EVALUATING THE GspC PROTEIN IN SUBSTRATE SPECIFICITY OF *RALSTONIA SOLANACEARUM* TYPE II SECRETION

by

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(Under the Direction of Timothy P. Denny)

ABSTRACT

The type II secretion (T2S) system in *Ralstonia solanacearum* secrets multiple plant cell wall-degrading enzymes and other proteins that are important for pathogenesis. GspC is an essential component of the T2S system, and is one of two proteins thought to help determine substrate specificity. In *R. solanacearum* GspC lacks a predicted C-terminal protein-protein interaction domain, and this may contribute to its secreting more proteins than other bacteria. To investigate the role of GspC in the T2S system, I deleted *gspC* in *R. solanacearum* GMI1000 and tested various plasmid-borne genes for their ability to restore secretion of polygalacturonase, endoglucanase, pectin methylesterase, and trehalase enzymes. GspC protein from *Cupriavidus metallidurans* fully restored secretion of the tested enzymes, while *Burkholderia thailandensis* GspC only partially restored secretion of some enzymes. These results suggest that general recognition of secreted proteins by the *R. solanacearum* T2S system lies in both the secreted protein and GspC function.

INDEX WORDS: *Ralstonia solanacearum*, type II secretion system, GspC protein

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# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

## Introduction

*Ralstonia solanacearum* is a Gram-negative, aerobic rod in the β-subdivision of Proteobacteria (Stackebrandt et al. 1988) and has been the subject of numerous studies on host-pathogen interactions. The organism is one of the world's most important phytopathogenic bacteria because of its lethality, persistence, wide host range, and broad geographic distribution (Denny 2006). The pathogen enters plant roots via wounds or where secondary roots emerge, invades xylem vessels and rapidly spreads throughout the vascular system. The bacterial population within the vascular system can reach higher than $10^9$ colony-forming units (CFU) $gram^{-1}$ fresh weight, producing large amounts of plant-cell-wall-degrading enzymes and high-molecular-mass extracellular polysaccharide, which eventually lead to vascular dysfunction and cause the plant to wilt and die (Schell 2000).

The type II secretion (T2S) system is a protein secretion mechanism that is widely conserved among Gram-negative bacteria. The T2S secretion apparatus is a membrane-associated protein complex that requires the general-secretion pathway (Gsp) proteins encoded by at least 12 genes. This system is responsible for transporting selected proteins from the periplasm to the extracellular environment (Filloux 2004). Many of the secreted proteins are important virulence factors in both plant and animal pathogens. The proteins to be secreted initially reach the periplasm via the Sec-dependent pathway or the Tat pathway (González et al. 2007), where they fold into their mature form before being secreted. However, of the many

proteins present in the periplasm, only a relative few are recognized as a substrate by the T2S system.

The number of proteins secreted by the T2S system varies between bacterial species. Additionally, exoproteins from one bacterial species may or may not be secreted by other species (Filloux 2004). Comparison of the primary amino acid sequences of diverse proteins secreted by the many T2S has not revealed any obvious regions of similarity. Instead, research suggests that the secretion signal is a "patch signal" in the tertiary structure of exoproteins comprising different parts of the linear sequence (Lindeberg et al. 1998; Py et al. 1991). These and other aspects of T2S substrate specificity are still not clearly understood.

GspC is an essential component of the T2S system, and along with GspD is one of the two proteins thought to help determine substrate specificity (Bouley et al., 2001; Lee et al. 2004). GspD forms a stable multimeric complex within the outer membrane that functions as a gated pore (Chami et al. 2005). Although GspC has its N-terminus embedded in the inner membrane, most of the protein is in the periplasm. The N-terminal half of GspC is somewhat conserved, but the C-terminal half usually has one of several putative protein-protein interaction domains. The exact function of GspC remains unclear, but research with *Erwinia* and *Pseudomonas* species has shown that the C-terminal half of GspC is involved with recognizing some substrate proteins (Bouley, et al. 2001, de Groot et al. 2001), Surprisingly, the *R. solanacearum* GspC lacks a predicted C-terminal protein-protein interaction domain, and this may contribute to this pathogen secreting more proteins than other bacteria.

Here, I studied the contribution of GspC from *R. solanacearum* and two other β-proteobacteria in determining function and substrate specificity of the *R. solanacearum* T2S system. I first created a *R. solanacearum gspC* non-polar deletion mutant and showed that it has

a nonfunctional T2S system. I then cloned wild-type *gspC* from *R. solanacearum*, *Cupriavidus metallidurans* and *Burkholderia thailandensis* on various broad-host-range plasmid vectors, some of which I created for this project. I also created a C-terminally truncated *R. solanacearum gspC* allele that is structurally comparable to the 'naturally truncated' allele in *B. thailandensis*. These constructs were tested for their ability to complement the *R. solanacearum gspC* deletion mutant by qualitative and quantitative assays for secretion of four *R. solanacearum* exoproteins: endoglucanase, polygalacturonase, trehalase, and pectin methylesterase.

## Literature Review

Gram-negative bacteria have several different systems to transport extracellular proteins across their phospholipid membranes. The extracellular proteins are involved with important cellular functions such as nutrient acquisition, virulence, and other toxins (Kostakioti et al. 2005). Export of these proteins to the bacterial surface involves transport across the inner membrane (IM), periplasm, and outer membrane (OM).

### The type II secretion system

T2S is a two step process. In the first step, most of the proteins to be secreted are expressed with signal peptides that lead them to the Sec-dependent general export pathway (Filloux 2004). The signal peptide is removed during transport across the cytoplasmic membrane, and the protein becomes fully folded in the periplasm. In the second step, the folded proteins are recognized and transported from the periplasm across the outer membrane by components of the T2S apparatus (Filloux 2004, Kostakioti et al. 2005). In addition to the Sec system, the twin-arginine translocation (TAT) system, which usually transports previously folded protein across

the IM, may also serve as the first step for T2S substrates. For example, phospholipases in *Pseudomonas aeruginosa* (Voulhoux et al. 2001) and the exo-polygalacturonase PehC in *Ralstonia solanacearum* (González et al. 2007) are exported by the TAT system and secreted by the T2S system.

The T2S apparatus (the secreton) is composed of 12 to 16 different proteins. The genes are clustered and often organized into one large operon. The components of the T2S system are located in both the inner and outer membranes and assemble into a multi-protein complex that spans the cell envelope (Filloux 2004); the essential and widely conserved proteins are listed in Table 1.1 along with their subcellular location.

GspD is the only protein in the secreton associated with the OM. It is a member of the conserved secretin family, which form stable multimeric complexes within the OM (Chen et al. 1996; Nouwen et al. 2000). It has a highly conserved C-terminal half predicted to have multiple transmembrane spanning regions that form the pore through which the substrates are exported (Chami et al. 2005). The more variable and possibly species-specific N-terminal domain extends into the periplasm and seems to be important for recognizing the substrates, interacting with the cytoplasmic components of the secretion apparatus, and possibly gating the formed channel (Nouwen et al. 2000).

GspE, GspF, GspL, and GspM form an IM platform and provide energy by hydrolyzing ATP to support the active process of protein secretion. Though direct interaction between ATP and GspE has not yet been demonstrated, a motif similar for NTP-binding has been found in GspE. Mutation within the conserved NTP-binding motif of GspE from *Pseudomonas aeruginosa, Klebsiella oxytoca, Erwinia chrysanthemi* or *Vibrio cholerae* causes the bacteria to become secretion-defective (Sandkvist et al. 1995; Turner et al. 1993; Py et al. 1999; Possot et al.

1994). The abundance of GspM in the cell depends on GspL, indicating that these two Gsp components interact with each other (Possot et al. 2000). The GspM protein may determine the membrane location of the secretion site, recruit the GspL component, and bring GspE into the membrane. In *E. chrysanthemi* the N-terminus of GspF interacts both with GspE and GspL (Py et al. 2001). GspF may strengthen the association of GspE with the membrane, and/or be involved in pore formation in the IM, allowing pseudopilin translocation.

The pseudopilin is composed of five different proteins: GspG to GspK. Their N-terminus is similar to type IV pilins, but the C-terminal domain is different. They are bitopic proteins with a single hydrophobic N-terminal segment, and are able to form a pilus-like structure that spans the periplasmic part of the T2S secreton (Hu et al. 2002). In *P. aeruginosa*, overproduction of GspG, the major pseudopilin results in formation of cell-surface pseudopili and reduced protein secretion, possibly because the pseudopili occupy the secretin channel (Durand et al. 2003). Similar results have also been demonstrated in *K. oxytoca* (Possot et al. 2000; Vignon et al. 2003).

The function of GspC protein is unclear. In *E. chrysanthemi*, the C-terminal portion of GspC has a predicted PDZ domain. PDZ domains, which are named after the three eukaryotic proteins (post-synaptic density protein, disc large and zo-1 proteins) in which they were first found, are often involved in protein-protein interactions. In *Pseudomonas* species the PDZ domain is replaced with a predicted coiled-coil domain, which also can promote protein-protein interactions. In *Xanthomonas campestris* pv. *campestris*, its GspC also has a weakly predicted coiled-coil domain in the C-terminal region. An interaction of GspC with GspD has been difficult to demonstrate. This first report was by Lee et al. (2000) who showed GspC-GspD interaction in *X. campestris* pv. *campestris* by coimmunoprecipitation and coelution on affinity

chromatography. Such interaction was recently reported also in *E. chrysanthemi* (Login et al. 2010).

**Specificity of the type II secretion system**

There are many proteins that function in the periplasm, but only a relatively few are recognized as substrates by the T2S secreton and transported across the OM (Filloux 2004). Even though the T2S apparatus is widely conserved, secretion of exoprotein is often species specific. For example, the exoprotein from *K. oxytoca*, pullulanase, is not secreted by the T2S system of *P. aeruginosa* (de Groot et al. 1991). In phytopathogenic *E. chrysanthemi* and *Erwinia carotovora*, multiple exoproteins without any sequence similarity are secreted by their respective T2S systems. Even though both bacteria secrete homologous proteins using very similar T2S systems, cellulase Cel5 (ex-EGZ) and CelV are recognized exclusively by their own secretion machinery (He et al. 1991; Py et al. 1991). However, in a few bacteria, exoproteins from one species can be secreted by another T2S system. The best example is *Pseudomonas alcaligenes* lipase that is secreted by *P. aeruginosa*, whereas *P. aeruginosa* elastase is secreted by *P. alcaligenes* (de Groot et al. 2001). Sequence analyses of the secreted proteins have not identified a common secretion motif (Filloux 2004).

