Regulatory pathways controlling cell division after DNA damage in *Caulobacter crescentus*

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ABSTRACT

All cells must coordinate DNA replication with cell division in order to faithfully propagate whole chromosomes to daughter cells. During episodes of DNA damage, cells often delay division until the lesions have been repaired and replication has completed. The paradigm for the bacterial response to DNA damage is the transcriptional induction of “SOS” genes, and many organisms encode an SOS-induced cell division inhibitor. However, the mechanistic details of division inhibition are understood only in the γ-proteobacterium *E. coli*, and it is unclear whether there are SOS-independent modes of division inhibition.

I have studied the DNA damage response in the α-proteobacterium *Caulobacter crescentus* and identified two damage-induced cell division inhibitors. *sidA* is an SOS-induced division inhibitor whereas *didA* is induced in an SOS-independent fashion. Unlike most division inhibitors, SidA and DidA do not disrupt the localization of the cell division scaffold FtsZ or any other component of the cell division machinery or “divisome”. Instead, SidA and DidA target the late-acting division proteins FtsW, FtsI, and FtsN to prevent divisome constriction, demonstrating that divisome components other than FtsZ can serve as regulatory targets. I have characterized mutations in *ftsW* and *ftsI* which suppress the activities of both inhibitors, likely by causing cells to divide hyperactively. These results suggest that the FtsW/FtsI/FtsN subcomplex serves as an important regulatory node and may play an unexpected role in triggering divisome constriction in *Caulobacter*. I show that cells require at least one inhibitor to properly delay division following DNA damage, as cells lacking both inhibitors divide prematurely and suffer a viability defect in the presence of the DNA damaging agent mitomycin C (MMC). This finding suggests that some degree of redundancy exists within the *Caulobacter* response to MMC. Finally, I describe ongoing experiments which explore the origins of the SOS-independent induction of *didA*.

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Chapter 1

Introduction
Introduction

My grandfather always told me to “be fruitful and multiply.” Whether this suggestion was meant for the cells in my seminiferous tubules or the bacteria in my gut, the task is easier said than done. In order to multiply, cells must grow and divide, endowing each daughter cell with all the components necessary for survival and future growth. Specifically, each daughter must receive an intact cellular envelope, a full genome and the macromolecular machinery needed to perform repair, regulatory and biosynthetic functions. As division events are energetically costly and irreversible, nearly all cells have mechanisms to ensure that division is tightly regulated, occurring only after the accumulation of enough of these essential goods. However, even in the most well-studied model organisms, the genetic networks responsible for spatially and temporally controlling cell division remain incompletely understood.

One of the most crucial tasks for a dividing cell is the faithful partitioning of a complete set of chromosomes to each daughter cell. A failure in genome partitioning that causes certain chromosomal regions to be absent or over-represented could result in lethality or, in higher organisms, oncogenesis (reviewed in (Holland and Cleveland, 2012)). Cells must therefore divide only after the DNA has been fully replicated and each copy spatially segregated to create a DNA-free division plane. This task is challenging owing to the difficulties inherent in packaging and manipulating a genome which, when unwound, is orders of magnitude longer than the cell in which it resides. Furthermore, cells frequently encounter endogenous and environmental DNA damaging agents which cause lesions that can necessitate lengthy periods of repair. As a result, the timing of cell division must be somewhat flexible, and the decision to divide must be intimately linked to the status of DNA replication and repair.

How do cells ensure that division occurs only after a successful round of DNA replication? Hartwell and Weinert described two general strategies for establishing a dependent relationship, whereby the initiation of event B requires the completion of a prior event A (Hartwell and
Weinert, 1989). In a “substrate-product” relationship, event B is triggered by an intrinsic property of event A revealed only upon its completion. For instance, the successful completion of replication could expose a chromosomal structure that serves as a substrate or physical component of the division machinery. Alternatively, some dependencies are enforced by dedicated control mechanisms or “checkpoints”, in which extrinsic factors that do not participate in the completion of either step nonetheless provide regulatory control. In this case, incomplete or stalled DNA replication could activate an extrinsic surveillance system that prevents cell division; or, the completion of DNA replication could inactivate a system that otherwise constitutively prevents division.

Examples of both substrate-product and checkpoint models are abundant, but in eukaryotes, DNA replication and segregation are coordinated with division mainly through a series of checkpoints (reviewed in (Tyson and Novak, 2008)). Eukaryotic cells divide by a process known as “mitosis”, during which replicated chromosomes are spatially segregated by spindle microtubules, and a contractile ring of actin and myosin filaments drives constriction of the envelope and splitting of the cell (reviewed in (Fededa and Gerlich, 2012; Glotzer, 2005)). Two closely related checkpoints ensure that cells initiate mitosis only once the DNA has been fully replicated and all lesions have been repaired. While stalled replication forks and damaged DNA structures are likely recognized by distinct classes of sensor proteins, both ultimately activate signal transducers that inhibit the activity of a cyclin-dependent kinase (CDK) involved in mitotic entry (reviewed in (Hurley and Bunz, 2007)). Cyclin-dependent kinases belong to a family of regulatory proteins whose substrates perform essential cell cycle steps in a phosphorylation-dependent manner. The mitotic CDK complex, mCDK, phosphorylates targets involved in chromosome condensation and spindle formation which initiate the mitotic program (Andersen, 1999; Kimura et al., 1998). Activation of the DNA replication or DNA damage checkpoints stabilizes inhibitory phosphorylations on mCDK and thus prevents mitosis (reviewed in (Harper and Elledge, 2007)). A third checkpoint system, known as the mitotic
checkpoint complex (MCC), surveys the appropriate attachment of chromosomes to the segregation machinery (reviewed in (Musacchio and Salmon, 2007)). Only after every chromosome pair is securely attached does the MCC activate the anaphase-promoting complex (APC), a ubiquitin ligase whose targets enable further progression through mitosis (Hwang et al., 1998; Kim et al., 1998). The mCDK and MCC thus stand as gate-keepers of two crucial mitotic decision points (Figure 1.1). Furthermore, each complex is extrinsic to the process it regulates and not a component of the machinery that actually executes the biochemistry of chromosome segregation and division. Perhaps for these reasons, mCDK and MCC are widely conserved mediators of eukaryotic checkpoint control.

Figure 1.1. Mitotic checkpoints in eukaryotes. The entry into prophase, the first phase of mitosis, is enabled by the mitotic cyclin-dependent kinase (mCDK) in complex with a cyclin. DNA damage or stalled replication forks prevent mitotic entry by activating a signaling pathway, which includes the kinase chk1, that introduces inhibitory phosphorylations on mCDK. A second checkpoint system prevents progression through mitosis in response to improper chromosome attachment to the segregation machinery. Once the chromosomes are firmly attached, a tensile force inactivates the mitotic checkpoint complex (MCC) which normally represses activity of the anaphase promoting complex (APC). Once activated, APC causes the degradation of securin which releases separase to free the sister chromatids and allow chromosome segregation during anaphase. Figure adapted from (Harper and Elledge, 2007; Musacchio and Salmon, 2007).
The relationship between DNA replication and cell division in bacteria is much less well understood. The bacterial DNA damage response has been studied for decades, and there are now examples in several species of DNA damage checkpoints that prevent cell division. However, these checkpoint systems are poorly conserved and are mechanistically understood only in the γ-proteobacterium *E. coli*. Thus, it remains unclear (1) how bacteria outside the γ-proteobacteria regulate division in response to DNA damage and (2) whether the general strategies and principles of regulatory control are conserved even though the regulators themselves are not. I have pursued these questions in the α-proteobacterium *Caulobacter crescentus*, identifying and subsequently characterizing two damage-induced cell division inhibitors which are functionally distinct from those found in *E. coli* and other organisms. Before describing these new regulators, I will first introduce bacterial cell division, known examples of how bacterial division is temporally regulated, the canonical DNA damage response systems in bacteria, and finally the model organism employed in my work, *Caulobacter*.

**Bacterial cell division**

Bacteria display tremendous morphological diversity, taking the form of spheres, rods and spirals and varying in size by over three orders of magnitude. The bacterial envelope is similarly diverse, as evolutionary pressures to survive and interact with different environments have yielded variations in the thickness and composition of the cell wall, the presence or absence of an outer membrane and the type and number of accessory proteins and lipids. Despite these differences, most bacteria divide in generally the same fashion: A division site at mid-cell is chosen, a multi-protein cell division complex is constructed, and constriction of one or both cell membranes is accompanied by the synthesis of a new peptidoglycan cell wall, known as the "septum", which divides the cytoplasm into two compartments and serves as the new poles for daughter cells following cytokinesis (Figure 1.2; reviewed in (Egan and Vollmer, 2013; Lutkenhaus et al., 2012)). Here, I will describe the constituents, assembly and function of the cell division complex.
division machine which will provide a foundation for understanding known and prospective modes of division regulation.

Figure 1.2. An overview of bacterial cell division. The first step in bacterial cell division involves polymerization of the divisome scaffold FtsZ into a "Z-ring" at mid-cell on the cytoplasmic surface of the inner membrane (IM). Next, divisome components are recruited to the Z-ring forming a multi-protein cell division machine. Each divisome component is labeled with the last letter in its gene name such that "Z" denotes FtsZ, "A" denotes FtsA, etc.... Following divisome assembly in Gram-negative bacteria, the IM, peptidoglycan (PG) cell wall and outer membrane (OM) constrict sharply at mid-cell, and cytokinesis ultimately produces two daughter cells. Adapted from (Vicente and Lüwe, 2003).

Identification of cell division genes

Members of the division machinery, known as the "divisome", were originally identified in *E. coli* through screens for thermosensitive mutations that prevented division and caused filamentation without disrupting DNA synthesis or chromosome segregation at the restrictive temperature. Genes harboring these mutations were called filamentous temperature-sensitive or "fts" genes. The first division genes to be identified in this fashion were *ftsA* (Van de Putte et al., 1964) and *ftsZ* (Hirota et al., 1968), which were later mapped to neighboring locations near the 2 minute position on the *E. coli* linkage map (Wijsman, 1972). Subsequently, *ftsI* (Suzuki et al.,
1978), \textit{ftsQ} (Begg et al., 1980), \textit{ftsW} (Ishino et al., 1989), and \textit{ftsL} (Guzman et al., 1992) were mapped to the same 2 minute cluster, while \textit{ftsB} (Ricard and Hirota, 1973), \textit{ftsN} (Dai et al., 1993) and \textit{ftsK} (Begg et al., 1995) were found elsewhere in the genome. Remarkably, despite vast differences in envelope properties across bacterial species, these core division genes are conserved in most bacteria that have not undergone significant periods of genome reduction. Many bacterial genomes contain additional division proteins whose essentiality and/or conservation differ, but the bulk of the divisome is derived from a common origin.

Several early lines of evidence suggested that among the many division genes, FtsZ was a critical player. Genetic experiments in \textit{E. coli} demonstrated that FtsZ participated in an early cell division stage as \textit{ftsZ} mutants were unable to form membrane invaginations at mid-cell unlike mutants in other division genes (Begg and Donachie, 1985). Subsequently, FtsZ levels were determined to be rate-limiting for division (Bi and Lutkenhaus, 1990), and apparent FtsZ rings, or “Z-rings”, were found localized to mid-cell by immuno-electron microscopy (Bi and Lutkenhaus, 1991). It is now appreciated that Z-ring formation on the cytoplasmic surface of the inner membrane is the earliest known event in bacterial cell division. At mid-cell, the Z-ring determines the future division site by serving as a scaffold upon which the remainder of the divisome is constructed (reviewed in (Goehring and Beckwith, 2005)). Below, I will discuss the mechanisms by which cells position the Z-ring and the general stages of divisome assembly.

\textit{FtsZ Positioning}

FtsZ is a homolog of the eukaryotic microtubule-forming monomer, tubulin (Nogales et al., 1998). Although they share little sequence identity, both proteins polymerize into long head-to-tail filaments in the presence of GTP (Mukherjee and Lutkenhaus, 1994). Despite its initial appearance as a continuous closed loop, superresolution microscopy in \textit{E. coli}, \textit{B. subtilis}, and \textit{Caulobacter} has revealed a Z-ring that is formed by discontinuous bundles of proto-filaments with extensive lateral associations (Fu et al., 2010; Li et al., 2007; Strauss et al., 2012). In order
to correctly position Z-rings, many bacteria employ a strategy whereby concentrations of FtsZ inhibitors are highest at the cell poles and lowest at mid-cell. In *E. coli* and *B. subtilis*, MinC prevents polymerization of FtsZ at the poles, and its localization is mediated by the membrane tether MinD which associates with a third topological factor (de Boer et al., 1989; Lee and Price, 1993). In *E. coli*, this factor is MinE, which shuttles from pole-to-pole and stimulates the membrane release and inactivation of MinD (Fu et al., 2001; Hale et al., 2001; Raskin and de Boer, 1999). These rapid oscillations result in time-averaged MinC/MinD concentrations that are lowest at mid-cell. In *B. subtilis*, MinD is stably recruited to both cell poles by DivIVA, which accumulates in regions of high membrane curvature (Edwards and Errington, 1997; Lenarcic et al., 2009). *Caulobacter* do not encode MinC/MinD homologs, but a functional analogue exists in the Z-ring inhibitor MipZ which is directed to the poles by an association with the origins of replication, which are themselves polarly localized (Thanbichler and Shapiro, 2006). Each of these systems is essential for directing division events away from the poles, and mutants lacking components of the MinC/MipZ complexes display an increased frequency of polar division events that result in nucleoid-free "mini-cells".

A second, complementary system for correctly positioning the Z-ring is known as ‘nucleoid occlusion’ whereby Z-ring assembly is prevented on top of nucleoid-dense regions (reviewed in (Wu and Errington, 2012)). In *E. coli* and *B. subtilis*, the nucleoid occlusion factors SlmA and Noc, respectively, each contain DNA-binding domains that mediate an association with the chromosome and effector domains that prevent proper Z-ring assembly (Cho et al., 2011; Wu et al., 2009). Together, mini-cell prevention and nucleoid occlusion reinforce the mid-cell assembly of Z-rings such that cells lacking both systems display lethal division defects (Wu, Errington, 2004; Bernhardt, de Boer, 2005). However, in addition to its role in spatial organization, nucleoid occlusion may also partially mediate the temporal coordination of cell division with DNA replication. As DNA replication and segregation occur concomitantly in bacteria, a nucleoid-free, and thus Noc/SlmA-free, region at mid-cell is only revealed during late stages of
replication and segregation. These nucleoid occlusion effectors could therefore serve as a type of DNA replication checkpoint, although it could be argued that they are part of a hybrid substrate-product system given their close association with the nucleoid.

There are no obvious homologs of \textit{noc} or \textit{slmA} in \textit{Caulobacter}, and a nucleoid occlusion mechanism may be wholly absent given that Z-rings form during a cell cycle stage when DNA still occupies the mid-cell region. However, it is formally possible that a novel nucleoid occlusion factor regulates a step in division downstream from FtsZ polymerization, as will be discussed further below. Interestingly, \textit{noc} and \textit{slmA} were both identified in screens for genes that are synthetically lethal with mini-cell mutants, but a similar screening strategy is not possible in \textit{Caulobacter} given that \textit{mipZ} is essential. It thus remains unclear whether nucleoid occlusion or yet other unidentified systems affect the positioning and temporal control of division in \textit{Caulobacter}.

\textit{Divisome Assembly}

The construction of fluorescently-labeled translational reporters has allowed the subcellular localization of divisome members to be visualized in real-time in live bacterial cells, revealing unique sets of assembly times and recruitment dependencies for each component. In \textit{E. coli}, the divisome assembles in two general stages (reviewed in (Goehring and Beckwith, 2005)). First, a set of FtsZ-interacting proteins are recruited to the mid-cell where they promote the formation of the Z-ring. The essential FtsZ interactors FtsA and ZipA tether FtsZ proto-filaments to the inner membrane while the non-essential FtsZ association proteins (ZapA-D) may promote Z-ring bundling and integrity (Dajkovic et al., 2010; Durand-Heredia et al., 2012; Ebersbach et al., 2008; Galli and Gerdes, 2010; Gueiros-Filho and Losick, 2002; Hale and de Boer, 1997; Low et al., 2004; Pichoff and Lutkenhaus, 2005). The \textit{Caulobacter} genome harbors no ZipA homolog, but does contain FzlA, a protein conserved in \textit{\alpha}-proteobacteria with a role in promoting the curvature of FtsZ filaments (Goley et al., 2010b). FtsE is another early-arriving FtsZ interactor
that, together with FtsX, may coordinate the activity of peptidoglycan-remodeling amidases with FtsZ dynamics (Meisner et al., 2013; Yang et al., 2011). The FtsEX complex is essential in Caulobacter (Christen et al., 2011) but in E. coli is required only under low-salt conditions (de Leeuw et al., 1999).

Following the establishment and stabilization of the Z-ring, the second stage of divisome assembly involves the recruitment of the remaining divisome components, most of which are single or multi-pass transmembrane proteins (reviewed in (Errington et al., 2003)). Studies in E. coli that assessed the ability of individual components to localize in the presence or absence of other components, suggested a linear recruitment hierarchy in the order FtsZ > ZipA/FtsA > FtsK > FtsQ > FtsB/FtsL > FtsW > FtsI > FtsN (Goehring et al., 2006; Goehring et al., 2005; Vicente and Rico, 2006). In this hierarchy, the recruitment of each divisome member requires the proper assembly of all upstream factors. In B. subtilis, assembly may follow a more cooperative rather than sequential model (Errington et al., 2003), and even in E. coli, there is evidence that sub-complex formation may in some cases precede recruitment to mid-cell (Buddelmeijer and Beckwith, 2004; Fraipont et al., 2011). Furthermore, in all organisms surveyed, the pair-wise interaction map of divisome components, as revealed by bacterial two-hybrid experiments, appears not as a linear sequence but as a complex web (Daniel et al., 2006; Di Lallo et al., 2003; Karimova et al., 2005; Maggi et al., 2008). While some of these interactions may be required for downstream steps in divisome constriction and septum-synthesis, others may reflect a more nuanced reality of divisome assembly. In Caulobacter, divisome components assemble in roughly the same order as E. coli, although FtsA is recruited late in the cell cycle, at a time when the Z-ring has already been well established (Goley et al., 2011). Localization dependencies in Caulobacter were also observed to be much less strict, as very few proteins other than FtsZ and FtsL were individually required for the recruitment of any downstream component. Nonetheless, the assembly of the bacterial divisome likely requires a combination of sequential and cooperative protein-protein interactions.
The execution of cytokinesis

Following site-selection and assembly, the divisome must somehow orchestrate the final stage of division which involves constriction of the membrane(s), synthesis of the septal cell wall and the separation of daughter cells. While most, if not all, division proteins play some role in divisome assembly by recruiting downstream components, it is less clear which proteins possess the additional activities that are specifically required for cytokinesis. In contrast to the steady accumulation of data describing the dynamics of divisome assembly, assigning functions to individual divisome components has progressed more slowly, owing in part to the difficulty in designing and performing biochemical assays on a multi-component and membrane-bound machine. Nonetheless, many enzymatic and other functional domains have been characterized and domains of unknown but essential functions have been isolated. Here, I will summarize the known and putative functions for individual divisome components and speculate on how they might cooperate to perform cytokinesis.

FtsZ and Z-interactors: As mentioned, FtsZ is the first recruit to the divisome and is thus involved in division site selection and the recruitment of all subsequent division proteins. ZipA (in *E. coli*) and FtsA tether FtsZ to the inner membrane, and ZapA-D, FzIa, FzIC have been implicated in regulating Z-ring properties such as stability, lateral interactions and filament curvature.

FtsK: FtsK is a four-pass transmembrane protein with a C-terminal DNA translocase domain that facilitates the segregation of replicated chromosomal termini (Aussel et al., 2002). In *E. coli*, this translocation function is non-essential (Wang and Lutkenhaus, 1998), and may only be required to resolve the formation of chromosome dimers or during certain stressful conditions (Britton and Grossman, 1999; Sivanathan et al., 2009; Yu et al., 1998). The essential function of FtsK resides within its transmembrane domains (Draper et al., 1998; Dubarry et al., 2010) and can be bypassed by activating mutations in FtsA or overexpression of the *ftsQAZ* operon (Geissler and...
Margolin, 2005). In *Caulobacter*, the C-terminal translocase domain is essential, possibly because it is involved in recruiting the topoisomerase IV subunit ParC to the replisome and thus coordinating DNA segregation and replication (Wang et al., 2006).

**FtsQLB:** In *E. coli*, FtsQLB is thought to form an essential subcomplex prior to its recruitment to the mid-cell (Buddelmeijer and Beckwith, 2004). Each component is a bitopic membrane protein with cytoplasmic and periplasmic domains, and together, they have been shown by bacterial-two-hybrid experiments to form multiple interactions with most other divisome components (Di Lalio et al., 2003; Karimova et al., 2005). None of these proteins has a known enzymatic or other function, but in *B. subtilis*, FtsL is highly unstable and rate-limiting for division, and it has been implicated in mediating division regulation during replication stress (Bramkamp et al., 2006; Breier and Grossman, 2009; Goranov et al., 2005). Due to its placement within the recruitment hierarchy and its ability to serve as a protein interaction hub, it is thought that FtsQLB could somehow coordinate constriction of the Z-ring with the synthesis of septal cell wall.

