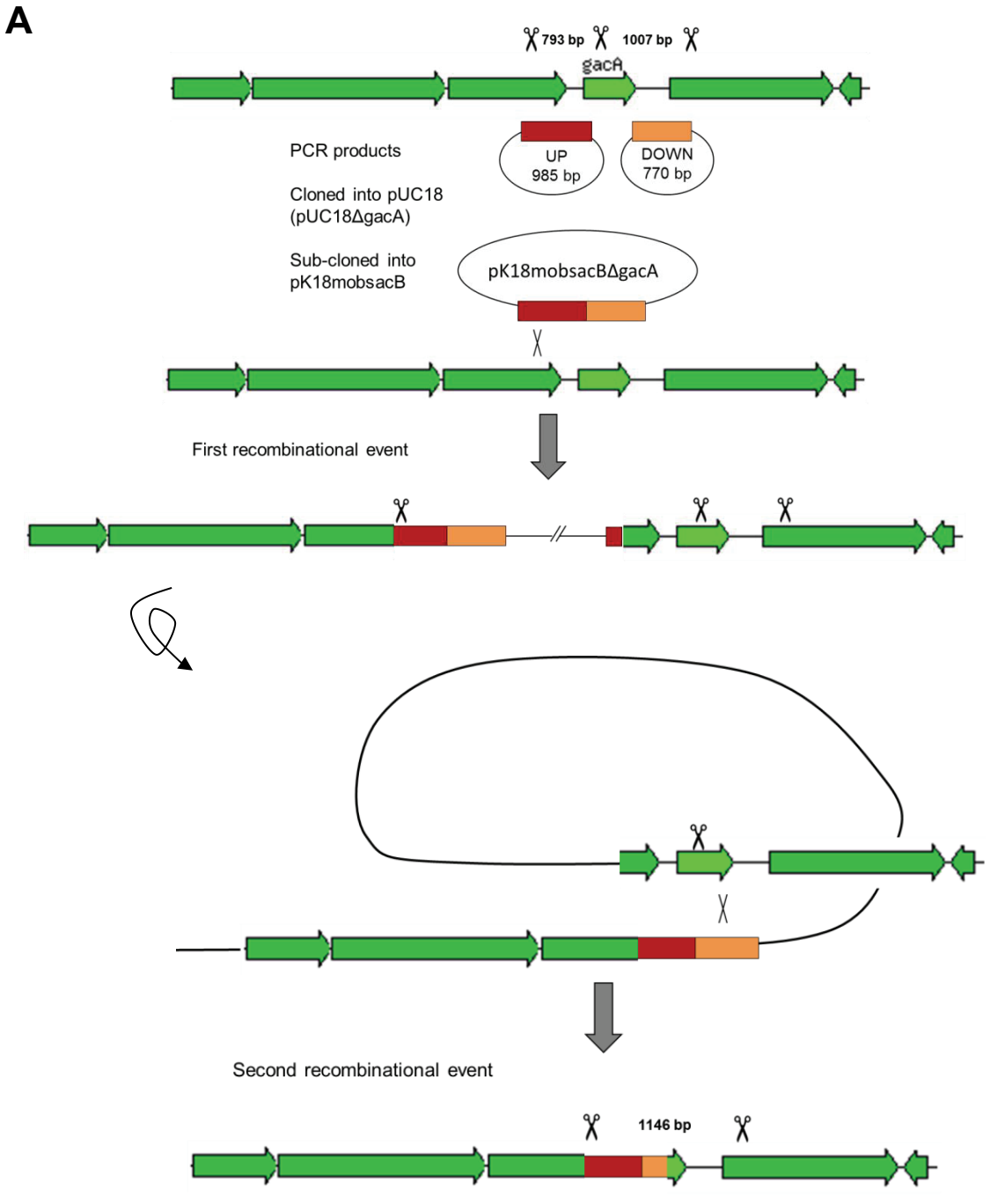

A
793 bp
1007 bp
gacA
PCR products
UP
985 bp
DOWN
770 bp
Cloned into pUC18
(pUC18ΔgacA)
Sub-cloned into
pK18mobsacB
pK18mobsacBΔgacA
First recombinational event
Second recombinational event
1146 bp
B
1 2
1146 bp
800 bp

Figure 45. Construction and the Southern blot analysis of BHΔgacA. (**A**) Schematic of the construction of the in-frame deletion mutant BHΔ3885. Chromosomal DNA is depicted in green and the light and dark brown are PCR fragments amplified from genomic DNA. Restriction sites are indicated by scissors above the DNA. (**B**) In a Southern blot analysis, 3 μg genomic DNA from *Azoarcus* sp. strain BH72 (lane 1), BHΔgacA (lane 2) were digested with *PstI*. DNA fragments and molecular size marker, *Pst*I digested lambda phage DNA (200 ng/μl), were separated by agarose gel electrophoresis, transferred to a nylon membrane and probed with DIG-labeled 'up' fragment. Hybridized probe was detected using CDP-star Chemiluminescent Substrate (1:25,000) (Sigma, Aldrich, St. Louis, MO, USA). The expected band sizes are indicated in kilobases (kb) on the right.

III. F. 9. It could not be concluded from the initial screening of BHΔgacA that GacA functions as a transcriptional regulator of T6SSs in *Azoarcus* sp. strain BH72.

Expression of Hcp by BHΔgacA was compared to *Azoarcus* sp. strain BH72. Total protein was extracted from the strains grown in SM semi-solid media and in SM+N semi-solid media after two days. There was no apparent difference in Hcp detected between the wild type and GacA mutant strains grown in non-fixing conditions in semi-solid media supplemented with ammonium chloride (Figure 46, lanes 1 and 2). Also in SM+N broth, there was not a difference in Hcp detected in the total cell protein extracts (Figure 46, lanes 8 and 9). Only when cells were grown under conditions promoting nitrogen fixation (SM-N semi-solid media) were some differences seen. Initially the expression of Hcp seemed greatly reduced in the GacA mutant compared to wild type (Figure 46, lanes 3 and 5). However, the Colloidal Coomassie Brilliant Blue stained gel run in parallel suggests (CCBB stain, 46) the reduced amount of Hcp detected was primarily due to an overall inefficient protein extraction. Additionally, these initial results could not be replicated in subsequent trials. From these findings when initially screening BHΔgacA, it was decided to no longer pursue GacA as a transcriptional regulator of the T6SS and efforts focused on Azo0559.

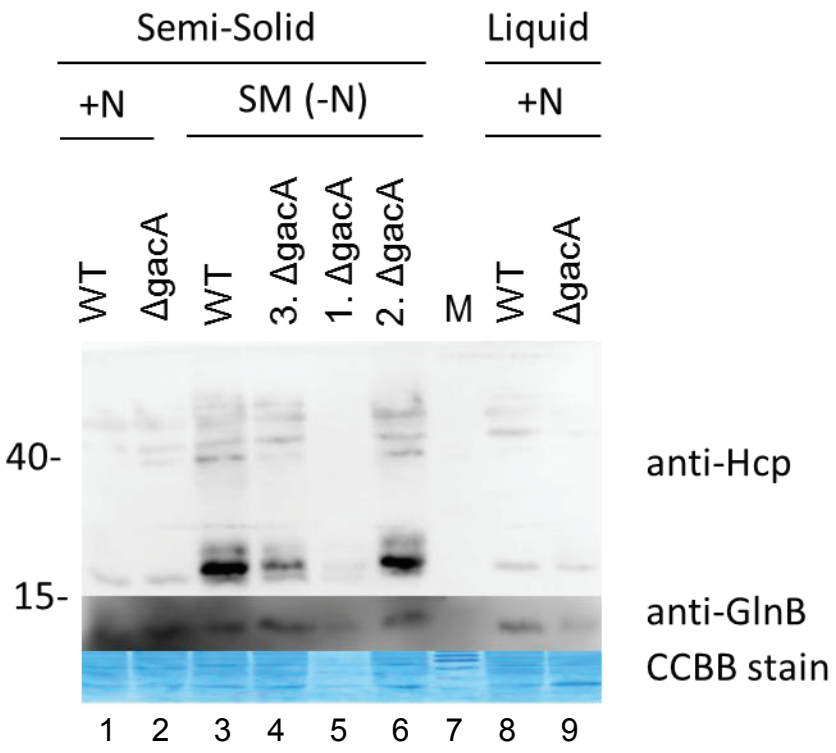


Figure 46. Detection of Hcp in total cell protein extracts of *Azoarcus* sp. strain BH72 and BHΔgacA. Total cell protein extracts were obtained from *Azoarcus* sp. strain BH72 (WT, lanes 1, 3 and 8) and BHΔgacA (ΔgacA, lanes 2, 4-6 and 9). Cells grown in semi-solid SM media (-N, lanes 3-6), semi-solid SM media with 9.3 mM NH_4Cl (+N, lanes 1 and 2) or SM broth with 9.3 mM NH_4Cl (Liquid +N). Total proteins were seperated using a 12% polyacrylamide SDS-PAGE gel and transferred onto a nitrocellulose membrane. Western blots were probed with anti-Hcp serum. Lumi-Light^{PLUS} western blotting substrate (Roche, Rockford, IL, USA) was used for detection. A parallel gel stained with Colloidal Coomassie Brilliant Blue Stain (CCBB, a portion is pictured) controlled for equal loading. GlnB (~12 kDa) was detected by anti-GlnB (1:1000, 1% BSA; 1X TBS) to control equal loading. Protein band sizes were estimated using PageRuler™ Prestained Protein Ladder (lane 7) (Fermentas, ThermoFisher, Rockford, IL, USA).

IV. Discussion

It has been speculated that microbes have been interacting with plants since their movement to land (Chisholm et al., 2006). Despite the plethora of microbes present in the environment, most microbes do not develop an association while the plants are resistant or immune to microbial advances. Only through the co-evolution of precise signaling pathways do the lines of communication even open between interacting partners, allowing the impeding microbe to breech several levels of plant defense. The arsenal of each side goes back and forth with increasing specificity for each organism involved. Structural boundaries must be penetrated, and MTI and ETI responses must be overcome. The subsequent plant-microbe interaction lies on a spectrum of associations ranging from pathogenic to beneficial. Occasionally, a balance is found, but even then the scales are vulnerable to tipping in one direction or the other as organisms adapt to ever-changing niches. In a pathogenic interaction microbes feed off their host plant as bio-, necro- or hemibiotrophs with a disproportionately low benefit reciprocated to the host, if any. In this interaction the host is at a disadvantage, which in some cases eventually leads to the detriment of the microbe. (reviews: Ahmadjian and Paracer, 2000; Thordal-Christensen, 2003; Kogel et al., 2006; Pel and Pieterse, 2013; Muthamilarasan and Prasad, 2013).

More intriguing is the maintenance of a sustainable, non-pathogenic interaction, such as a mutualistic one. Well-studied examples of mutualistic, endosymbiotic microbes include AMF and legume-nodulating rhizobia, where continual crosstalk is required for both parties to persist and benefit (Harrison, 2005; Gyaneshwar et al., 2011; Jones et al., 2007). A lesser-studied, non-pathogenic lifestyle is the endophytic one, such as the occurrence of *Azoarcus* sp. strain BH72 colonizing rice without rejection (Egener et al., 1999; Hurek et al., 1997). Wild type strain BH72 has been shown to invade rice in a mode similar to pathogens without invoking a visible defense response (Egener et al., 1999; Hurek et al., 1997). So far, it has been determined endogluconase, type IV pili and flagella are bacterial features promoting colonization (Reinhold-Hurek et al., 2006; Reinhold-Hurek et al., 1993b; Dörr et al., 1998; Buschart et al., 2012). However, it remains unclear how *Azoarcus* sp. strain BH72 actively evades or overcomes the plant's immune response. In ETI, which requires more specificity in signaling than MTI (Schulze-Lefert, 2010; Pel and Pieterse, 2013), an effector is usually delivered via a T4SS or T3SS, secretion systems lacking in *Azoarcus* sp. strain BH72 (Krause et al., 2006). The discovery of T6SS(s), and their involvement in bacterial interactions with a

eukaryotic host, was a promising avenue to pursue, because two T6SS gene clusters had been identified in the genome of *Azoarcus* sp. strain BH72 (Krause et al., 2006). In this study the T6SS of *Azoarcus* sp. strain BH72 was investigated, expanding findings from previous work with the primary goal to determine the function of T6SS in *Azoarcus* sp. strain BH72 and if it plays a role in establishing the endophytic lifestyle in rice.