Analysis of the ability of individual *gsp* genes of *E. carotovora* to restore secretion in the *E. chrysanthemi* T2S system demonstrated that GspD and GspC are the only components that are not functionally interchangeable between these two secretons (Lindeberg et al. 1996). Additionally, the C-terminal PDZ protein-protein interaction domain of GspC in *Erwinia* species determines its secretion specificity towards certain exoproteins. In the absence of the PDZ domain, the T2S still secretes pectin methylesterase PmeA and cellulase Cel5, but not most of

the pectate lyases. This indicates that the PDZ domain could be necessary for the recognition of some exoproteins but not others (Bouley et al. 2001). Furthermore, replacement of the PDZ domain of *E. carotovora* GspC with that from *E. chrysanthemi* changes the specificity of the protein, making it functionally equal to *E. chrysanthemi* GspC (Bouley et al. 2001), again indicating the importance of the protein-protein interaction domain of GspC proteins in recognizing some substrates. Similar to *E. chrysanthemi* C-terminal PDZ deletion, when *Xanthomonas campestris* pv. *campestris* GspC lacks the coiled-coil protein interacting domain due to removal of amino acids 159 to 261, it still secretes protease, pectate lyase, cellulase, and α-amylase (Lee et al. 2004). However, deletion of two more residues makes the protein nonfunctional, suggesting that the N-terminal 158 residues probably hold the minimal information for the functional GspC.

Overall, these results suggest that the N-terminal conserved region of GspC is sufficient to maintain the structure of the T2S secreton and the function of T2S in different bacteria species. The different C-terminal protein-protein interaction domains are more relevant to protein-specific substrate recognition than basic T2S functions.

**Type II secretion in *Ralstonia solanacearum***

*Ralstonia solanacearum* causes lethal wilting diseases on many crop and wild plants in warm climates. The pathogen has a wide host range and broad geographic distribution. Many of its most important hosts, such as tomato, potato, pepper, banana, tobacco, and eggplant have economic value (Denny 2006). In *R. solanacearum*, there appear to be 12 proteins involved in assembling the T2S system. The genes encoding Gsp proteins are all in one cluster, and all but *gspC* appear to be organized in one large operon. In addition to the conserved proteins listed in

Table 1.1, *R. solanacearum* has a GspN protein. GspN is predicted to be a bitopic IM protein that previously had been identified only in some bacteria, including *K. oxytoca*, *E. carotovora* (but, surprisingly, not *E. chrysanthemi*), and *Burkholderia pseudomallei*. The exact function of GspN is unknown, but in *K. oxytoca*, GspN is not required for T2S (Possot et al. 2000), while in *B. pseudomallei* it increases secretion efficiency (DeShazer et al. 1999).

In *R. solanacearum* most of the plant cell-wall-degrading enzymes (CWDEs) and other abundant extracellular proteins are transported across the inner membrane through the Sec translocase and the outer membrane via the T2S system (Liu et al. 2005). CWDEs are involved in virulence of *R. solanacearum*. Six extracellular enzymes have been identified: a β-1,4-endoglucanase (Egl), an exoglucanase (CbhA), an endo-polygalacturonase (PehA or PglA), two exo-polygalacturonases (PehB and PehC), and a pectin methylesterase (Pme). All but CbhA have detectable in vitro enzyme activity (Denny 2006). Inactivation of single CWDE genes showed that none of these exoenzymes is essential and their relative importance to cause disease varies with different strains (Liu et al. 2005). However, a mutant of *R. solanacearum* strain GMI1000 that lacks a functional T2S does not secrete any of the CWDEs and is much less virulent than either a Egl-CbhA double mutant or a mutant that lacks all six CWDEs (Liu et al. 2005). By comparing the secretion profile between wild-type *R. solanacearum* strain GMI1000 and T2S secretion mutant, about 44 proteins are probably secreted by the T2S system based on quantitative information on their relative abundance (Zuleta 2007). No other bacterium has been reported to have this many type II-secreted proteins.

Unlike most of the known GspC proteins, amino acid sequence analysis showed that there is no obvious protein-protein interacting domain in *R. solanacearum* GspC protein's C-terminal region (Fig 1.1). Besides lacking the protein-interaction domain, *R. solanacearum* GspC

also potentially possess another transmembrane domain in the C-terminal region. Since the C-terminal region of GspC proteins from other bacteria is involved with substrate recognition, the absence of such a domain in *R. solanacearum* GspC might contribute to its ability to secrete a greater number of exoproteins than other bacteria.

**GspC in related β-proteobacteria genera**

Protein BLAST using the *R. solanacearum* GspC as a query showed that it has high homology with the N-terminus of the GspC protein in *Cupriavidus* and *Burkholderia* species (Fig. 1.1), which, like *R. solanacearum*, belong to the β-proteobacteria. In *C. metallidurans*, which has many orthologous genes in *R. solanacearum*, sequence analysis of the GspC protein showed that it also lacks a protein-protein interacting domain in the C-terminus. Previous research has demonstrated that endoglucanase; polygalacturonase and pectin methylesterase from *R. solanacearum* can be secreted by *C. metallidurans* (Denny, unpublished data). In *B. pseudomallei*, *gspC* is essential for T2S (DeShazer et al. 1999), but GspC completely lacks a C-terminal region. The 130 amino acids in its functional protein align with the N-terminal conserved region of other GspC proteins. This again indicates that the N-terminal conserved region of GspC is sufficient to maintain the structure and function of the T2S system.

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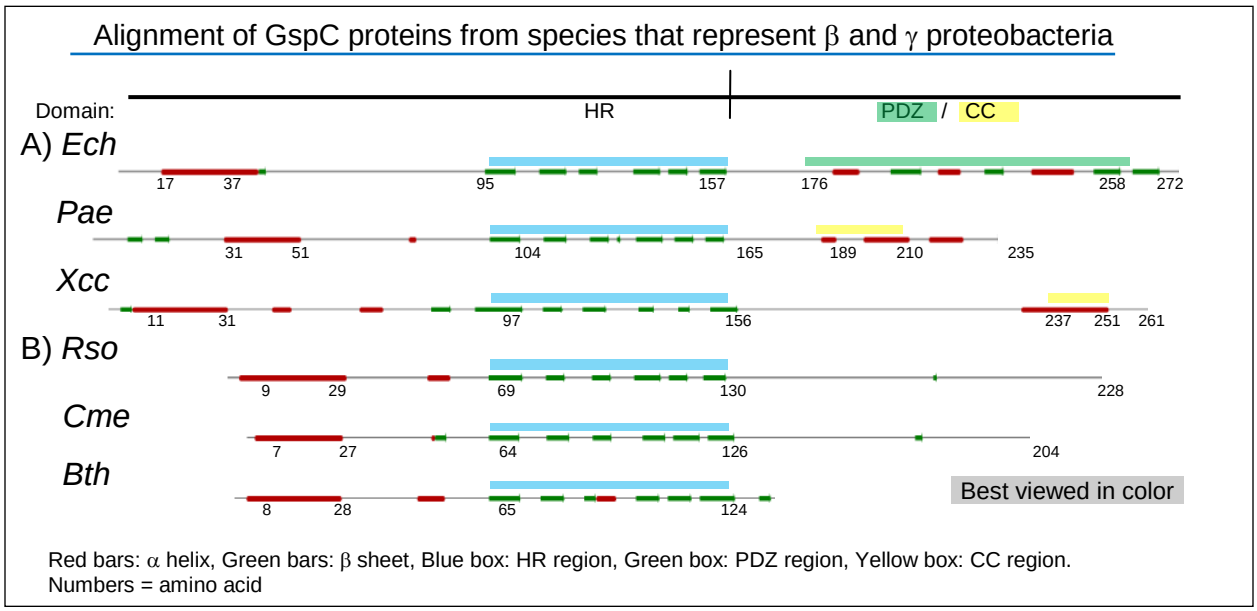


Fig 1.1. Comparison of representative GspC proteins. HR = homology region; PDZ/CC indicates the protein-protein interacting domains. Amino acids are numbered below each line. (A) Selected γ-proteobacteria: *Ech* = *Erwinia chrysanthemi*, *Pae* = *Pseudomonas aeruginosa*, *Xcc* = *Xanthomonas campestris* pv. *campestris*, (B) Selected β-proteobacteria: *Rso* = *Ralstonia solanacearum*, *Cme* = *Cupriavidus metallidurans*, *Bth* = *Burkholderia thailandensis.* Note how GspC in β-proteobacteria lack a protein-protein interacting domain.

Table 1.1. Main component proteins of the type II secretion apparatus.

| Protein function | Protein |
|---|---|
| Outer membrane secretin | GspD |
| Cytoplasmic ATPase | GspE |
| Inner transmembrane protein | GspF |
| ATPase facilitator | GspL, GspM |
| Pseudopilins | GspG, GspH, GspI, GspJ, GspK |
| Substrate recognition and/or secretin interaction | GspC |

# CHAPTER 2

## Evaluating the GspC protein in substrate specificity of *Ralstonia solanacearum* type II secretion

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## Introduction

The type II secretion (T2S) system is a protein secretion mechanism that is widely conserved among Gram-negative bacteria. The T2S secretion apparatus is a membrane-associated protein complex that requires at least 12 proteins encoded by what are often referred to as general-secretion pathway (*gsp*) genes. This secreton is responsible for transporting selected proteins from the periplasm to the extracellular environment (Filloux 2004). Many of the secreted proteins are important virulence factors in both plant and animal pathogens. The proteins to be secreted initially reach the periplasm via the Sec-dependent pathway or the Tat pathway (González et al. 2007), where they fold into their mature form before being secreted. Interestingly, of the many proteins present in the periplasm, only a relative few are recognized as substrates by the T2S system.

The T2S system was first identified in *Klebsiella pneumoniae* (later renamed *K. oxytoca*) (d'Enfert et al. 1987) and virtually all research has been with this and other organisms in the γ-proteobacteria, such as *Erwinia, Pseudomonas*, *Vibrio*, and *Xanthomonas* (Lindeberg et al. 1996; Bouley et al. 2001; de Groot et al. 2001; Robert et al. 2005; Korotkov et al. 2006; Lee et al. 2004). In comparison, very little research has been published describing work with the T2S system in β-proteobacteria. The genetic loci required for T2S in *Burkholderia pseudomallei* were identified by transposon and targeted mutagenesis and found to contribute little to pathogenesis (DeShazer et al. 1999). In *Ralstonia solanacearum*, an important plant pathogen that causes lethal wilting on a wide variety of important crop plants (Denny 2006), the T2S system is crucial for secreting a large variety of extracellular proteins that contribute to infection via the roots, as well as wilting and killing host plants (Kang et al. 1994; Liu et al. 2005). Sequence analysis

indicates that these and other β-proteobacteria have all the essential *gsp* genes that have been better studied in the γ-proteobacteria.