**FtsWIN:** FtsWIN are also thought to form an essential subcomplex within the divisome, and they are generally among the latest-arriving divisome components (Fraipont et al., 2011; Mercer and Weiss, 2002; Wissel and Weiss, 2004). Each of these proteins possesses a known or putative function in constructing or modifying the septal peptidoglycan cell wall. FtsW is a predicted 10-pass transmembrane protein that translocates the peptidoglycan precursor Lipid II from the cytoplasm into the periplasm at mid-cell (Mohammadi et al., 2011). Once in the periplasm, septal Lipid II is joined into chains by an unknown transglycosylase and then cross-linked by the transpeptidase activity of FtsI into a rigid, 3D structure (Adam et al., 1997). The function of FtsN is less clear, although it is thought to both stabilize the divisome and recruit peptidoglycan-modifying enzymes to mid-cell (Goehring et al., 2007; Möll et al., 2010; Peters et al., 2011).

**Peptidoglycan-modifiers:** Just as new cell wall must be synthesized at the septum, likely by FtsW and FtsI among other proteins, it must also be simultaneously remodeled and cleaved to allow
new pole formation and daughter cell separation. In *E. coli*, the amidases AmiA, AmiB and AmiC are thought to selectively remove peptidoglycan cross-links and facilitate cell wall remodeling and cleavage (Heidrich et al., 2001). Although these amidases are individually dispensable, cells lacking all three are defective for separation and exhibit a chaining phenotype. In *Caulobacter*, homologs of AmiABC are absent, but a putative endopeptidase DipM is involved in remodeling septal peptidoglycan (Goley et al., 2010a; Möll et al., 2010; Poggio et al., 2010).

**Tol-Pal**: In Gram-negative bacteria, the outer membrane must invaginate in tandem with the cell wall and inner membrane. In *E. coli*, the Tol-Pal system is a five protein complex that localizes to mid-cell and forms contacts with all three envelope layers (Gerding et al., 2007). Although the Tol-Pal system is not essential in *E. coli*, it is essential in *Caulobacter* and likely facilitates proper invagination of the outer membrane in both organisms (Yeh et al., 2010).

*What triggers cytokinesis?*

After, or perhaps shortly before, the division machine is fully constructed, it must shift from a state of assembly into one of septum-synthesizing activity and envelope constriction. In tandem with these cytokinetic activities, the divisome must remodel itself in order to account for its own shrinking circumference and the dynamic nature of its substrate. The molecular mechanism that triggers this change from static assembly to dynamic activity is largely unknown. In *E. coli*, the onset of constriction temporally coincides with the arrival of the latest recruited divisome component FtsN (Gerding et al., 2009), and logically, the arrival of a late-arriving component would serve as a useful trigger, indicating that the assembly stage is near completion. However, it remains unclear what set of protein-protein interactions and conformational changes within the divisome constitute this triggering pathway.
Once the decision to divide has been made, the divisome is faced with the task of cutting the cell in half, an energy-intensive process that must be tightly controlled to avoid envelope rupturing. As mentioned, some divisome components likely play roles in septum synthesis, membrane constriction and DNA segregation, while others may be required only for structural support and divisome assembly. An overlapping and perhaps broader question is which of these proteins, subcomplexes and/or activities actually provides the force and directionality for constriction? Theories as to which divisome component(s) is in the driver’s seat can be generally grouped into two categories (Errington et al., 2003):

**FtsZ-centric:** FtsZ forms proto-filaments in the presence of GTP that bend following GTP hydrolysis *in vitro* (Lu et al., 2000). It has therefore been proposed that FtsZ could simultaneously translate the force supplied by GTP hydrolysis into the directionality of inward invagination. In *Caulobacter*, FzIA may enhance the constrictive force by increasing FtsZ bending during successive rounds of FtsZ assembly, GTP hydrolysis, filament breakage and reassembly (Goley et al., 2010b). In support of these models, a membrane targeted FtsZ fusion can itself form Z-rings and mediate constrictions in lipid vesicles (Osawa et al., 2008, 2009). Furthermore, Mollicutes and artificially derived L-form bacteria retain functional FtsZ but possess no cell walls (Margolin, 2005; Onoda et al., 2000; Siddiqui et al., 2006), perhaps together suggesting that FtsZ itself provides the cytokinetic force. In a related model, FtsZ proto-filaments could slide inwards against the ATPase FtsA which would serve as a motor (Feucht et al., 2001; Paradis-Bleau et al., 2005). Intriguingly, gain of function mutations in *ftsA* have been identified which cause a small-cell phenotype, indicative of a propensity to hyperactively divide (Geissler et al., 2007). These results suggest that FtsZ, perhaps in association with FtsA, could drive constriction and effectively drag the rest of the divisome along with it.

**Cell wall-centric:** It is alternatively possible that division is advanced by the inward growth of the septal cell wall. Accordingly, FtsI and the peptidoglycan remodeling proteins could provide
the motive force for division while the Z-ring would be required mainly for correct positioning of the divisome. GTP hydrolysis-defective \textit{ftsZ} mutants are still capable of division suggesting that other components may indeed provide the constrictive force (Bi and Lutkenhaus, 1992; Dajkovic and Lutkenhaus, 2006; Mukherjee et al., 2001). Interestingly, the chlamydiae lack FtsZ, and while they also lack a cell wall, some peptidoglycan synthesis proteins are retained, and division can still be inhibited by the \textit{FtsI} transpeptidase inhibitor penicillin (Ghuysen and Goffin, 1999; McCoy and Maurelli, 2006). It is unclear whether the existence of Z-less bacteria indicates a less integral role for FtsZ in divisome constriction in other clades.

**The temporal regulation of cell division**

Cell division must be temporally regulated such that it follows DNA replication and chromosome segregation during each cell cycle. Additionally, division must be transiently delayed during stressful conditions that perturb the natural replication cycle or otherwise disfavor division. In order to achieve such temporal control, regulation could theoretically occur at any step in divisome assembly, such that the recruitment of essential downstream components is prevented until division is desired. Alternatively, any of the many essential enzymatic, biosynthetic or other divisome activities could serve as targets for temporal regulation, either by inhibition until, or activation at, the appropriate time. Many temporal regulators of division have now been found in several model systems, and indeed, assembly and activity steps can both serve as regulatory nodes. Here, I will summarize our understanding of temporal division regulators and discuss the common regulatory strategies that have emerged.

*Control of cell division during the cell cycle*

The means by which bacterial cells coordinate cell division with DNA replication and the broader cell cycle are poorly understood. One potential mechanism could be the cell-cycle controlled expression of division genes such that divisome components are only made available
when needed. In *E. coli*, *ftsZ* is transcribed by at least six promoters (Flärdh et al., 1997), and its mRNA levels oscillate with the cell cycle, peaking during the initiation of DNA replication when Z-rings are formed (Garrido et al., 1993). Because FtsZ polymerization is concentration-dependent *in vitro* (Romberg and Levin, 2003), titrating FtsZ levels during the cell cycle could be an effective mode of control. However, FtsZ protein levels are largely unchanged during steady-state growth in *E. coli* and *B. subtilis* (Weart and Levin, 2003), and artificially increasing or decreasing FtsZ levels does not significantly affect the timing of division (Palacios et al., 1996; Ward and Lutkenhaus, 1985; Weart and Levin, 2003). Furthermore, a dependency of FtsZ levels on DNA replication has not been observed in *B. subtilis* (Rowland et al., 1997), and while a slight dependency may exist in *E. coli*, its relevance and mechanism are unclear (Liu et al., 2001). In *B. subtilis*, a detailed analysis of transcriptional fusions to nine divisome components revealed minimal cell cycle-dependent oscillations (Trip et al., 2013), and similar results have been observed at the protein level for several components in *E. coli* (Rueda et al., 2003). These results together suggest that the division cycle is unlikely to be enforced by oscillations in divisome component availability.

A more likely model is that during each cell cycle, division proteins are regulated at the level of activity, localization, or by post-translational modification. The nucleoid occlusion proteins SlmA and Noc prevent Z-ring formation until late stages of DNA segregation and could in theory serve as cell cycle checkpoints that regulate FtsZ post-translationally (Bernhardt and de Boer, 2005; Wu and Errington, 2004). However, cells lacking either factor are unaffected during vegetative growth, suggesting that other factors can compensate to correctly coordinate Z-ring formation with the cell cycle. Intriguingly, nucleoid occlusion may prevent division during replication fork arrest in *B. subtilis* cells in a Noc-independent manner suggesting the existence of additional nucleoid occlusion proteins (Bernard et al., 2010). Another attractive candidate for enforcing the dependency of division on replication is the division protein FtsK whose C-terminal cytoplasmic domain participates in late stages of chromosome segregation. FtsK could
thus coordinate the status of DNA segregation with divisome assembly given that it is required for the localization of several downstream components (Chen and Beckwith, 2001). Despite these features, there is no evidence of an actual replication-division dependency enforced by FtsK, and the dispensability of its C-terminal domain suggests that a role in checkpoint control is unlikely. It thus remains unclear whether bacteria employ substrate-product relationships, checkpoints or some other form of control to ensure the proper timing of division within the cell cycle.

**Conditional control of cell division**

One remarkable feature of bacterial physiology is the ability to rapidly adjust intracellular activities in response to a changing environment. Bacterial cells are equipped with sensory proteins that can detect a wide variety of internal and external cues and mount the appropriate regulatory response through mediators and effectors. Many of these regulatory systems modulate the bacterial cell cycle, and some impinge directly on the cell division machinery. For instance, many cells regulate their size in response to nutrient availability. In *B. subtilis* and *Caulobacter*, nutrient-rich conditions activate cell division inhibitors resulting in longer division cycles and larger cells. In *B. subtilis*, the metabolite uridine-5’-diphosphoglucose (UDP-Glc) binds and activates UgtP which in turn prevents FtsZ assembly (Weart et al., 2007). Similarly, *Caulobacter* cells detect the metabolite NAD(P)H by a sensor unrelated to UgtP called KidO, which somehow targets FtsZ to delay division (Radhakrishnan et al., 2010). In each case, a single protein surveys the levels of an internal metabolite and appropriately regulates cell division through an interaction with FtsZ. Nutrient-influenced size control has also been documented in *E. coli*, although the metabolite sensed and mode of regulation are unknown (Donachie and Begg, 1989; Reeve et al., 1984).

Many Gram-positive bacteria can produce dormant but resilient cells known as “spores” in response to starvation. The developmental program resulting in sporulation applies several
unique constraints on cell division, the first of which involves an asymmetric division event that creates a small forespore and a large mother cell (reviewed in (Errington, 1993)). In *B. subtilis*, FtsZ is redistributed from its normal position at the mid-cell to the cell poles by an association with the multi-functional membrane protein SpoIIE (Beall and Lutkenhaus, 1991; Khvorova et al., 1998; Levin et al., 1997; Lucet et al., 2000). Following the asymmetric division, the mother cell engulfs and nurtures the spore until its ultimate lysis when the spore is released. Importantly, the mother cell is terminally differentiated and its activities are dedicated solely to spore development. To help ensure this exit from the cell cycle, *B. subtilis* encodes a division inhibitor mciZ, which specifically inhibits FtsZ polymerization in the mother cell (Handler et al., 2008). *S. coelicolor* is another sporulating bacterium which lives normally as a multi-nucleate filament that does not require FtsZ for normal growth and viability (McCormick et al., 1994). However, following nutrient limitation, *S. coelicolor* filaments assemble Z-rings at multiple division sites in order to create chains of spores (Schwedock et al., 1997). Intriguingly, the membrane-associated factor SsgB recruits FtsZ to these division sites and is the only known example of a divisome component whose localization precedes that of FtsZ (Willemse et al., 2011).

Many types of stress that do not result in a new developmental program like sporulation can nonetheless require cell division to be regulated. The oxidative stress response in *M. tuberculosis* causes FtsZ-interacting protein A, FipA, to bind FtsZ, allowing division to occur and sustaining growth during macrophage engulfment (Sureka et al., 2010). In *E. coli*, the non-essential divisome component FtsP may stabilize the divisome during oxidative stress (Samaluru et al., 2007). Although *ftsP* mutants can be suppressed by mutations that stabilize Z-ring assembly, its direct binding partners are not known. FipA and FtsP are examples of proteins that promote division during oxidative stress, a condition where protein damage poses a barrier to division. A more common stress response involves the prevention of division until the stress has been resolved. During the stringent response to amino acid starvation, the alarmone (p)ppGpp may somehow prevent FtsZ transcription in *E. coli* (Powell and Court, 1998). During stationary
phase in *E. coli*, a condition when cells are likely starved and subjected to changing pH, the RNA-binding protein Hfq prevents minicell formation by down-regulating FtsZ expression (Takada et al., 1999). Similarly, during stationary phase in *Caulobacter*, FtsZ levels are kept low although the mechanism is unknown (Wortinger et al., 1998). Division is likely regulated by the heat shock response machinery (Tsuchido et al., 1986), and there are many other types of stress during which cells arrest the division cycle, although the regulators and their targets have not been identified.

![Diagram of FtsZ regulation](image)

**Figure 1.3.** Temporal and spatial regulators of division converge on FtsZ. Each of the Z-ring regulators are grouped based on their proposed function and/or regulatory category. Z-ring inhibitors are shown above FtsZ and positive regulators are shown below. Figure adapted from (Kirkpatrick and Viollier, 2011).

Within single bacterial cells, multiple regulators of cell division together ensure the proper timing of divisions both during the normal cell cycle and in response to a wide variety of changing environmental conditions. Although we have likely found only a small fraction of these regulators, it is clear that the most common regulatory strategy involves targeting FtsZ at the transcriptional, translational, or post-translational levels (Figure 1.3). Many division inhibitors
prevent Z-ring formation and thus disrupt all subsequent steps in divisome assembly. This is perhaps the surest way to completely abolish any chance of division. However, many stresses are ephemeral and cells requiring a transient delay in cell division might not want to invest in the complete disassembly and re-assembly of the division machine. DNA damage is one such stress given that cells frequently encounter many types of DNA lesions, some of which can be quickly repaired. The bacterial DNA damage response has been studied extensively, and indeed, several division inhibitors have unique mechanisms compared to those already discussed. In the next section, I will introduce the DNA damage response in bacteria and examine its associated modes of division regulation.

The DNA damage response

DNA damage poses a direct challenge to the replication-division dependency, because it causes replication to slow or stall during periods of repair. It is formally possible that cells could employ a single regulatory system to coordinate division with replication during both normal cell cycles and episodes of DNA damage. For instance, nucleoid occlusion might suffice to accommodate DNA damage and any stress that similarly prolongs replication and leaves unreplicated DNA at mid-cell. However, the canonical bacterial response to DNA damage, known as the ‘SOS response’ (reviewed in (Erill et al., 2007)), typically includes the induction of a specialized cell division inhibitor (Figure 1.4). These inhibitors are not required for normal viability suggesting that their roles are specifically reserved for encounters with DNA damage. There is also a growing appreciation that some cells have additional, SOS-independent mechanisms for damage-induced division control, although these are poorly understood. Here, I will introduce the bacterial response to DNA damage and discuss the known modes of division regulation.
The SOS regulators RecA and LexA

The first hints of a multifunctional bacterial DNA damage response came nearly fifty years ago in a series of experiments in *E. coli*. Cells subjected to DNA damage by UV irradiation formed long filaments (Green et al., 1969), underwent prophage induction (Hertman and Luria, 1967) and exhibited increased rates of mutagenesis (Weigle, 1953). Mutants that mapped to the *lexA* and *recA* loci were sensitive to UV irradiation and defective in each of these UV-induced activities (Gudas and Pardee, 1975). It was soon proposed that these genes orchestrate an inducible DNA damage response that requires *de novo* protein synthesis for full function (Radman, 1975).

Years of rigorous experimentation have solidified the roles of RecA and LexA as the gatekeepers of SOS induction. LexA is a transcriptional repressor whose N-terminal DNA-binding domain silences the promoters of SOS genes in undamaged cells (Brent and Ptashne, 1980; Little and Harper, 1979; Little et al., 1981). Following DNA damage, LexA undergoes a RecA-dependent autocatalytic cleavage which de-represses the regulon and allows the induction of SOS genes (Lin and Little, 1989; Little et al., 1980). RecA is a DNA-dependent ATPase and homolog of the eukaryotic repair gene Rad51 (Ogawa et al., 1979; Roberts et al., 1979). Following DNA damage, RecA is activated by the presence of single-stranded DNA (ssDNA) which can arise in several ways (reviewed in (Indiani and O'Donnell, 2013)). When DNA polymerase is paused at lesion sites, continued activity of the replicative helicase could unwind DNA revealing stretches of ssDNA. Alternatively, the replisome could skip lesion sites and resume replication downstream, leaving behind ssDNA gaps. DNA repair can also generate ssDNA ends following the enzymatic processing of double-strand break ends or ssDNA gaps during excision repair. Once bound to ssDNA, RecA polymerizes into filaments along the DNA whose assembly requires ATP binding (Lee and Cox, 1990). The ATP-bound RecA filament represents its active form (RecA*) which performs two essential functions in DNA repair. As a
recombinase, RecA* catalyzes strand exchange and mediates homology searching during the recombination-based repair of double-strand breaks and damaged single-strand gaps (McEntee et al., 1979). Its second function, likely located at a distinct site within RecA* (Adikesavan et al., 2011), is to bind LexA and promote its cleavage.

**The SOS regulon**

Microarray experiments indicate that hundreds of genes are regulated by DNA damage, but only a subset are *bona fide* SOS members (Au et al., 2005; Fernández De Henestrosa et al., 2000). The *E. coli* SOS regulon is comprised of 40 genes that are directly repressed by LexA (reviewed in (Kelley, 2006)). Among these, RecA and LexA themselves are SOS-induced. As mentioned, RecA mediates recombination repair and, with Ssb, stabilizes stalled replication forks (Buss et al., 2008), while newly synthesized, uncleaved LexA is required to shut off the SOS system when the damage has been fully repaired (Little, 1983). Many SOS genes are involved in DNA damage repair, as UvrABCD and RuvCAB participate in nucleotide excision and recombination-based repair pathways respectively (Sancar and Rupp, 1983; Seigneur et al., 1998). PolB, DinB, and UmuDC constitute another class of SOS genes involved in translesion DNA synthesis (Yeiser et al., 2002). These polymerases catalyze error-prone base additions that sacrifice fidelity for an ability to proceed through DNA lesions. The final class of SOS genes are the checkpoint genes which prevent cell cycle progression until the damage has been removed. In *E. coli*, SulA prevents cell division and will be discussed further below, while UmuDC delays DNA replication before it undergoes a RecA-mediated autocleavage that activates its function in translesion synthesis (Opperman et al., 1999).

Most sequenced bacterial genomes contain homologs of LexA, although LexA binding motifs are widely divergent between clades (Erill et al., 2007). The motif in most Gram-positive bacteria, GAAC-N4-GTTC, and that for the β- and γ-proteobacteria, CTGT-N8-ACAG, are both palindromic sequences that bind LexA dimers with varying hinge angles to accommodate the
different spacer lengths (Cheo et al., 1991; Erill et al., 2003). Unexpectedly, the motif in α-proteobacteria, GTTC-N7-GTTC, is a direct repeat instead of a palindrome, indicating a completely novel dimer interface and binding mechanism (Fernández de Henestrosa et al., 1998). Other oddities have emerged as more LexA binding motifs have been sequenced, including some that are asymmetrical and imperfectly palindromic (Campoy et al., 2003; Campoy et al., 2002; Yang et al., 2002). Despite these marked differences in binding sites, a similar set of core SOS genes are commonly found throughout the bacterial kingdom, including \textit{lexA}, \textit{recA}, \textit{uvrA}, \textit{ruvCAB} and \textit{ssb} (Erill et al., 2007). Interestingly, very few bacteria contain all of these core genes, and more recently, the in silico analysis of a highly conserved SOS-inducible operon encoding the mutagenesis genes \textit{imuAB} and \textit{dnaE2} has led to questions regarding which genes are the oldest SOS members (Abella et al., 2004). Although \textit{imuAB-dnaE2} are not found in \textit{B. subtilis} or \textit{E. coli}, their tight genetic and physical linkage with \textit{lexA} in most other phyla suggests that the SOS response may have originally been an inducible translesion synthesis system (Erill et al., 2006).

Regardless of what the ancestral SOS regulon looked like, it is clear that many bacteria respond to DNA damage by inducing a similar set of regulatory and effector genes. The SOS-induced division inhibitors are a notable exception as those characterized to date show very little sequence homology. Given that these genes are likely unrelated functional analogs, have they nevertheless converged to inhibit division in similar ways, by targeting the same components and/or activities? And, do they, like most of the known positional and conditional regulators of division, also target FtsZ and prevent formation of the Z-ring?