Prior to this study T6SS gene clusters were identified, but details about the majority of components remained unknown. Since this time several advancements have been made in the field, elucidating detailed structures and functions. Upon close examination of the *Azoarcus* sp. strain BH72 genome, both T6SS gene clusters showed little deviation from the contents of typical T6SS(s). In 2009 Boyer et al. divided the T6SSs into 5 sub-groups (I-V) based primarily on presence of conserved accessory proteins (in addition to core components). T6SS-2 was assigned to sub-group III and T6SS-1 to sub-group IV (Boyer et al., 2009).

IV. A. Characterization of T6SS components encoded by the *Azoarcus* sp. strain BH72 genome

IV. A. 1. The Membrane Complex

TssM, the IcmF-like remnant of the T4bSS of *L. pneumophilia*, has long been recognized as a core component of the T6SS, essential for function (Zheng and Leung 2007; Schwarz et al., 2010a; Silverman et al., 2012), and was found encoded in both T6SS gene clusters of the *Azoarcus* sp. strain BH72 genome. In 2009 Boyer et al. performed a genome wide *in silico* analysis of 176 T6SS in 92 bacteria. They found 6% of the TssM had three transmembrane segments and 84% had at least one (Boyer et al., 2009). TssM proteins of *Azoarcus* sp. strain BH72 have a short N-terminus (probably cytoplasmic) followed by three transmembrane segments with a Walker A motif between the second and third transmembrane segment. Similar structural predictions were made for *Agrobaterium tumefaciens* str. C58 as illustrated in Figure 47 (adapted from Erh-MinLai et al. 2009, http://ipmb.sinica.edu.tw/index.html/?q=node/209). The majority of T6SS TssM proteins contain a Walker A motif (GXXXXGKT) at about residue 140 (Ma et al., 2009). The lysine binds α- and γ- phosphoryl groups of nucleotide triphosphates, critical for ATPase activity (Walker, 1982). However, evidence for Walker A activity necessity is conflicting. In *A. tumefaciens* replacing Lys with Ala reduced T6SS activity, and T6SS was abolished by additionally replacing the adjacent Gly with an Ala and when deleting the entire Walker A motif (Ma et al., 2009). Contradictory to this, in

an *E. tarda* EvpO (TssM) deletion mutant, complementation with EvpO where the Lys was replaced with Ala produced the same extracellular proteins as did complementation with a wild type EvpO. Replacement of other conserved residues and double mutations also produced secretion profiles similar to complementation with wild type (Zheng and Leung 2007). The necessity of Walker A motifs in T6SS secretion by *Azoarcus* sp. strain BH72 was not determined, but the motif was identified in both TssM1 and TssM2. In future work, mutation to the Walker A motifs may reveal its requirement for function in one, both or neither of the T6SSs.

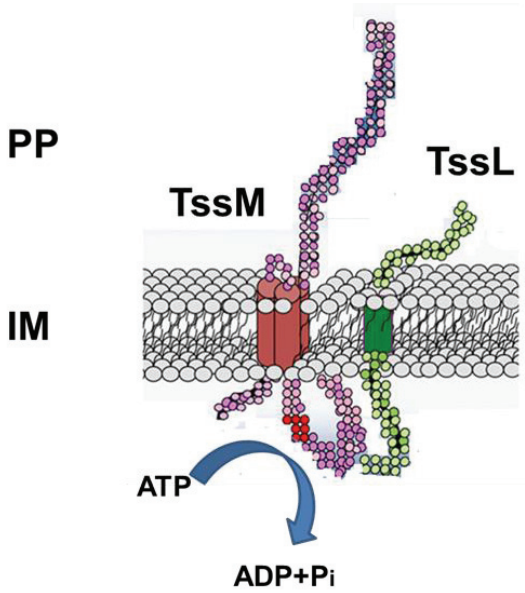


Figure 47. Predicted positioning of TssM and TssL in the inner membrane (IM). Adapted from an original figure by Erh-Min Lai et al. of Imp_K and Imp_L of *A. tumefaciens* str. C58. TssM (purple) is represented with three transmembrane helices with a Walker A motif (red) between the second and third transmembrane segment. The C-terminus of both TssM and TssL (green) is predicted to face the periplasm (PP). This figure is adapted from an original image found at http://ipmb.sinica.edu.tw (Copyright © 2009 Institute of Plant and Microbial Biology, 128 Sec. 2, AcademiaRd, Nankang, Taipei 11529 Taiwan, R.O.C.)

TssL (IcmH/DotU), another remnant of the T4bSS, stabilizes TssM (Durand et al., 2012). In other genomes these genes are highly conserved and usually occur in a pair (Robb et al., 2012). In the *Azoarcus* sp. strain BH72 genome, *tssM1* and *tssL1* were in opposing open reading frames and *tssM2* and *tssL2* were organized in tandem (Julia Herglotz, Diplomarbeit, 2007). TssL typically has a periplasmic peptidoglycan binding domain at its C-terminus, a single transmembrane segment and a cytoplasmic N-terminus, where the bulk of the protein resides and interacts with TssM (Durand et al., 2012). In *Azoarcus* sp. strain BH72, TssL1 lacks a peptidoglycan binding domain (PGB), such as the OmpA/MotB-like one of TssL2.

However, the C-terminal of the gene product of *azo1306* was predicted to be OmpA/MotB-like. In other organisms, if not a part of TssL, a PGB domain containing accessory protein (TagL, TagP, TagN or TagW) is usually present that binds TssL and peptidoglycan (Aschtgen et al., 2010; Cascales and Cambillau, 2012; Filloux et al., 2008; Ma et al., 2009). Aschtgen et al. proposed a 'fusion hypothesis', in which TssL proteins that include a PGB domain are 'evolved TssL' resulting from the fusion of TssL and TagL (Aschtgen et al., 2010). It is probable that Azo1306 anchors the T6SS apparatus to the peptidoglycan. Still, despite evidence that anchorage to the cell wall is an absolute requirement for some T6SSs, some T6SS gene clusters do not encode for a PGB domain containing protein. The well characterized and functional T6SSs of *V. cholerae* and *E. tarda* lack a PGB domain (Aschtgen et al., 2010). A previous student from this laboratory attempted localization studies with a strep-tagged Azo1306, but because of antibody cross-reactivity in all cellular fractions, nothing could be concluded about localization (Anna Tomhardt, Diplomarbeit, 2009). It would be interesting to determine if Azo1306 does in fact bind to TssL1. In future work, the binding of TssL1 to strep-tagged Azo1306 immobilized in a sepharose column could be assessed by checking for the expected protein sizes in an SDS-PAGE gel.

The final component of the membrane complex is TssJ (SciN), an outer membrane lipoprotein that faces the periplasm and interacts with TssM. TssJ homologs of *Azoarcus* sp. strain BH72, TssJ1 (*azo1300*) and TssJ2 (*azo3893*), have a predicted lipobox (LAAC) without an aspartate in the +2 position, which is a requirement for localization (Aschtgen et al., 2008; Cascales and Cambillau, 2012). Additionally, the genome of *Azoarcus* sp. strain BH72 encodes for proteins involved in proper localization of TssJ, signal peptidase II (LspA, *azo1204*), LolA (*azo1366*) and LolB (*azo0757*). In enteroaggregative *Escherichia coli*, the proper localization of TssJ is a requirement for function (Aschtgen et al., 2008). Lipoproteins of the outer membrane contain an N-terminal lipobox (L-A/S-G/A-C) that is cleaved by signal peptidase II at the inner membrane. After removal of the consensus signal sequence, the N-terminal α-amino group of the cysteine residue is fatty acylated, and the mature lipoprotein is carried to the outer membrane through the periplasm by carrier protein LolA, as long as there is not an aspartate at the +2 position. LolB then anchors the lipoprotein into the outer membrane via the acylated cysteine (Aschtgen et al., 2008; Cascales and Cambillau, 2012). Further studies on localization or function of these predicted proteins, as they pertain to the T6SS, may reveal their role in proper localization of the membrane complex. It would

be interesting to determine if mutation to the signal peptidase II, LolA or LolB affected both T6SSs. However, this aspect of T6S was not assessed in this work.

IV. A. 2. The bacteriophage-like injectisome

TssB and TssC cognates are core T6SS components also found encoded within both T6SS gene clusters of the *Azoarcus* sp. strain BH72 genome. They are similar to the injectisome components of a bacteriophage, forming the outer sheath of the Hcp tubule and believed to function in a similar way (Cascales and Cambillau, 2012; Kanamaru et al., 2002; Bönemann et al., 2009). The structure and interaction between TssB, TssC and Hcp proteins was determined by Cascales and Cambillau (2012) and Aksyuk et al. (2009) as seen in Figure 48. It is believed that contraction of TssB/TssC tubules provides the energy to push the Hcp syringe with a VgrG needle tip across the membranes and into the target cell (Basler et al., 2012). Like most T6SSs discussed in literature (Boyer et al., 2009), TssB and TssC proteins of *Azoarcus* sp. strain BH72 are highly conserved and co-organized with their respective Hcp(s) (TssD). Essential for their action is TssE, a protein similar to the bacteriophage gp25 protein. Like gp25, TssE proteins form a baseplate or hub required for secure anchoring of the apparatus to the cytoplasmic side of the inner membrane (Lossi et al., 2011). Unlike gp25, TssE does not have lysozyme activity. In *P. aeruginosa*, mutations led to loss of T6SS function (Lossi et al., 2011). TssE homologs were also identified within the *Azoarcus* sp. strain BH72 genome, but their necessity for T6SS was not evaluated.

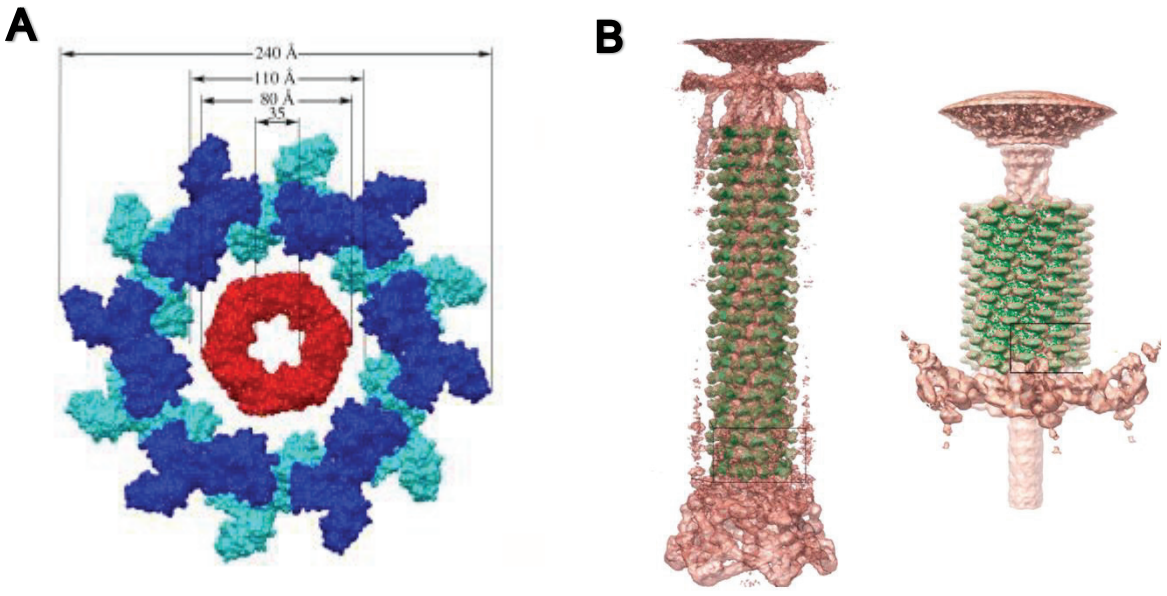


Figure 48. TssB and TssC form a sheath around Hcp hexamers. (**A**) A molecular surface model of a hexameric Hcp ring (red) was produced by Cascales and Cambillau (2012) and the predicted

organization of TssB and TssC (light and dark blue) modeled phage T4 tail sheath structure. (**B**) Aksyuk et al. (2009) produced a model of extended (left) and contracted (right) tail sheath structures of bacteriophage T4 using cryo-electron microscopy (Aksyuk et al., 2009).