The number of proteins secreted by T2S systems varies widely between bacterial species. *K. oxytoca* secretes only the pullulanase lipoprotein, an enzyme that stays associated with the outer membrane (d'Enfert et al. 1987; Possot et al. 2000). In contrast, *R. solanacearum* appears to secrete at least 35 proteins, among which are multiple enzymes that help degrade plant cell walls and promote virulence (Zuleta 2007; Liu et al. 2005). How bacteria differentiate between the majority of proteins that remain in the periplasm and those destined for secretion by the T2S system (exoproteins) is poorly understood. Comparison of the sequences of diverse exoproteins has not revealed any obvious regions of similarity (Filloux 2004). Instead, research has suggest that the secretion signal is a "patch signal" in the tertiary structure of exoproteins comprising different parts of the linear sequence (Lindeberg et al. 1998; Py et al. 1991). However, just having the correct tertiary structure does not guarantee that an exoprotein will be secreted when it is produced by a heterologous organism, indicating a level of species specificity in substrate recognition. For example, cellulases from *E. chrysanthemi* and *Erwinia carotovora* are recognized exclusively by their own secretons (Py et al. 1991).

Work with *Klebsiella*, *Erwinia*, and *Pseudomonas* species shows that most of the essential secreton proteins do not determine substrate specificity, because they restore secretion of native exoproteins when introduced into heterologous bacteria (Hazes and Frost 2008; de Groot et al. 2001; Lindeberg et al. 1996). In contrast, the function of GspC and GspD is usually species specific. For example, when *E. chrysanthemi* has its native GspC and GspD (better known as OutC and OutD, respectively) replaced by those from *E. carotovora*, the bacterium no longer secretes its native pectate lyase, PelC (Lindeberg et al. 1996). Later research demonstrated

that the C-terminal PDZ domain of GspC and the N-terminal region of GspD help to determine this secretion specificity in *E. chrysanthemi* (Bouley et al. 2001). In contrast, when *Pseudomonas alcaligenes* GspC and GspD (known as XcpP and XcpQ, respectively) were expressed simultaneously in a *Pseudomonas aeruginosa gspCD* deletion mutant, secretion of a native protease was observed on agar plates (de Groot et al. 2001). However, the complementation was imperfect, because the composite secreton did not function when bacteria were grown in liquid culture. Furthermore, *P. alcaligenes* GspC and GspD did not function when produced individually by their respective *P. aeruginosa* mutants.

GspD is a member of the secretin family (Bitter 2003) that assembles as a multimeric complex within the outer membrane and functions as a gated pore. This complex is thought to resemble an inverted cup and saucer with the variable N-terminus in the periplasm and more conserved C-terminus, which has 10-14 transmembrane segments, embedded in the outer membrane (Chami et al. 2005; Nouwen et al. 2000). GspC, which is the least conserved of the essential secreton proteins, is inserted into the inner membrane by a single transmembrane segment near the N-terminus, while most of the protein is in the periplasm. One stretch of about 90 amino acids in the N-terminal half of GspC is known as the Homology Region (HR), based on its moderate conservation in some *Erwinia* and *Pseudomonas* species (Fig. 2.1.) (Bouley et al. 2001; Robert et al. 2005). Login et al. (2010) recently showed that a 20 amino acid region of the HR in *E. chrysanthemi* OutC interacts with several N-domains in OutD, and that some amino acids in this region are essential for OutC function. The C-terminal half of GspC is more variable, but in γ-proteobacteria it always has one of two putative protein-protein interaction domains (Fig. 2.1.). Research on GspC from *Erwinia* and *Pseudomonas* species has shown that the C-terminal

half of GspC is involved with recognizing some, but not all, substrate exoproteins (Bouley, et al. 2001, de Groot et al. 2001).

In contrast, analysis of sequence data for multiple β-proteobacteria genomes revealed that they all have a GspC that is predicted lack a protein-protein interaction domain in the C-terminus (Fig. 2.1). The C-terminal half of GspC in *R. solanacearum* strain GMI1000 and *Cupriavidus metallidurans* strain CH34 are similar in length (78 to 98 amino acids) but are otherwise poorly conserved (only 13% identical). Interestingly, GspC proteins in 14 *Burkholderia* species (20 strains total) are predicted to lack a C-terminal region (Fig. 2.1). Nevertheless, DeShazer et al. (1999) showed that the *B. pseudomallei* GspC is functional and essential for T2S.

In this study, we tested the contribution of GspC from *R. solanacearum* and two other β-proteobacteria in determining function and substrate specificity of the *R. solanacearum* T2S system. We found that, in contrast to work with γ-proteobacteria, GspC of *C. metallidurans* fully complemented an *R. solanacearum gspC* deletion mutant for secretion of four exoproteins, and that GspC from *Burkholderia thailandensis* partially complemented this mutant. In addition, a C-terminally truncated *R. solanacearum* GspC fully complemented the *gspC* deletion mutant, confirming that the C-terminal portion of this protein is not essential.

## Material and Methods

**Bacterial strains, media and growth conditions.** Strains and plasmids used in this study are listed in Table 2.1. *R. solanacearum* mutants and *E. coli* DH5α strains containing plasmids with the complementing *gspC* alleles were stored in 15% glycerol at -80$^{o}$C. *R. solanacearum* strains were grown routinely in BG broth (1% Bacto peptone, 0.1% casamino acid, 0.1% yeast

extract, and 0.5% glucose) or BG agar (BG broth + 1.6% agar) at 30°C. BS broth (BG broth made with 0.5% sucrose instead of glucose, 1 mM $MgSO_4$ and 10 μg/ml of kanamycin to maintain the plasmids) was used for growing the strains for enzyme assays. Minimal medium (MM) (Clough et al. 1994) amended with 1.0% or 1.5% (w/v) filter-sterilized sucrose was used during unmarked mutagenesis. The basal medium for exoenzyme assay plates contained liquid MM (without glucose), 0.1% (w/v) yeast extract, 0.5% (w/v) glycerol, and 1.6% (w/v) agar. For Egl and Pme activities, the basal medium was supplemented with 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and respectively, 0.5% (w/v) carboxymethylcellulose (Sigma) or 0.5% (w/v) pectin (Sigma). For Pgl activity, only 0.5% (w/v) polygalacturonic acid (J.T. Baker) was added to the basal medium. *E. coli* DH5α strains were grown in Luria-Bertani broth or Luria-Bertani agar at 30-37°C (Miller 1972). Except where noted, kanamycin (50 μg/ml), spectinomycin (50 μg/ml), or tetracycline (5 μg/ml) were added to the culture media when needed.

**DNA manipulation, transformation and sequence analysis.** Cloning, competent cell preparation and heat-shock transformation of *E. coli* followed standard protocols (Ausubel et al. 1989). Plasmid DNA was isolated using a Qiagen Mini kit (Qiagen Inc., Valencia, CA). Restriction enzymes and T4 DNA ligase were from New England Biolabs (Beverly, MA). PCR was performed with the MasterAmp Tfl DNA polymerase kit as recommended by the manufacturer (Epicentre Technologies, Madison, WI). PCR primers and other oligonucleotides were synthesized by Integrated DNA Technology (Coralville, IA). For direct PCR from cells, about $10^8$ cells were suspended in 50 μl of deionized water, heated to 100 °C for 10 min, and then 1 μl was used as the template in a 25-μl reaction. *R. solanacearum* electroporation-competent cells were prepared using standard procedures (Sambrook et al. 1989). Plasmid DNA was introduced using a BioRad electroporator (0.25 μFD, 200 Ω, 10,000 V $cm^{-1}$) after which

cells were shaken in BG broth for 1-3 hours at 30$^{o}$C before plating on BG agar containing antibiotics.

**Creation of *R. solanacearum gspC* deletion mutants.** Splice overlap extension (SOE) PCR (Horton et al. 1993) was used to create an unmarked 'deletion allele' of *gspC* (Rsc3105) as described previously (Liu et al 2005). Briefly, four PCR primers (GspCsoe1, 2, 3, and 4) were designed to amplify approximately 500-bp DNA fragments flanking *gspC* and to add restriction endonuclease sites. GspCsoe2 and GspCsoe3 share homologous extensions and GspCsoe3 also adds a *Bam*HI restriction site. The gel-purified PCR products (prepared using the Qiagen gel purification kit) were mixed and a SOE PCR reaction joined them to produce an approximately 1-kb chimeric deletion allele. This SOE product was digested with *Kpn*I and *Hin*dIII, gel purified, and ligated to similarly digested pEX18Tc to create plasmid pS3105, which was then cloned in *E. coli* DH5α. Diagnostic digests of purified plasmid DNA confirmed that the construct was correct. The deletion allele was introduced into the *R. solanacearum* GMI1000 genome by a two-step, SacB-assisted homologous recombination process as previously described with minor modifications (Liu et al. 2005). Briefly, pS3105 was first introduced into the GMI1000 chromosome by a single homologous recombination event. Subsequent selection of sucrose resistant colonies enriched for mutants that had undergone a second homologous recombination that resulted in *gspC* being replaced by the deletion allele. The desired GMI-ΔC mutant was identified by colony PCR using GspCsoe1 and GspCsoe4 primers.

A marked *gspC* deletion allele was created to simplify introducing a *gspC* deletion into other *R. solanacearum* strains. The Ω interposon, which encodes streptomycin resistance, was released from pUC8-Ω by digestion with *Bam*HI, gel purified, ligated to similarly digested pS3105 to create pS3105Ω and cloned in DH5α. DNA of pS3105Ω was introduced into GMI-31

by electroporation and mutants where homologous recombination replaced the wild-type *gspC* with the *gspC*::Ω allele were recovered by selecting for spectinomycin resistance followed by screening for tetracycline sensitivity and defective secretion of selected exoenzymes.

**Cloning *gspC* alleles on broad host range plasmid vectors.** Wild-type *gspC* from *R. solanacearum*, *C. metallidurans* (GenBank locus ABF10253), and *B. thailandensis gspC* (GenBank locus ABC36853) were cloned on the broad host range vector pBBR1MCS-2. PCR primers (GspCsoe1 and GspCsoe4; CmeGspCF and CmeGspCR; BthGspCF and BthGspCR) were designed to amplify the target genes including 300-500 base regions upstream presumed to contain their native promoters, with addition of a *Kpn*I site the on 5' end and a *Hin*dIII site on the 3' end of the amplicon. The PCR fragments were digested with these restriction enzymes, ligated to similarly digested vector DNA, and cloned in *E. coli* DH5α to create pGspC-MCS2, pCmeC-MCS2, and pBthC-MCS2. Diagnostic restriction enzyme digests of purified plasmid DNA confirmed that the constructs were correct, and pBthC-MCS2 was sequenced.

To modify *R. solanacearum gspC* to encode a protein lacking the C-terminal domain, a new reverse primer, Ctru136, was designed to terminate translation after 136 amino acids and again add a *Hin*dIII site. This primer and the GspCsoe1 upstream primer were used to amplify the partial open reading frame with the native *gspC* promoter. The fragment was cloned as described for the wild-type genes to create pCtru136. Sequencing confirmed that the C-terminal deletion occurred at the desired codon.