\textit{SOS-induced division inhibitors}
The *E. coli* SOS member *sulA* has long been the lone example of a bacterial DNA damage-induced cell division inhibitor. *sulA* mutations were originally identified as suppressors of loss-of-function mutants in the protease *lon*, which normally exhibit irreversible filamentation following exposure to UV light (George et al., 1975). The same screen yielded suppressor mutations in *ftsZ*, which encodes the target of SulA. During SOS-induction, SulA accumulates and prevents division by sequestering the free ends of FtsZ monomers or proto-filaments and blocking the polymerization of additional subunits (Bi and Lutkenhaus, 1993; Cordell et al., 2003; Mukherjee et al., 1998). *De novo* Z-ring formation is thus prevented, and extant Z-rings are dissolved due to the highly dynamic nature of FtsZ filament assembly and disassembly (Stricker et al., 2002). Following DNA repair, Lon degrades SulA and allows division cycles to resume (Figure 1.4; (Mizusawa and Gottesman, 1983)).

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**Figure 1.4.** SOS induction of the division inhibitor *sulA*. In undamaged cells, the repressor LexA silences transcription at the promoters of SOS genes. Following DNA damage, RecA forms active filaments on single-stranded DNA and causes the autocatalytic cleavage of LexA. SOS genes are de-repressed, and SulA, an SOS-induced division inhibitor in *E. coli*, prevents polymerization of FtsZ. After the damage is repaired, the Lon protease degrades SulA allowing Z-ring formation.
The existence of SulA initially supported the notion that most, if not all regulators of bacterial cell division target FtsZ. However, in the last decade, several SOS-induced division inhibitors with seemingly novel mechanisms have been identified in Gram-positive bacteria. YneA is a strongly SOS-induced cell division inhibitor in \textit{B. subtilis} that shares few other characteristics with its \textit{E. coli} counterpart (Kawai et al., 2003). While SulA is a cytoplasmic protein like its target FtsZ, YneA is a single-pass transmembrane protein with an extracellular LysM peptidoglycan-binding domain. There is no evidence that YneA interacts with FtsZ \textit{in vitro}, and Z-ring formation is reduced but not abolished during YneA overproduction. While its precise target and mechanism are unknown, the transmembrane and extracellular domains are required for its function suggesting that it may interact with the many divisome components downstream from FtsZ that have transmembrane domains or the septal cell wall itself (Mo and Burkholder, 2010). The SOS-regulated division inhibitors ChiZ and DivS have been identified in the Gram-positive Actinobacteria \textit{M. tuberculosis} and \textit{C. glutamicum} respectively, and both of these are single-pass transmembrane proteins like YneA, although they share limited sequence similarity (Chauhan et al., 2006; Ogino et al., 2008). ChiZ contains a LysM domain, interacts with FtsI and FtsQ by bacterial-two-hybrid analysis and exhibits cell wall hydrolase activity; similar mechanistic insights are lacking for DivS (Vadrevu et al., 2011). Both inhibitors may have mild effects on Z-ring formation, but as with YneA, evidence for a direct interaction with FtsZ is lacking.

Although detailed mechanisms are lacking for the Gram-positive division inhibitors, it is clear that they do not completely disrupt Z-ring assembly like SulA. This distinction could be a testament to the inherent differences between the envelopes of Gram-negative and Gram-positive bacteria. The Gram-positive cell wall is two- to eight-fold thicker than that of Gram-negatives, and it is enriched in teichoic acids which are polysaccharides that provide the cell wall with increased rigidity (Wicken and Knox, 1975). Division in Gram-positive bacteria may thus follow a more cell wall-centric mode of constriction, and inhibitors of division could target divisome...
components and activities more intimately linked to peptidoglycan synthesis and remodeling. Given that so few division inhibitors have been identified, it is difficult to say whether distinctions among the current group reflect fundamental differences in bacterial physiology. As described in Chapter 2, I have sought to identify and characterize the SOS-induced division inhibitor in the α-proteobacterium *Caulobacter crescentus*, in part to determine whether SulA and its target FtsZ truly serve as a paradigm for regulatory control in Gram-negative bacteria.

**SOS-independent regulation**

Several lines of evidence have suggested that in *E. coli*, SulA may not be the sole means of damage-induced division inhibition. Cells lacking *sulA* and those unable to induce SOS genes still exhibit filamentation following replication arrest or DNA damage (Burton and Holland, 1983; Hill et al., 1997; Howe and Mount, 1975; Huisman et al., 1980; Ishioka et al., 1997; Jaffé et al., 1986; Liu et al., 2001). Similar results have been observed in *B. subtilis* (Love and Yasbin, 1984), and in *M. tuberculosis*, ChiZ induction is not completely eliminated in cells lacking *recA* (Rand et al., 2003). These results suggest that SOS-independent modes of division inhibition likely exist, but the associated regulators and inhibitors have not been identified.

How do cells respond to DNA damage if not by the RecA-ssDNA-mediated induction of SOS genes? Exposed stretches of ssDNA are one of many abnormal DNA structures that could serve as DNA damage signals for regulatory systems. In *B. subtilis*, the di-adenylate cyclase DisA monitors genome integrity and likely binds to branched DNA structures that arise during recombination-based repair (Bejerano-Sagie et al., 2006). When paused at such sites, the di-adenylate cyclase activity of DisA is inhibited causing decreases in cellular levels of cyclic-di-AMP (c-di-AMP). c-di-AMP is a secondary messenger that in undamaged cells activates Spo0A, a master regulator involved in triggering sporulation (Oppenheimer-Shaanan et al., 2011; Witte et al., 2008). DisA is thus an example of a checkpoint system whose activity is regulated by a non-canonical DNA damage-associated structure.
A second possibility is that a separate regulatory system is tasked with responding directly to replication stress which is intimately but not exclusively linked to DNA damage. For instance, the universally conserved replication initiation factor DnaA has been proposed to function as both a sensor of replication stress and a mediator of response elements. During replication initiation, DnaA-ATP multimers promote double-stranded DNA melting at the origin and recruit replisome components to the nascent replication forks (Bramhill and Kornberg, 1988). DnaA-ATP also functions as a transcription factor for genes involved in replication, transcription, translation, division and, in *B. subtilis*, sporulation inhibition (Burkholder et al., 2001; Goranov et al., 2005; Hottes et al., 2005; Messer and Weigel, 1997). Following replication initiation, DnaA activity is inhibited by members of the elongation complex in order to prevent overinitiation (Camara et al., 2005; Katayama and Sekimizu, 1999). When replication is stalled, transcriptional activities of DnaA are reinstated. Intriguingly, it has been proposed that DnaA mediates a replication checkpoint on cell division by preventing *ftsL* transcription in *B. subtilis* (Goranov et al., 2005).

In summary, there are likely damage-induced, SOS-independent regulatory modules and modes of division inhibition in many bacteria. In Chapter 3, I have used *Caulobacter* to identify and characterize an SOS-independent inhibitor of cell division.

*Caulobacter crescentus* as a model system

Several unique features of the *Caulobacter* life cycle make it an ideal system for studying the coordination between DNA replication and cell division (reviewed in (Skerker and Laub, 2004)). Unlike many bacteria, which in rich media can initiate multiple rounds of replication between consecutive divisions, *Caulobacter* replicates once and only once per cell division resulting in distinct G1, S and G2 cell cycle stages (Marczynski, 1999). Additionally, pure populations of G1 cells are readily obtained by density centrifugation, facilitating the analysis of individual cell cycle stages and their associated dependencies (Evinger and Agabian, 1977).
Finally, as a genetically tractable $\alpha$-proteobacterium, *Caulobacter* allows principles gleaned from the $\gamma$-proteobacterium *E. coli* to be placed in a broader evolutionary context.

![Diagram of the Caulobacter cell cycle](image)

Figure 1.5. The *Caulobacter* cell cycle. Phosphorylated CtrA (CtrA~P) is indicated by blue shading. The Z-ring is indicated in green, and the fully assembled divisome is indicated in purple. The Z-ring inhibitor MipZ is localized to the cell poles through an association with chromosomal origins of replication.

*Caulobacter* has long been studied as a model system for understanding the molecular events that drive a bacterial cell cycle (Fig. 1.5). Its mandatory progression through distinct cell cycle stages with respect to replication is reminiscent of the eukaryotic cell cycle. It is therefore perhaps not surprising that the *Caulobacter* cell cycle is orchestrated by the oscillating activity of a phosphorylated regulator, CtrA, much as the eukaryotic cell cycle is orchestrated by cyclin-dependent kinases (Quon et al., 1996). CtrA is a transcription factor that, when activated by phosphorylation, binds to DNA and regulates the expression of nearly 100 genes (Domian et al., 1997; Laub et al., 2002). In G1 'swarmer' cells, CtrA represses replication initiation by binding to and silencing the origin, which is located at the flagellated cell pole (Quon et al., 1998). At the end of G1, CtrA is dephosphorylated and degraded allowing replication to initiate. Concomittantly, the flagellum is shed and in its place a 'stalk' is built, which facilitates surface adsorption and nutrient uptake (Stoveoindexter and Cohen-Bazire, 1964). The newly replicated
origin is immediately shuttled to the opposite cell pole which establishes the bipolar localization of the origin-associated Z-ring inhibitor MipZ (Thanbichler and Shapiro, 2006). As S phase proceeds and the stalked cell elongates, the Z-ring is established at mid-cell where concentrations of MipZ are lowest (Kelly et al., 1998). Mid-way through S phase, CtrA again accumulates in its phosphorylated form, allowing the transcription of genes necessary for cell division and other late cell cycle events. As replication nears completion, the fully assembled divisome causes cells to constrict at mid-cell during the ‘pre-divisional’ cell cycle stage (Goley et al., 2011). Finally, an asymmetric cell division produces a flagellated swarmer cell which resides in G1 and a stalked cell which immediately begins another round of replication.

The regulation of cell division in Caulobacter

Microarray experiments on synchronized Caulobacter cultures revealed that nearly 500 genes varied during the course of the cell cycle (Laub et al., 2000). Furthermore, many of these genes were expressed during the cell cycle stage when their activity was required, a phenomenon termed ‘just-in-time’ transcription. In early predivisional cells, CtrA transcribes the essential division genes, \( ftsQ, ftsA \) and \( ftsW \) at a time when divisome assembly is just underway. Furthermore, the \( ctrA \) loss-of-function phenotype includes extensive filamentation, suggesting that the transcriptional activation of these divisome components in predivisional cells could be a prerequisite for division (Quon et al., 1996). CtrA is thus well situated to serve as a cell division checkpoint, and indeed, its activity appears to be regulated by genome integrity and the status of DNA replication. Replication initiation is required to somehow activate the hybrid histidine kinase CckA (Iniesta et al., 2010) which in turn phosphorylates and stabilizes CtrA through the histidine phosphotransferase ChpT (Biondi et al., 2006). Furthermore, cells treated with DNA damaging agents or hydroxyurea, which depletes nucleotides pools and stalls replication, demonstrate reduced CtrA activity (Wortinger et al., 2000).
Taken together, these results suggest that in *Caulobacter*, DNA damage and replication stress could prevent division by decreasing the activity of the cell cycle regulator CtrA. However, it is unknown whether the inhibition of CtrA is necessary or sufficient to prevent division during these conditions.

An alternative and perhaps complementary mode of division control could be provided by direct division inhibitors activated during the *Caulobacter* DNA damage response. Following damage, *Caulobacter* induces an SOS regulon similar to that of *E. coli* in its size and composition (da Rocha et al., 2008). However, no homologs of *sulA* or any other division inhibitor are present, and SOS-induced division inhibitors have not been identified in any α-proteobacteria. It thus remains unclear whether α-proteobacterial genomes encode a novel class of damage-induced division inhibitors, or whether CtrA or other cell cycle genes instead provide the necessary control.

**Research Summary**

The subsequent chapters describe a series of experiments that explore the mechanisms of division inhibition during the *Caulobacter* DNA damage response. Chapter 2 details the identification and characterization of *sidA*, an SOS-induced cell division inhibitor that is conserved in many α-proteobacteria. *sidA* encodes a short, 29 amino acid protein that is localized to the membrane at mid-cell following DNA damage. I provide evidence that SidA does not prevent Z-ring formation or divisome assembly, but instead inhibits division through a direct interaction with the late-arriving divisome component FtsW. I show that SidA does not disrupt the cell wall translocase activity of FtsW suggesting that FtsW likely performs an additional role in executing division. My work demonstrates that divisome components other than FtsZ can be targeted by division inhibitors and identifies FtsW as a critical regulatory node in *Caulobacter*. 
During our work with *sidA*, I noticed that cells incapable of mounting an SOS response can nonetheless delay cell division during DNA damage. In chapter 3, I identify and characterize *didA*, an SOS-independent division inhibitor. Like *sidA*, *didA* encodes a short transmembrane protein that does not prevent any step in divisome assembly. DidA interacts with the late-arriving divisome component FtsN, and intriguingly, mutations in *fisW* and *fisI* can suppress the activities of both SidA and DidA. My work suggests that these suppressor mutations do not reduce the affinities of SidA and DidA for their divisome targets, but instead hyperactively promote cell division. Finally, I show that cells require at least one of these inhibitors to properly regulate division during DNA damage illustrating a degree of redundancy within the *Caulobacter* DNA damage response. My work uncovers an SOS-independent DNA damage response in *Caulobacter* and provides further support for the importance of the FtsW/FtsI/FtsN complex in triggering division.

In chapter 4, I discuss the implications of our work for understanding bacterial cell division and its regulation. I also present ongoing work which explores the molecular mechanisms underlying the SOS-independent regulation of *didA*. 
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Chapter 2

A DNA damage checkpoint in Caulobacter crescentus inhibits cell division through a direct interaction with FtsW

This work was published as Modell, J. W., Hopkins, A. C., and Laub, M. T. 2011, A DNA damage checkpoint in Caulobacter crescentus inhibits cell division through a direct interaction with FtsW: Genes Dev, v. 25, p. 1328-43.

JWM, ACH and MTL conceived and designed the experiments. JWM and ACH performed the experiments.
Abstract

Following DNA damage, cells typically delay cell cycle progression and inhibit cell division until their chromosomes have been repaired. The bacterial checkpoint systems responsible for these DNA damage responses are incompletely understood. Here, we show that Caulobacter crescentus responds to DNA damage by coordinately inducing an SOS regulon and inhibiting the master regulator CtrA. Included in the SOS regulon is sidA (SOS-induced inhibitor of cell division A), a membrane protein of only 29 amino acids that helps to delay cell division following DNA damage, but is dispensable in undamaged cells. SidA is sufficient, when overproduced, to block cell division. However, unlike many other regulators of bacterial cell division, SidA does not directly disrupt the assembly or stability of the cytokinetic ring protein FtsZ, nor does it affect the recruitment of other components of the cell division machinery. Instead, we provide evidence that SidA inhibits division by binding directly to FtsW to prevent the final constriction of the cytokinetic ring.
Introduction

Cells frequently experience genotoxic stresses with potentially mutagenic or fatal consequences. To maintain genome integrity, organisms respond by producing or activating proteins with roles in translesion DNA synthesis, recombination, and repair. In addition, organisms may delay the cell cycle and inhibit cell division until DNA repair and replication are complete. In eukaryotes, these responses are orchestrated by elaborate checkpoint systems that are well understood at the molecular level (Harper and Elledge, 2007). The bacterial mechanisms of cell cycle and division control following DNA damage remain only partially understood, and the extent to which different bacterial species use checkpoint systems is unresolved.

In bacteria, DNA damage triggers a change in gene expression called the SOS response (Butala et al., 2009; Little and Mount, 1982). This gene expression program is initiated by the autocatalytic cleavage of the transcriptional repressor LexA, an event stimulated by RecA, a multifunctional recombinase and Rad51 homolog. Cleavage of LexA induces the SOS regulon, which includes a battery of DNA repair genes, some of which are widely conserved in bacteria (Erill et al., 2007). In addition, the *Escherichia coli* SOS regulon includes *sulA*, which encodes a cell division inhibitor (Huisman and D'Ari, 1981; Mukherjee et al., 1998). SulA directly inhibits polymerization of FtsZ, a GTP-binding tubulin homolog that polymerizes at mid-cell and is crucial to cytokinesis. As SulA is not conserved outside the γ-proteobacteria, additional mechanisms for inhibiting cell division likely exist in other bacteria.

Cytokinesis in bacteria requires the assembly of a large multicomponent complex of proteins called the divisome. In *E. coli*, these components assemble in an ordered fashion. The first protein to assemble is FtsZ, which polymerizes at mid-cell into a ring-like structure that is tethered to the cytoplasmic surface of the inner membrane (Adams and Errington, 2009; Lutkenhaus and Addinall, 1997). In addition to its direct role in constriction, the Z-ring also nucleates assembly of the rest of the divisome, which ultimately coordinates constriction and
membrane invagination with construction of a septum, a process that requires localized peptidoglycan synthesis (Errington et al., 2003). The initial formation of a Z-ring is mediated by the proteins FtsA and ZipA, which stabilize FtsZ polymers and anchor the ring to the inner membrane (Adams and Errington, 2009). In *E. coli*, additional components are then assembled in a step-wise manner in the order FtsK, FtsQ, FtsL/FtsB, FtsW, FtsI, and FtsN (Goehring and Beckwith, 2005; Vicente and Rico, 2006). The order of assembly is similar, but not identical, in *Caulobacter* (Goley et al., 2011). Although the assembly of cell division proteins is relatively well characterized, the execution and regulation of cell division remain poorly understood.

Previous studies of cell division regulation have focused almost exclusively on proteins that modulate Z-ring assembly and stability. For instance, in *E. coli*, the regulators MinCD and SlmA inhibit FtsZ polymerization at the poles and in proximity to chromosomal DNA, thereby restricting Z-ring formation to mid-cell (Bernhardt and de Boer, 2005; Lutkenhaus, 2007); MinCD and Noc play analogous roles in *Bacillus subtilis* (Wu and Errington, 2004). FtsZ is also a focal point of regulation following cellular stress. In *B. subtilis*, Ugp coordinates Z-ring assembly with glucose availability (Weart et al., 2007), while MciZ inhibits FtsZ polymerization during sporulation (Handler et al., 2008). In *E. coli*, DNA damage induces SulA, which inhibits FtsZ polymerization and GTP hydrolysis to prevent cell division (Justice et al., 2000; Mukherjee et al., 1998).

It is unclear whether the numerous essential components of the cytokinetic ring other than FtsZ can also serve as points of control. The SOS-induced inhibitors YneA, DivS, and Rv2719c in *B. subtilis*, *Corynebacterium glutamicum*, and *Mycobacterium tuberculosis*, respectively, appear not to inhibit Z-ring formation (Chauhan et al., 2006; Kawai et al., 2003; Ogino et al., 2008). However, the direct targets of these inhibitors remain unknown.

The α-proteobacterium *Caulobacter crescentus* is an excellent model system for understanding the regulation of cell division. *Caulobacter* cells are synchronizable, and the cell
cycle is accompanied by a series of easily tracked morphological transitions (Fig. 2.1A). Motile swarmer cells reside in a G1 phase and cannot initiate DNA replication. Swarmer cells then differentiate into stalked cells and, concomitantly, initiate DNA replication. As replication proceeds, cells grow and elongate, build a Z-ring at mid-cell, and eventually form a visible constriction at this future site of cell division. Once S phase completes, the cell can divide asymmetrically to produce a stalked cell that immediately reinitiates DNA replication and a swarmer cell that must again differentiate before initiating.

How Caulobacter delays cell division after DNA damage is unknown. As noted, Caulobacter does not encode a homolog of SulA or other known SOS cell division inhibitors. The disruption of DNA replication was shown to down-regulate the activity of CtrA, a cell cycle-regulated transcription factor that normally promotes the expression of ftsA and ftsQ (Wortinger et al., 2000). However, it is unknown whether the inhibition of CtrA is either necessary or sufficient to delay cell division following DNA damage.

Here, we identify sidA (SOS-induced inhibitor of cell division A), which encodes a small inner membrane protein that is strongly up-regulated following DNA damage. Overproducing SidA is sufficient to inhibit cell division, suggesting it plays an analogous role to SulA in E. coli. However, unlike SulA, SidA does not interfere with the assembly of the Z-ring or the recruitment of other essential cell division components. Instead, SidA inhibits constriction by interacting directly with FtsW and FtsN. Furthermore, mutations that suppress the lethality of overproducing SidA map to ftsW and ftsI. We present evidence that FtsW, FtsI, and FtsN form a subcomplex within the cytokinetic ring. Although these proteins have been implicated in septal peptidoglycan synthesis, SidA does not appear to inhibit this activity. Our results instead suggest a second role for these proteins in triggering the final constriction of the cytokinetic ring, an activity that is inhibited by SidA. The FtsW/I/N subcomplex thus represents a key regulatory
node within the cell division machinery, and SidA is, to our knowledge, the first endogenous cell division inhibitor with a known binding target other than FtsZ.
Results

DNA damage induces cell cycle arrest and a change in global transcription patterns

To investigate the mechanisms coupling DNA integrity and replication status with cell division, we first analyzed the response of *Caulobacter* cells to mitomycin C (MMC) and ultraviolet light (UV), which damage DNA, and to hydroxyurea (HU), which disrupts DNA replication by depleting nucleotide pools. Each treatment caused cells to transiently arrest the cell cycle; cells continued to elongate but failed to divide (Fig. 2.1B). Using whole-genome microarrays, we analyzed global changes in gene expression after each perturbation. Wild-type cells were grown to mid-exponential phase in either rich medium or minimal medium and were exposed to one of the three agents (MMC, UV, or HU). Samples were collected immediately prior to treatment and every 20 min up to 80 min. RNA from each time point was compared with RNA from the pretreatment sample on DNA microarrays.