At the tip of the Hcp tubule, extending into the ECM or the point of entry at the target cell, is another bacteriophage-like protein, VgrG. VgrG (TssI) proteins form a trimer that resembles a fusion of gp27 and gp5 of the bacteriophage T4 needle-like protein complex $(gp27)_3$-$(gp5)_3$ and is necessary for Hcp secretion (Pukatzki et al., 2007; Zheng and Leung 2007; Shalom et al., 2007; Boyer et al., 2009). Multiple VgrG proteins are sometimes encoded by a genome containing a single T6SS, and in *V. cholerae* it was shown that mulitmeric complexes can form; VgrG1 pulled down VgrG2 and VgrG3 in immunoaffinity assays (Pukatzki et al., 2007). Three VgrG-like proteins are encoded by the *Azoarcus* sp. strain BH72 genome, but only one was identified in the secretome, Azo3876 (TssI2). In this study, nothing was determined about their interactions with one another, but the appearance of only one in the secretome argues that the three VgrG proteins of *Azoarcus* sp. strain BH72 do not interact with one another during the secretion process, or at least not under these growth conditions.

It is not uncommon for the multiple genes encoding VgrG and/or Hcp proteins to be located distant from their respective T6SS gene cluster (Filloux et al., 2008). It has been proposed that unlinked Hcp and/or VgrG loci may contain secreted effectors (Coulthurst, 2013). Interestingly, Russel et al. discovered a diverse superfamily of bacterial phospholipases that are often encoded adjacent to VgrG homologs and are typically antagonistic to bacteria. Using bioinformatics, they found 377 putative lipases that could be divided into five families, type VI lipase effectors 1-5 (Tle 1-5). They found two homologs of Tle3 encoded within the *Azoarcus* sp. strain BH72 genome, *azo2942* and *azo3471*, with VgrG (*azo3470*) being linked to the latter (Russell et al., 2013). Due to time constraints, the possible role of the putative Tle3 homologs in T6S was not tested. However, neither of the Tle3 homologs nor the accompanying VgrG, Azo3470 was detected in the secretome of *Azoarcus* sp. strain BH72.

IV. A. 3. T6SS-2 might include additional genes with yet unknown functions.

Although it is true that VgrG and Hcp proteins are routinely found encoded outside of T6SS gene clusters, Azo3876 (TssI2/VgrG) should arguably still be considered as part of T6SS-2. Most of the core components are encoded by the genes *azo3890-azo3903*, but upstream of this are multiple *tag* genes that are now known to encode for accessory proteins including TagH/Fha (*azo3884*), TagG (*azo3885*), TagE (*azo3888*), TagF (*azo3889*) and maybe more. Between genes *azo3876* and *azo3884*, *azo3877-azo3883* encode for hypothetical proteins–

except Azo3878 is a 3-oxoacyl-(acyl carrier protein) synthase. Interestingly, the T6SS gene cluster of *P. aeruginosa* PAO1, HSI-1, encodes for proteins PA0076-PA0091, and just downstream of these, genes encoding for PA0096-PA0101 (but not PA0100) showed a high degree of similarity to proteins encoded by the genes upstream of the T6SS-2 gene cluster, Azo3877-Azo3883 (but not Azo3879 or Azo3880). Using NCBI blastp the scores ranged from 41.2-83.6, with sequence coverage 29-98% and e-values always below 5e-06. The function of these proteins remains unknown in both *Azoarcus* sp. strain BH72 and *P. aeruginosa* PAO1, but the similarity in sequence and gene organization with proximity to T6SSs that potentially utilize a threonine phosphorylation pathway could be indicative of these genes also playing a role in T6SSs.

Although the significance is unknown, it is also interesting that Azo3886 was identified in the secretome. It is predicted to be a periplasmic protein, which would increase the chance that it would be found in the supernatant fraction regardless of function. It was more abundant in the BHΔimpLsciO mutant, which is not the criteria for a secreted substrate, but could mean that accumulation occurs when secretion is turned off. Additionally, Azo3875 was predicted to be a homolog of the σ^{54} enhancer binding protein, VasH, which is required for expression of *V. cholerae*, *P. aeruginosa* and *A. hydrophila* T6SSs (Miyata et al., 2013). Azo3875 was determined to not play a role in the regulation of the T6SS-1 gene, *azo1305*, which encodes for the Hcp protein TssD1 (Teja Shidore, PhD thesis, 2012). However, there have not yet been any studies testing its potential role in the regulation the T6SS-2. In future work, transcription of T6SS-2 genes by BHΔ3875 will need to be tested. Taken together, there is the potential that the T6SS-2 gene cluster utilizes all gene products encoded by genes *azo3875-azo3903*.

IV. A. 4. Differences between the two T6SS gene clusters

TssH (ClpV) is thought to energize the propulsion of the Hcp tubule out of the bacterial cell and into the target cell (Schlieker et al., 2005; Bönemann et al., 2009). The T6SS-1 gene cluster encoded by the *Azoarcus* sp. strain BH72 genome contains all core components of a T6SS, TssA-M, except TssH. In a genome wide *in silico* analysis by Boyer et al. (2009) the T6SS-1 gene cluster was the only T6SS from the sub-group IV to lack a gene encoding for TssH (Boyer et al., 2009), but TssH is lacking in a number of T6SS systems from other sub-groups including the fully functioning T6SS of *Campylobacter jejuni* and *R. leguminosarum* (Bleumink-Pluym et al., 2013; Boyer et al., 2009). Also, in *V. cholera*, it was non-essential

for killing *E. coli* (Zheng et al., 2011). It is thought these systems use a different member of the ClpB family of ATPases or ClpV or ClpB homologs encoded elsewhere in the genomes (Bleumink-Pluym et al., 2013; Boyer et al., 2009). There might be even more examples of T6SSs lacking TssH, since the analysis performed by Boyer et al. could not always distinguish between ClpV and ClpB encoding genes (Boyer et al., 2009; Schlieker et al., 2005). Close examination of TssH2/ClpV (*azo3903*) encoded by the *Azoarcus* sp. strain BH72 genome revealed that its protein sequence included all of the residues unique to ClpV. It may be that this gene is necessary for the function of both T6SSs. Ideally, assessing Hcp expression and secretion of a TssH2 mutant would have provided insight into the necessity of the protein for the function of the T6SSs. However, little could be determined when using Western blot analysis. Hcp was still detected in the supernatant of BHazo3903 cultures. Considering the BHazo3903 construct, four additional genes were also potentially disrupted. These genes included a carboxypeptidase, regulatory domain containing protein (*azo3904*), a putative lipase (*azo3905*), putative phospholipase C (*azo3906*) and a tetratricopeptide repeat containing protein (*azo3907*). There lay the potential that one of the downstream genes of *azo3903*, especially *azo3904* and *azo3907*, played a role in negative regulation of the T6SS. However, upon further review it seemed more likely that Hcp arose in the supernatant as a result of cell lysis.

Unique to T6SS-2 were accessory genes just upstream of the gene cluster, encoding for TagE, TagG, and TagH/Fha. These were not found encoded within or near the T6SS-1 gene cluster. However, these are missing from the majority of studied T6SSs (Boyer et al., 2009). These are similar to components of the threonine phosphorylation pathway regulation well-documented in *P. aeruginosa* (Mougous et al., 2007). The online tool for classification and secondary structure prediction of membrane proteins, SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui), did not predict TagE (*azo3888*) to include a transmembrane segment like *ppkA* of *P. aeruginosa* H1-T6SS. Also, in the *Azoarcus* sp. strain BH72 genome, there were only a few genes with very limited similarity to TagR. Taken together, this suggested that regulation of TagH/Fha phosphorylation in *Azoarcus* sp. strain BH72 differs from threonine phosphorylation pathway of *P. aeruginosa* (Mougous et al., 2007). Still, lipoprotein transporter, permease protein, LolE (*azo1509*), and lipoprotein releasing system ATP-binding protein, LolD (*azo1510*), are also encoded by the *Azoarcus* sp. strain BH72 genome, which may be the homologs of TagS and TagT. In *P. aeruginosa* these Lol-like lipoproteins form a membrane complex with ATPase activity. They are believed to detect environmental cues

and transmit the signal by modulating TagR activity (Casabona et al., 2013). A TagF homolog (*azo3889*) was also found near the T6SS-2 gene cluster. In *P. aeruginosa* TagF represses T6SS post-translationally, independent of threonine phosphorylation pathway stimulation (Silverman et al., 2011).

In addition to the upstream genes, a difference between the two clusters is that SciE (*azo3899*) was found encoded by the T6SS-2 gene cluster and not the T6SS-1. A similar protein is found within the HSI-1 T6SS of *P. aeruginosa* but not within the other two gene clusters, HSI-2 or HSI-3. In *P. aeruginosa* and *S. marcescens*, this protein was identified as TagJ and shown to be non-essential. It interacts with TssB, modulating its incorporation into the T6SS (Lossi et al., 2012). Based on COG assignments in an *in silico* analysis by Boyer et al. (2009), TagJ (COG4455) was only present in sub-groups III and V. Therefore, its absence in T6SS-1 was not surprising.

In addition to OmpA/MotB-like, Azo1306 (TagL), and VgrG-like, Azo1307 (TssI), the only other protein unique to the T6SS-1, not found in the T6SS-2, was Azo1301, which was predicted to be a metallopeptidase with one transmembrane segment. Two of the effectors secreted by *P. aeruginosa*, Tse1 and Tse3, are also peptidases, which are involved in peptidoglycan degradation and recycling (Benz et al., 2012). It is tempting to speculate that Azo1301 plays a role in T6SS, possibly in peptidoglycan degradation, like Tse proteins of *P. aeruginosa*, acting as a toxic effector targeted to bacteria. The Tse proteins and Azo1301 showed no sequence similarity, but that is typical. There is little similarity between most T6SS effector proteins of different organisms (Hood et al., 2010; Li et al., 2012). Still, nothing has been determined about the function of Azo1301. Azo1301 is also interesting because it and Azo1305 (Hcp protein TssD1) were the only T6SS-1 proteins identified in the secretome of *Azoarcus* sp. strain BH72. Because of cellular contaminants in the secretome, future work should look at localization of a tagged version of Azo1301 to determine if it is a legitimate secreted protein. Determining if Azo1301 affords *Azoarcus* sp. strain BH72 with a growth advantage over other bacteria would also be interesting. If it functioned as a peptidase in bacterial competition, an immunity protein may also be found to co-precipitate with Azo1301 in a pull down assay. Whether or not Azo1301 is determined to be involved in bacterial competition in future work, it would also be worthwhile to check for influences on the interaction of *Azoarcus* sp. strain BH72 with rice by comparing colonization efficiencies

of an Azo1301 mutant to *Azoarcus* sp. strain BH72 and by assaying for ethylene production of rice cell culture suspensions in response to purified Azo1301.

Considering the T6SS components encoded by the genome of *Azoarcus* sp. strain BH72 and the predicted structures of homologs expressed by other organisms found in literature, a depiction of the predicted T6SS apparatus can be seen in Figure 49 (adapted from Filloux et al., 2008). TagL is marked with an asterisk because it is only expressed by T6SS-1, and components only expressed by T6SS-2 are in parentheses.