The three wild-type *gspC* open reading frames also were cloned on two new vectors so that they would be expressed by the same *R. solanacearum* promoters. To create a vector carrying the native *R. solanacearum gspC* promoter, we used GspCsoe1 and GMIupCR, to amplify a 500 base region upstream of *gspC* and add *Kpn*I to the 5' end and *Nde*I and *Eco*RV

sites to the 3' end of the amplicon. The fragment was digested with *Kpn*I and *Eco*RV, ligated to similarly digested pBBR1MCS-2 to create pUpC-MCS2 and cloned in *E. coli* DH5α. The ATG triplet within the *Nde*I site can be used as the start codon when introducing a promoterless open reading frame. To test expression driven by the *gspC* promoter, a 3-kb fragment containing *lacZ* without its start codon was amplified from pINT104 (Mukaihara et al. 2004) with primers LacZF-NA and LacZR-Xba, which add both *NdeI* and *Apa*I sites to the 5' end and an *Xba*I site to the 3' end of the amplicon. The *lacZ* coding sequence has an *Nde*I site near its 3' end, so this amplified fragment could not simply be digested with *Nde*I and *Xba*I. Therefore, the amplicon was digested only with *Xba*I, ligated to similarly digested pUpC-MCS2 and the linear molecule was transformed into *E. coli* DH5α. Selection for kanamycin resistance and screening for β-galactosidase activity recovered colonies that contained pUpC-LacZ, a circularized plasmid resulting from in vivo homologous recombination between the 5' end of the amplicon and the vector.

To over-express the various *gspC* genes to the same level, the promoter region plus the ribosome binding site from *R. solanacearum gapA* (RSc2749), which encodes the housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase, was amplified using primers Pgap-ForKH and Pgap-RevAN that added both *Kpn*I and *Hin*dIII sites to the 5' end and both *Nde*I and *Apa*I sites to the 3' end of the amplicon. This fragment was digested with *Kpn*I and *Apa*I, ligated to similarly digested and gel purified pUpC-LacZ to create pPgap-LacZ and cloned in *E. coli* DH5α.

To add the *Strep*-tag® II epitope (Schmidt & Skerra 2007) to the N-terminus of GspC, two oligonucleotides (S-tagTop and S-tagBot) were designed to make an adaptor that encodes the desired amino acids with single-strand overhangs compatible with vector DNA digested with *Nde*I and *Apa*I. The two oligonucleotides were mixed in a 1:1 molar ratio in a PCR reaction tube

and annealed at 70 $^{o}$C for 10 min in a PCR machine. The PCR tube was then transferred to a 1.5-ml microcentrifuge tube containing 1 ml of 70$^{o}$C water and allowed to slowly cool to room temperature. pPgap-LacZ has two *Nde*I sites, so plasmid DNA was partially digested with *NdeI* and gel-purified full-length linear products were then completely digested with *ApaI.* Full-length linear products were again gel purified, ligated to the *Strep*-tag adaptor to create the plasmid pPgap-Tag and cloned in *E. coli* DH5α.

*R. solanacearum*, *C. metallidurans*, and *B. thailandensis gspC* open reading frames were amplified by PCR with primer pairs (PgapGmiC-F and GmiCtagR; PgapCmeC-F and CmeCtagR; PgapBthC-F and BthCtagR, respectively) that amplified from the second codon to about 150 bases after the stop codon of the structural gene. The primers also added an *Apa*I restriction site on the 5' end and *Xba*I site on the 3' end of the amplicons. The *gspC* fragments were digested with *Apa*I and *Xba*I, ligated to similarly digested pPgap-LacZ and pPgap-Tag and cloned in *E. coli* DH5α to create both native and epitope-tagged fusion GspC proteins, respectively. Sequencing both strands of the constructs from vector primer sites confirmed that they were correct.

**Enzyme Assays.** Secretion of four different CWDEs (Endoglucanase (Egl), polygalacturonases (Pgl), trehalase (Tre), and pectin methylesterase (Pme)) was evaluated either qualitatively on agar assay plates or quantitatively using cells and supernatants from broth cultures. Egl, Pgl, and Pme were tested both on plates and in broth culture; Tre could only be tested for in broth culture. For agar plate assays, 5-µl drops containing approximately 5 x $10^5$ bacteria suspended in water were applied to agar media supplemented with the desired substrates and incubated at 30$^{o}$C for one to two days. Bacteria then were washed off the plates, and results of Pgl and Pme activity was detected by flooding the plates with 2 N HCl; results of Egl activity

were detected by flooding plates with 0.1% (w/v) Congo Red followed by bleaching with 1 M NaCl (Liu et al. 2005).

For quantitative enzyme assays, 1 x $10^5$ cells from freshly grown BG Km plates were cultured in BS Km broth shaken (250 rpm) at 30$^o$C for 40 – 48 hours. One milliliter of culture was removed and the cells and supernatant were separated by centrifugation. Cell pellets were washed with 0.1 M NaCl, suspended in 1 ml of 10 mM NaKPO4 buffer (pH 7). Both culture supernatants and cell suspensions were stored at -20$^o$C until needed. Frozen cell suspensions were thawed and a 150-µl aliquot was permeabilized by adding 7.5 µl of 0.1% SDS and 15 µl of chloroform, vortexing for 10 seconds, the tube was stored at room temperature for several minutes to allow the chloroform to settle to the bottom. Assays for Egl, Pgl, and Tre activities were essentially as described previously (Brumbley and Denny, 1990); 100 µl of the culture supernatants or permeabilized cell suspensions were mixed with suitable substrates (1.5% wt/vol carboxymethylcellulose, 0.5% polygacturonic acid, or 0.5% wt/vol trehalose) and reducing sugars liberated by hydrolysis were quantified and normalized for total cellular proteins. Pme activity was quantified as described by Clough et al. (1994) except that Bromocreosol Purple (50 ng/ml) was used as the pH indicator instead of Phenol Red.

**Gel electrophoresis and immunoblotting.** To examine cellular proteins, bacteria were grown in BG broth to $OD_{600}$ = 1 (approximately $10^9$ cells $ml^{-1}$) and a 0.33-ml sample was removed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Bacteria were pelleted by centrifugation, washed twice with 0.33 ml of 0.1 M NaCl and, 0.32 ml of the supernatant were drawn off and discarded. After the cells were suspended in the remaining liquid, 20 µl of 2X Laemmli sample buffer (BioRad, Hercules, CA) were added, and the samples were heated at 95 $^o$C for 5 min. The entire sample was loaded on a BioRad Criterion Gel (10-20%

gradient) and electrophoresis was performed as recommended by the manufacturer. Proteins were transferred from the gel to a nitrocellulose membrane in a Criterion Blotter (BioRad) using Tris-glycine buffer (25 mM Tris-base, 0.2M glycine, 15% (v/v) methanol) at 50 volts for 1 hour. The membrane was blocked using 20 ml of PBS buffer (4 mM $KH_2PO_4$, 16 mM $Na_2HPO_4$, 115 mM NaCl, pH 7.4) containing 3% (w/v) bovine serum albumin and 0.5% (v/v) Tween 20 for 1 hour at room temperature with gentle agitation, followed by three washes with 20 ml of PBS-Tween (PBS with 0.1% Tween 20) for 5 minutes each. Native biotin-binding proteins were blocked by incubating the membrane in 10 ml of PBS-Tween with 10 µl of Biotin Blocking Buffer (IBA GmbH, Goettingen, Germany) for 10 minutes at room temperature. Five microliters of Strep-Tactin alkaline phosphatase conjugate (IBA GmbH) was added and incubation continued for 1 hour, after which the membrane was washed twice for one minute with 20 ml of PBS buffer and Western Blue® alkaline phosphatase substrate (Promega, Madison, WI) was used for chromogenic detection according to the manufacturer's instructions.

## Results

**Creating *gspC* deletions in two *R. solanacearum* strains.** Two-step SacB-assisted site-directed mutagenesis was used to delete *gspC* in *R. solanacearum* GMI1000 resulting in the unmarked mutant strain GMI-ΔC. Sucrose resistant, tetracycline sensitive colonies were screened by colony PCR using primers GspCsoe1 and GspCsoe4 to identify those with a genomic 1-kb deletion allele rather than the wild-type 1.8-kb amplicon that includes both the flanking region and the *gspC* open reading frame (Fig. 2.2). Selected mutants were then tested for extracellular endoglucanase and polygalacturonase activities using enzyme assay plates. In contrast to the wild-type parent, the mutant selected as GMI-ΔC produced no detectable

extracellular Egl activity during the 48-h incubation period and it produced much less extracellular Pgl activity (Fig. 2.2). Complementation of GMI-ΔC with plasmid-borne wild-type *R. solanacearum gspC* restored secretion of both Egl and Pgl, and confirmed the lack of enzyme secretion by the mutant was due to deletion of *gspC* (Fig 2.3).

Accurate assessment of pectin methylesterase activity using enzyme assay plates required using a strain that lacks Pgl activity, because these hydrolases (especially PglA) break down the polygalacturonic acid produced when Pme demethylates pectin. Therefore, I first inserted the spectinomycin resistance Ω interposon into a *Bam*HI site in the middle of the *gspC* deletion allele and then used standard allelic exchange to introduce this marked deletion into the genome of GMI-31 (Δ*pglA*, Δ*pglB*, Δ*pglC*) to create GMI-31ΔC. On enzyme assay plates GMI-31 produced a large hazy zone and the Ω marked *gspC* deletion mutant, GMI-31ΔC, was negative for extracellular Pme activity (Fig. 2.4) Deletion of *gspC* was confirmed by complementing GMI-31ΔC with plasmid-borne wild-type *gspC*, which fully restored extracellular Pme activity (Fig. 2.4).

**Complementation tests with *gspC* driven by native promoters.** To test whether wild-type *C. metallidurans* and *B. thailandensis gspC* could restore secretion of CWDEs by the *R. solanacearum* Δ*gspC* mutant, plasmid-borne *gspC* driven by their respective native promoters (pCmeC-MCS2 and pBthC-MCS2, respectively) were introduced into GMI-ΔC and tested for secretion of Egl and Pgl. The same plasmids were introduced into GMI-31ΔC to assay for Pme secretion on enzyme assay plates. As shown in Figures 2.3 and 2.4, pCmeC-MCS2 restored secretion of all three enzymes, but while pBthC-MCS2 almost fully restored both Pgl and Pme activities it barely improved Egl secretion.

Native GspC protein of *R. solanacearum* has 228 amino acid residues the N-terminal conserved region ends at residue 130. Since no obvious protein interacting domains are in the C-terminal region, this part of the protein was eliminated by truncating the *gspC* open reading frame at codon 136. The plasmid carrying the truncated *gspC*, pCtru136, was used to complement *gspC* deletion mutants and tested for secretion of the CWDEs. On enzyme assay plates, the pCtru136 complemented strains secreted Egl, Pgl, and Pme to almost the same level as GMI-ΔC complemented with wild-type *gspC* (Fig. 2.3 and 2.4). This result indicated that, for *R. solanacearum*, the C-terminus of GspC protein is not required for a functional T2S system.