A total of 160 genes, or nearly 5% of the annotated genes in *Caulobacter*, were significantly induced or repressed during at least one of these six time courses (for complete data, see Supplemental Table S1 in (Modell et al., 2011)). To identify candidate regulators of cell division, we focused on the gene expression changes that were common across all conditions. We found that 74 genes changed consistently across the six time courses, with 28 being down-regulated and 46 being up-regulated (Fig. 2.1C; Fig. 2.2). Of the 28 genes consistently repressed, 10 were identified previously as direct targets of the master cell cycle regulator CtrA, and nine others are likely indirect targets, as they showed significant decreases in expression in *ctrA* strains (Laub et al., 2002). A previous study demonstrated a similar down-regulation of two CtrA targets, *ftsQ* and *ftsA*, after HU treatment (Wortinger et al., 2000). We also found that *ftsQ* mRNA levels dropped after DNA damage, but just missed the thresholds set for inclusion, while the probe for *ftsA* did not provide reliable data.
A. Diagram showing cell cycle stages: swarmer, stalked, predivisional, cell division inhibition.

DNA damage

B. Images showing effects of UV, HU, MMC, and MMC (160°) on cell morphology.

C. Heat map showing gene expression changes under different conditions: HU, MMC, UV, LexA, with fold change indicated.

D. Graph showing mRNA levels of sidA, recA, lexA, and ssb over time.
Figure 2.1. DNA damage induces global changes in gene expression and inhibits cell division. (A) Schematic of the Caulobacter cell cycle with and without DNA damage. (B) Wild-type CB15N grown to mid-exponential phase was exposed to UV light, HU, or MMC for 80 min or left untreated, and was imaged by differential interference contrast microscopy. Cells exposed to MMC were also examined after 160 min. Bar, 2 μm. (C) Gene expression profiles of DNA damage-regulated genes in Caulobacter. Profiles are shown for 74 genes significantly changed in expression level after treatment with HU, MMC, or UV light in rich (PYE) and minimal (M2G) media. The column labeled “LexA” indicates with a red box whether a gene has a LexA box upstream. The columns labeled “CtrA-direct” and “CtrA-indirect” indicate with a green or black box, respectively, whether a gene is a direct target of CtrA or is indirectly affected by CtrA. For annotation of individual genes and complete data, see Figure 2.2 and Supplemental Table S1 in (Modell et al., 2011). Expression ratios are shown relative to untreated cells and are represented using the color scale shown. Gray blocks indicate missing data. (D) Graph showing induction of sidA after DNA damage, relative to other members of the SOS regulon: recA, lexA, and ssb. Response curves are the average across all six time courses in C.
Figure 2.2. Annotated gene expression profiles. Transcriptional profiles for the 74 genes regulated during DNA damage (see Figure 2.1) are shown with the corresponding CC numbers and NA1000 annotation.
Identification of sidA, a novel SOS-induced cell division inhibitor

Inhibiting CtrA may help block cell division by decreasing the expression of ftsQ and ftsA. However, many bacteria induce genes—such as sulA in *E. coli* and yneA in *B. subtilis*—following DNA damage that post-transcriptionally inhibit cell division (Huisman and D’Ari, 1981; Kawai et al., 2003). As noted, the *Caulobacter* genome does not contain homologs of these genes. To identify a damage-inducible cell division inhibitor, we examined our expression data for genes that (1) are rapidly and strongly up-regulated following DNA damage, (2) are predicted members of the SOS regulon based on the presence of a consensus LexA-binding site (da Rocha et al., 2008), and (3) have no known or predicted role in DNA repair. Based on these criteria, we identified CC1927, which we named sidA, which has a predicted LexA-binding site in its promoter region and is induced nearly 14-fold within 20 min of DNA damage (Fig. 2.2D). *sidA* is predicted to encode a highly hydrophobic 40-amino-acid protein lying 39 bases upstream of the replicative DNA polymerase III α subunit (*dnaE*). Despite their close proximity, *dnaE* and *sidA* are likely not cotranscribed, as the expression levels of *dnaE* and *sidA* are not strongly correlated in the experiments here or reported previously (Laub et al. 2000).

SidA is the principal SOS-induced cell division inhibitor

To explore the function of *sidA*, we created a strain in which all but the first and last three amino acids of the originally annotated coding sequence were deleted. This ΔsidA strain showed no obvious growth or morphological defects in standard rich (Fig. 2.3A–B) or minimal medium, suggesting that *sidA* is dispensable in undamaged cells.

To assess the ability of ΔsidA cells to withstand DNA damage, we first tested the growth of wild type and ΔsidA on plates containing increasing concentrations of the damaging agent MMC. Under such conditions of DNA damage, ΔsidA cells showed no major viability defect (data not shown). To better characterize the DNA damage response of ΔsidA, we used time-lapse
microscopy to monitor cells grown on agarose pads containing MMC (Fig. 2.3C). For both wild-type and ΔsidA, we tracked >250 individual cells from three independent movies, noting for each cell its initial cell cycle stage and subsequent cell division behavior. Among cells that had yet to initiate division when first placed on the pad, 24.9% of ΔsidA cells divided compared with 15.1% of wild-type cells. We also observed a sixfold increase in the number of minicells in ΔsidA populations, which resulted from ectopic cell divisions occurring near the cell poles (Fig. 2.3C). SidA thus appears to help prevent inappropriate cell division events following DNA damage at both mid-cell and the poles.

Figure 2.3. sidA is the primary SOS-induced cell division inhibitor. (A,B) Micrographs (A) and growth curves (B) of wild-type, ΔlexA, ΔsidA, and ΔlexA ΔsidA cells grown in rich medium. Bar in the top left panel, 2 μm. (C) ΔsidA (tetR) cells were placed on agarose pads containing MMC and imaged for up to 600 min. Examples of minicell formation resulting from division near a cell pole or division at mid-cell are shown. The percentage of cells that produce minicells or divide medially are shown on the right and are compared with wild-type cells treated identically.
Although these data indicate that SidA inhibits cell division following DNA damage, most AsidA cells still eventually formed long filaments (Supplemental Movie S1 in (Modell et al., 2011)), indicating that an alternative means of division inhibition also functions following DNA damage. Note that sulA in E. coli behaves similarly; it is the primary SOS-induced inhibitor of cell division, but not all cells lacking sulA divide prematurely following DNA damage, presumably due to the action of another cell division inhibitor (Gottesman et al., 1981; Hill et al., 1997).

To determine whether SidA is the primary division inhibitor within the Caulobacter SOS regulon, we created a strain harboring deletions in both lexA and sidA. The lexA deletion alone results in extreme cellular filamentation and a reduction in growth rate owing to constitutive expression of the SOS regulon, even in the absence of DNA damage (Fig. 2.3A,B; (da Rocha et al., 2008)). If SidA is the primary SOS-induced inhibitor of cell division, a deletion of sidA should eliminate or alleviate the phenotypes of a ΔlexA strain. Strikingly, we found that deleting sidA in a ΔlexA strain completely suppressed cellular filamentation (Fig. 2.3A), demonstrating that SidA is the principal SOS-regulated division inhibitor in Caulobacter. Whereas ΔlexA cells were often 10–20 times the length of wild-type cells, AsidA ΔlexA cells were nearly indistinguishable from wild type. Deleting sidA also partially suppressed the growth phenotype of a ΔlexA strain (Fig. 2.3B). A complete rescue of growth is likely not possible, owing to the constitutive activation of DNA repair and recombination genes in the ΔlexA strain. Taken together, our data thus far suggest that sidA (1) is strongly induced following DNA damage as part of the Caulobacter SOS regulon and (2) plays a role in preventing cell division post-damage.

SidA is sufficient, when overproduced, to inhibit cell division

Next, we wanted to test whether SidA was sufficient to inhibit cell division in the absence of DNA damage by overexpressing sidA in undamaged, mid-exponential-phase cells. We fused the
annotated *sidA* coding sequence to the xylose-inducible promoter \( P_{xyl} \) on a high-copy plasmid and transformed this construct into the wild-type strain CB15N. To overproduce SidA, cells were grown in medium containing xylose. Within 1.5 h, cells were elongated relative to wild type or cells harboring an empty vector. After 3 and 6 h, nearly every cell overproducing SidA had formed a long filament, often with multiple pinch sites that likely represent nascent cell division sites (Fig. 2.4A,B). These data indicate that SidA is sufficient to inhibit cell division even in the absence of DNA damage, and that *sidA* may participate in a DNA damage checkpoint similar to *sulA*.

Homologs of *sidA* are often predicted to start at a position corresponding to the second methionine of the originally annotated *sidA*. Additionally, we noted that, unlike the annotated start site, the second methionine in *sidA* lies downstream from an apparent Shine-Delgarno sequence, suggesting that it is the primary translational start site. To test this prediction, we mutated the first methionine on the \( P_{xyl}:sidA \) overexpression plasmid from ATG to AAG. For cells harboring this construct, growth in xylose also led to cell division inhibition, suggesting that the second start site is functional (Fig. 2.4B). The cellular filamentation phenotype was, however, diminished when overexpressing the shorter version of *sidA*. The more pronounced phenotype seen when overproducing the longer version may indicate that the additional 11 amino acids at the N terminus either stabilize the protein or somehow render SidA more potent. We favor the former, as overproducing SidA with an N-terminal M2-epitope tag, which is unlikely to enhance potency, led to severe cellular filamentation, as with the original \( P_{xyl}:sidA \) construct (Fig. 2.4B). We could not, however, directly test the stability and size of the native SidA protein, as it is difficult to raise antibodies against such short, hydrophobic proteins.

To more firmly identify the translational start site, we fused the coding sequence for green fluorescent protein, *gfp*, to the *sidA* promoter at each of the two putative translational start sites on a low-copy plasmid. Following MMC treatment, only cells bearing the fusion of GFP to the
second methionine exhibited significant fluorescence (Fig. 2.4C). We conclude that SidA translation initiates primarily, and perhaps exclusively, from the second site to produce a 29-amino-acid protein; hereafter, references to SidA imply this shorter version and SidA* refers to the longer form.

Figure 2.4. Overproducing SidA is sufficient to inhibit cell division. (A) Growth curve in rich medium of cells carrying a high-copy plasmid on which the xylose-inducible promoter drives expression of the M2 epitope only; sidA*, the originally annotated CC1927 ORF; sidA*(T2A), which harbors a mutation in the first annotated start codon; or M2-sidA, in which the M2 epitope is fused to the second start site in the originally annotated CC1927 ORF. (B) Each strain from A was grown to mid-exponential phase in rich medium supplemented with glucose to repress expression of the plasmid-encoded construct. Expression was then induced by adding xylose, and cells were imaged by differential interference contrast (DIC) microscopy after 3 and 6 h. (C) The promoter and leader region of CC1927, up through either the first or second annotated methionine, were fused to the coding region of EGFP to generate P_ssidA*.egfp and P_ssidA:egfp, respectively. Strains carrying each construct on a low-copy plasmid were grown in the presence or absence of MMC, and expression was examined by epifluorescence microscopy. Bars, 2 μm.
SidA does not disrupt divisome assembly or localization

How does SidA inhibit cell division? We first tested whether overproducing SidA affected patterns of gene expression. We compared RNA from cells overproducing M2-SidA for 45 or 90 min with cells grown in noninducing conditions on whole-genome DNA microarrays, but did not observe any significant changes to the SOS or CtrA regulons (data not shown). Overproducing SidA also did not affect chromosome content, as measured by flow cytometry, indicating that SidA does not inactivate CtrA. Instead, SidA likely acts post-transcriptionally to inhibit cell division.

In *E. coli*, the SOS-induced regulator SulA disrupts polymerization of the cytokinetic ring protein FtsZ (Mukherjee et al., 1998). To determine whether SidA functions similarly, we introduced the P_{xy}:M2-sidA overexpression plasmid into a strain in which \textit{ftsZ-eyfp} is expressed from the chromosomal vanillate promoter P_{van}. Vanillate was added to cells 1.5 h prior to the addition of xylose to allow FtsZ-EYFP to accumulate to sufficient levels for visualization by epifluorescence microscopy. After growth in xylose for 1.5 h, the majority of cells had begun to elongate, but FtsZ foci were still visible at the constriction site (Fig. 2.5A), indicating that SidA did not disrupt the cytokinetic Z-ring. After 3 and 4.5 h in xylose, cellular filamentation became more severe, but FtsZ foci were still present (Fig. 2.5A). In some cells, the FtsZ ring appeared to move a short distance from the original constriction site (Fig. 2.5A), indicating that FtsZ rings are also able to assemble de novo even in the presence of high levels of SidA. To confirm these results, we used time-lapse microscopy of this same strain grown on an agarose pad containing xylose (Supplemental Movie S2 in (Modell et al., 2011)). This analysis confirmed that FtsZ rings typically remain at constriction sites in individual cells well beyond the time normally necessary for septation, although occasionally the Z-ring relocated to a new position along the long axis of the cell body. Collectively, these results demonstrate that SidA does not disrupt the polymerization, assembly, or stability of FtsZ rings.
A

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91% (n=123)

B

\[ \text{gfp-ftsl} + P_{xyI}^{M2-sidA} \]

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79% (n=172)

C

\[ \text{gfp-ftsl} + P_{xyI}^{M2-sidA} \]

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67
Figure 2.5. SidA does not prevent assembly of the cell division machinery. (A) Subcellular localization of FtsZ was examined in a strain expressing *ftsZ-eyfp* from the vanillate-inducible promoter P_{van} and overexpressing *M2-sidA* from a xylose-inducible promoter on a high-copy plasmid. Cells were grown to mid-exponential phase in rich medium with glucose and then shifted to xylose. Vanillate was added 1.5 h before shifting to xylose. At the times indicated, samples were taken and cells were imaged by DIC and epifluorescence microscopy (top two rows). The white asterisk indicates a FtsZ ring that is no longer associated with a constriction site and so likely moved and reassembled. (Bottom two rows) Localization of FtsN was examined in a strain expressing an *egfp-ftsN* fusion at its native chromosomal locus and overexpressing *M2-sidA* from a high-copy plasmid. The percentage of cells with foci after 4.5 h in xylose is shown below the last panel. (B) Localization of FtsI was examined in a strain expressing *gfp-ftsl* at its native chromosomal locus and overexpressing *M2-sidA* from a xylose-inducible promoter carried on a high-copy plasmid. Cells were grown to mid-exponential phase in rich medium with glucose and then placed on agarose pads containing xylose to produce M2-SidA. Individual cells were followed by time-lapse microscopy, with phase and epifluorescence images captured at the time points indicated. (C) Cells expressing *gfp-ftsl* and xylose-inducible *M2-sidA* were grown in the presence of xylose for 4.5 h to inhibit cell division and induce cellular filamentation. Cells were then placed on an agarose pad containing glucose to repress *M2-sidA* expression. Individual cells with localized GFP-FtsI were then followed by time-lapse microscopy, with phase and epifluorescence images captured at the time points indicated. Bars, 2 μm.

SidA thus acts downstream from FtsZ assembly to affect cell division. In *E. coli* and *Caulobacter*, the cell division machinery assembles in a stepwise manner, with the recruitment of each component to mid-cell requiring recruitment and assembly of prior components (Goehring and Beckwith, 2005; Goley et al., 2011; Vicente and Rico, 2006). Once the divisome is fully assembled, FtsZ constriction and cell envelope septation proceed, although the signals and mechanisms driving these processes are poorly understood. As FtsZ recruitment is one of the earliest steps of cell division, SidA could block the recruitment of a late-arriving cell division protein. Alternatively, SidA may not affect divisome assembly and may instead inhibit the constriction process.

To distinguish between these two possibilities, we examined the localization of the cell division protein FtsN, which is one of the late-arriving essential components recruited to the division site (Costa et al., 2008; Goley et al., 2011; Möll and Thanbichler, 2009). The P_{xy}:*M2-sidA* overexpression plasmid was introduced into a strain expressing *egfp-ftsN* from its native, chromosomal locus. When grown in the presence of glucose, EGFP-FtsN foci were seen at mid-cell only in very late, deeply pinched predivisional cells. When grown in xylose to induce SidA production, these FtsN foci remained intact, always localizing at or near the constriction site (Fig. 2.5A). Similar results were obtained with Venus-FtsW (see below; Fig. 2.9B). The
persistence of FtsZ, FtsW, and FtsN foci suggests that SidA does not disrupt the assembly of the cell division apparatus; instead, SidA likely inhibits the active process of constriction.

Despite the persistence of FtsN foci, SidA could somehow prevent the localization of another divisome component while still allowing FtsN assembly. We therefore tested whether SidA overproduction affected the localization of FtsI, another essential, late-arriving cell division protein that participates in septal peptidoglycan synthesis. For these experiments, the $P_{xyf}:M2$-sidA overexpression plasmid was introduced into a strain expressing gfp-ftsI from its chromosomal locus. Cells were grown initially in glucose and then shifted to xylose to induce sidA and were followed by time-lapse microscopy. As expected, cells became filamentous owing to the accumulation of SidA. GFP-FtsI foci were seen localized to mid-cell and were maintained through the duration of the 4.5 h cells were imaged (Fig. 2.5B).

Taken together, our data suggest that, in cells overproducing SidA, the cell division machinery is fully assembled but somehow inactivated. To test whether cell division can occur once SidA is removed, we examined the behavior of cells harboring the $P_{xyf}:M2$-sidA plasmid and expressing gfp-ftsI. Cells were grown initially in xylose for 4.5 h to inhibit cell division and were then returned to medium containing glucose to shut off sidA expression. Within 30 min, many cells were noticeably more pinched at the same sites initially containing GFP-FtsI foci, and, after an hour, these cells had divided (Fig. 2.5C). Importantly, at time points during and immediately preceding division, the GFP-FtsI foci did not appear significantly brighter than the first time point, regardless of initial intensity. These analyses suggest that SidA does not drive delocalization of FtsI and that cells are poised, once SidA is cleared, to complete cell division.

Mutations in ftsW and ftsI suppress the effects of overproducing SidA

To identify the protein(s) targeted by SidA, we screened for mutations that suppress the growth defect of sidA overexpression by isolating mutants that form colonies despite SidA overproduction, which is normally lethal. Wild-type cells were transformed with a high-copy
plasmid harboring P_{xyI}:M2-sidA and grown on plates containing xylose. This process yielded 80 colonies; an identical transformation with an empty vector yielded tens of thousands of colonies, indicating that suppressors of SidA overproduction are rare. To ensure that suppression did not result from mutations on the sidA overexpression plasmid, we tested whether plasmids from each colony could, in a clean background, still disrupt cell division. We also confirmed by Western blotting that putative suppressor strains still produced high levels of M2-SidA in xylose. In total, we identified nine strains that supported growth despite the production of high levels of M2-SidA. Strikingly, six of the nine suppressors mapped to ftsI or ftsW, both of which encode late-acting, transmembrane components of the cell division machinery. FtsI participates directly in the synthesis of septal peptidoglycan as a periplasmic transpeptidase, while FtsW is a polytopic membrane protein that likely translocates peptidoglycan precursors from the cytoplasm to the periplasm (Errington et al., 2003; Hölte, 1998; Mohammadi et al., 2011). The amino acid substitutions in FtsW that suppress sidA overexpression—A31K, F145L, and T180A (found twice)—are predicted to lie adjacent to the cytoplasmic end of transmembrane helix 1, the cytoplasmic end of transmembrane helix 4, and the periplasmic end of transmembrane helix 6, respectively (Fig. 2.6A). The substitutions identified in FtsI—I45V and F58V—are located in the cytoplasmic tail and the center of FtsI's single-transmembrane helix, respectively (Fig. 2.6A).

For the remaining three suppressor strains, no mutations were found in any known divisome/fts gene.