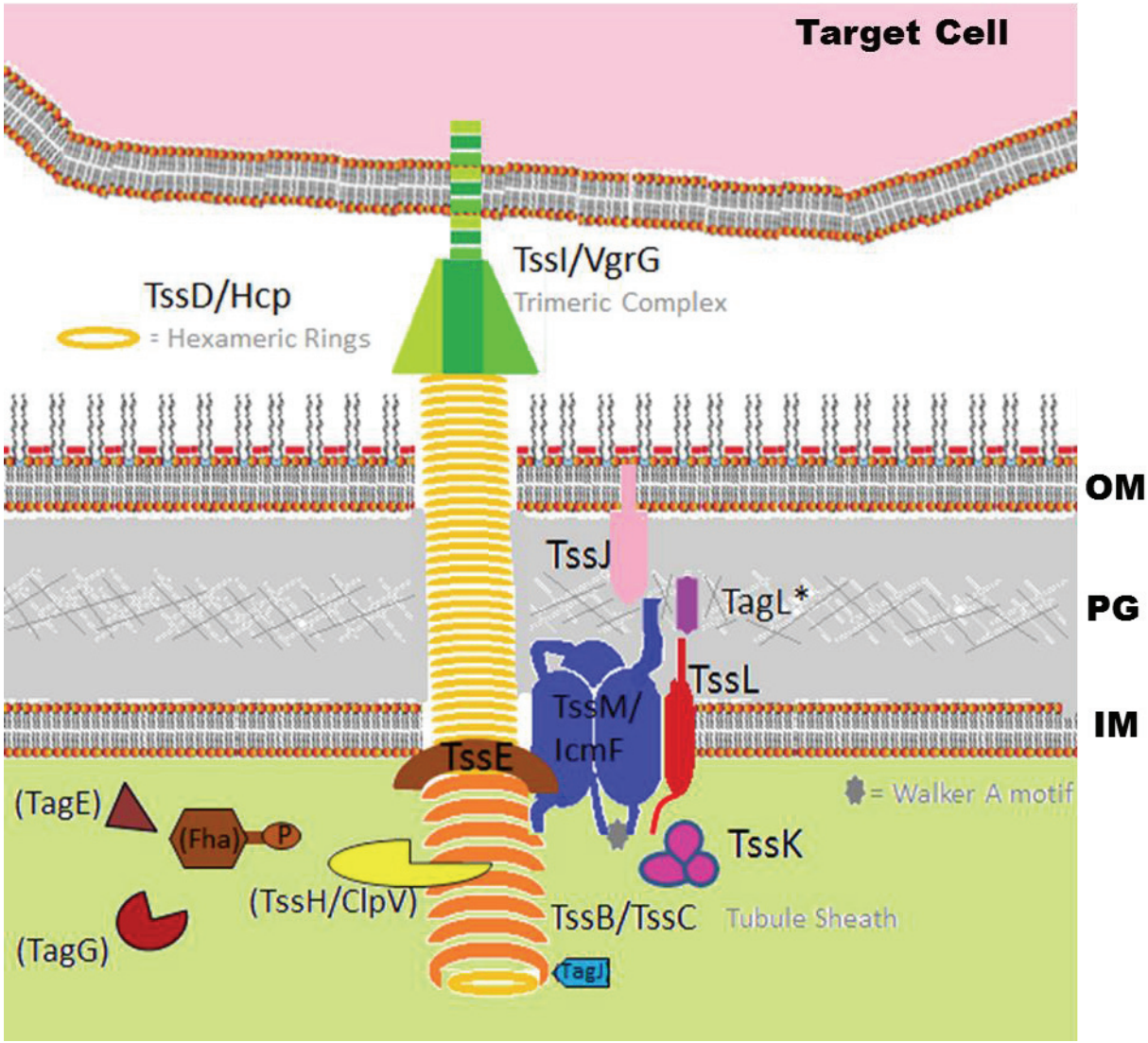


Figure 49. Diagram of the proposed structure of the type VI secretion apparatus and accessory components. Components of a functional T6SS are depicted on the left with their positions relative to the bacterial outer membrane (OM), peptidoglycan (PG), inner membrane (IM) and the target cell. TagL, encoded only by T6SS-1, is marked with an asterisk. Proteins encoded only in or near T6SS-2 are labeled in parenthesis. The figure was adapted from Filloux et al., 2008.

IV. B. Secretion and Detection of Hcp

IV. B. 1. Cross-reactivity of Hcp antibodies used in this study

Antibodies against each of the Hcp proteins encoded by the *Azoarcus* sp. strain BH72 genome had been generated in a previous study (Johannes Öhrlein, Diplomarbeit, 2009). Unfortunately, each antibody showed cross-reactivity and detected each Hcp. As attempts at antibody purification failed, the inability to decipher which Hcp was detected in the supernatant limited the interpretation of several studies to simply addressing whether secretion from either or both systems was on or off. Despite this, some clues as to which Hcp protein was detected were given based on size. Of the three Hcp proteins produced by *Azoarcus* sp. strain BH72, Hcp proteins of about 17 kDa encoded by the T6SS-2 gene cluster (Azo3897/TssD2 and Azo3898/TssD3) and a 20 kDa Hcp protein (Azo1305/TssD1) by the T6SS-1 gene cluster were predicted by GenDB (Table 6). These sizes were different enough to be distinguished from one another using the standard SDS-PAGE protocol used in this study, but both protein sizes were not always detected and usually only in the total cell protein extractions. Another program predicted TssD1 to be the same size as the 17 kDa Hcp proteins expressed from T6SS-2 (web.expasy.org). If there was no discernable difference in size between the Hcp expressed, the different sizes of protein bands detected by the Hcp antibodies could arise from the Hcp phosphorylation status. When Hcp was first described in 1996, it was said to run aberrant in gels (Williams et al., 1996). Another explanation for an additional protein band, close to the monomeric size of Hcp, in Western blots of the total cell fraction (Figure 17) is antibody cross-reactivity with a non-specific cellular component.

Focusing solely on the protein band detected in culture supernatants by Hcp antibodies, it is unlikely that non-specific extracellular proteins were cross-reacting with the Hcp antibodies used in this study. In most trials, a single Hcp protein band was detected in the supernatant fraction of strains grown with a functioning T6SS using Western blot analyses. Additionally, the T6SS was vulnerable to manipulation. Mutation to predicted core components for secretion successfully abrogated detection of protein bands between 17-20 kDa in the supernatant, while these bands were still detected in the total cell fraction (Figure 12 and Figure 10). This was a good indication that these proteins were still expressed and that their appearance in the supernatant was not an artifact, but rather the result of secretion. Additionally, of the multiple proteins detected by the Hcp antibodies in the cellular fraction, protein bands of the monomeric Hcp sizes were the most differentially expressed in response

to mutations made to the T6SS, advocating that protein bands near 17kDa and 20 kDa in size were Hcp and not unknown cellular components cross-reacting with the antibodies used.

In most Western blots, several larger protein bands were also detected in the total cell fractions. Although the protein band of about 50 kDa likely appeared from a cross-reaction of Hcp antibodies with flagella, this cannot account for the remaining protein bands regularly detected. To perform Western blots in this study, protein samples were reduced and boiled before loading SDS-PAGE gels. The additional larger bands could possibly result from various multimers of Hcp. Hcp monomers consist of anti-parallel β-sheets with a β-hairpin extension. Hexameric rings are stabilized by an extension acting as an inter-subunit belt (Cascales and Cambillau, 2012). In an in vitro study, Hcp nanotubes were stabilized by engineered disulfide bonds. These stacked ring structures remained stable for weeks at room temperature but were easily reduced and disassembled in the presence of reducing agent, 5 mM DTT. The hexameric ring structures were not reduced to monomers with DTT treatment (Ballister et al., 2008). It is possible that the treatment of samples for SDS-PAGE using β-mercaptoethanol was not denaturing enough to completely dissociate all hexamers. However, additional protein bands were seen predominately in total cell fractions (Figure 8) where there was an increased chance of unspecific antigens to be present. Additionally, the majority of other larger and potentially unspecific bands detected were typically consistent in abundance regardless of the mutant type, suggesting their detection was independent of Hcp expression. However, distinguishing each Hcp from one another in Western blot analyses is desirable. In future work, the addition of different protein tags to each of the Hcp proteins might prove useful so long as the antibodies used for detection do not cross-react with the other tags used.

IV. B. 2. Manipulating the on and off status of Hcp secretion

Detection of Hcp in the supernatant, not only in the total cell fractions, was a promising indicator of a functional T6SS. In a previous study, it was found the GUS activity of two T6SS-2 genes (*azo3901* and *azo3892*) and two genes of T6SS-1 (*azo1302* and *azo1299*) with transcriptional fusions was higher in stationary phase than in the exponential growth phase when cells were grown in full media (Julia Herglotz, Diplomarbeit, 2007). In this study, primarily cells in the exponential phase grown in minimal media were used, and Hcp was still detected in the supernatant and total cell fractions. Detection of Hcp in *P. aeruginosa* PA01 liquid culture supernatants required use of a ΔRetS (**R**egulator of **e**xopolysaccharide and **t**ype

III secretion) mutant strain, a ΔPppA strain (unable to dephosphorylate the TagH/Fha protein) or contact stimulation (Mougous et al., 2006; Silverman et al., 2011; Kapitein and Mogk, 2013). RetS is a signaling kinase that positively regulates the T3SS but represses T6SS gene expression (Mougous et al., 2006). The ability to detect Hcp secretion under standard growth conditions proposes this is a very active and important system for the survival of *Azoarcus* sp. strain BH72.

The means necessary to turn off secretion were unexpected. The up-regulation of T6SS-1 genes and constitutive expression of T6SS-2 genes under nitrogen fixing growth conditions observed previously in GUS assays and microarray studies led to the assumption that turning off T6SS-1 alone would be sufficient to turn off all Hcp secretion under nitrogen fixing conditions (Julia Herglotz, Diplomarbeit, 2007; Sarkar and Reinhold, 2014). However, the secretion of Hcp appeared unaltered by mutation to TssM1 (Figure 16) despite, in literature, TssM consistently being seen as a requirement for T6S. It was necessary to mutate both TssM1 and TssM2 to cease detection of Hcp proteins in the supernatant (Figure 12).

Interestingly, knocking out *azo3888* alone was sufficient to eliminate detection of Hcp proteins in the supernatant using Western blot analysis (Figure 19). The majority of trials assessed secretion of Hcp by BHazo3888 under non-fixing conditions. It remained possible that TssD1 was not detected in the supernatant because non-fixing growth conditions were used. However, the lack of any Hcp secretion was also demonstrated in a single trial using nitrogen fixing conditions. Azo3888 shares a high degree of sequence similarity to the accessory protein TagE, which is a well-documented component of the threonine phosphorylation pathway in *P. aeruginosa* (Mougous et al., 2007). Azo3888 is not predicted to include a transmembrane segment like *ppkA* of *P. aeruginosa* H1-T6SS, and there were only a few proteins encoded by *Azoarcus* sp. strain BH72 with very limited sequence similarity to TagR. These predictions would suggest regulation in *Azoarcus* differs from the threonine phosphorylation pathway of *P. aeruginosa*. However, TagE was critical for detection of Hcp proteins in culture supernatants, implying that the threonine phosphorylation pathway does indeed play a role in T6S by *Azoarcus* sp. strain BH72.