**Complementation tests with *gspC* driven by the *gapA* promoter.** The inability of Bth *gspC* to fully complement GMI-ΔC could be due to poor expression from its native promoter or to reduced stability of the foreign protein in *R. solanacearum*. To address the first possibility, we sought to express all the *gspC* genes from the same *R. solanacearum* promoter on a broad-host-range plasmid. The first vector created for this purpose, pUpC-LacZ, carried the native *R. solanacearum gspC* promoter region and a promoterless *lacZ* gene. However, when this plasmid was introduced into GMI1000 no β-galactosidase activity was detected when transformed colonies were cultured on agar medium amended with 5-bromo-4-chloro-3-indolyl galactopyranoside (X-Gal) (not shown), which indicated that the *gspC* promoter drives low-level expression. Therefore, the promoter region and ribosome binding site from *R. solanacearum* glyceraldehyde phosphate dehydrogenase (*gapA*), a constitutively expressed housekeeping gene, was used to replace the *gspC* promoter in pUpC-LacZ to create pPgap-LacZ. After transforming GMI1000, colonies carrying pPgap-LacZ exhibited uniformly strong β-galactosidase activity on X-Gal plates. To monitor stability of over-expressed proteins, we then created pPgap-Tag by inserting a Strep-tag adaptor in frame with the start codon so that N-terminal fusion proteins

could be analyzed by immunoblotting. GMI1000 colonies carrying pPgap-Tag also were β-galactosidase positive on X-Gal plates.

PCR fragments with promoterless open reading frames of *R. solanacearum*, *C. metallidurans*, and *B. thailandensis gspC* were cloned into both pPgap-LacZ and pPgap-Tag (by replacing *lacZ*) to create plasmids that over express either native (untagged) or Strep-tagged versions of GspC. These six plasmids (pG-RsoC, pG-CmeC, pG-BthC, pGT-RsoC, pGT-CmeC, and pGT-BthC) were first tested for their ability to complement the *gspC* deletion mutants GMI-ΔC and GMI31-ΔC on enzyme assay plates. There was no obvious difference between the untagged and tagged GspC proteins in their ability to restore enzyme secretion (Fig. 2.5. and 2.6.). Both *R. solanacearum* GspC (expressed from pG-RsoC and pGT-RsoC) and *C. metallidurans* GspC (expressed from pG-CmeC and pGT-CmeC) restored Egl and Pgl secretion in GMI-ΔC to wild-type level, and also fully restored Pme secretion in GMI-31ΔC. Unexpectedly, *B. thailandensis* GspC (expressed from pG-BthC and pGT-BthC) was nonfunctional, because extracellular enzyme activity from the transformants was the same as that from the *gspC* deletion mutants (Fig. 2.5 and 2.6).

To quantify the amount of enzymes being secreted and to determine the proportion of enzymes retained inside the cell, the complemented strains were grown in broth and the activity of Egl, Pgl, Pme, and Tre in the culture supernatant and cell pellets were determined (Table 2.2). For wild-type *R. solanacearum* GMI1000, about 80-90% of the enzyme activities were found in the culture supernatant, whereas in GMI-ΔC, about 11-21% of the enzyme activities were extracellular. The total units of enzyme activity were about the same in both GMI1000 and GMI-ΔC, so the lack of enzyme activity in the culture supernatant of the *gspC* mutant was due to the non-functional T2SS rather than due to altered enzyme production (Table 2.2).

As expected, both untagged and tagged *R. solanacearum* GspC fully restored enzyme secretion by GMI-ΔC grown in broth (Table 2.2) and there was no obvious effect on total enzyme activity. However, in contrast to the assay plate results, *C. metallidurans gspC* did not fully restore the secretion of Pgl (37% extracellular), Egl (44% extracellular), or Tre (32% extracellular). Similar to the plate assays, over-expression of *B. thailandensis* GspC did not restore secretion of any enzymes and normal enzyme activities were found within cells (Table 2.2).

**Gel electrophoresis and immunoblotting.** To examine the production and stability of the foreign GspC proteins in *R. solanacearum*, total cellular proteins from GMI-ΔC carrying pGT-RsoC, pGT-CmeC, and pGT-BthC were separated by SDS-PAGE and analyzed by immunoblotting. As shown in Fig. 2.7, all three Strep-tagged GspC proteins were detected by the Strep-Tactin AP conjugate at the expected protein size with similar band density, indicating that the difference in the ability of these proteins to restore enzyme secretion was because of their differential function, not due to variation of protein stability.

## Discussion

This work focused on the molecular mechanisms that allow the *R. solanacearum* T2S system to distinguish which exoproteins are to be secreted. In particular, we studied the role of GspC in secretion specificity by deleting *gspC* in this pathogen to inactivate the T2S system and testing various plasmid-borne genes for their ability to restore secretion of four extracellular enzymes. We found that there are significant similarities and differences in the structure and function of GspC in *R. solanacearum* when compared to the better characterized T2S systems in γ-proteobacteria.

*R. solanacearum* is one of the few examples where a heterologous GspC protein by itself can function normally to restore secretion of native proteins, because GspC from *C. metallidurans* fully restored secretion of all four exoenzymes by GMI-ΔC on enzyme assay plates regardless of whether gspC was expressed from its native promoter or over-expressed by the *gapA* promoter. Although early work with *E. chrysanthemi* and *E. carotovora* (now renamed *Dickeya dadantii* and *Pectobacterium carotovorum subsp. carotovorum*, respectively) indicated that their T2S secretion is species specific (Py et al. 1991; Lindeberg et al. 1996), Bouley et al. (2001) later reported that that native *E. chrysanthemi* OutC is essential only for secretion of five pectate lyases, and that *E. carotovora* OutC partially restores secretion of a sixth pectate lyase and fully restores secretion of a cellulase and a Pme. Species specific secretion of the pectate lyases is determined in part by the PDZ motif in the C-terminal half of OutC. Similarly, de Groot et al. (2001) reported that the *P. alcaligenes* XcpP (the GspC ortholog) only slightly restored secretion of elastase (a protease) by a *P. aeruginosa xcpP* mutant. The basis for this specificity has not been reported, but it is not due to differences in the C-terminus, since a *P. aeruginosa* chimeric protein with a C-terminus from *P. alcaligenes* was even more effective at restoring secretion of elastase than was native GspC. Interestingly, when chimeras of *P. aeruginosa* and *E. chrysanthemi* were created, only a 35 amino acid region located between the transmembrane domain and the HR in the N-terminal half of OutC was not functional in inserted into an XcpP background (Gerard-Vincent, 2002).

However, *C. metallidurans* GspC did not behave exactly like *R. solanacearum* GspC, because the heterologous protein only partially restored secretion of Egl, Pgl and Tre by GMI-ΔC when strains were cultured in liquid medium. That culture conditions can affect secretion was reported in *P. aeruginosa,* where secretion of elastase by a secreton containing both XcpP

and XcpQ from *P. alcaligenes* is normal on plates but negative in liquid culture (de Groot el al. 2001). This defect in secretion, which was more severe that what observed in *R. solanacearum*, was attributed to improper assembly of the composite secreton due to instability of XcpQ in the OM that can be overcome by mutations that alter *P. aeruginosa* LPS or by increasing the concentration of divalent cations in the medium (de Groot 2001; Bitter et al. 2007).

The results for complementation by *B. thailandensis gspC* were more complicated, since expression from its native promoter restored secretion of only Pme and Pgl. That GspC from *B. thailandensis* functions the least well is understandable, because its amino acid sequence is the most divergent of the three GspC proteins tested. However, this fact does not easily explain why over-expression of *B. thailandensis* gspC by the *gapA* promoter, resulted in a composite secreton that was completely nonfunctional. Excess production of *B. thailandensis* Step-tagged GspC fusion protein did not result in unexpected protein degradation, because immunoblotting showed that it was present at levels similar to the Strep-tagged *R. solanacearum* and *C. metallidurans* proteins. However, in other bacteria GspC reportedly interacts both with some other inner membrane proteins (i.e., GspL, GspM, and GspG) and with GspD secretin in the outer membrane (Possot et al. 2000; Lee et al. 2005; Login et al. 2010), so it is possible that an increased ratio of Bth GspC to the other Gsp proteins interfered with secreton assembly or function.

To this point our results supported the idea that the C-terminus of GspC proteins from β-proteobacteria is not essential, and our results showing that *R. solanacearum* GspC136, which is analogous to *B. thailandensis* GspC in lacking a C-terminus, fully restored secretion confirmed this. Similar results were first reported for *P. aeruginosa*, where removal of the C-terminal 61 amino acids actually increased the secretion efficiency of elastase (Bleves et al. 1999). In

addition, for *Xanthomonas campestris* pv. *campestris* GspC the N-terminal 158 amino acid residues are sufficient for its ability to restore secretion of amylase, protease, pectate lyase, and cellulase (Lee et al. 2004). Furthermore, *E. chrysanthemi* with a GspC lacking its C-terminal PDZ domain still secretes a Pme and cellulase at wild-type levels, and 50 to 80% of normal amounts of pectate lyase I (PelI), but not the other five pectate lyases (Bouley et al. 2001). This suggests that in *E. chrysanthemi* there are at least two categories of exoproteins: those like most pectate lyases that are PDZ dependent and those like Pme that are not (Bouley et al. 2001). Interestingly, *R. solanacearum* naturally lacks pectate lyase enzymes, and similar to *E. chrysanthemi* the secretion of Pme and Egl is C-terminus-independent. Moreover, by comparing the secretion profile between wild-type *R. solanacearum* strain GMI1000 and a T2S secretion mutant, about 35 proteins are probably secreted by the T2S system based on quantitative information on their relative abundance (Zuleta 2007). No other bacterium has been reported to have this many type II-secreted proteins. It is puzzling how R. solanacearum GspC, although it lacks a protein-protein interacting domain in GspC, still recognizes this large number of exoproteins. One possibility is that the lack of a protein-protein interaction domain resulted in evolution of exoproteins not dependent on interaction with such a domain or vice versa. Regardless, that *R. solanacearum* GspC functions without any protein-protein interacting domain suggests either that the necessary domains for recognizing all the T2S substrates are within the more conserved N-terminal region or that recognition of substrates is determined by other secreton proteins, with GspD being the obvious choice (Bouley et al. 2001). There are no reports of GspC physically contacting an exoprotein substrate, but this might be due to very transient interactions. Conversely, although some studies did not detect interaction of GspC and GspD, Lee et al. (2005) reported that in a functional *Xanthomonas campestris* pv. campestris T2S

secreton, the association of the major pseudopilin XpsG with the XpsD secretin appears strongly dependent on the existence of XpsN, its GspC ortholog. More recently, Login et al. (2010) demonstrated that N-terminal domains of *E. chrysanthemi* OutD interact both with a region of OutC containing the transmembrane domain and C-proximal residues and also with a 20 amino acid region at the right end of the HR. Therefore, it is possible that features of the N-terminal region of GspC could have an indirect influence on substrate specificity of GspD.