Each ftsW and ftsI suppressor mutation was introduced into a clean wild-type genetic background by allelic replacement, and no growth or morphological phenotypes were seen during growth in rich medium (Fig. 2.7). We then transformed each strain with the M2-sidA overexpression plasmid and inoculated serial dilutions on rich-medium plates containing xylose. Each suppressor mutation conferred a significant growth advantage on xylose as compared with wild-type cells transformed with the same plasmid (Fig. 2.6B), and each mutation significantly reduced the cellular filamentation that results from overexpressing sidA (Fig. 2.6C).
Figure 2.6. Mutations in ftsW and ftsI suppress the SidA overproduction phenotype. (A) Summary of a sidA overexpression suppressor screen. The location of mutations identified in FtsW and FtsI are indicated on schematics representing the domain structure of each protein. (B) Each suppressor mutation was introduced into a clean wild-type background by allelic replacement, followed by transformation with the M2-sidA overexpression plasmid. Each strain was then grown to mid-exponential phase, and serial dilutions were plated on PYE supplemented with chloramphenicol to maintain the plasmid and with xylose to induce SidA. (C) Cellular morphology of strains
harboring each suppressor mutation and overexpressing M2-sidA for 6 h. Bars, 2 µm. (D) Cellular morphology of strains harboring each suppressor mutation and a deletion of lexA. The doubling time of each strain in rich medium is indicated below the corresponding micrograph. (E) The location of a predicted transmembrane domain within sidA is shown schematically at the top and directly below in an alignment of the coding region of SidA orthologs. The location of a single predicted transmembrane domain within ftsI is shown schematically at the bottom and directly above in an alignment of the transmembrane domains of FtsI orthologs. The alignments are aligned to each other, using the last predicted amino acid as an anchor point. The arrow indicates a conserved phenylalanine in both alignments. Black and gray shading indicate residue conservation and similarity, respectively, found at that position in >50% of aligned sequences. (F) Subcellular fractionation of cells overexpressing M2-sidA from a xylose-inducible promoter on a high-copy plasmid for 1.5 h and expressing cckA-gfp from the chromosome. Samples from cells grown in either glucose or xylose, as indicated, were fractionated into soluble (S) and membrane (M) fractions. Samples were separated by SDS-PAGE, transferred to a PVDF membrane, cut into three pieces, and probed with antibodies specific for GFP, CtrA, or the M2 epitope.

Each mutation also significantly reduced the cellular filamentation of a AlexA strain (Fig. 2.6D), which, as noted (see Fig. 2.3), is strictly dependent on sidA. The FtsW(F145L) and FtsI(I45V) mutations led to the most complete suppression of both growth and morphological phenotypes (Fig. 2.6C,D). These results suggest that SidA inhibits cell division at a late stage by modulating an activity of FtsW or FtsI.

![Figure 2.7](image)

Figure 2.7. ftsW and ftsI suppressor mutations display wild-type morphology during growth in rich medium. Each suppressor mutation was introduced into a clean wild-type background by allelic replacement. Cells were grown in log phase and imaged by DIC microscopy.

**SidA resides in the inner membrane and interacts directly with FtsW and FtsN**

As the suppressors of sidA overexpression mapped near or within the transmembrane domains of FtsW and FtsI, we considered whether SidA is also localized to the inner membrane. An analysis using TMHMM (Krogh et al., 2001) predicted that residues 9–29 of SidA constitute a transmembrane domain. In the middle of this domain is a highly conserved phenylalanine; a conserved phenylalanine is also found in the middle of the transmembrane domain of FtsI, which was also the site of the mutation F58V in FtsI that suppressed sidA overexpression (Fig. 2.6E).
To determine the subcellular localization of SidA, we fractionated cells overproducing M2-SidA and probed both membrane and soluble fractions with an α-M2 antibody. SidA was found exclusively within the membrane fraction after 1.5 h of overproduction (Fig. 2.6F). As controls, we confirmed that the cytoplasmic response regulator CtrA was found predominantly in the soluble fraction, while the sensor histidine kinase CckA was found exclusively in the membrane fraction.

To determine whether SidA interacts physically with FtsW, FtsI, or any other divisome component, we used a bacterial two-hybrid system based on the reconstitution of a split adenylate cyclase (Karimova et al., 2005). We found that, of the essential divisome components FtsZ/A/K/Q/L/B/W/I/N, SidA interacted most strongly with FtsW and to a lesser extent with FtsN (Fig. 2.8A). Interestingly, FtsW and FtsN also interacted strongly with each other, as reported recently in *E. coli* (Alexeeva et al., 2010). Although SidA did not interact with full-length FtsI, neither did FtsW (data not shown), as expected based on studies in *E. coli* (Mercer and Weiss 2002). We therefore tested a truncated version of FtsI (FtsIΔC) lacking its periplasmic catalytic domain. This construct did interact weakly with FtsW and FtsN, but did not interact with SidA. However, an interaction between FtsIΔC and SidA was observed when full-length, untagged *ftsW* was cotranscribed with *ftsIΔC*, suggesting that FtsIΔC and SidA can bind FtsW simultaneously and likely do not compete for the same site. The strength of the SidA–FtsW and FtsW–FtsN interactions were approximately fivefold lower than a soluble zip–zip homodimerization-positive control. However, divisome components must interact in the membrane, where protein levels and molecular orientations may be more limited than with the cytoplasmic zip–zip interactions. Collectively, these interaction studies demonstrate that (1) FtsW binds to FtsI and FtsN, with these three proteins likely forming a subcomplex within the cytokinetic ring, and (2) SidA directly binds FtsW and FtsN and is brought in close proximity to FtsI via FtsW. We also found that, in cells synthesizing low levels of M2-EGFP-SidA, fluorescent foci were frequently seen at pinch sites within cells (Fig. 2.9A). These foci were
often relatively dim, similar to the foci of Venus-FtsW observed in cells synthesizing M2-SidA (Fig. 2.9B) and in contrast to FtsZ-YFP foci (Fig. 2.5A). These data are consistent with a model in which SidA binds FtsW, a low-abundance divisome component.

Figure 2.8. SidA interacts with the late-arriving divisome components FtsW and FtsN. (A) Bacterial two-hybrid analysis of interactions between M2-SidA and cell division proteins fused to T18 and T25, as indicated. Interactions were quantified using a Miller assay and are reported relative to empty vector controls, which yielded 60 Miller units. Each interaction was measured in triplicate; error bars represent the standard error of the mean. FtsA# indicates the FtsA-MalF(TM) fusion described in the Materials and Methods. FtsIAC was tested alone and while producing untagged FtsW, as indicated. Asterisks indicate a statistically significant difference (P < 0.05, one-sided t-test) relative to empty vector controls. (B,C) Bacterial two-hybrid analysis of interactions between M2-SidA (B) or FtsN (C) fused to T18 and mutants of FtsW fused to T25. Interactions are reported as a percentage of that measured for wild-type FtsW with M2-SidA (B) or with wild-type FtsN (C).
Figure 2.9. SidA and FtsW localize to constriction sites. (A) Subcellular localization of SidA was examined in a strain expressing \textit{M2-egfp-sidA} from a xylose-inducible promoter on a high-copy plasmid. Cells were grown to mid-exponential phase in rich media supplemented with glucose and imaged by phase and epi-fluorescence microscopy. (B) Localization of FtsW was examined in a strain expressing \textit{yfp-ftsW} from the vanillate promoter \textit{P_{van}} and overexpressing \textit{M2-sidA} from a xylose-inducible promoter carried on a high-copy plasmid. Cells were grown to mid-exponential phase in rich media with glucose, shifted to xylose and vanillate for 4.5 hours and imaged by phase and epifluorescence microscopy.

We also assessed the ability of the FtsW suppressor mutants to interact with SidA. The suppressor mutations F145L and T180A in FtsW both resulted in a weaker interaction with SidA (Fig. 2.8B), but did not disrupt interaction with FtsN (Fig. 2.8C). The FtsW mutant A31K did not interact with SidA or FtsN, suggesting that it may be misfolded or mislocalized in \textit{E. coli}.

Notably, the substitution F145L significantly reduced the binding of FtsW to SidA, and this
substitution yielded the most complete suppression of *sidA* overexpression (Fig. 2.6C,D). We conclude that SidA likely inhibits cell division through its direct interaction with FtsW, and that mutations in FtsW suppress this inhibition by decreasing its affinity for SidA.

**SidA prevents constriction of the Z-ring without disrupting synthesis of nascent septal peptidoglycan**

Collectively, our data indicate that SidA targets an activity, but not the localization, of the late-stage cell division proteins FtsW, FtsI, and FtsN. Each of these proteins is thought to participate in the synthesis of septal peptidoglycan, a critical aspect of cell division. FtsW likely translocates peptidoglycan precursors into the periplasm, where they are incorporated into the nascent septum by transglycosylases and the transpeptidase FtsI (Mohammadi et al., 2011). The role of FtsN is less clear, although it has been proposed to stabilize the divisome and recruit several nonessential proteins involved in cell wall remodeling (Gerding et al., 2009; Rico et al., 2010). Given its affinity for FtsW, SidA could inhibit cell division by preventing the translocation of septal peptidoglycan precursors into the periplasm. To test this possibility, we examined cells stained with fluorescein-tagged vancomycin (Van-FL), which is thought to label sites of nascent peptidoglycan synthesis by binding peptidoglycan precursor monomers and growing chains in the periplasm (see the Materials and Methods; Fig. 2.11; (Daniel and Errington, 2003; White et al., 2010)). In wild-type cells, Van-FL weakly stained the cell periphery, with strong foci visible at deep constriction sites in late predivisional cells and weaker transverse bands at mid-cell in early predivisional cells (Fig. 2.10A). No transverse bands or bright foci of Van-FL were seen in cells (*n* = 57) depleted of *ftsZ* and hence lacking an assembled cytokinesis complex (Fig. 2.10A). These results are consistent with Van-FL recognizing nascent septal peptidoglycan in *C. crescentus*.
Figure 2.10. Overproducing SidA does not inhibit the translocation of septal peptidoglycan precursors. (A) Van-FL staining of wild-type, ftsZ depletion, and ftsW depletion. Wild-type cells were grown to mid-exponential phase in rich medium. The ftsZ depletion strain was grown to mid-exponential phase in the presence of xylose, washed, and then grown in the presence of xylose or glucose for 1.5 h before imaging. The ftsW depletion strain was grown to mid-exponential phase in the presence of vanillate, washed, and then grown without vanillate for 7.5 h before imaging. (B) The M2-SidA overproduction strain was synchronized, released into rich medium containing either glucose or xylose, and imaged at the times indicated. (C) A mixed population of the M2-SidA overproduction strain was imaged after growth in xylose for 4.5 h. In all panels, cells were stained with Van-FL and were imaged by DIC or phase and epifluorescence microscopy. Arrowheads indicate Van-FL staining in transverse bands and foci that are at least 1.5-fold over the cell background (see the Materials and Methods; Fig. 2.11). Bars, 2 μm.
To test whether mid-cell Van-FL foci are dependent on the presence of FtsW, we examined a \( \textit{ftsW} \) depletion strain in which the only copy of \( \textit{ftsW} \) is controlled by the vanillate-inducible promoter \( P_{\text{van}} \) on the chromosome. Cells grown initially in the presence of vanillate were washed and then grown in the absence of vanillate for 7.5 h to deplete FtsW. As expected, these cells showed a marked cell division defect, forming long filaments by 7.5 h that were comparable in length with cells overproducing SidA for 4.5 h (Fig. 2.10A). However, unlike cells overproducing SidA, cells depleted of FtsW were noticeably less pinched and formed long, smooth filaments. Accordingly, Van-FL foci were observed only in 10% of cells \( (n = 70) \) after 7.5 h without vanillate (Fig. 2.10A, arrowheads); these rare foci may arise from incomplete FtsW depletion or compensation by another translocase. We conclude that FtsW is required for cell pinching, cell division, and mid-cell staining with Van-FL, consistent with its proposed role in translocating peptidoglycan precursors to the periplasm.

To examine the effects of SidA on Van-FL staining, we synchronized cells containing the \( M2\text{-sidA} \) overexpression plasmid and released swarmer cells into either glucose or xylose. In glucose, transverse bands and foci of Van-FL were seen in predivisional cells; after 90 min, these cells had divided and the daughter cells did not have foci, as expected (Fig. 2.10B). In xylose, bands and foci of Van-FL were also seen in predivisional cells despite the overproduction of
SidA (Fig. 2.10B). After 95 min, cells grown in xylose had not divided and most cells still contained a focus of Van-FL at mid-cell. We also examined a mixed population of cells overproducing SidA for 4.5 h and found that Van-FL foci were seen at pinched sites in 70% (n = 83) of these filamentous cells (Fig. 2.10C). Although we cannot rule out that SidA has a mild effect on the flippase activity of FtsW, these data suggest that SidA likely does not inhibit cell division by abolishing the synthesis of septal peptidoglycan. Instead, our results collectively suggest that the FtsW/I/N subcomplex is critical in triggering the final stages of cytokinesis, and that SidA blocks this step of cell division through a direct interaction with FtsW.
Discussion

Following DNA damage, cells typically must inhibit cell division to allow time for DNA repair. In bacteria, the paradigm for damage-induced inhibition of cytokinesis has been the depolymerization of FtsZ by SulA (Justice et al., 2000; Mukherjee et al., 1998). However, sulA homologs are found only in γ-proteobacteria, and it has been unclear whether other damage-induced inhibitors also target FtsZ. Here, we identified sidA and showed that it encodes the primary SOS-induced cell division inhibitor in C. crescentus. Unlike SulA in E. coli, as well as many other known regulators of cell division, SidA does not directly disrupt FtsZ polymerization or stability. Cells overproducing SidA retain a clear FtsZ ring at mid-cell, but cannot constrict (Fig. 2.5). Overproducing SidA also did not affect the subcellular localization of FtsW, FtsI, or FtsN, suggesting that SidA does not interfere with divisome assembly. Instead, our results indicate that SidA inhibits constriction by targeting an activity of the late-acting cell division proteins FtsW, FtsI, and FtsN (Fig. 2.12). Mutations in both ftsW and ftsI were identified in an unbiased screen for suppressors of SidA overproduction. In addition, SidA was found to bind directly to FtsW and FtsN, but, importantly, not to the FtsW suppressor mutants.

What are the activities of FtsW, FtsI, and FtsN in bacterial cytokinesis and how does SidA affect them? These three essential cell division proteins are among the last components recruited to the cytokinetic ring, where they likely form a subcomplex. Furthermore, each component has been implicated in septal peptidoglycan synthesis. FtsW is a polytopic membrane protein and has been proposed to translocate or “flip” peptidoglycan precursors from the cytoplasm to the periplasm, where they are incorporated into existing peptidoglycan strands (Mohammadi et al. 2011). FtsI, also known as penicillin-binding protein 3 (PBP3), harbors transpeptidase activity in its periplasmic domain and cross-links septal peptidoglycan (Spratt, 1977). The precise role of FtsN in cytokinesis is unknown, but its essential function resides within its periplasmic linker domain (Möll and Thanbichler, 2009). Our results suggest that SidA does not inhibit the putative flippase activity of FtsW. In cells overexpressing sidA, the presence of Van-FL staining at...
constriction sites indicates that septal peptidoglycan precursors are present in the periplasm. SidA could target septal peptidoglycan modification activities of FtsW, FtsI, or FtsN that occur in the periplasm after precursor flipping. However, this possibility seems unlikely, given that SidA comprises only 29 amino acids and so lies almost entirely within the inner membrane. Moreover, the mutations in ftsW and ftsI that suppress sidA overexpression are all found within the transmembrane or cytoplasmic domains.

Figure 2.12. A DNA damage checkpoint regulating cell division in C. crescentus. Model for regulation of cell division by SidA following DNA damage. The schematic at the top shows the Caulobacter cell cycle and indicates the progression of cell division, beginning with assembly and initial stabilization of FtsZ rings in stalked cells, followed by constriction in late predivisional cells, and resulting finally in cell division. When DNA damage occurs, the FtsZ ring is still assembled, but cell division is inhibited while cells continue to elongate. SidA inhibits cell division by inserting into the membrane and binding FtsW and FtsN. The expression of sidA is under SOS control, and hence is induced following DNA damage and cleavage of the LexA repressor.
Our results suggest that, in addition to controlling septum synthesis, FtsW, FtsI, and FtsN may also play central roles in triggering the final constriction of the cytokinetic ring, and that SidA may disrupt this function to inhibit cell division. As noted, we identified mutations in \( ftsW \) and \( ftsI \) that suppress the effects of overproducing SidA. The substitutions in FtsW likely suppress by preventing the binding of SidA to the cytokinetic ring. However, a similar explanation does not hold for the substitutions in FtsI, as SidA did not bind FtsI in our two-hybrid system. Instead, these mutations may allow FtsI to help trigger constriction even when SidA is bound to FtsW, thereby bypassing cell division inhibition. FtsI has also been suggested to serve as a checkpoint signal that triggers Z-ring constriction in \( E. \ coli \), possibly via interactions with FtsA (Corbin et al., 2004), and FtsW and FtsI form a ternary complex with FtsZ in \( M. \ tuberculosi s \) (Datta et al., 2006). Although we did not observe an interaction between \( Caulobacter \) FtsI and FtsA or FtsZ, FtsI may act through an as-yet-unidentified component of the cytokinetic ring. The identification of \( sidA \) overexpression suppressor mutations that do not map to the \( fts \) genes should prove illuminating in this regard. Finally, we note that FtsW, FtsI, and FtsN may not trigger constriction, and SidA could simply be using FtsW and FtsN as a docking site from which it directly inhibits the activity of another divisome component, such as FtsZ. Although we did not observe strong interactions between SidA and any of the other essential Fts cell division proteins, this possibility cannot be ruled out completely.

The identification of SidA underscores how little is known about the execution of cytokinesis in bacteria. The hierarchical dependencies underlying assembly of the cell division apparatus have been well documented in recent years, largely through the study of fluorescently tagged proteins. While a map of localization dependencies is useful, it remains a significant challenge to define the biochemical activities of each cell division protein and to understand their interdependencies and their roles in regulating cell division.
Is SulA an outlier?

SulA and the depolymerization of FtsZ has long been the paradigm for regulated cell division inhibition in bacteria. Consequently, the characterization of other SOS-induced cell division inhibitors has focused on Z-ring assembly and stability. However, in many cases, such as YneA in *B. subtilis* (Kawai et al., 2003), DivS in *C. glutamicum* (Ogino et al., 2008), and Rv2719c in *M. tuberculosis* (Chauhan et al., 2006), a direct interaction with FtsZ has not been documented. Moreover, in none of these cases did inhibitor overproduction result in complete Z-ring dissociation, as with SulA. In light of our results, we speculate that these inhibitors could target cell division steps downstream from Z-ring assembly or stability, as has been proposed recently for YneA and Rv2719 (Chauhan et al., 2006; Kawai and Ogasawara, 2006; Mo and Burkholder, 2010). Consistent with this possibility, YneA, DivS, and Rv2719c are each single-pass transmembrane proteins like SidA but in contrast to the cytoplasmic SulA. In addition, Rv2719c and YneA contain periplasmic domains with putative peptidoglycan-binding motifs, and Rv2719c exhibits cell wall hydrolase activity in vitro. It is thus quite plausible that these inhibitors also target FtsI, FtsW, or another late-acting divisome component, almost all of which possess transmembrane and/or periplasmic domains. The three-protein complex SpoLID, SpoIM, and SpoIIP in *B. subtilis* is also thought to block cytokinesis at a late stage in mother cells during sporulation (Eichenberger et al., 2001).

Why target divisome components other than FtsZ? We speculate that the regulation of late cell division events may be more effective at inhibiting division in cells that already have fully formed, stable FtsZ rings. Targeting a late-acting divisome component may be easier for the cell than dismantling an existing cytokinetic ring. Interestingly, Z-ring assembly occurs much earlier during the cell cycle in *Caulobacter* than in *E. coli*, perhaps necessitating a target other than FtsZ (Quardokus et al., 2001). Alternatively, this strategy may prevent the wasteful disassembly and reassembly of the cell division machinery during periods of transient DNA damage. Modulating the activity of cell division proteins may also be a more effective and reliable means of inhibiting
cytokinesis than blocking component localization or assembly. The efficacy of inhibiting the activity of late-stage cell division proteins is reinforced by the fact that many antibiotics, such as cephalexin, target FtsI activity to effectively disrupt cell division in many bacteria.

Redundancies in cell division regulation

Is SidA the only mechanism for inhibiting cell division following DNA damage in Caulobacter? The fact that a sidA deletion almost completely suppressed the cellular filamentation of ΔlexA mutants argues that SidA is the primary SOS-induced cell division inhibitor. However, the increase in aberrant cell divisions following DNA damage in ΔsidA cells was modest and the majority of ΔsidA cells did not divide, suggesting that additional mechanisms for inhibiting cell division must exist. As noted, a similar situation exists in E. coli, where sulA mutations suppress the filamentation of ΔlexA strains but sulA mutants still filament following DNA damage (Hill et al., 1997).

One possible additional mechanism is the damage-dependent down-regulation of CtrA, which controls the expression of the cell division genes ftsQ and ftsA (Fig. 2.1B; Wortinger et al., 2000). The down-regulation of CtrA may prevent divisome assembly in cells that have yet to synthesize FtsQ or FtsA, while SidA may block constriction in damaged cells that have already assembled the divisome. Consistent with this model, we found that mixed populations of cells treated with DNA-damaging agents exhibited varying levels of pinching, possibly reflecting whether a divisome was fully formed when DNA damage occurred (Fig. 2.1B).