IV. B. 3. Threonine phosphorylation pathway activity in *Azoarcus* sp. strain BH72

In some organisms using the threonine phosphorylation pathway, T6S is dependent on cell-cell or cell-surface contact (Schwarz et al., 2010b; Silverman et al., 2011; Bleumink-Pluym et al., 2013). In *P. aeruginosa* PA01 it was previously thought that a ΔRetS or ΔPppA mutant

was a prerequisite for secretion of Hcp by the gene cluster HSI-1 (Mougous et al., 2006; Silverman et al., 2011), which is most similar to T6SS-2. It was later realized that stimulation of the threonine phosphorylation pathway was contact dependent. It was proposed that T6SS effectors remained elusive because of the lack of intimate contact in standard culturing conditions (Silverman et al., 2011; Kapitein and Mogk, 2013). Stimulation of changes in gene regulation by surface contact is a well-documented event. One-third of the genes in *Salmonella typhimurium* were altered in a global gene expression analysis during surface growth (Silverman et al., 2012; Wang et al., 2004). The effect of surface growth on *Azoarcus* sp. strain BH72 secretion of Hcp was also tested, but none of the Hcp proteins were stimulated in expression or secretion compared to liquid grown cultures (Figure 20) in this study.

There were possible faults with the assay used in this study. In the assays used to examine surface stimulation of *P. aeruginosa*, 3 mL of a 1:100 dilution of overnight culture was spotted onto a 0.2 mM polycarbonate membrane placed on LB agar for 4 h then resuspended in 2 mL of LB with 5 minutes of shaking (Silverman et al., 2011). *C. jejuni*, which also showed stimulation of T6S upon surface growth, was grown on agar plates for 7 d and then in broth for 16 h (Bleumink-Pluym et al., 2013). In this study 1 ml or 2 ml of culture with an OD_{578} of 0.5 was incubated on agar plates or the glass bottom of an Erlenmeyer flask, respectively. The liquid cultures were grown at 37°C in 100 ml of VME broth, overnight growth, without agitation. The liquid cultures from seven Erlenmeyer flasks were pooled, and cells were immediately pelleted out. Cells were scraped from the plates using a minimal amount of liquid media. Cells were then immediately pelleted out of this suspension before further processing of the proteins in supernatant and total cell fractions. There was never an incubation period for secreted proteins to accumulate in the media. This might account for the lack of surface contact stimulation observed, or it could be that T6S by *Azoarcus* sp. strain BH72 is not stimulated by surface contact. The genome of *Serratia marcescens* also encodes for threonine phosphorylation pathway components (TagE, TagG, TagH/Fha), and activation of its serine threonine kinase activity is independent of surface contact (Fritsch et al., 2013). However, the current lack of evidence for contact stimulated Hcp expression should not exclude the chance that it plays a role in the T6S by *Azoarcus* sp. strain BH72. Considering the presence of genes encoding for components of the threonine phosphorylation pathway near the T6SS-2 gene cluster and that delivery of effectors relies on the close proximity of its target, performing more assays testing contact dependent induction of T6SS-2 genes

specifically would be worthwhile. Also, it remains possible that T6SS-1 Hcp protein TssD1 secretion is stimulated by surface contact, but differences may be too minor for detection via Western blot analysis. Looking at induction of gene expression would be a more sensitive method for determining if surface contact stimulated either T6SS. Still, other environmental cues remain to be tested that may stimulate T6S.

Early identification of and findings for T6SSs were strongly correlated to associations with eukaryotes, and secreted effectors were assumed to target and manipulate their host. T6SSs were also found to function in bacterial competition, targeting other prokaryotes for toxin injection. Recently, evidence of bacterial targets is beginning to outnumber eukaryotic ones (Kapitein and Mogk, 2013; Silverman et al., 2012; Fritsch et al., 2013; Hood et al., 2010). If one or both of the T6SS(s) played a role in interbacterial competition, stimulation of T6SS assembly or secretion via cell density is likely. In research looking at the quorum sensing activity of *Azoarcus* sp. strain BH72, a 2-fold increase in expression of *azo3895* (TssB), *azo3896* (TssC) and *azo3898* (Hcp) was seen (Lena Hauberg, PhD thesis, 2010). Given that it is under nitrogen fixing conditions that *Azoarcus* sp. strain BH72 interacts with its host and potentially competes with bacteria for plant colonization, it would be interesting to see the effects of quorum sensing, or cell-cell or cell-surface contact, on Hcp secretion (or T6SS gene transcription) in combination with growth under nitrogen fixing conditions.

It is interesting that components of the threonine phosphorylation pathway are all together lacking in T6SS-1, but TagE is necessary for secretion of any detectable levels of Hcp in Western blot analysis. When multiple T6SS gene clusters are encoded within a single organism, it has thus far seemed that the clusters typically function and are regulated independent of each other. However, there are reports of crosstalk between T6SSs and other secretion systems with regards to regulation. Crosstalk between two of three *P. aeruginoa* T6SSs has been speculated because of common σ^{54}–dependent transcriptional regulators and σ^{54} consensus binding sites found (Leung et al., 2011). There is limited evidence of one T6SS's dependence on components of another within an organism. In *P. aeruginosa*, three different VgrG proteins (TssI) have been shown to mulitmerize into the T6SS tip puncturing device, but they are co-regulated by the same T6SS gene cluster (H1-T6SS) (Hachani et al., 2011). The potential remains that this interdependence is an oversight by groups working on organisms with multiple T6SSs. Several studies focus on only one or two of the up to six T6SSs within an organism. However, only 30% of the identified T6SS loci encode for

threonine phosphorylation pathway components, even when only a single T6SS was encoded, such as in the *Edwardsiella tarda* genome (Zheng and Leung 2007; Boyer et al., 2009). To test if the threonine phosphorylation pathway functions to regulate T6SS post-translationally for both clusters, assessment of Hcp secretion by a mutant lacking a core structural component of T6SS-2 (e.g. TssM2) should be done. Detection of Hcp in the supernatant of this mutant would indicate that secretion by T6SS-1 requires threonine phosphorylation pathway components of T6SS-2. This sort of interdependence between systems would be a novel finding for T6SSs.

With the objective to identify the T6SS effector protein(s), construction of a hyper-secreting mutant was desired to augment effector presence in the supernatant. If the threonine phosphorylation pathway functioned in *Azoarcus* sp. strain BH72, it was anticipated that mutation to the antagonizing phosphatase, TagG (*azo3885*), would leave the T6SS in a constant on state and result in 'hyper-secretion', as this had been seen in *P. aeruginosa* (Silverman et al., 2011). Upon construction of BHΔ3885, the desired, hyper-secreting effect was seen with regards to Hcp presence in the supernatant. Unfortunately, this was constructed too late for a more comprehensive analysis. These trials should be repeated, and the presence of cellular protein in the supernatant should be tested for. Identifying which Hcp protein(s) were hyper-secreted would also be valuable in future work. If it were determined that TssD1 was hyper-secreted, this would support the notion of interdependence between T6SSs. Nevertheless, observations in this study support the assumption that *Azoarcus* sp. strain BH72 uses the threonine phosphorylation pathway.

IV. C. The *Azoarcus* sp. strain BH72 secretome

The primary aim of this study was to determine the role of the T6SS in crosstalk occurring between rice and *Azoarcus* sp. strain BH72 when establishing and maintaining an endophytic relationship. Finding the secreted T6SS effector was highly desired, but not accomplished. There was little to no success using MALDI-TOF for protein identification. Protein samples sent away for LC-MS/MS analysis proved useful in protein identification. However, contrary to what was desired, no evident effector protein(s) was revealed in the wild type secretome that was absent from the secretome of BHΔimpLsciO. The secretome included cell wall components such as OMP porin, pilus associated proteins and flagellar proteins, but the most abundant proteins were cellular ones. Identification of an actual effector protein was clouded

by contamination of supernatants with cellular proteins likely arising from cell lysis. Whether legitimate extracellular proteins or not, the majority of the 20 most abundant proteins were found equally abundant in wild type and mutant supernatants, and several proteins were found more abundant in the supernatant samples of BHΔimpLsciO. Only two of the 20 most abundant proteins showed a significant difference in presence: Azo1466 (Mg/Fe SOD) and Azo3897 (Hcp/TssD2). Considering proteins only in the wild type cultures (and completely lacking in the mutant strain) would ideally increase the stringency of the search, but this search criterion was potentially misleading. The majority of these were in such low abundance they did not even appear in the supernatant of all four wild type replicates. Of these 20 proteins, only six were found in amounts with a statistically significant difference from BHΔimpLsciO supernatants: Azo3876 (TssI2/VgrG), Azo0522 (SodC), Azo1906 (Frr), Azo2635 (unknown), Azo3898 (TssD3/Hcp) and Azo2168 (phosphotransferase).

It is notable that two different SOD (superoxide dismutase) proteins had a significantly greater presence in the wild type supernatant. The secretion of SOD could play a role in colonization by overcoming the plants MTI response. As discussed in Section IB1, plant MTI defense responses include production of microbiocidal reactive oxygen species (ROS) Lamb and Dixon, 1997; Muthamilarasan and Prasad, 2013). Classification of SOD proteins is based on their metal cofactor (e.g. Mn-SOD (SodA), Fe-SOD (SodB), Cu/Zn-SOD (SodC) and Ni-SOD (SodN)), but each functions to dismutase superoxides into oxygen and hydrogen peroxide (Fridovich, 1995). SodA and SodB are located in the cytoplasm but are secreted by some bacterial strains (Fridovich, 1995; Lynch and Kuramitsu, 2000). If SOD played a role in the colonization of rice by *Azoarcus* sp. strain BH72, it would likely be by overcoming an MTI plant response. In this secretome data, significantly less Azo1466 was found in the supernatant of BHΔimpLsciO, but this is not sufficient evidence that an SOD is the secreted T6SS effector. As with all findings in the secretome data, further studies assessing the genuine secretion of identified proteins need to be performed.

In an effort to expand the search for potential effectors and maintain some stringency, only proteins with a statistically significant difference in presence were considered. In a list of 36 proteins, it was promising that over half, 22, were more abundant in the wild type supernatants. This list included some of the proteins that were not detected in the BHΔimpLsciO supernatant (Azo3876 (TssI2/VgrG), Azo0522 (SodC) Azo1906 (Frr), Azo2635 and Azo2168), T6SS component Azo3897 (TssD2/Hcp), expected extracellular

proteins and some were likely cellular contaminants. Of the proteins of interest, some had unknown functions (Azo2255, Azo1102, Azo2635 and Azo2168). Azo1464, a transaldolase, was one protein identified that stood out. In a microarray study, *azo1464* was up-regulated after 1 h exposure to root exudates (Shidore et al., 2012) and had a significantly greater presence in the wild type supernatant than the supernatant of BHΔimpLsciO. From the root exudate microarray study, the only gene known to be associated with the T6SS and differentially regulated was *azo3888*. It was up-regulated almost 2-fold after one hour of exposure to root exudates (Shidore et al., 2012).

Of the T6SS components, T6SS-2 proteins were the most represented in the secretome despite all supernatants being acquired from cultures grown under nitrogen fixing conditions. This was not expected, because in previous findings T6SS-1 genes were up-regulated under nitrogen fixing conditions and T6SS-2 genes were constitutively expressed (Julia Herglotz, Diplomarbeit, 2007; Sarkar and Reinhold-Hurek, 2014). Little attention has been given to the expression of T6SS-2, but some lines of evidence advocate that T6SS-2 is the more active secretion system. Mutation of a single T6SS-2 affiliated accessory gene (*azo3888*) was sufficient to turn off all detectable secretion of Hcp. In a proteomic study of all cellular proteins found in *Azoarcus* sp. strain BH72 grown in full media (VME), T6SS-2 proteins were identified, but T6SS-1 proteins were not (Hauberg et al., 2010). However, T6SS-1 genes are clearly expressed and up-regulated under nitrogen fixing conditions (Julia Herglotz, Diplomarbeit, 2007; Sarkar and Reinhold-Hurek, 2014). Furthermore, the T6SS-1 Hcp protein TssD1 was indeed present in the secretome of BHΔimpLsciO and wild type strains (Table 7). Although evidence is lacking for the importance of T6SS-2 under nitrogen fixing conditions, future studies assessing transcriptional activities of T6SS-2 genes under a broader range of growth conditions and stimuli may reveal that they too are up-regulated by conditions faced at the rhizosphere. It has already been observed that the T6SS-2 affiliated accessory gene *azo3888* was up-regulated in a microarray study examining the response of *Azoarcus* sp. strain BH72 to rice root exudates after one hour (Shidore et al., 2012), and quorum sensing activity produced a 2-fold increase in expression of *azo3895* (TssB), *azo3896* (TssC) and *azo3898* (TssD3/Hcp) (Lena Hauberg, PhD thesis, 2010).