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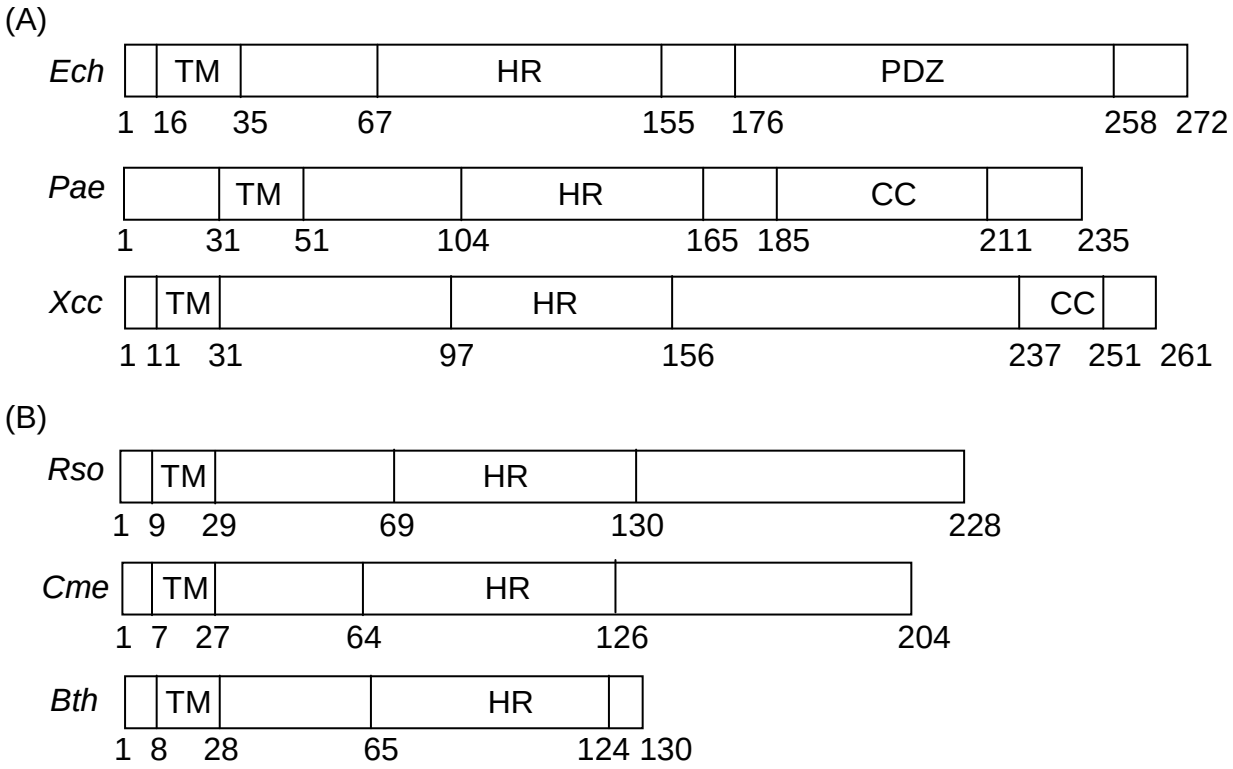


Fig 2.1. Comparison of representative GspC proteins. TM = transmembrane domain; HR = homology region; PDZ/CC indicates the protein-protein interacting domains. Amino acids are numbered below each line. (A) Selected γ-proteobacteria: *Ech = Erwinia chrysanthemi*, *Pae = Pseudomonas aeruginosa*, *Xcc = Xanthomonas campestris* pv. *campestris*, (B) Selected β-proteobacteria: *Rso = Ralstonia solanacearum*, *Cme = Cupriavidus metallidurans*, *Bth = Burkholderia thailandensis.* Note how GspC in β-proteobacteria lack a protein-protein interacting domain.

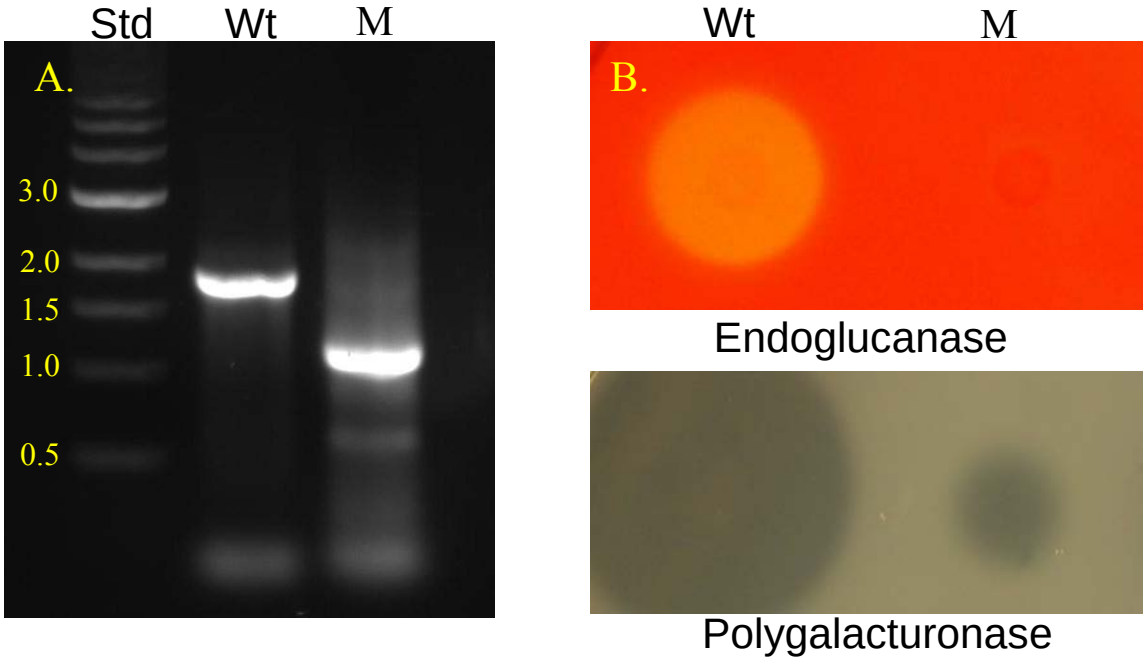


Fig 2.2. Confirmation of the *gspC* deletion in *R. solanacearum* GMI-ΔC. (A) PCR primers GspCsoe1 and GspCsoe4 were used to amplify the 1.8-kb region containing *gspC* from wild-type (Wt) and the altered 1.0-kb region from *gspC* mutant GMI-ΔC (M) templates. The DNA size standard (Std) was the 1-kb ladder from New England Biolabs. (B) Enzyme assay plates showing the decreased extracellular Egl (top) and Pgl (bottom) activities of GMI-ΔC (M) compared to wild type (Wt). The assay plates were developed after 48 hours incubation. Yellow halos shows where the carboxymethylcellulose substrate had been degraded by Egl activity. Clear zone on a hazy background shows where the polygalacturonic acid had been degraded by Pgl activity.

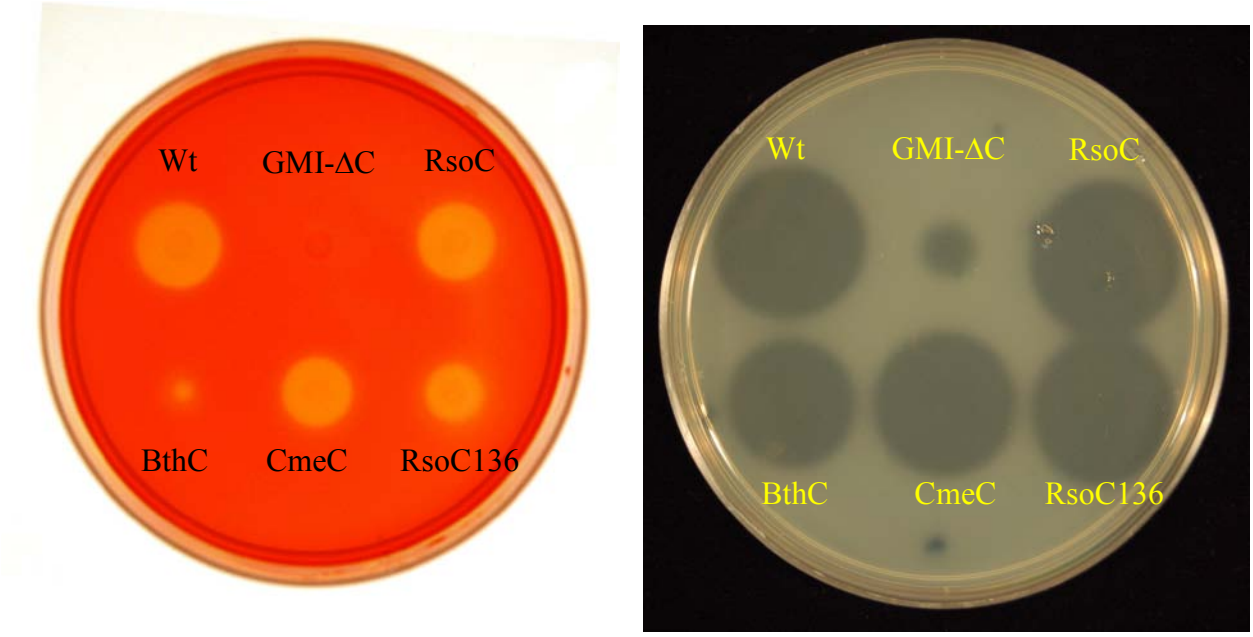


Fig 2.3. Enzyme assay plates showing endoglucanase and polygalacturonase activities of wild-type GMI1000 (Wt), the *gspC* mutant GMI-ΔC, and GMI-ΔC complemented with four different *gspC* alleles. Wild-type alleles driven by native promoters were from *R. solanacearum* (RsoC), *B. thailandensis* (BthC), *and C. metallidurans* (CmeC). A modified *R. solanacearum gspC* that encodes only the first 136 amino acids (RsoC136) was also driven by its native promoter.