The presence of unreplicated DNA at mid-cell following DNA damage may also prevent cell division. Such a mechanism, termed “nucleoid occlusion,” has been described in E. coli and B. subtilis, where the proteins SlmA and Noc, respectively, bind throughout the chromosome to inhibit FtsZ assembly in nucleoid-proximal areas (Bernhardt and de Boer, 2005; Wu and Errington, 2004). In Caulobacter, the nucleoid occlusion model was initially dismissed because Z-rings form when the nucleoid is still present at mid-cell. However, a nucleoid
occlusion-like mechanism could exist that inhibits later stages of cell division rather than FtsZ assembly.

**Final perspectives**

The discovery of SidA also underscores the increasingly recognized role of small proteins in bacterial regulatory responses. Bacterial genomes encode scores of small (<50 amino acids) proteins that have been missed in genome annotation projects or eluded identification in genetic and proteomic screens. Recent studies have begun to systematically identify these small proteins, and, intriguingly, a disproportionate number are predicted to be single-pass transmembrane proteins that accumulate in stress conditions (Hemm et al., 2010; Hemm et al., 2008). In nearly all cases, the functions of these small proteins are unknown, but some could target the cell division machinery like SidA does.

Finally, while cell division in *Caulobacter* has been known to depend on DNA replication and chromosome segregation (Degnen and Newton, 1972), dependencies are not necessarily enforced by dedicated surveillance or checkpoint systems (Hartwell and Weinert, 1989). The identification of SidA demonstrates that *Caulobacter* does, in fact, employ checkpoint systems to regulate its cell cycle under times of stress and DNA damage. In *E. coli*, SulA and UmuD mediate DNA damage checkpoints (Huisman and D'Ari, 1981; Opperman et al., 1999), and, in *B. subtilis*, a developmental checkpoint couples the status of DNA replication to the initiation of sporulation (Burkholder et al., 2001). Cell cycle checkpoints that coordinate the timing and order of cell cycle events to maintain genome integrity thus appear to be a common and powerful regulatory strategy in bacteria, as in eukaryotes.
Materials and Methods

Strains, plasmids, and growth conditions

Strains and plasmids used are listed in Supplemental Table S2 in (Modell et al., 2011), with construction details and growth conditions provided in the Supplemental Material.

DNA microarrays

RNA expression profiling was done as described previously (Biondi et al., 2006). Complete data sets are provided in Supplemental Table S1 in (Modell et al., 2011). Genes selected for Figure 2.1 had expression values that increased or decreased by twofold or greater in at least four of the six conditions tested.

Microscopy

For time-lapse microscopy, cells were immobilized on 1% agarose pads made with PYE medium. Images were acquired on a Zeiss Axiovert 200M microscope with a 63× phase or αFluar 100×/1.45 objective using an Orca II ER camera. For epifluorescence, illumination was provided by an HBO103 arc lamp using the following emission/excitation filters: YFP, S500/20x and S535/30m; GFP, S470/40x and 520/40m; mCherry, S572/35x and S632/60m. All image capture and processing was done through Metamorph software (Universal Imaging Group).

Immunoblots and biochemical fractionations

Immunoblots were performed as follows: Samples were normalized in sample buffer to 0.5 OD\textsubscript{600}/50 μL, resolved on 12% sodium dodecyl sulfate-polyacrylamide gels, and transferred to polyvinylidene difluoride transfer membrane (Pierce). Membranes were probed with polyclonal rabbit α-CtrA and α-GFP (Invitrogen) at a 1:5000 dilution and monoclonal mouse α-Flag (Sigma) at a 1:1000 dilution. Secondary HRP-conjugated α-rabbit (Pierce) or α-mouse (Pierce) were used at a 1:5000 dilution.
Biochemical fractionation was performed as described (Möll and Thanbichler, 2009) with several modifications. Samples from each culture were adjusted to total 8 mL*OD₆₀₀ units, washed once with 0.2 M Tris-HCl (pH 8), and resuspended in 1 mL of 60 mM Tris-HCl (pH 8), 0.2 M sucrose, 0.2 mM EDTA, 200 µg mL⁻¹ lysozyme, and 5 U of DNase I. The resuspension was incubated for 10 min at room temperature, frozen at -80°C, thawed, and sonicated for 10 sec. Intact cells and chromosomal DNA were pelleted by centrifugation at 4000g for 10 min. Membranes were then pelleted by centrifugation at 126,000g for 1 h at 4°C. Two-hundred microliters of the supernatant representing the soluble fraction was diluted in 4× SDS sample buffer. The membrane pellet was washed once with 0.2 M Tris-HCl (pH 8) and resuspended in 200 µL of 1× SDS sample buffer. Equal volumes from each fraction were loaded for immunoblot analysis.

Identification of SidA overproduction suppressors

Wild-type cells were transformed with plasmid pML1716-sidA and plated on PYE supplemented with chloramphenicol and xylose. Single colonies were grown overnight in PYE containing chloramphenicol and xylose and samples were taken for immunoblots, plasmid preparations, and archiving. To isolate chromosomal suppressor mutations and eliminate mutations arising in the sidA overexpression plasmid, we screened for colonies that met three criteria. (1) We used immunoblotting to check that SidA production in each suppressor strain was similar to that seen in wild-type cells transformed with the pML1716-sidA plasmid and grown in xylose for 1.5 h. (2) Plasmids from the suppressor strains were transformed into wild-type cells and plated on PYE supplemented with xylose or glucose. The presence of thousands of colonies on glucose plates and few colonies on xylose indicated a functional plasmid. (3) Plasmids from the suppressor strains were sequence-verified.
Nine strains fulfilled the three criteria above and were deemed likely to harbor chromosomal suppressors. We sequenced the essential fts genes from each strain. Mutations identified in ftsW and ftsI were then engineered into a clean wild-type background by allelic replacement.

**Bacterial two-hybrid analysis**

Two-hybrid complementation assays were performed essentially as described (Karimova et al., 2005). OD\textsubscript{420} readings were obtained with a Spectramax 340PC\textsuperscript{384} plate reader (Molecular Devices), and the average rate (dOD\textsubscript{420}/dt) during the 10- to 20-min time points was used to calculate enzyme activity.

**Van-FL labeling**

Van-FL (Molecular Probes) was prepared in DMSO at 3 mg/mL and cells were labeled at a final concentration of 3 \(\mu\)g/mL for 20 min. Cells were then pelleted at 13,000 rpm for 8 sec, washed in PBS, and imaged directly by DIC or phase and epifluorescence microscopy. Fluorescent bands and foci were measured in ImageJ (http://rsbweb.nih.gov/ij), and those with intensities at least 1.5-fold over the cell background were scored as significant (see also Fig. 2.11).
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Note added in proof

In B. subtilis, the cell division inhibitor Maf was also shown recently to directly target a protein other than FtsZ, called DivIVA (Briley et al., 2011).
References


Chapter 3

A DNA damage-induced, SOS-independent checkpoint regulates cell division in *Caulobacter crescentus*

This work is in submission as Modell, J. W., Kambara, T. K., and Laub, M. T. A DNA damage-induced, SOS-independent checkpoint regulates cell division in *Caulobacter crescentus*.

JWM, TKK and MTL conceived and designed the experiments. JWM and TKK performed the experiments.
Abstract

Cells must coordinate DNA replication with cell division, especially during episodes of DNA damage. The paradigm for cell division control following DNA damage in bacteria involves the SOS response where cleavage of the transcriptional repressor LexA induces a division inhibitor. However, in Caulobacter crescentus, cells lacking the primary SOS-regulated inhibitor, sidA, can often still delay division post-damage. Here we identify didA, a second cell division inhibitor that is induced by DNA damage in an SOS-independent manner. DidA does not disrupt assembly of the division machinery and instead binds the essential division protein FtsN to block cytokinesis. Intriguingly, mutations in FtsW and FtsI, which drive the synthesis of septal cell wall material, can suppress the activity of both SidA and DidA, likely by causing the FtsW/I/N complex to hyperactively initiate cell division. Finally, we demonstrate that DidA and SidA act synergistically to inhibit division as cells lacking both inhibitors divide prematurely following DNA damage, with lethal consequences.
Introduction

Progress through the cell cycle requires the sequential execution of three fundamental processes: DNA replication, chromosome segregation, and cell division. Maintaining the precise order of these events is crucial to preserving genomic integrity, as any attempt to divide before completing DNA replication or chromosome segregation could result in the scission of DNA and a failure to endow each daughter cell with a complete genome. Coordinating DNA replication and cell division is particularly challenging when cells encounter DNA damaging agents that necessitate lengthy periods of chromosome repair before replication can finish and cell division can resume. To ensure the order of cell cycle events and preserve genome integrity, many cells employ checkpoints that actively halt cell cycle progression until DNA damage has been repaired. While checkpoints are prevalent and well-characterized in eukaryotes (Harper and Elledge, 2007), their role and significance in governing the bacterial cell cycle is less clear.

The α-proteobacterium Caulobacter crescentus, which replicates once and only once per cell division, is an excellent system for understanding the bacterial cell cycle. In particular, cells are easily synchronized and DNA replication initiates only once per cell division, resulting in distinguishable G1, S, and G2 phases. As with most bacteria, cell division in Caulobacter involves the assembly of a large multi-protein complex at mid-cell that drives constriction of the cell envelope and separation of daughter cells (Goley et al., 2011). The position of the division machinery is established by the tubulin homolog FtsZ, which forms a ring-like structure at mid-cell and subsequently recruits other essential cell division proteins (Goley et al., 2011; Quardokus et al., 2001; Wortinger et al., 2000). Once assembled, how these proteins coordinate the various steps of cytokinesis is unclear and the factor(s) that ultimately trigger cytokinesis are unknown.
Like eukaryotes, bacteria can inhibit cell division following DNA damage. The best-studied mechanism involves the so-called ‘SOS response’ (Erill et al., 2007; Little, 1991) in which DNA damage stimulates the recombinase RecA to trigger an auto-catalytic cleavage of the transcriptional repressor LexA. This cleavage leads to induction of SOS genes, many of which are involved in DNA recombination and repair (Butala et al., 2009; Little, 1991). The SOS regulon also typically includes a cell division inhibitor that can delay cytokinesis until after damage is cleared. The best characterized SOS-induced division inhibitor, *E. coli* SulA, disrupts polymerization of FtsZ and thus inhibits assembly of the cell division machinery, known as the “divisome” (Huisman and D’Ari, 1981; Mukherjee et al., 1998). However, *sulA* is not widely conserved beyond the γ-proteobacteria and recent studies have indicated that the SOS-induced division inhibitors from several Gram-positive species do not target FtsZ, although in most cases the direct target remains unknown (Chauhan et al., 2006; Kawai et al., 2003; Ogino et al., 2008).

In *Caulobacter* the primary SOS-induced division inhibitor is a 27 amino acid inner membrane protein called SidA that inhibits division by interacting directly with the late-arriving division protein FtsW (Modell et al., 2011).

Although *sidA* is the primary SOS-induced division inhibitor in *Caulobacter*, cells lacking *sidA* can still arrest division when grown in the presence of the DNA damaging agent mitomycin C (MMC). An SOS-regulated endonuclease called BapE may indirectly contribute to inhibiting division (Bos et al., 2012), but we conjectured that *Caulobacter* likely encodes another direct cell division inhibitor that is induced by DNA damage but in an SOS-independent manner. Here we identify such an inhibitor, now named *didA*, that is induced following DNA damage even in strains that cannot mount an SOS response, demonstrating the existence of an SOS-independent transcriptional response to DNA damage. Like *sidA*, the overexpression of *didA* in undamaged cells is sufficient to prevent cell division. DidA does not disrupt FtsZ ring formation or divisome
assembly and instead likely inhibits division through a direct interaction with the divisome component FtsN. Intriguingly, point mutations in *ftsW* and *ftsI* suppress the lethality that results from overproducing either SidA or DidA. Our results suggest that these mutations hyperactivate the cell division process and implicate the protein complex FtsW/I/N in the triggering of cytokinesis. Finally, we show that *Caulobacter* cells are highly sensitized to MMC only when both *sidA* and *didA* are deleted indicating that under certain conditions of DNA damage, these inhibitors are functionally redundant.
Results

Identification of didA, a DNA damage-induced, SOS-independent cell division inhibitor

Our previous work demonstrated that sidA is the primary SOS-induced division inhibitor in Caulobacter. However, many ΔsidA and ΔrecA cells exposed to the DNA damaging agent mitomycin C still become filamentous suggesting that another, SOS-independent inhibitor may also prevent division following DNA damage (Fig. 3.1; (Modell et al., 2011)). To identify candidate SOS-independent division inhibitors, we examined global gene expression changes following mitomycin C (MMC) treatment of a ΔrecA strain, which cannot induce SOS genes. Wild-type and ΔrecA cells were grown to mid-exponential phase in rich medium and exposed to MMC for 30 minutes. RNA was then isolated and compared to mock treated cells on whole-genome DNA microarrays (Table S1 in submitted manuscript).

Figure 3.1. Filamentation in sidA and recA mutants. Wild-type, ΔsidA and ΔrecA cells were grown to mid-exponential in PYE and treated with 1 μg/mL MMC or left untreated. After 3 hours, cells were imaged by phase microscopy.
Of the 50 most up-regulated genes following MMC treatment in wild-type cells, 44 were recA-dependent, including 31 that are directly regulated by LexA (da Rocha et al., 2008; Modell et al., 2011) (Fig. 3.2A, 3.3A). The remaining 6 damage-regulated genes showed similar induction levels in both wild-type and ΔrecA backgrounds (Fig. 3.2A) and are thus likely controlled by an SOS-independent mechanism. One of these genes, CCNA03212 in the NA1000 (CB15N) genome, encodes a previously uncharacterized 71 amino acid protein with a single predicted transmembrane helix flanked by short cytoplasmic and periplasmic domains (Fig. 3.2B). The open reading frame of CCNA03212 overlaps with the C-terminus of the open reading frame of CC3114 from the annotated genome of CB15. In our expression profiling experiments, only those probes lying within the CCNA03212 coding sequence were significantly upregulated by MMC in wild-type cells (Fig. 3.3B-C), suggesting that the NA1000 annotation is correct. Based on the studies described below, we named this gene didA for damage-induced cell division inhibitor A.

To confirm that the didA locus encodes a damage-inducible protein, we created a strain in which the chromosomal didA coding sequence was fused at its C-terminus to the coding region of the 3xFLAG (3xF) epitope. DidA-3xF was barely detectable in the absence of DNA damage, but was strongly induced following MMC treatment with protein levels increasing nearly 20-fold after 1 hour (Fig. 3.2C). Western blotting indicated a band at the size predicted for DidA-3xF (~11 kDa) and not CC3114-3xF (~25 kDa) indicating that the larger gene product annotated in CB15 is likely not produced at significant levels. To test the SOS-dependence of DidA-3xF synthesis following MMC treatment, we examined DidA-3xF production in a ΔrecA strain and in a strain harboring lexA(K203A), which encodes a non-cleavable form of LexA that blocks the induction of an SOS response. In each case, DidA-3xF was slightly elevated in untreated cells, likely due to increased basal levels of damage in the absence of SOS-mediated repair (Fig. 3.2D). Following MMC treatment, DidA-3xF was strongly induced in all strains (Fig. 3.2D), consistent with an SOS-independent mode of regulation.
Figure 3.2. *didA* is induced by DNA damage and is not SOS regulated. (A) Wild-type and Δ*recA* cells were grown in a rich medium (PYE) to mid-exponential phase and treated with 1 μg/mL MMC for 30 minutes. Expression values, calculated from the average of two biological replicates, are shown for the 50 most upregulated genes in wild-type cells with fold-change ratios calculated in comparison to mock treated cells. Expression values in wild-type cells are plotted on the x-axis and those in Δ*recA* cells are plotted on the y-axis. The dashed line corresponds to fold-change values that are identical in wild-type and Δ*recA* cells. For complete data, see Figure 3.3 and Table S1 in submitted manuscript. (B) CC3114 and CCNA03212 (*didA*) are shown schematically in their genomic context. Nucleotide positions relative to the annotated CC3114 start site are shown below. The gray shaded region represents a predicted transmembrane domain (TMHMM). (C) Western blot of cells expressing *didA* fused to a C-terminal 3xFLAG epitope at the native *didA* chromosomal locus. Cells were grown in PYE to mid-exponential phase and treated with 1 μg/mL MMC for the times indicated. (D) Western blot of wild-type, Δ*recA* and lexA(K203A) cells expressing *didA-3xFLAG* from its native locus treated with 1 μg/mL MMC for 1 hour. Membranes (C-D) were blotted with the α-FLAG antibody.
Figure 3.3. Annotated gene expression profiles. (A) Transcriptional profiles for the 50 most up-regulated genes during DNA damage in wild-type cells (see Fig. 3.2A) are shown with their corresponding CC numbers and NA1000 annotation. The ‘LexA’ column shows genes whose upstream region contains a sequence match to 7 of the 8 bases in the *Caulobacter* LexA consensus binding site (GTTCN-GTTC) (da Rocha et al., 2008). Genes whose log-fold changes post-damage in ΔrecA cells are below 50% of those in wild-type cells are marked as ‘RecA-dependent’. All other genes are marked as ‘RecA-independent.’ (B) The positions of microarray probes representing
CC3114 and CCNA03212 are shown below the genes as horizontal bars. The four right-most probes were used to calculate expression values for CCNA03212 (didA). (C) The transcriptional profiles for each probe in panel B are shown.

To test whether DidA is a cell division inhibitor, we fused the didA coding sequence to the vanillate-inducible promoter P\textsubscript{van} and cloned this construct into both a low- and medium-copy plasmid. We transformed wild-type cells with each plasmid and then grew cells in the presence of vanillate to induce didA in the absence of a DNA damaging agent. Synthesis of DidA from a low-copy plasmid resulted in mild cellular filamentation and a modest growth defect, while overproduction from the medium-copy plasmid caused a more pronounced division defect with nearly all cells demonstrating severe filamentation after 6 hours (Fig. 3.4A-B). Thus, DidA, like SidA, is sufficient to inhibit cell division in the absence of DNA damage. Taken together, our results suggest that following DNA damage, DidA accumulates in an SOS-independent fashion to help prevent cell division.
DidA interacts with the late-arriving divisome component FtsN

We next sought to establish whether DidA interferes with cell division directly, through an interaction with the divisome, or indirectly by inducing the SOS regulon or inhibiting the cell cycle regulator CtrA. To investigate the possibility of the latter, indirect mechanisms, we isolated RNA from cells overproducing DidA from a medium-copy plasmid for 45 minutes and compared it on DNA microarrays to RNA from similarly treated cells grown in the absence of inducer. No significant gene expression changes were observed in the SOS or CtrA regulons (data not shown) suggesting that DidA acts post-transcriptionally, and possibly directly, to inhibit cell division.