Performing the LC-MS/MS analysis on culture supernatants again would be very useful. In future attempts, a hyper-secreting strain should be used to enhance effector presence in the supernatant. BHΔ3885 or a mutant of another putative post-translational repressor of T6S,

TagF (BHΔ3889), could be used to enhance effector presence in the supernatant. However, it remains possible that use of these strains may only augment secretion from T6SS-2. Regardless of strains tested, extra care in handling should be taken to prevent unnecessary cell lysis in order to avoid contamination of the supernatant fraction with cellular proteins. Stirring cultures at a slower speed during growth and removing the supernatant from pelleted cells more quickly might decrease the amount of cellular contamination. Additionally, if in future work preparation of samples and LC-MS/MS studies could be optimized where only truly secreted proteins were detected, comparing the secretomes of a mutant with only T6SS-1 turned off to one with only T6SS-2 turned off would help match the secreted effector to their respective T6SS.

IV. D. The T6SS in Plant-Microbe Interactions

Root hairs are elongated outgrowths of specialized cells in the epidermis, trichoblasts, formed by cytoskeletal rearrangement and cell wall reassembly. They provide a large surface area for the uptake of water and nutrients and interactions with microbes (Lan et al., 2013). Microbe-associated molecular patterns (MAMPs), potentially originating from a pathogen, are sensed by the plant and trigger MAMP triggered immunity (MTI). MTI responses include production of reactive oxygen species (ROS) and callose. Callose is a polysaccharide polymer usually deposited at the plasmadesmata during development but as a defense mechanism also between the cell wall and plasma membrane at the site of invasion. The cell wall is composed of a complex polysaccharide, cellulose, and branched polysaccharides such as cross-linking glycans and pectins that 'cement' the cells together (Stone and Clark, 1992; Freeman and Beattie, 2008). ROS are microbiocidal but also strengthen the cell wall through oxidative cross linking of cell wall polymers (Lamb and Dixon, 1997). It has long been established that *Azoarcus* sp. strain BH72 can efficiently colonize rice in high numbers without the plant showing signs of a defense response (Hurek et al., 1994b; Reinhold-Hurek et al., 1993a). In this and previous studies, rice seedlings post-inoculation with T6SS-1 mutants were affected. This was a promising indicator of the importance of T6S in the plant microbe interactions, so the putative role in silencing the plant response was further examined.

In assays measuring ethylene production by rice cell cultures, the Hcp protein Azo1305 (TssD1) appeared to elicit a response from the plant. Recognition of pathogen-associated

molecular patterns triggers a signaling cascade, leading to biochemical defense mechanisms including the production of ethylene. In this study, assays were performed where production of stress hormone ethylene was measured after rice cell suspension cultures were exposed to potential elicitors. Two known elicitors, Pen1 (Thuerig et al., 2006) and Chitin (Felix et al., 1999), were used to control the sensitivity of the assay. Incubation of rice cell cultures with Pen1 or Chitin produced an increase in ethylene production in these assays, but because of the variability in values measured, only exposure of rice cell cultures to 5 µg of Pen1 showed a significantly different production of ethylene compared to cells not exposed to an elicitor ($P<0.05$ in a two-tailed paired t-test). Of the different Hcp proteins tested, only 5 µg of TssD1/Azo1305 showed a significantly different production of ethylene compared to cells not exposed to an elicitor ($P<0.05$ in a two-tailed paired t-test) over the six trials using seven and eight day old cell cultures.

In addition to the limited results showing statistical significance, the purity of the Hcp proteins used was questionable. Proteins used for testing were overexpressed in *E. coli* cells and then purified using Strep-Tactin sepharose columns (Section IIB4a-c). The potential for co-purification of additional *E. coli* proteins cannot be disregarded. Additionally, the presence of a Strep-tag could have elicited the response from cell cultures. However, the different elicitation seen by the different Strep-tagged Hcp proteins was an indication that this was not the case. A former student (Martin Schaefer, Master thesis, 2012) performed additional assays using synthetic peptides, a 25 amino acid peptide from the N-terminal region of TssD1/Azo1305 (N25-Azo1305) and a 20 amino acid peptide from the central region of TssD1/Azo1305 (C20-Azo1305), which were said by the manufacturer to be 37% and 54% pure, respectively (Martin Schaefer, Master thesis, 2012). These 'crude' preparations produced little or no elicitation compared to the negative controls. However, increasing the concentration of the high purity grade peptides (about 96%) increased ethylene production by cell culture suspensions (Martin Schaefer, Master thesis, 2012). Additionally, M. Schaefer tested other Strep-tagged proteins. Strep-tagged ColR and GlnB, elicited a very high response. GlnB was an even stronger elicitor than Azo1305 (Martin Schaefer, Master thesis, 2012). Although it could not be concluded if the Strep-tag itself was responsible for some of the elicitation seen by Strep-tagged proteins, there was substantial evidence that the Hcp protein, Azo1305 (TssD1), did elicit a response in rice cell culture suspensions.

Interestingly, colonization of rice seedlings by BHΔimpLsciO led to the formation of aggregates on root hairs. It had appeared that without secretion of any Hcp or T6SS effectors, a plant response was invoked. In line with this, inoculating rice seedlings with a TssK1 mutant strain, BHimpJ::pK18GGST, also resulted in aggregate formation on rice root hairs (Julia Herglotz, Diplomarbeit, 2007) similar to what had been seen after inoculating seedlings with BHΔimpLsciO (TssM1 and TssM2 mutant). The root hair phenotype post-inoculation with BHsciO::pK18GGST was not noticeably different than wild type (Julia Herglotz, Diplomarbeit, 2007). This appearance of root hair aggregates seemed to be due to the lack of secretion by T6SS-1 in particular. Recall, of Hcp proteins tested, TssD1 (Azo1305) elicited the greatest response from rice cell cultures in ethylene production assays. It is tempting to reason that without initial signaling by TssD1 (Azo1305), or a T6SS-1 effector, a stronger defense response was invoked by the plant leading to root hair aggregates. Treatment of plants with chitin, or chitooligosaccharides, has been used to promote plant resistance to fungal and bacterial pathogens through chitin-mediated plant innate immunity (Stoner et al., 2000). Triggering of a LysM receptor-like protein (LysM RLK1) induces genes that lead to a defense response in *Arabidopsis thaliana*. (Wan et al., 2008a; Wan et al., 2008b). A rice LysM domain–containing protein was shown to bind chitin (Kaku et al., 2006), and treatment with chitooligosaccharide also resulted in enhanced fungal resistance (Tanabe et al., 2006). Although it is highly speculative, TssD1 might participate in somehow priming rice for an endophytic colonization.

With regards to colonization of rice seedling by T6SS mutants, T6SS-1 deficient strains were less efficient than the wild type strain in single infection experiments. BHimpL::pK18GGST and BHimpJ::pK18GGST were 19.9% and 59% less efficient, respectively (Julia Herglotz, Diplomarbeit, 2007). In competition assays using a TssM1 mutant strain, BHimpL::pK18GGST, and wild type strain, only 5% of the bacteria colonizing roots were the mutant strain (performed by Shanmugam Solaiyappan, 2013). Of the colonizing bacteria, 95% were the wild type strain. From the secretome data it was clear that the T6SS-1 Hcp protein, TssD1 (Azo1305), was secreted at much lower levels than TssD2 (Azo3897) by wild type *Azoarcus* sp. strain BH72 when grown in a bioreactor with conditions promoting nitrogen fixation. It could also be that that the secretion activity of T6SS-1 was so much lower than T6SS-2 that TssD1 was secreted at undetectable levels in Western blot analyses. Despite the potentially lower levels of secretion, T6SS-1 may be the more important system for interactions with rice.

Colonization of rice seedlings by T6SS-2 deficient mutant strains was not limited. These strains colonized in higher numbers than the wild type strain. BHsciC::pK18GGST and BHsciO::pK18GGST were 36.4% and 98.4% more efficient than the wild type strain, respectively (Julia Herglotz, Diplomarbeit, 2007). In 3 d competition assays, Shidore et al. had similar findings when inoculating *Oryza sativa cv. Nipponbare* seedlings with wild type *Azoarcus* sp. strain BH72 and BHazo3888. A greater percentage of the colonizing bacteria were BHazo3888 (Figure 32) (Shidore et al., 2012). In these assays the inoculum was comprised of an equal amount of mutant and wild type strains of bacteria and was added to the top of a gel rite plant medium. In these competition assays rhizosphere competence was also assessed, as the bacteria compete as they adapt to the rhizosphere and colonize the root tip (Teja Shidore, PhD thesis, 2012). BHazo3888 outcompeting the wild type strain in competition assays could not be explained by different growth rates; these were the same for both strains. The more efficient colonization of rice by BHazo3888 might be a direct result of disrupting *azo3888* or the lack of secretion by T6SS-2.

Considering the strain BHsciO::pK18GGST, integration of pK18GGST at *azo3892* also potentially disrupted transcription of *azo3888* (like BHazo3888), which encodes for the serine/threonine kinase. It is possible that this serine/threonine kinase regulates other unknown components that normally limit colonization independent of T6SS effectors. Pleiotropic effects of PppA and PpkA (TagG and TagE) have been demonstrated in global analyses of *P. aerugin*osa PAO1 and PppA/PpkA deletion mutant transcriptomes. In addition to manipulating the phosphorylation status of the TagH/Fha protein, PppA and PpkA sensed external stress signals and regulated a response. The response was believed to be connected with the stationary phase σ-factor (RpoS) and quorum sensing regulons (Goldová et al., 2011). If Azo3888 had additional regulatory functions limiting colonization, this might contribute to the reason why BHsciO::pK18GGST was 98.4% more efficient colonizing rice seedlings than the wild type strain and BHsciC::pK18GGST (disruption of *azo3901*) was only 36.4% more efficient (Julia Herglotz, Diplomarbeit, 2007).

There are other examples where T6SSs function to limit infections. In *R. leguminosarum* the T6SS was shown to impair the ability to nodulate peas, and in *Salmonella enterica* serovar *Typhimurium*, it was determined using an IcmF mutant that the T6SS limited intracellular replication (Records, 2011; Parsons and Heffron, 2005; Bladergroen et al., 2003).

It is necessary to acknowledge that in competition assays, seedlings were inoculated with both mutant and wild type strains. It is assumed that wild type T6SS effectors were always present to some extent in the media of these assays. Still, single infections using the quartz system were also performed using BHazo3888, and the results still suggested that the T6SS-2 deficient strain was more efficient at colonizing rice seedlings than wild type. After inoculating *O. sativa* cv. IR-36, roots were harvested 14 d post inoculation; 69,666 CFU of BHazo3888 per mg fresh root weight were averaged over four trials compared to 27,609 CFU of *Azoarcus* sp. strain BH72 per mg fresh root weight. However, using a two-tailed paired t-test, the difference was not significant. Also considering the TssM1 mutant, BHimpL::pK18GGST, colonization by this strain was less than wild type in both competition assays and single infections. Presence of effectors secreted by the wild type strain in competition assays was not sufficient enough to compensate for the lack of secretion by the mutant strain tested.

In this study, colonization of *O. sativa* cv. IR-36 rice seedlings by BHΔimpLsciO, where both T6SSs were shut off, was tested in single infections and compared to colonization by the wild type strain. The BHΔimpLsciO strain colonized rice seedlings in higher numbers than wild type. Again, the differences seen could not be explained by different growth rates; these were the same for both strains. BHΔimpLsciO was constructed by recombining pK18GGST into the chromosome at *azo3892*, potentially disrupting transcription of *azo3888* (TagE). The more efficient colonization of rice by BHΔimpLsciO might be a result of mutation to T6SS-2 or, once again, TagE (*azo3888*) may be directly responsible.