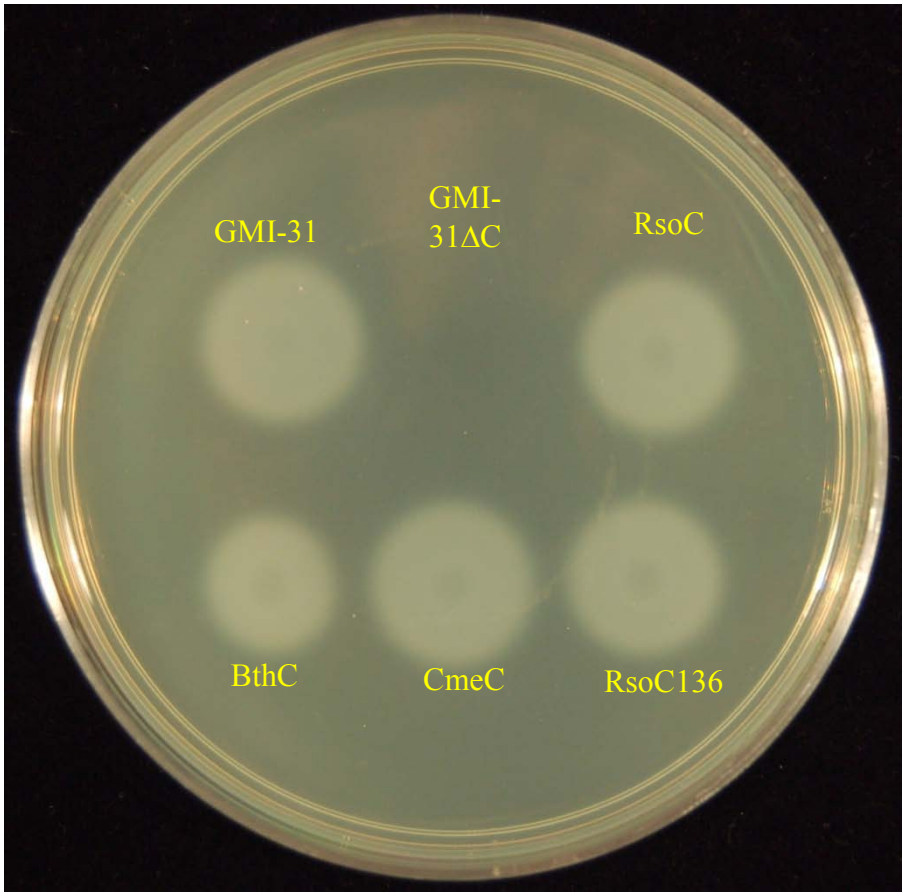


Fig 2.4. Enzyme assay plate showing pectin methylesterase activity of the polygalacturonase negative mutant GMI-31, the *gspC* deletion variant of this strain (GMI-31ΔC), and GMI-31ΔC complemented with four different *gspC* alleles as described in Fig. 2.2. Hazy zones show where the pectin substrate had been demethylated by Pme activity.

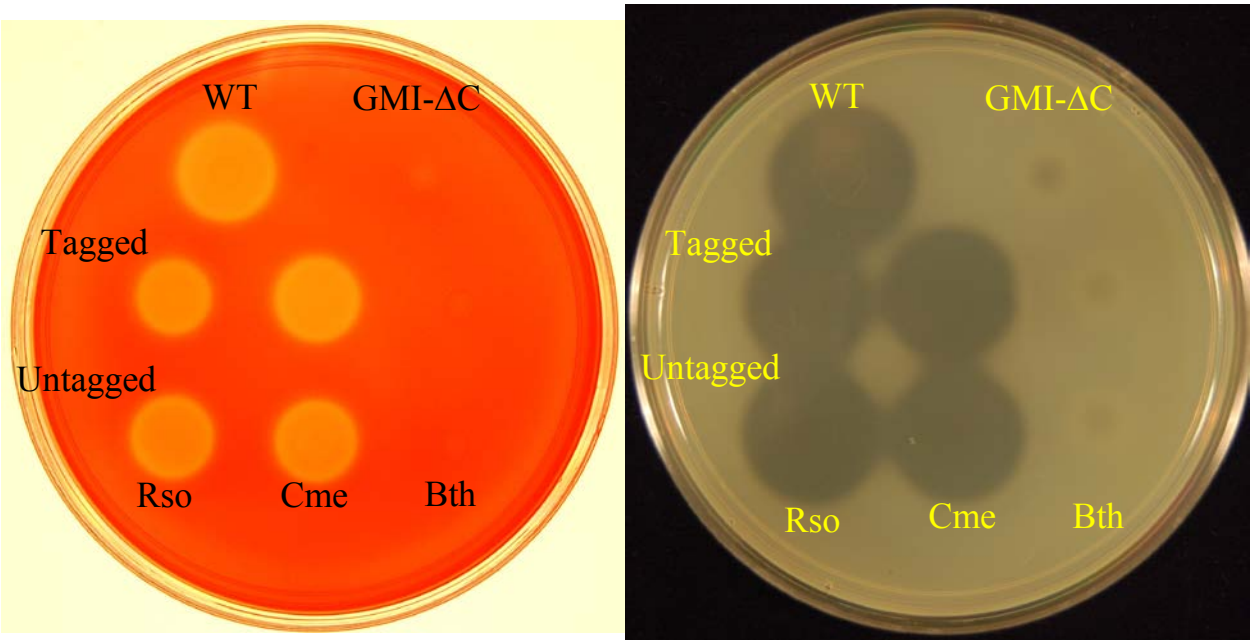


Fig. 2.5. Enzyme assay plate showing endoglucanase and polygalacturonase activities of wild-type GMI1000 (Wt), the *gspC* mutant GMI-ΔC, and GMI-ΔC complemented with six different *gspC* alleles. Wild-type alleles driven by *gapA* promoter were from *R. solanacearum* (RsoC), *C. metallidurans* (CmeC) and *B. thailandensis* (BthC), either with or without the Strep-tag.

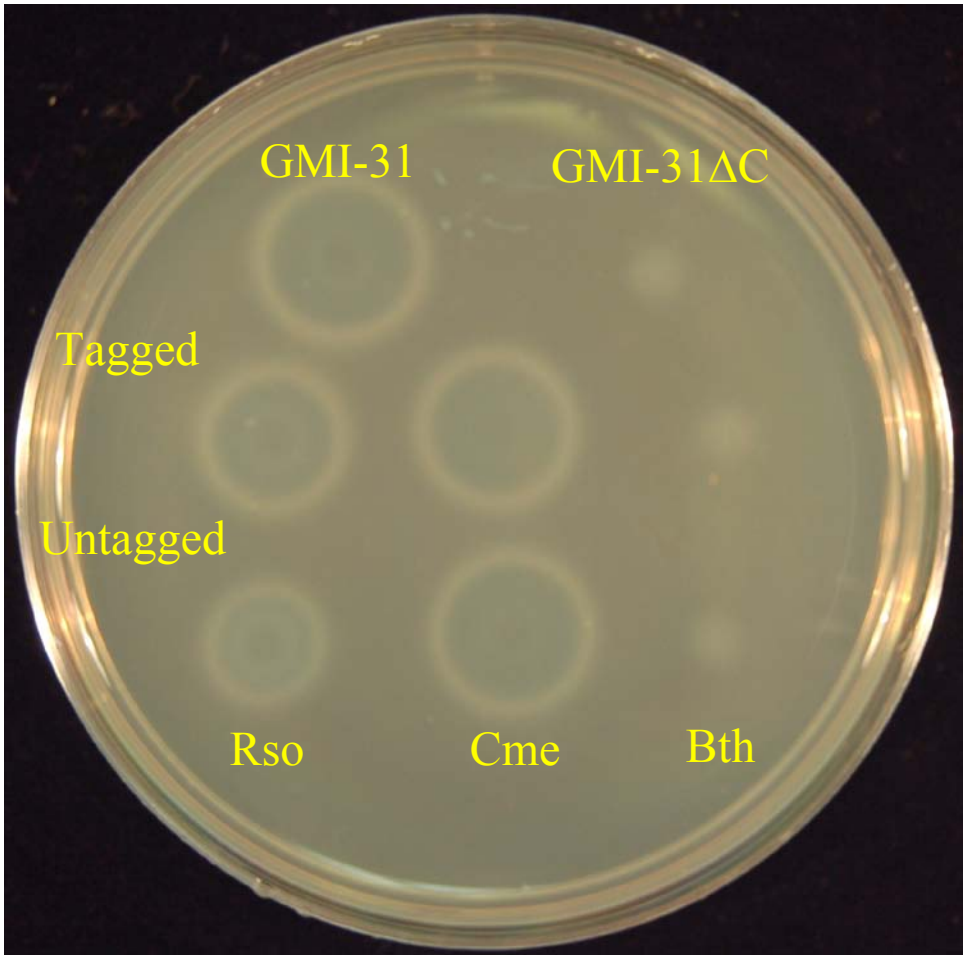


Fig. 2.6. Enzyme assay plate showing pectin methylesterase activities of GMI-31, the *gspC* mutant GMI-31ΔC, and GMI-31ΔC complemented with six different *gspC* alleles. Wild-type alleles driven by *gapA* promoter were from *R. solanacearum* (RsoC), *C. metallidurans* (CmeC) and *B. thailandensis* (BthC), either with or without the Strep-tag.

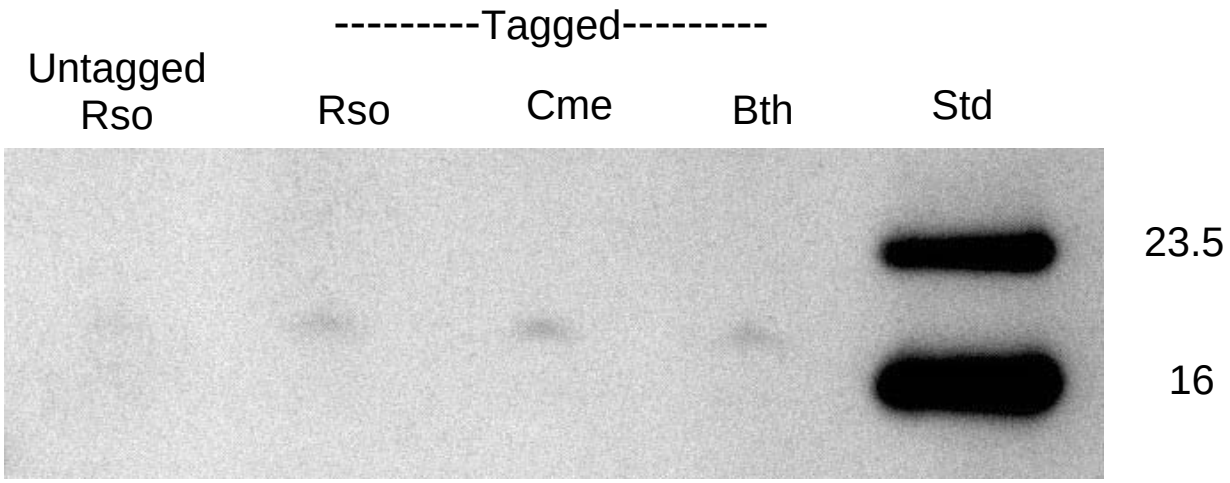


Fig. 2.7. Immunoblot demonstrating comparable production of all three Strep-tag GspC fusion proteins. Total cell proteins were separated by SDS-PAGE, transferred to a NC membrane and probed with Strep-Tactin alkaline phosphatase conjugate. Wild-type alleles driven by *gapA* promoters were from *R. solanacearum* (RsoC), *C. metallidurans* (CmeC), *and B. thailandensis* (BthC). The protein size standard (Std) was the Strep-tag® Protein Ladder from IBA.