To further explore how DidA inhibits cell division, we examined its subcellular localization. In predivisional cells, the major components of the cell division machinery are located at mid-cell (Goley et al., 2011) where they synthesize a septum and drive invagination of the cell envelope. To assess DidA localization, we transformed wild-type cells with a low-copy plasmid harboring an M2-yfp-didA fusion under the control of a xylose-inducible promoter, P_{xyt}. After induction for 3 hours, cells were typically more filamentous than those expressing untagged didA from the same plasmid for 6 hours (Fig. 3.4B, 3.5A), suggesting that the YFP-DidA fusion is functional and perhaps more stable or potent than DidA alone. Notably, YFP-DidA foci were frequently observed at pinch sites near mid-cell (Fig. 3.5A) placing it in close proximity to the cell division machinery. Further, the fractionation of cells overproducing M2-DidA from a plasmid indicated that DidA is strongly enriched in the membrane where many of the middle- and late-arriving cell division components also reside (Fig. 3.5B). These data are consistent with a model whereby DidA inhibits division through a direct interaction with a component of the division apparatus.
Figure 3.5. DidA is a small, inner-membrane protein that interacts directly with FtsN. (A) The subcellular localization of DidA was examined in a strain expressing m2-yfp-didA from the xylose-inducible promoter P\textsubscript{xy} on a low-copy plasmid. Cells were grown to mid-exponential phase in PYE with glucose and then shifted to xylose. At the times indicated, cells were imaged by phase and epifluorescent microscopy. (B) Subcellular fractionation of cells overexpressing 3xFLAG-didA from the P\textsubscript{van} promoter on a medium-copy plasmid for 1.5 hours and expressing the transmembrane protein cckA-gfp from the chromosome. Samples were fractionated into soluble (S) and membrane (M) fractions and analyzed by Western blot. The membrane was cut into three pieces, indicated by dashed lines, and probed with antibodies specific for the GFP, CtrA, or M2 epitope. (C) Bacterial two-hybrid analysis of interactions between T25-DidA and cell division proteins fused to T18, as indicated. The interacting pair T18-M2-SidA and T25-FtsN was included for comparison. E. coli strains harboring each pair of fusions were plated on LB, and colonies were restruck on MacConkey plates containing maltose. Red streaks indicate positive interactions. (D) Subcellular localization of FtsZ, FtsW, FtsI, and FtsN were examined in strains expressing ftsZ-yfp from the chromosomal P\textsubscript{van} promoter, or venus-ftsW, gfp-fsi or gfp-ftsN from its native chromosomal locus. Each strain was transformed with a medium-copy plasmid expressing didA from the P\textsubscript{van} promoter. Strains were grown to mid-exponential phase in PYE and samples imaged by phase and epifluorescent microscopy after addition of vanillate for 4.5 hours. In the fluorescent images, cell outlines were drawn based on the phase micrographs. Bars, 2 μm. (E) The strains from panel D were grown to mid-exponential phase and 10-fold serial dilutions were plated on PYE supplemented with vanillate to induce didA expression.
Although many regulators of cell division disrupt positioning or assembly of the FtsZ ring, several SOS-induced inhibitors, including SidA, do not. To test for direct interactions of DidA with the known set of critical Caulobacter cell division components (Goley et al., 2011), we performed a bacterial two-hybrid analysis with the reconstituted adenylate cyclase system used previously for SidA (Karimova et al., 2005; Modell et al., 2011). When expressed from the low-copy plasmid pKT25, a T25-DidA fusion interacted almost exclusively with the late-arriving cell division protein fusion T18-FtsN (Fig. 3.5C, 3.6). Identical results were obtained with a T18-DidA fusion on the high-copy plasmid pUT18C and individual division proteins produced from the low-copy plasmid pKT25, although the DidA-FtsN interaction was slightly weaker in this orientation (data not shown). SidA, whose primary target is FtsW, also interacts, to some extent, with FtsN; this interaction was at least as strong as the DidA-FtsN interaction, as judged by this two-hybrid assay (Fig. 3.5C). In sum, our data suggest that DidA is an integral membrane protein that localizes to mid-cell where it may disrupt cell division through a direct interaction with FtsN.

Figure 3.6. DidA interacts directly with FtsN. Bacterial two-hybrid analysis of interactions between T25-DidA and cell division proteins fused to T18, as indicated. Each pair was plated on LB, and colonies were restruck on MacConkey plates containing maltose.
FtsN is among the last cell division proteins to arrive prior to cytokinesis. Although its precise molecular function is unknown, FtsN interacts with multiple division proteins and is thought to play a key role in stabilizing the assembled divisome (Goehring et al., 2007; Karimova et al., 2005; Rico et al., 2010). To ask whether DidA destabilizes or blocks assembly of the cell division machinery through its interaction with FtsN, we examined the localization of early- and late-arriving division proteins during DidA overproduction. Cells producing fluorescently tagged FtsZ, FtsW, FtsI, or FtsN were transformed with a plasmid for overexpressing didA and then grown in the presence of vanillate to induce DidA synthesis. After 4.5 hours of induction, cells expressing ftsZ-yfp, venus-ftsW, or gfp-ftsI were inhibited for cell division, but 89%, 95% and 85% of cells, respectively, contained fluorescent foci at or near visible pinch sites. These results indicate that DidA likely does not disrupt the localization of cell division proteins or drive the disassembly of division protein complexes (Fig. 3.5D).

Additionally, we noted that many cells displayed multiple foci of the FtsZ, FtsW, or FtsI fluorescent fusions, a pattern rarely seen in the absence of DidA; this result suggests that DidA also does not prevent the formation of new division assemblies.

Intriguingly, cells expressing gfp-ftsN were noticeably shorter and more pinched than those expressing the other fluorescent fusions (Fig. 3.5D). Further, cells expressing gfp-ftsN were able to robustly form colonies despite DidA overproduction, in contrast to cells expressing the other fluorescent fusions or the wild type (Fig. 3.5E). The GFP-FtsN fusion therefore acts as a DidA suppressor, possibly by decreasing its affinity for DidA or by stabilizing FtsN and thereby increasing FtsN levels. Whatever the case, these data further support a model in which DidA interacts with FtsN to block cell division but without disrupting assembly of a full, intact division apparatus.
Mutations in *ftsW* can suppress the division inhibition caused by either SidA or DidA

To further explore the mechanism by which DidA inhibits division, we screened for spontaneous mutations that suppress the lethality of overproducing DidA. Wild-type cells carrying a medium-copy plasmid expressing 3xF-*didA* from P<sub>van</sub> were grown on plates containing vanillate to induce M2-DidA synthesis. Because wild-type cells overproducing M2-DidA cannot form colonies (Fig 3.7B), those rare colonies arising on vanillate plates represent strains harboring putative suppressor mutations. From roughly 3 x 10<sup>7</sup> plated cells, 34 suppressors were identified but only one strain retained high levels of functional M2-DidA. Whole genome resequencing identified a putative suppressor mutation in *ftsW*, which would produce the substitution A246T in the predicted large periplasmic loop of FtsW (Fig. 3.7A). This *ftsW* mutation was created *de novo* in a clean CB15N background and confirmed to suppress the lethality of overproducing DidA. As noted, no interactions between DidA and FtsW were observed in our two-hybrid analysis; thus FtsW(A246T) likely suppresses DidA overproduction, not by preventing binding of the inhibitor, but by promoting an activity of FtsW (Modell et al., 2011).

Intriguingly, we had previously found mutations in *ftsW* that suppress the lethality of overproducing SidA. We therefore reasoned that SidA and DidA may function similarly to inhibit cell division. To explore this possibility, we asked whether other, previously identified suppressors of SidA overproduction could also suppress DidA overproduction, and *vice versa*. Two mutations primarily suppressed the lethality of only one of the inhibitors; the FtsW(T180A) strain strongly suppressed overproduction of M2-SidA but not DidA, whereas the strain producing a GFP-FtsN fusion suppressed the activity of DidA but not M2-SidA. These inhibitor-specific suppressors may prevent binding of their respective inhibitors. The other mutations examined showed varying abilities to suppress the lethality associated with overproducing either inhibitor. In particular, the strains producing FtsW(F145L) or FtsW(A246T) showed robust suppression of both inhibitors.
Figure 3.7. Mutations in \( ftsW \) suppress SidA and DidA overproduction phenotypes. (A) Schematic showing the membrane topology of the division proteins FtsW, FtsI, and FtsN, as well as the division inhibitors SidA and DidA. Missense mutations and the GFP-FtsN fusion that suppress the activities of SidA or DidA, or both, are listed in red. (B) The suppressor mutations from panel A were introduced into a wild-type strain by allelic replacement followed by transformation with a medium-copy plasmid expressing either M2-sidA from the \( P_{ery} \) promoter or didA from the \( P_{van} \) promoter. To induce M2-sidA, strains were grown in PYE supplemented with glucose and then plated on PYE supplemented with xylose. To induce didA, strains were grown in PYE and plated on PYE containing vanillate. Each strain was plated in 10-fold dilutions.

The ability of these single substitutions, F145L and A246T, to suppress the lethality of overproducing either SidA or DidA could indicate that the inhibitors share a binding site within FtsW that is disrupted by the suppressor mutations. However, we viewed this as unlikely given that (1) DidA does not bind FtsW in our bacterial two-hybrid system and (2) M2-SidA binds to FtsW(A246T) as strongly as it does to wild-type FtsW (Fig. 3.8). As an alternative explanation, we hypothesized that the sub-complex of late-arriving division components FtsW, FtsI, and FtsN could exist in one of two conformations: an active conformation that promotes constriction of the inner membrane and septum, and an inactive conformation that is promoted or stabilized by
SidA and DidA. In this model, the suppressor mutations in *ftsW* and *ftsI* would promote the active conformation and thus enable cell division even in the presence of SidA and DidA.

![Figure 3.8](image)

**Figure 3.8.** SidA interacts directly with FtsW. Bacterial two-hybrid analysis of interactions between T18-M2-SidA and FtsW mutants fused to T25 as indicated. Colonies were grown to exponential phase in LB and 5 μL aliquots plated on MacConkey agar containing maltose.

**SidA and DidA suppressor mutations drive hyperactive cell division**

If the FtsW(F145L) and FtsW(A246T) mutations promote an active conformation of a subcomplex of cell division proteins, cells harboring these mutations, but not producing SidA or DidA, may attempt division at an earlier stage of the cell cycle than wild-type cells, even in the absence of DNA damage. To explore this possibility, we grew strains harboring one of the suppressor mutations in *ftsW*, or in *ftsI*, into mid-exponential phase in rich medium and measured cell lengths in a large population of cells. Indeed, many of the suppressor mutations resulted in cells that were significantly shorter on average than the wild type even though their growth rates were not substantially different (Fig. 3.9A-B, 3.10A-B). The degree of shortening roughly correlated with the strength of suppression, as cells harboring the mutations *ftsW(A246T)*, *ftsW(F145L)*, and *ftsI(I45V)* that were best able to suppress both SidA and DidA were also the shortest. For *ftsW(A246T)*, which yielded the strongest suppression, all cell types were, on average, ~0.3 microns shorter than wild-type cells (Fig. 3.10B). These observations suggest that the *ftsW(A246T)* strain is not simply enriched for swarmer cells, the shortest *Caulobacter* cell type, but that these mutant cells divide as shorter predivisional cells resulting in shorter swarmer and stalked daughter cells.
Figure 3.9. Mutations that suppress *sidA* and *didA* overexpression likely hyperactivate cell division. (A-B) The *sidA* and *didA* suppressor mutations, each introduced into a clean wild-type background by allelic replacement, were grown to mid-exponential phase in PYE and phase images were taken. Bar (A), 2 µm. Cell lengths, relative to wild-type, were calculated for each strain (n > 440 for each strain); error bars (B) represent standard error of the mean. (C) Wild-type, *ftsW(A246T)*, and Δ*sidA ΔdidA* cells were grown to mid-exponential phase and plated in 10-fold dilutions on PYE containing 0.35 µg/mL MMC, 0.35 µg/mL novobiocin, or 7.5 µg/ml cephalexin, as indicated.

Figure 3.10. Suppressor mutant growth properties. (A) Wild-type and *ftsW(A246T)* cells were grown to mid-exponential phase and imaged by phase microscopy. Cell lengths were quantified from 491 wild-type and 610 *ftsW(A246T)* cells using MicrobeTracker and summarized as a histogram with the maximum frequency for each strain normalized to 1. (B) Growth curves for the strains from Fig. 3.9C grown in PYE.
Another prediction of our model is that cells with the mutations causing hyperactive division may be sensitive to other stresses that, like DNA damage, require a transient inhibition of cell division. Novobiocin inhibits DNA gyrase and causes replication arrest without directly damaging DNA. Cells must therefore inhibit division to prevent scission of unreplicated DNA that remains at mid-cell. Cephalexin inhibits penicillin-binding proteins and prevents the crosslinking of septal cell wall material. An attempt to activate divisome constriction without the corresponding synthesis of septal cell wall could result in membrane or envelope stress. We focused on \textit{ftsW(A246T)}, the strongest suppressor of SidA and DidA overproduction, and found that cells expressing this allele exhibited a viability defect when grown on plates containing MMC, novobiocin, or cephalexin (Fig. 3.9C). These results are consistent with a model in which \textit{ftsW(A246T)} cells cannot properly delay division when stressed.

\textbf{SidA and DidA redundantly regulate division during MMC treatment}

Given that SidA and DidA are induced concomitantly following MMC treatment, what are the relative contributions of each inhibitor toward regulating cell division? Our previous work demonstrated that \textit{\Delta sidA} cells show modest division defects during DNA damage, perhaps because DidA can compensate functionally. To examine the roles of SidA and DidA during DNA damage, we constructed a strain in which all but the first and last three amino acids of \textit{didA} were deleted. As with the \textit{sidA} deletion strain, \textit{\Delta didA} cells grown on plates containing MMC showed no major viability defects (Fig. 3.11A). However, a strain lacking both \textit{sidA} and \textit{didA} showed a pronounced viability defect with a nearly 100-fold decrease in plating efficiency (Fig. 3.11A) suggesting that at least one of the inhibitors is necessary to properly control cell division post-damage. This viability defect was rescued by the presence of either inhibitor on a low-copy plasmid (Fig. 3.11B) confirming that in the presence of MMC, SidA and DidA are, to some extent, functionally redundant.
Figure 3.11. Cells lacking *sidA* and *didA* cannot properly regulate cell division following DNA damage. (A) Wild-type, *ΔsidA*, *ΔdidA*, and *ΔsidA ΔdidA* cells were grown to mid-exponential phase and plated in 10-fold dilutions on PYE containing 0.35 μg/mL MMC. (B) Wild-type and *ΔsidA ΔdidA* cells carrying an empty plasmid, and *ΔsidA ΔdidA* cells carrying a plasmid with either *sidA* or *didA* driven by their native promoters were plated as in panel A. (C-E) Synchronous populations of swarmer cells from the strains in panel A were placed on agarose pads containing PYE and MMC and then imaged for 8 hours by time-lapse microscopy. (C) The time to first mid-cell division and (D) the percentage of cells that stopped growing following division are shown (for criteria on calling division events and growth cessation, see Supplemental Materials and Methods). Asterisks represent a statistically significant (*p* < 0.01) difference relative to the wild type. Error bars represent standard error of the mean. (E) Representative fields of wild-type and *ΔsidA ΔdidA* swarmer cells grown on pads containing MMC are shown at the time points indicated in hours. Black arrows indicate cells that divided. Red arrows indicate cells arrested for growth following division. Bar, 2 μm.

To better understand the nature of MMC sensitivity in *ΔdidA ΔsidA* cells, we used time-lapse microscopy to examine synchronous populations of swarmer cells during growth on agarose pads containing MMC. Wild-type swarmer cells grown at this concentration of MMC
did not divide for ~5 hours on average, which is significantly longer than the standard doubling time of ~90 minutes in MMC-free media (Fig. 3.11C, 3.11E). Roughly 5% of wild-type cells arrested growth following a cell division event (Fig. 3.11D), indicating that division may have been premature or inappropriately executed and consequently was lethal. The single deletion strains, ΔsidA and ΔdidA, were also able to delay division in the presence of MMC, although they divided slightly earlier than the wild type by ~30 (p = 0.02) and ~15 (p = 0.34) minutes, respectively. These single deletion strains also had twice as many growth arrested cells following division events compared to the wild type, although these defects were apparently insufficient to produce a gross viability defect (Fig. 3.11A). In contrast, ΔdidA ΔsidA cells lacking both inhibitors divided ~1.25 hours earlier than wild-type (p = 6.9 x 10^-10), and more than 25% of cells exhibited growth defects following a division event (Fig. 3.11C-E). Taken together, our data suggest that the lethality experienced by ΔdidA ΔsidA cells in the presence of MMC results from an inability to appropriately delay cell division.

We also assessed the viability of ΔdidA ΔsidA cells on plates containing novobiocin or cephalixin. Neither of these conditions directly damage DNA nor do they strongly induce sidA or didA expression (data not shown). Accordingly, ΔdidA ΔsidA cells were not sensitive to novobiocin and were only weakly sensitive to cephalixin suggesting that DidA and SidA do not play significant roles during these stress responses (Fig. 3.9C). These findings are consistent with our model in which the sensitivity of ftsW(A246T) cells during these same stresses results from an abnormal propensity to divide and not a reduced affinity for DidA or SidA.


Discussion

**SOS-independent regulation of the DNA damage response**

During episodes of DNA damage, cells often use checkpoint systems to transiently inhibit the cell cycle and prevent cell division (Hartwell and Weinert, 1989). In bacteria, the regulatory paradigm for responding to DNA damage has long been the *E. coli* SOS system in which cleavage of the repressor LexA drives the transcription of DNA repair genes as well as the cell division inhibitor *sulA* (Huisman and D'Ari, 1981; Mukherjee et al., 1998; Opperman et al., 1999). SOS-induced division inhibitors have subsequently been identified in a range of other bacteria, including *sulA* homologs in γ-proteobacteria and the unrelated genes *yneA, divS, chiZ* and *sidA* in various other species (Chauhan et al., 2006; Dullaghan et al., 2002; Kawai et al., 2003; Modell et al., 2011; Ogino et al., 2008). Although these SOS-dependent regulators are often assumed to be the primary, or even sole, mechanism for inhibiting division post-damage, there have been hints of SOS-independent division regulation. For instance, in *E. coli, B. subtilis,* and *Caulobacter,* cells lacking their SOS-induced inhibitors, or that cannot induce an SOS response, can still become filamentous following DNA damage indicating an alternative means of blocking cell division (Hill et al., 1997; Howe and Mount, 1975; Huisman et al., 1980; Jaffé et al., 1986; Liu et al., 2001). However, to the best of our knowledge, no damage-induced, SOS-independent division regulators have been previously documented. Here, we identified *didA* in *Caulobacter* as one such regulator.

How do *Caulobacter* cells recognize and respond to DNA damage to induce *didA* if not through the canonical derepression of SOS genes? One possibility is that cells monitor the same signal, but with a different protein sensor. The SOS system measures the accumulation of ssDNA, which stimulates RecA to trigger the autocatalytic cleavage of LexA (Craig and Roberts, 1980; Little and Mount, 1982; Slilaty et al., 1986). Another protein, such as the RecA homolog RadA, could also recognize ssDNA, but ultimately affect the activity of a different transcription
factor. Alternatively, a DNA damage sensor unrelated to RecA could recognize a distinct type of DNA damage or DNA structure. In *B. subtilis*, the diadenylate cyclase DisA monitors genome integrity and may recognize branched DNA structures that arise during the recombination-based repair of double-strand breaks (Witte et al., 2008). When paused at such DNA structures, DisA is prevented from synthesizing cyclic-di-AMP (c-di-AMP), a diffusible molecule required for the activation of the transcription factor Spo0A, thereby coupling DNA damage with transcription (Bejerano-Sagie et al., 2006; Oppenheimer-Shaanan et al., 2011; Witte et al., 2008). It remains unclear precisely how c-di-AMP affects Spo0A activity in *B. subtilis* and whether a c-di-AMP-based response to DNA damage extends to other organisms. Nonetheless, *didA* transcription could follow a similar regulatory strategy that relies on c-di-AMP, or another damage-regulated second messenger.

Another potential mechanism driving the SOS-independent induction of *didA* could involve the conserved replication protein DnaA that also serves as a transcription factor. In *B. subtilis*, replication stress somehow activates DnaA to transcriptionally regulate ~50 genes (Breier and Grossman, 2009; Goranov et al., 2005). In *E. coli*, DnaA may also help respond to replication stress by inhibiting cell division in an SOS-independent manner, but this pathway remains poorly characterized (Fujimitsu et al., 2008; Gon et al., 2006). It is unclear whether the transcriptional activity of *Caulobacter* DnaA is affected by DNA damage and it is unknown whether DnaA regulates *didA*. Nonetheless, *didA* transcription could, in principle, be driven by DnaA or another transcription factor that responds to replication stress and stalled forks, which often accompany DNA damage.

*The execution and regulation of cell division*

Many cell division inhibitors, including *E. coli* SulA, block cell division by disrupting FtsZ polymerization. FtsZ is an effective target as it recruits most other cell division proteins. However, neither DidA nor SidA affect the assembly or stability of FtsZ rings in *Caulobacter*. 
Instead, these inhibitors appear to block cell division by targeting FtsW, FtsI, and FtsN. Bacterial two-hybrid studies indicated that DidA interacts with FtsN. Additionally, a chromosomally encoded GFP-FtsN fusion was able to suppress the effects of DidA overproduction, supporting a model in which DidA inhibits cell division by binding to FtsN. SidA interacts with FtsW and FtsN in a two-hybrid system, and the lethality of overproducing SidA can be suppressed by mutations in either FtsW or FtsI (Modell et al., 2011). Although DidA and SidA bind different proteins, these two inhibitors may ultimately inhibit division in similar ways as two mutations in \textit{ftsW}, and one in \textit{ftsI}, can suppress the effects of overproducing either SidA or DidA.

FtsW, FtsI and FtsN are among the last essential proteins recruited to the cytokinetic ring. These proteins physically interact with each other and likely form a sub-complex within the divisome that drives the synthesis and remodeling of the septal cell wall. FtsW translocates cell wall precursors into the periplasm at mid-cell which are then incorporated into a mature septum by transglycosylases and the transpeptidase domain of FtsI (Mohammadi et al., 2011; Tipper and Strominger, 1965; Wise and Park, 1965). The function of FtsN is less clear, although in \textit{Caulobacter}, its essential activity is located within a periplasmic linker domain (Möll and Thanbichler, 2009). In both \textit{Caulobacter} and \textit{E. coli}, FtsN also recruits proteins involved in cell wall remodeling to the division site (Bernhardt and de Boer, 2003; Goley et al., 2010; Möll et al., 2010; Peters et al., 2011; Poggio et al., 2010).