The importance of T6SS-1 for interactions with rice has been well supported. Turning off secretion by T6SS-1 reduced the colonization efficiency and resulted in formation of root hair aggregates. Of the Hcp proteins tested, TssD1 (Azo1305) was the strongest elicitor of ethylene production by rice cultures. Also, expression of T6SS-1 genes was up-regulated under nitrogen fixing conditions, which coincides nicely with the lifestyle of *Azoarcus* sp. strain BH72 as an endophyte. However, the function of T6SS-2 is less clear. Turning off T6SS-2 secretion increased the colonization efficiency. T6SS-2 gene expression was unaltered under nitrogen fixing conditions (Julia Herglotz, Diplomarbeit, 2007; Teja Shidore, PhD thesis, 2012; Sarkar and Reinhold-Hurek, 2014), but the potential that T6SS-2 genes are up-regulated by other conditions faced at the rhizosphere cannot be disregarded. Still, under nitrogen fixing conditions T6SS-2 encoded proteins were the most represented in the

secretome data. Also, in a proteomic study of all cellular proteins found in *Azoarcus* sp. strain BH72 grown in full media (Hauberg et al., 2010), T6SS-2 proteins were identified, but T6SS-1 proteins were not (Hauberg et al., 2010). Inoculating rice seedlings with a strain where both T6SSs were turned off (BHΔimpLsciO) resulted in the formation root hair aggregates, similar to those resulting from inoculation with a T6SS-1 mutant (BHimpL::pK18GGST), supporting the idea that without initial signaling by the T6SS-1 the rice seedlings are affected. However, unlike T6SS-1 mutant strains, colonization efficiency was improved, which resembled the colonization by a T6SS-2 mutant strain. The substantial expression of T6SS-2 components might be necessary for effectively limiting the colonization of *Azoarcus* sp. strain BH72. Even when T6SS-1 secretion is turned off, without secretion by T6SS-2 (or without TagE), strains were capable of colonizing rice seedlings and in higher numbers than the wild type strain. Although it is challenging to speculate why, these assays revealed that in terms of colonization, the consequence of disrupting T6SS-1 secretion was overshadowed by disruption of T6SS-2 (or TagE).

IV. E. Transcriptional regulation of genes encoding for T6SS components

The great number of different regulatory mechanisms (histone-like nucleoid-structuring proteins, σ^{54}, quorum sensing, two component systems, etc.) matches the diverse number of functions and bacteria that utilize the T6SS (Bernard et al., 2010). Environmental cues that stimulate T6S found to date include but are not limited to: cell density, temperature, pH, salinity, osmolarity, iron, phosphate and magnesium (Bernard et al., 2010; Miyata et al., 2013). In *Azoarcus* sp. strain BH72, the majority of T6SS-1 genes were up-regulated under nitrogen fixing conditions (in a bioreactor, SM medium, 0.6% O_2) compared to non-fixing conditions (in a bioreactor, SM+N medium, 0.6% O_2), and sometimes greater than 20-fold (*azo1299* and *azo1300*), in a microarray study (Sarkar and Reinhold-Hurek, 2014). Additionally, in GUS assays performed in a previous study, *azo1299* (TssK1) was transcriptionally up-regulated 20-fold under nitrogen fixing conditions (SM medium, batch cultures set with 1.6% O_2) compared to non-fixing conditions (SM+N medium, aerobic batch cultures) (Julia Herglotz, Diplomarbeit, 2007); and in semi-quantitative RT-PCR studies, *azo1299*, *azo1302* and *azo1305* were up-regulated 14, 12 and 9 -fold, respectively, under conditions promoting nitrogen fixation (SM medium with 20 mM glutamate, batch cultures set with 1.8% O_2, ≥10 h growth) compared to non-fixing conditions (SM+N, batch cultures set with 1.8% O_2, ≥10 h growth) (Teja Shidore, PhD thesis, 2012). In the current study, using

a transcriptional fusion of the promoter region upstream of *azo1301* with *gusA*, a 4-fold induction was observed when cultures were grown under nitrogen fixing conditions (SM medium, batch cultures set with 1.2% O_2, 6 h) compared to non-fixing conditions (SM+N medium, batch cultures set with 1.2% O_2, 6 h). There was also induction when cultures were grown under conditions promoting nitrogen fixation with glutamate present (SM+20 mM glutamate, batch cultures set with 1.2% O_2, 6 h) but to a lesser extent (Figure 44). The different magnitudes of induction could possibly be accounted for by the different promoters tested or differences in how assays were performed.

Only one of two T6SS-1 genes tested in GUS assays from the previous study (Julia Herglotz, Diplomarbeit, 2007), *azo1299* (TssK1), showed an up-regulation from about 50 Miller Units under non-fixing conditions to 850 Miller Units under nitrogen fixing conditions. In an opposite facing operon, *azo1302* (TssM1) showed no modulation with respect to nitrogen fixing conditions, remaining consistent with about 900 Miller units (Julia Herglotz, Diplomarbeit, 2007). In semi-quantitative RT-PCR studies on RNA extracted from exponentially growing cells under conditions promoting nitrogen fixation (SM+20 mM glutamate, batch cultures set with 1.8% O_2), *azo1299* was up-regulated, but the difference was less dramatic than previous GUS assays. Also differing from the earlier GUS assays, in the semi-quantitative RT-PCR study, *azo1302* was up-regulated when grown in conditions promoting nitrogen fixation (Teja Shidore, PhD thesis, 2012). GUS assays in this study showed an up-regulation of *azo1301* (which potentially shares a promoter with *azo1302*) when grown under nitrogen fixing conditions, but less than the up-regulation observed in the semi-quantitative RT-PCR studies. Which genes were modulated and the degree of modulation in the microarray study also differed from these studies. Clearly, the sensitivity of methods used to measure transcriptional activity varied, but the putative role of growth phase and/or oxygen availability was intriguing.

In the former study using GUS assays (Julia Herglotz, Diplomarbeit, 2007), a growth phase comparison had been done with cells grown aerobically on full media (VME). Transcriptional activities were on average 1.7-fold higher in the stationary phase for both T6SS-2 and both T6SS-1 genes tested (Julia Herglotz, Diplomarbeit, 2007). GUS assays from the earlier study were performed on cells grown to an OD_{578} of 0.4-0.6, still in exponential phase. In this study cells were harvested after six hours of induction. The average OD_{578} of cells used was 0.57, but in a few trials an OD_{578} of about 1 was reached. Growth phase could

account for some of the variation in gene expression seen. With respect to oxygen availability, cells grown under non-fixing conditions in the previous work were aerobic cultures, while in this study they were grown in the same microaerobic conditions used to establish nitrogen fixing conditions. The effects of oxygen concentration might account for some of the variation seen when comparing gene expression under nitrogen fixing conditions to non-fixing conditions. However, in a microarray study, gene transcription of *Azoarcus* sp. strain BH72 when grown in a microaerobic environment (0.6% O_2) was compared to aerobic growth (20% O_2), both in SM media supplemented with ammonium chloride in a bioreactor. Of the T6SS genes, only modulation of *azo3879* (down 2-fold) and *azo3903* (down 2.2-fold) was observed (Abhijit Sarkar, unpublished data). It could be that growth phase and/or oxygen availability impact the regulation of T6SS expression to some extent and explain some of the variation seen between assays. Nonetheless, taken en bloc, the T6SS-1 gene cluster was clearly up-regulated under nitrogen fixing conditions.

Because T6SS genes were up-regulated under nitrogen fixing conditions, it had been presumed that expression would be, similar to *nifHDK*, dependent on the transcriptional activator, NifA. In semi-quantitative RT-PCR studies, transcription of *azo1299*, *azo1302* and *azo1305* in a *nifLA*$^-$ strain, BHLAO, was tested (Teja Shidore, PhD thesis, 2012). The differences in transcription levels of these genes in BHLAO compared to wild type strain BH72 was not statistically significant, suggesting there was a different transcriptional activator than NifA (Teja Shidore, PhD thesis, 2012). Confounding this, GUS assays in this study showed reduced activity from the promoter region upstream of *azo1301* in the *nifLA*$^-$ strain under nitrogen fixing conditions. In the semi-quantitative RT-PCR studies, glutamate was added to the media when promoting nitrogen fixation. However, glutamate cannot account for the differences seen between assays. In the GUS assays from this study, transcription from the promoter region of *azo1301* in BHLAO was less than in wild type when strains were grown in SM media with or without the addition of glutamate. This data advocated that some aspects of T6SS-1 regulation were shared with *nif* genes. The differences between each assessment are perplexing. In both studies, gene transcription was tested under microaerobic conditions with glutamate. In the previous study cells were grown in batch cultures, like this one, and harvested when cultures reached an OD_{578} of 0.4-0.6 after >10 h (Teja Shidore, PhD thesis, 2012). In this study cells reached cell densities close to those of cells used in the semi-quantitative PCR study. Another consideration is that the assays used to assess transcription were different: one tested the *azo1301* promoter region

activity in GUS assays using a transcriptional fusion, and the other tested mRNA quantities of *azo1299*, *azo1302* and *azo1305* using semi-quantitative RT-PCR. Although similar regulation of the T6SS-1 genes tested was anticipated, different genes were tested in each assay. As with any RT-PCR analysis, it remains possible that a bias was created from RNA integrity or that presence of reaction inhibitors affected the efficiency of reverse transcription or PCR steps. Reviewing the methods used in the previous study, substantial consideration was given to all standard concerns in obtaining reliable results in a semi-quantitative RT-PCR reaction (Nolan et al., 2006; Teja Shidore, PhD thesis, 2012). Once again, the sensitivity of methods used to measure transcriptional activity varied, but considering the observed GUS activities in this study, it could not be concluded that NifA does not regulate the transcription of T6SS-1 to some extent.

In a microarray study, transcription of T6SS genes by the strain BHLAO was compared to the wild type strain BH72. Cells were grown in SM media with 10 mM glutamate in a bioreactor (0.6% O_2). Under these conditions that promote nitrogen fixation, *azo1300*, *azo1301*, *azo1306*, *azo1307* and *azo3872* were up-regulated 2.7, 2.0, 5.3, 3.0 and 3.7-fold, respectively; while T6SS-2 genes, *azo3884*, *azo3890*, *azo3895* and *azo3910* were down-regulated 3.4, 2.9, 3.9 and 2.3-fold, respectively (Sarkar and Reinhold-Hurek, 2014). Repeating assays using identical conditions and comparing transcript levels from the same genes may help resolve discrepancies between results and to determine whether or not expression of some T6SS-1 genes are regulated by NifA. However, despite a GenDB-PatScan search predicting a σ^{54} binding consensus sequences (Abhijit Sarkar, unpublished data), upon closer examination, the T6SS-1 gene cluster lacked a σ^{54} binding motif (Teja Shidore, PhD thesis, 2012). This was strong evidence that T6SS-1 regulation is independent of the σ^{54} transcriptional activator, NifA (Teja Shidore, PhD thesis, 2012).

A transcriptional activator other than that encoded by the *nifLA* operon was sought. After screening several transcriptional regulators, RseC-like Azo0559 showed potential as a positive regulator of T6SS genes expression. RseC is a positive regulator of alternative sigma factor E. Sigma factor E (σ^E/ σ^{24}) is an alternative sigma factor of the σ^{70} family. It is essential for *E. coli*, but not for all bacteria (Hayden and Ades, 2008). Its activation is thought to be signaled by accumulation of outer membrane proteins in the periplasm (Rowley et al., 2006). Once activated, σ^E controls the expression of genes necessary for pathogenesis and resistance to environmental stresses (Rowley et al., 2006). Gene products include those that

function outside of the cytoplasm, and some are involved in the folding or degradation of polypeptides in the periplasm (Rowley et al., 2006). Upon activation by extra cellular stress, σ^E is released from RseA (Missiakas et al., 1997). RseA is a negative transcriptional regulator that functions by sequestering σ^E to the inner membrane. σ^E is freed after the DegS homolog, RseD/MucD, degrades RseA in the periplasm and RseP (putatively *azo1902*) degrades the transmembrane domain (Flynn et al., 2004). In *E. coli*, it was shown DegS is stimulated by the interaction of three C-terminal ends of unfolded proteins (only exposed when unfolded) with its PDZ domain (Hasselblatt et al., 2007). RseB is a dimeric, periplasmic protein that recognizes the secondary structure of outer membrane proteins (OMP), and in *E.coli*, the sequence of a β-strand motif of the OMP is believed to modulate the strength of the response (Kulp and Kuehn, 2011). RseB acts as a negative regulator of σ^E by binding RseA at the DegS binding site (periplasmic C-terminal domain), inhibiting the cleavage (Kim et al., 2010). How RseC positively modulates σ^E activity is unknown, but it is thought to act on RseA or RseB (Missiakas et al., 1997). The predicted function of Rse proteins are diagrammed in Figure 50, adapted from Hayden and Ades (2008).