Table 2.1. Strains and plasmids used in this study.

| Strain | Characteristics[a] | Source |
|---|---|---|
| ***R. solanacearum*** | | |
| GMI1000 | Wild type, race 1, biovar 3, phylotype I | (Salanoubat et al. 2002) |
| GMI-ΔC | GMI1000 Δ*gspC* | This study |
| GMI-31 | GMI1000 Δ *pglA pglB pglC* | (Liu et al. 2005) |
| GMI-31ΔC | GMI-31 Δ*gspC*::Ω, $Sp^r$ | This study |
| ***Escherichia coli*** | | |
| DH5α | Φ80*dlac*ZΔm15 *recA*1 *endA*1 *gyrA*96 *thi*-1 *hsd*R17 ($rk^-$ $mk^+$) *supE*44 *relA*1 *deoR* Δ(*lacZYA-argF*)U169 | Invitrogen |
| **Plasmids** | | |
| pEX18Tc | gene-replacement vector, *oriT sacB*, $Tc^r$ | (Hoang et al. 1998) |
| pBBR1MCS-2 | Broad-host-range vector, *rep mob*, $Km^r$ | (Kovach et al. 1995) |
| pUC8-Ω | Source of Ω interposon, $Sp^r$ | (Prentki & Krisch, 1984) |
| pINT104 | Source of *lacZ*, $Ap^r$, $Tc^r$ | (Mukaihara et al. 2004) |
| pS3105 | 1-kb *gspC* deletion allele in pEX18Tc, $Tc^r$ | This study |
| pS3105Ω | Ω–marked *gspC* deletion allele in pEX18Tc, $Tc^r$, $Sp^r$ | This study |
| pGspC-MCS2 | Wild-type *R. solanacearum gspC* with native promoter in pBBR1MCS-2, $Km^r$ | This study |
| pCmeC-MCS2 | Wild-type *C. metallidurans gspC* with native promoter in pBBR1MCS-2, $Km^r$ | This study |

| Strain | Characteristics[a] | Source |
|---|---|---|
| pGspCtru136 | Truncated *R. solanacearum gspC* at 136 amino acid with native promoter in pBBR1MCS-2, $Km^r$ | This study |
| pUpC-Mcs2 | *R. solanacearum gspC* promoter region in pBBR1MCS-2, $Km^r$ | This study |
| pUpC-LacZ | 3-kb full length *lacZ* cloned in pUpC-MCS2, $Km^r$ | This study |
| pPgap-LacZ | 320-bp *gapA* promoter region in pUpC-MCS2, $Km^r$ | This study |
| pPgap-Tag | 320-bp *gapA* promoter region and Strep-tag adaptor in pPgap-LacZ, $Km^r$ | This study |
| pG-RsoC | Wild-type *R. solanacearum gspC* with *gapA* promoter in pPgap-LacZ, $Km^r$ | This study |
| pGT-RsoC | Wild-type *R. solanacearum gspC* with *gapA* promoter with N-terminal Strep-tag in pPgap-Tag, $Km^r$ | This study |
| pG-CmeC | Wild-type *C. metallidurans gspC* with *gapA* promoter in pPgap-LacZ, $Km^r$ | This study |
| pGT-CmeC | Wild-type *C. metallidurans gspC* with *gapA* promoter with N-terminal Strep-tag in pPgap-Tag, $Km^r$ | This study |
| pG-BthC | Wild-type *B. thailandensis gspC* in *gapA* promoter in pPgap-LacZ, $Km^r$ | This study |
| pGT-BthC | Wild-type *B. thailandensis gspC* with *gapA* promoter with N-terminal Strep-tag in pPgap-Tag, $Km^r$ | This study |

[a] $Km^r$, $Sp^r$, $Tc^r$ = kanamycin, spectinomycin, and tetracycline resistant, respectively.

Table 2.2. Enzyme activities of various tagged and untagged *gspC* complemented strains.

| | | Pme[a] | | Pgl[b] | | Egl | | Tre | |
|---|---|---|---|---|---|---|---|---|---|
| Strain | Plasmid | Supt. | Cell | Supt. | Cell | Supt. | Cell | Supt. | Cell |
| GMI1000 | pBBR1-MCS2 | 7.33 | ND | 68.9 (91.1%) | 6.7 (8.9%) | 22.7 (85.9%) | 3.7 (14.1%) | 108.4 (79.4%) | 28.0 (20.6%) |
| GMI-ΔC | pBBR1-MCS2 | ND | 8.57 | 16.1 (21%) | 60.6 (79%) | 3.86 (11.2%) | 30.6 (88.8%) | 23.1 (18%) | 105.1 (82%) |
| GMI-ΔC | pG-RsoC | 7.69 | ND | 70.8 (94.4%) | 4.2 (5.6%) | 24.4 (89.4%) | 2.9 (10.6%) | 108.8 (75.2%) | 35.9 (24.8%) |
| GMI-ΔC | pGT-RsoC | 8.64 | ND | 152.5 (82%) | 33.7 (18%) | 30.2 (67%) | 14.9 (33%) | 255.8 (69.2%) | 113.8 (30.8%) |
| GMI-ΔC | pG-CmeC | 7.33 | ND | 39.2 (59.8%) | 26.4 (40.2%) | 17.6 (73.8%) | 6.3 (26.2%) | 88.2 (52.4%) | 80.0 (47.6%) |
| GMI-ΔC | pGT-CmeC | 7.68 | ND | 67.2 (37%) | 116.8 (63.5%) | 27.7 (44%) | 35.3 (56%) | 143.5 (32%) | 311.0 (68%) |
| GMI-ΔC | pG-BthC | ND | 8.16 | 0.8 (2.7%) | 30.8 (97.3%) | 2.2 (15.9%) | 11.7 (84.3%) | 10.0 (8.5%) | 108.5 (91.5%) |
| GMI-ΔC | pGT-BthC | ND | 6.82 | 11.0 (6%) | 167.6 (94%) | 6.0 (13%) | 40.3 (87%) | 26.3 (7.5%) | 322.1 (92.5%) |

[a]Numbers indicate the nmole $H^+$/min/mg protein; ND indicates enzyme activity was not detectable. Data are for single cultures from one experiment that was representative of two experiments.

[b]Numbers indicate units/mg protein; Percentage values are given in parentheses and were calculated by portion of total enzyme detected (i.e., [Supt./(Supt.+ Cell)]100 or [Cell/(Supt.+ Cell)]100). Data are the average for duplicate cultures from one experiment that was representative of two experiments.

Table 2.3. Primers or oligonucleotides used in this study.

| Gene target[a] | Primer Name | Primer Sequences (5' → 3')[b] | Restriction site |
|---|---|---|---|
| *Rso gspC* | GspCsoe1 | GAC***GGTAC***CTCAGGTACTGGTACGGGTGG | *Kpn*I |
| | GspCsoe2 | GCTGAGCAGACGGGACGACT | |
| | GspCsoe3 | CGTCCCGTCTGCTCAGC***GGATCC***CACTGATCCGT CCGCCTGT | *Bam*HI |
| | GspCsoe4 | GAC***AAGCTT***CGAAATGCCTGGCGAGCTTG | *Hin*dIII |
| *Rso gspC* | Ctru136 | ATG***AAGCTT***TCAGGCCACGGCGCTGGCCGGCA | *Hin*dIII |
| *Cme gspC* | CmeGspCF | ATG***GGTACC***GACGCCCGCTTCTATCTG | *Kpn*I |
| | CmeGspCR | ATG***AAGCTT***GATCTTGTTGAACAGCGCATCA | *Hin*dIII |
| *Bth gspC* | BthGspCF | ATG***GGTACC***CCGGGTTCAGGTACTTGTACGA | *Kpn*I |
| | BthGspCR | ATG***AAGCTT***GGCGCACGATGTTTCTCACGAG | *Hin*dIII |
| *Rso gspC* promoter | GMIupCR | GAC***GATATCCATATG***AGGCGGATCCACCG | *Eco*RV, *Nde*I |
| *E. coli lacZ* | LacZF-NA | CCGCCT***CATATGGGGCCC***ATTACGGATTCACTG | *Nde*I, *Apa*I |
| | LacZR-Xba | ATG***TCTAGA***TACGGGCAGACATGGCTTG | *Xba*I |
| *gapA* promoter | Pgap-ForKH | ATT***GGTACCAAGCTT***ACCGTGTTCGACAAG | *Kpn*I, *Hin*dIII |
| | Pgap-RevAN | AAT***GGGCCCCATATG***CAGTCTCCTGATAGC | *Apa*I, *Nde*I |
| *Strep-tag* adaptor | S-tagTop | TATGGCGTCGGCGTGGAGCCACCCGCAGTTCGA GAAG***ACTAGT***GGGCC | *Spe*I |
| | S-tagBot | C***ACTAGT***CTTCTCGAACTGCGGGTGGCTCCACGC CGTCGCCA | *Spe*I |
| *Rso gspC* | PgapGmiC-F | GA***GGGCCC***ACCGTTCAACAGTCGTCCCG | *Apa*I |
| | GmiCtagR | GA***TCTAGA***CCCGAGGATGCGCGCGCATT | *Xba*I |

| Gene target[a] | Primer Name | Primer Sequences (5' → 3')[b] | Restriction site |
|---|---|---|---|
| *Cme gspC* | PgapCmeC-F | GA***GGGCCC***CCTCGCCTGGCTGGCCTGGC | *Apa*I |
| | CmeCtagR | GA***TCTAGA***TTACGGGGCTCACAGAACAG | *Xba*I |
| *Bth gspC* | PgapBthC-F | GA***GGGCCC***AACGCGCTATCGATCCGGCT | *Apa*I |
| | BthCtagR | GA***TCTAGA***TGACGGAAGGCAAGATGATGTGC | *Xba*I |

[a] *Rso*, *R. solanacearum*; *Cme*, *C. metallidurans*; *Bth*, *B. thailandensis*. [b]Italic-bold letters indicate the restriction site added.

# CHAPTER 3

# CONCLUSION

*R. solanacearum* is a soil-borne pathogen that causes lethal wilt symptoms in over 200 plant species around the world, including tomato, potato, and banana (Denny 2006). This study demonstrated that the substrate recognition of the T2SS, which is essential for this pathogen to cause disease, is strongly influenced by the GspC protein. The T2SS is present in many Gran-negative bacteria, including both plant, animal, and human pathogens. Therefore, these results and subsequent studies with *R. solanacearum* can inform research on many bacteria, and hopefully lay the foundation for novel disease management strategies.

In this study, we tested the contribution of GspC from *R. solanacearum* and two other β-proteobacteria in determining function and substrate specificity of the *R. solanacearum* T2S system. We found that, in contrast to work with γ-proteobacteria (Bouley et al. 2001; de Groot et al. 2001), GspC of *C. metallidurans* fully complemented an *R. solanacearum gspC* deletion mutant for secretion of four exoproteins, and that GspC from *Burkholderia thailandensis* partially complemented this mutant. In addition, a C-terminally truncated *R. solanacearum* GspC fully complemented the *gspC* deletion mutant, confirming that the C-terminal portion of this protein is not essential.

## References

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