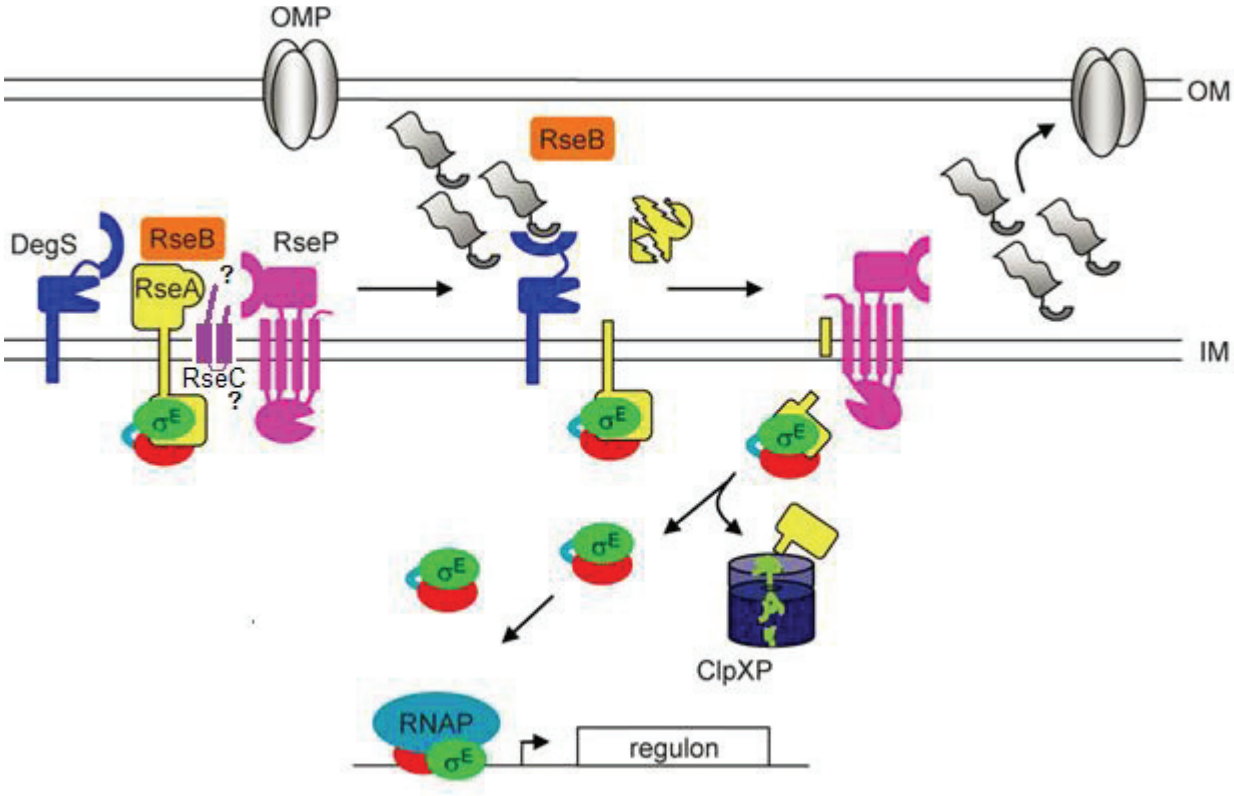


Figure 50. Diagram of the interaction of proteins regulating the activity of RpoE (σ^E) (adapted from Hayden and Ades, 2008). Negative transcriptional regulator RseA sequesters σ^E at the inner membrane. DegS degrades the periplasmic protein of RseA and RseP degrades the transmembrane

domain. RseB binds RseA at the DegS binding site inhibiting the cleavage of RseA. Three C-terminal ends of unfolded proteins interact with the PDZ domain DegS and degradation is stimulated. RseC is an inner membrane protein with two transmembrane segments thought to act on RseA or RseB to positively modulate σ^E activity (Flynn et al., 2004; Kim et al., 2010; Missiakas et al., 1997).

Interestingly, a σ^E binding site was predicted by BPROM (http: http://linux1.softberry.com) to be upstream of *azo1301* (Teja Shidore, PhD thesis, 2012). The *Azoarcus* sp. strain BH72 genome includes two genes that encode for RpoE, *azo0075* and *azo1630* (or *algU*). The gene name *algU* is derived from its derepression in *P. aeruginosa* resulting in the overproduction of alginate, partially for biofilm formation (Borgos et al., 2013). A homolog of AlgU was found encoded at the beginning of an operon containing σ^E transcription factors rseA, rseB, rseC and mucD (Figure 36) in the *Azoarcus* sp. strain BH72 genome.

Less Hcp was detected in the total cell protein fractions using Western blot analysis when *azo0559* was disrupted compared to wild type when cells were grown under nitrogen fixing conditions. Non-fixing conditions showed little or no difference in the small amount of Hcp detected (Figure 38). Despite *azo0559* being outside of the *rpoE-rseABCD* (*azo1630-azo1634*) operon, its gene product may function as an RpoE anti-anti sigma factor. Similar in function to other studied RseC proteins, regulation was not anticipated to be comprehensive enough to completely prevent the anti-σ^E activity of RseA or RseB. As seen in this and other studies, the action of RseC was only partially effective at positively regulating σ^E activity (Missiakas et al., 1997). Using GUS assays to assess transcription from the promoter region upstream of *azo1301*, in BHΔ0559, the activity was significantly less than in wild type when strains were grown under nitrogen fixing conditions. This strengthened the argument that Azo0559 acts as a transcriptional regulator of T6SS-1 genes. Interestingly, addition of glutamate to the medium resulted in an induction of *azo1301* expression by BHΔ0559 compared to growth in the other media used.

The effects of glutamate on T6SS gene expression were surprising. Also in BHLAO, of all the media used for growth, *azo1301* showed the highest level of expression in GUS assays when cells were supplemented with glutamate. In the wild type background, transcription of *azo1301* was higher when cells were grown under nitrogen fixing conditions without glutamate. Nevertheless, the presence of glutamate in media needs to be taken into account in T6SS studies. In previous semi-quantitative RT-PCR studies testing the expression of *azo1299*, *azo1302* and *azo1305*, glutamate was always added to the media. For those studies

T6SS-1 genes tested in the *nifLA*$^-$ strain (BHLAO) showed no significant modulation compared to expression in the wild type strain, but in this study, without the addition of glutamate, there was a significant reduction (4.5-fold). However, the different findings in the two studies cannot be explained by glutamate alone. When glutamate was added to the growth medium, there was still a statistically significant reduction observed, but less dramatic (1.5-fold). Additionally, in the *ntrBC*$^-$ strain (BNtrBsp) T6SS-1 genes *azo1299*, *azo1302* and *azo1305* were up-regulated when grown under conditions stimulating nitrogen fixing (with glutamate added to the medium) in the semi-quantitative RT-PCR studies (Teja Shidore, PhD thesis, 2012). Repeating the semi-quantitative RT-PCR analyses using RNA extracted from cells grown under nitrogen fixing conditions without glutamate would be interesting.

The effect of glutamate on T6S was also demonstrated in Western blots. The effect was not apparent in wild type, but the expression of Hcp by *azo0559* mutant strains appeared to be stimulated when media was supplemented with glutamate (Figures 39 and 42). As discussed above, GUS assays showed that addition of glutamate increased expression of *azo1301* from T6SS-1 in BHΔ0559 (Figure 44), but transcription of T6SS-2 genes was not measured. There was also an increase in the Hcp detected in Western blots, Hcp antibodies used in this study did not allow for a distinction between Hcp proteins detected. Expression of T6SS-2 Hcp proteins (TssD2 and/or TssD3) might also be induced with the addition of glutamate to the growth media of *azo0559* mutant strains, but this was not assessed. It would be interesting in future work to assess the transcription of T6SS-2 genes by an *azo0559* deletion mutant strain in the presence of glutamate.

It is tempting to speculate that the presence of glutamate plays a role in regulation of T6SS gene expression. However, in a microarray study, transcription of T6SS genes by *Azoarcus* sp. strain BH72 grown under nitrogen fixing conditions was compared to growth under the same conditions with media supplemented with glutamate. The addition of glutamate showed only a slight up-regulation of some T6SS-1 genes (*azo1298* +2.1, *azo1299* +1.9, *azo1302* +2.1 and *azo1303* +2.1) and no significant change in T6SS-2 genes (Sarkar and Reinhold-Hurek, 2014). In GUS assays and in Western blot analysis, Hcp expression by wild type strain BH72 was the least affected by glutamate relative to the transcriptional regulator mutants. These results provide little support for the notion that the presence of glutamate alters gene expression by wild type strain BH72. However, this study does provide evidence that adding glutamate to media of transcriptional regulator mutants, BHΔ0559 and BHLAO,

promoted expression of at least *azo1301* of T6SS-1, but possibly both T6SSs. Further investigations may yield glutamate itself or secondary effects of glutamate act to stimulate one or both T6SSs.

This study has further confirmed that both T6SS gene clusters encoded within the genome of *Azoarcus* sp. strain BH72 display strong homology to established T6SSs functioning in other organisms. There is a paradigm of thought that the function of T6SS is to provide a competitive advantage in free-living environments against other microbes, and after the bacterium transitions to be strictly host-associated, T6SSs become dispensable (Schwarz et al., 2010a). In *Azoarcus* sp. strain BH72, it has been clearly demonstrated that both T6SSs actively secrete. Additionally, there is strong evidence that the accessory proteins encoded near the T6SS-2 gene cluster are components of the threonine phosphorylation pathway. The secretome data obtained here was insufficient for identification of the secreted effector, but it did make clear that T6SS-2 proteins were more prevalent in the wild type secretome under growth conditions used. Despite the predominance of Azo3897 (TssD2) in the secretome, this does not eliminate the potential role of T6SS-1 being the more important system for endophytic interactions with rice, especially considering its up-regulation with growth under nitrogen fixing conditions and its importance for efficient colonization of rice. It remains possible that multiple effectors will be found secreted by each T6SS and that one or more effectors possibly functions in bacterial competition. The up-regulation of T6SS-2 genes in response to cell density hints at a role for this gene cluster in interbacterial competition. This and other environmental cues, such as exposure to root exudates, need to be tested further. Also, gaining a better understanding of how the addition of glutamate influences Hcp expression by BHLAO and *azo0559* mutant strains may prove to be an interesting lead. Utilizing the right environmental cues to promote secretion will help in identifying the effector protein(s) in secretome analyses, as might utilizing transcription factors. In future work, more potential transcriptional regulators need to be screened, and in addition to monitoring transcription of T6SS-1 genes, T6SS-2 gene expression should also be considered. Eventual identification of the T6SS effector protein(s) will prove to be a valuable tool in understanding communication between plant and microbes in an endophytic interaction. In addition to assessing plant-microbe interactions, testing the role of T6SS effectors in interbacterial competition will also be important. In the future, it might be demonstrated that *Azoarcus* sp. strain BH72 is in fact ‘armed’ and active in the race of co-evolutionary events that lead to endophytic interactions.

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