How do single mutations in FtsW and FtsI prevent the inhibition of cell division by both SidA and DidA? One possibility is that these mutations reduce the affinities of SidA and DidA for their division protein targets. However, SidA binding to FtsW was unaffected by the A246T mutation and DidA binds FtsN, not FtsW or FtsI, at least in our bacterial two-hybrid system. A second possibility is that SidA and DidA block the recruitment of even later arriving proteins. As noted, FtsN may help recruit cell wall remodeling factors such as the peptidase DipM and the peptidoglycan amidase AmiC (Bernhardt and de Boer, 2003; Möll et al., 2010). Although the
genes encoding such proteins are individually dispensible, it is formally possible that SidA and DidA disrupt the recruitment of multiple peptidoglycan remodeling factors, thereby preventing division. However, given that the inhibitory activity of both SidA and DidA can be suppressed by mutations in FtsW and FtsI, this model seems unlikely.

Instead, we favor a model in which the FtsW/FtsI/FtsN subcomplex exists in two conformations: an inactivate conformation that is stabilized by SidA or DidA, and an active conformation which drives cytokinesis (Fig. 3.12A). We propose that the substitutions that suppress both SidA and DidA, such as FtsW(A246T), may effectively lock FtsW/FtsI/FtsN in the active state allowing cells to bypass the block in division normally caused by an accumulation of these inhibitors. On their own, these suppressor mutations cause cells to initiate division hyperactively. In support of this model, cells with the suppressing mutations were reproducibly shorter than wild-type cells (Fig. 3.9A-B), likely because they divide at a slightly earlier stage of the cell cycle. Additionally, FtsW(A246T) cells are sensitive to novobiocin and cephalixin, treatments that do not cause DNA damage but nonetheless require cells to delay division.
Figure 3.12. Parallel pathways regulate cell division in *Caulobacter* following DNA damage. (A-B) Two cell division inhibitors are induced following DNA damage in *Caulobacter*: *sidA* is induced by cleavage of the SOS repressor LexA while *didA* is induced by unknown regulatory factors. SidA and DidA are both small transmembrane proteins that can block cell division by preventing the divisome sub-complex FtsW/I/N from assuming an active conformation, FtsW/I/N*. FtsW/I/N* could promote division by enhancing peptidoglycan synthesis and remodeling, by triggering FtsZ constriction, or by coordinating these activities.

Taken together, our results suggest that the DNA damage-induced division inhibitors in *Caulobacter* target the FtsW/FtsI/FtsN subcomplex to block constriction of the division machinery and cell envelope. Precisely how SidA and DidA block division is not yet clear, in part because the execution of cytokinesis remains poorly characterized at a molecular level. The synthesis of septal cell wall material could provide the force and directionality for cellular constriction, with FtsZ required mainly for mid-cell positioning of division proteins. In such a case, SidA and DidA could prevent division by directly blocking a critical or rate-limiting peptidoglycan modifying activity of the FtsW/FtsI/FtsN subcomplex. Alternatively, GTP hydrolysis by the FtsZ ring may provide the energy for, and directionality of, constriction, effectively pulling the rest of the cytokinetic ring along with it (Li et al., 2007). Assembly or activity of the FtsW/FtsI/FtsN sub-complex could somehow trigger FtsZ constriction, and the inhibitors SidA and DidA may block this step of division. Finally, it is possible that Z-ring constriction and septum synthesis combine to drive cytokinesis. As FtsW, FtsI, and FtsN are transmembrane proteins with cytoplasmic and periplasmic domains, they are each well positioned to coordinate the Z-ring and nascent septum, and SidA and DidA could disrupt this coordination. Distinguishing between these various models for cytokinesis and elucidating the precise mechanisms of action for SidA and DidA will ultimately require more detailed studies of the FtsW/I/N sub-complex; the mutants identified here, such as FtsW(A246T), may prove particularly useful in these efforts.
Final Perspectives

Our results highlight the FtsW/FtsI/FtsN subcomplex as an important regulatory node in the control of cell division. Following certain types of DNA damage, DidA and SidA appear to function together to prevent inappropriate cell divisions (Fig. 3.12A-B). Such redundancy may afford cells with a fail-safe survival mechanism. Alternatively, or perhaps in addition, SidA and DidA may be differentially induced following different types of DNA damage, providing independent routes to the inhibition of cell division under different conditions. Additionally, we note that although cells lacking both sidA and didA divide prematurely during DNA damage, many still filament to some degree, suggesting that yet other mechanisms of division inhibition exist in Caulobacter. Finally, DidA is the latest in a growing class of small, stress-induced membrane proteins that play critical regulatory roles (Fontaine et al., 2011; Hobbs et al., 2011). These proteins are often missed or incorrectly annotated in genome sequences, but many, like SidA and DidA, clearly play critical roles in regulating cellular processes, including cell division.
Materials and Methods

Strains, plasmids, and growth conditions

Strains and plasmids used in this study are listed in Table S2 in the submitted manuscript with construction details and growth conditions provided in the Supplemental Materials and Methods.

DNA microarrays

RNA expression profiling was done as described (Biondi et al., 2006). Expression experiments were performed in duplicate and the results for each gene were averaged.

Immunoblots and biochemical fractionations

Samples for immunoblots were normalized in sample buffer to 0.5 OD$_{600}$/50 $\mu$L, resolved on 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride transfer membrane (Pierce). Membranes were probed with polyclonal rabbit $\alpha$-CtrA and $\alpha$-GFP (Invitrogen) at a 1:5000 dilution and monoclonal mouse $\alpha$-FLAG (Sigma) at a 1:3000 dilution. Secondary HRP-conjugated $\alpha$-rabbit (Pierce) or $\alpha$-mouse (Pierce) were used at a 1:5000 dilution. Biochemical fractionation was performed as described (Modell et al., 2011).

Microscopy

All phase contrast images were acquired on a Zeiss Observer Z1 microscope with a 100x/1.4 oil immersion objective and an LED-based Colibri illumination system. For additional information on image analysis and time-lapse microscopy, see the Supplemental Materials and Methods.

Bacterial two-hybrid analysis

Two-hybrid complementation assays were performed essentially as described (Karimova et al., 2005). BTH101 cells harboring plasmids with the T25 and T18 fusion constructs were grown
to single colonies on LB agar plates and restruck or spotted on MacConkey agar plates supplemented with maltose for imaging.

**Identification of DidA overproduction suppressors**

Wild-type cells were transformed with a $P_{van}:3xF-didA$ overproduction plasmid and plated on PYE agar in absence of vanillate to allow colony formation. Single colonies were grown overnight in PYE and plated on PYE agar supplemented with vanillate at roughly $2 \times 10^6$ colony forming units per 10 cm plate. Rare colonies were grown overnight in PYE supplemented with vanillate and samples were taken for immunoblots, plasmid preparations and archival. To isolate chromosomal suppressor mutations and eliminate mutations arising in the $3xF-didA$ overproduction plasmid, we screened for colonies that met two criteria. (1) We used immunoblotting to check that 3xF-DidA production in each suppressor strain was similar to that seen in wild-type cells transformed with the same plasmid and grown in vanillate for 1.5 h. (2) Plasmids from the suppressor strains were transformed into wild-type cells and plated on PYE agar supplemented with or without vanillate. The presence of thousands of colonies on plain plates and few colonies on vanillate indicated a functional plasmid. The mutation in the $ftsW(A246T)$ suppressor was identified by whole-genome sequencing.
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References


Chapter 4

Conclusions and future directions
Conclusions

In this work, I identified and characterized two regulators that together allow *Caulobacter* to delay cell division following DNA damage (Fig. 3.12). *sidA* is an SOS-induced division inhibitor and functional analog of *sulA* in *E. coli* and *yneA* in *B. subtilis*. Given that a *sidA* deletion significantly reduced filamentation in a ΔlexA background, which causes constitutive SOS induction, we suspect that *sidA* is the primary, if not only division inhibitor within the SOS regulon. *didA* also accumulated following DNA damage, but its induction occurred even in strains lacking *recA* or harboring a non-cleavable allele of *lexA*, suggesting that its regulation is SOS-independent. Strains lacking either inhibitor still typically delayed division and remained viable during exposure to Mitomycin C (MMC). However, strains lacking both inhibitors divided prematurely and suffered a viability defect under the same conditions. These results illustrate some degree of redundancy within the *Caulobacter* DNA damage response and underscore the presence and importance of an SOS-independent transcriptional response to DNA damage.

Once induced, SidA and DidA are both inserted into the inner membrane at mid-cell where they prevent division. Unlike SulA which completely dissolves Z-rings or the Gram-positive SOS-induced inhibitors which have mild effects on Z-ring assembly, SidA and DidA did not noticeably disrupt Z-ring formation nor any subsequent step in divisome assembly. Instead I provided evidence that each inhibitor disrupts divisome constriction by targeting components of the late-arriving divisome sub-complex FtsW/FtsI/FtsN. By a bacterial two-hybrid analysis, I showed that SidA bound strongly to FtsW and secondarily to FtsN while DidA bound exclusively to FtsN. Furthermore, the *ftsW*T180A mutation and an N-terminal GFP fusion to FtsN selectively suppressed the activities of SidA and DidA respectively, likely by reducing the affinity of each inhibitor for their target. To our knowledge, these are the first examples of endogenous, regulatory division inhibitors with known targets other than FtsZ.
Strikingly, several mutations in \(ftw\) and \(ftsi\) suppressed the effects of both inhibitors with \(ftw(F145L)\) and \(ftw(A246T)\) demonstrating the most complete suppression. An explanation of reduced affinity is unlikely for these suppressors given that by our bacterial two-hybrid analysis, SidA bound FtsW(A246T) as strongly as wild-type FtsW and DidA did not bind FtsW. Instead, we hypothesize that these suppressors cause cells to divide hyperactively. In support of this model, cells harboring suppressor mutations divided prematurely in the absence of DNA damage resulting in a small cell phenotype. Importantly, \(ftw(A246T)\) cells were also sensitive to several additional stresses which likely require division inhibition but do not cause SidA or DidA induction. Taken together, our results highlight FtsW/FtsI/FtsN as a critical regulatory node in \(Caulobacter\) and suggest a new role for the sub-complex in triggering divisome constriction.

**New Roles for FtsW/FtsI/FtsN in cytokinesis**

What are the activities of FtsW and FtsN that are inhibited by SidA and DidA respectively? In *E. coli*, FtsW forms a complex with FtsI in the absence of other divisome components (Fraipont et al., 2011), and it is required for the proper recruitment of FtsI (Mercer and Weiss, 2002) and FtsN (Addinall et al., 1997). In *Caulobacter*, although FtsW may not be strictly required for the recruitment of FtsI or FtsN (Goley et al., 2011), all three proteins interact with each other and likely form a sub-complex within the divisome. The essential function of FtsN is unknown, but it may help to stabilize the assembled divisome (Goehring et al., 2007; Rico et al., 2010). I showed that during SidA or DidA overproduction, FtsZ, FtsW, FtsI and FtsN still localize to nascent division sites indicating that the assembly, recruitment and stabilization activities of FtsW and FtsN are unaffected. A second proposed function of FtsW is to translocate the cell wall precursor lipid II from the cytoplasm into the periplasm at mid-cell where it is incorporated into the invaginating septum. Biochemical evidence for this activity was recently provided as purified FtsW allowed the translocation of lipid II in artificially constructed single-membrane liposomes (Mohammadi et al., 2011). Using a fluorescently-labeled vancomycin reporter, I showed that SidA does not prevent the septal translocation of cell wall precursors into
the periplasm and thus does not inhibit the translocase activity of FtsW. Given that SidA and DidA do not disrupt any known function of FtsW or FtsN in divisome assembly or septum synthesis, we propose that these components likely play previously unappreciated roles in promoting constriction of the assembled divisome.

The identification of mutations in *ftsW* and *ftsI* that suppressed the effects of both inhibitors indicated that despite having distinct binding partners, SidA and DidA could prevent division by inhibiting the same general divisome function. Furthermore, our data suggest that these suppressor mutations do not simply decrease inhibitor affinity, but instead cause divisomes to execute this function hyperactively. We have proposed a unified model whereby this unknown, critical activity could be considered as a single output performed by the FtsW/FtsI/FtsN complex. Accordingly, the complex could exist in one of two conformations: an inactive conformation which is stabilized by the presence of SidA or DidA and an active conformation which is stabilized by the suppressor mutations in *ftsW* and *ftsI*. Understanding the precise nature of this divisome function should provide mechanistic insights into the molecular events that mediate cytokinesis and help to distinguish between the proposed models of divisome constriction.

**Characterizing the FtsW/FtsI/FtsN activity pathway**

In the cell wall-centric model of division, septum synthesizing proteins provide the force and directionality for divisome constriction and cytokinesis. Intriguingly, hyperactive suppressor mutations were found in *ftsI*, which encodes the only essential divisome component with a well-established catalytic activity in septum synthesis. The transpeptidase domain of FtsI introduces crosslinks into the peptide chains that project from the glycan backbone, resulting in mature septal cell wall (Adam et al., 1997). Accordingly, SidA and DidA could inhibit this transpeptidase activity through the intermediates FtsW and/or FtsN. Alternatively, the inhibitors could prevent FtsW, FtsI or FtsN from activating one or more septal transglycosylases or
amidases or from performing yet other functions that are required for synthesizing and shaping the septal cell wall. Several currently available techniques might afford a more complete assessment of peptidoglycan dynamics during SidA and DidA overproduction. Pulse chase experiments with D-Cysteine, which is readily incorporated into peptidoglycan peptide chains, have allowed new areas of cell wall growth to be monitored with high temporal resolution (Aaron et al., 2007). The fluorescent penicillin derivative Bocillin-FL has been used to directly assess of FtsI activity (Eberhardt et al., 2003). Another peptidoglycan labeling technique, using fluorescently tagged tripeptides, can be potentially used to determine regions of amidase activity (Olrichs et al., 2011). Each of these techniques monitor the synthesis of septal cell wall at different stages and might lend support to cell wall-centric regulation by SidA, DidA and the suppressor mutations.

In the FtsZ-centric model of division, FtsZ and perhaps FtsA drive divisome constriction and guide the inward synthesis of the septal cell wall. Under this model, SidA and DidA could prevent FtsW/FtsI/FtsN from triggering a divisome assembly checkpoint in which the late-arriving sub-complex could somehow enable FtsZ constriction. The FtsQ/FtsL/FtsB complex forms contacts with both early- and late-arriving divisome components and is thus an attractive candidate for mediating such a checkpoint (Di Lallo et al., 2003; Karimova et al., 2005). Interestingly, interactions within the divisome occur in part through contacts between the transmembrane domains (van den Berg van Saparoea et al., 2013), and most of the SidA and DidA suppressor mutations mapped within the transmembrane domains of FtsW and FtsI. Alternatively, FtsW/FtsI/FtsN could directly trigger Z-ring constriction and each component has demonstrated direct binding to FtsZ or FtsA in various organisms (Busick et al., 2012; Datta et al., 2002; Datta et al., 2006; Di Lallo et al., 2003; Karimova et al., 2005), although evidence for such interactions in Caulobacter is lacking. Few techniques exist to experimentally assess FtsZ dynamics in vivo. Fluorescence recovery after photobleaching with a fluorescently-labeled copy of FtsZ could allow Z-ring turnover to be examined during SidA and DidA overproduction.
However, it would be difficult to determine whether any observed effects were due to direct regulation on FtsZ or to an indirect consequence of inhibiting any step in cell wall synthesis.

The identification of additional, hyperactive suppressors of SidA and DidA could help to distinguish between the models of division. During our suppressor screens, I isolated only one suppressor twice (ftsW(T180A)) and thus have not screened to saturation. To more readily identify additional mutations that suppress both inhibitors, I have recently developed a plasmid which overproduces SidA from the $P_{xyl}$ promoter and DidA from the $P_{van}$ promoter. Colonies allowing growth under one inducing condition can be restruck on the other condition providing a fast and easy secondary screen for dual suppression. Using this method, I have already recovered one of the original, hyperactive suppressor mutations (ftsW(F145L)). If additional suppressors are found in the transpeptidase domain of FtsI or the periplasmic domains of known or novel divisome components, the cell wall-centric model of division and its inhibition would be supported. Alternatively, suppressors within FtsZ, FtsA or other cytoplasmic FtsZ binding proteins might support the FtsZ-centric model. Ultimately, such a screening strategy would more thoroughly clarify which genetic determinants, both in terms of proteins and domains, constitute the FtsW/FtsI/FtsN activity pathway.

A complementary approach to investigate the downstream effectors of FtsW/FtsI/FtsN activity would be to expand upon our bacterial two-hybrid (BACTH) analysis. The suppressor mutations in $ftsW$ and $ftsI$ could expose or enhance binding sites within the FtsW/FtsI/FtsN complex for other divisome components. SidA and DidA could then prevent division by obstructing these binding sites. To detect such interactions, the FtsW/FtsI/FtsN complex, with or without suppressor mutations, could be co-expressed and tested for interactions with each divisome component. Similarly, a three-hybrid approach could be used to determine whether SidA or DidA could disrupt any of these interactions. As with the saturating suppressor screen, the identities of these interactions could reveal which additional divisome components, if any,
participate in the SidA/DidA signaling pathway. A major caveat with these experiments is that it may not be possible to reconstitute activities of Caulobacter divisome components within the context of an E. coli host expressing its own divisome. It is also possible that additional components of the Caulobacter divisome would have to be co-expressed with FtsW/FtsI/FtsN in order to reveal yet other interactions or achieve proper membrane insertion or orientation.

Cryo-electron microscopy (Cryo-EM) would allow the direct visualization of the inner membrane and septum during SidA and DidA overproduction. Abnormalities in either of these layers could indicate a mechanism of action, although if both layers are similarly prevented from constricting, it would be difficult to determine which one was targeted for inhibition. A more informative Cryo-EM experiment would be one in which movements of the inner membrane and septum were uncoupled. For instance, I observed that strains harboring the hyperactive ftsW(A246T) mutation are sensitive to the FtsI transpeptidase inhibitor cephalexin (Fig). Cells treated with cephalexin cannot insert crosslinks into new septal cell wall and thus likely cannot support the growth of an invaginating septum. If FtsW(A246T) hyperactivity causes premature constriction of the Z-ring, the inner membrane could become uncoupled from growth of the cephalexin-inhibited septum. This result would potentially explain the viability defect of ftsW(A246T) cells treated with cephalexin and lend support to the model of a FtsW/FtsI/FtsN assembly checkpoint regulating a FtsZ-centric division event.

A puzzling aspect of our research is that suppressor mutations are found in divisome components that do not appear to be directly targeted by either inhibitor; mutations in ftsI suppress SidA overproduction, and mutations in ftsW and ftsI suppress DidA overproduction. However, given the close association of each subunit within the FtsW/FtsI/FtsN complex with the other two, it is not difficult to envision scenarios that explain suppressor activities and modes of inhibition (Fig. 4.1). If we consider FtsW, FtsI and FtsN as individual components of a linear activation pathway, an epistasis analysis would place FtsW and FtsI downstream from FtsN and
its inhibition by DidA. Similarly, FtsI would lie downstream from FtsW and its inhibition by SidA with the final pathway appearing FtsN > FtsW > FtsI. Alternatively, the FtsW/FtsI/FtsN complex could harbor a single active site composed of features from each subunit. The binding of SidA and DidA at distinct, possibly allosteric sites could ultimately inhibit the activity of a single active site. Structural studies of FtsW/FtsI/FtsN, in complex with SidA or DidA, would help to distinguish between these models. While it is still difficult to perform crystallographic or structural NMR studies on membrane proteins, several recent technological and analytical advances could facilitate such research (Ubarretxena-Belandia and Stokes, 2012).

Figure 4.1. Models of FtsW/FtsI/FtsN and SidA/DidA activity. On the left, FtsW, FtsI and FtsN are modeled as a linear activation pathway. \textit{ftsW}^* and \textit{fisI}^* denote hyperactive suppressor mutations. On the right, FtsW, FtsI and FtsN are modeled as a complex with a singular function.

**Why target FtsW/FtsI/FtsN?**

Before the identification of SidA and DidA, it was unclear whether the targeting of FtsZ by SulA in \textit{E. coli} was representative of damage-induced cell division inhibitors in Gram-negative bacteria. The Gram-positive SOS-induced division inhibitors, with transmembrane domains and peptidoglycan-binding motifs, seemed to represent a mechanistically distinct group acting downstream from \textit{Z}-ring assembly. As mentioned, it is perhaps logical that Gram-positive
bacteria would regulate later steps in division given the presence of cell walls that are considerably thicker and more complex that their Gram-negative counterparts. For these reasons, it was surprising to find that two damage-induced division inhibitors in Caulobacter, a Gram-negative proteobacterium like E. coli, target divisome components implicated in cell wall synthesis and remodeling, and thus may bear more similarity to the Gram-positive inhibitors.

Why do Caulobacter and E. coli employ different regulatory strategies to execute the same task within a similar, rod-shaped envelope? One possibility is that despite their structural similarities, the two proteobacteria execute division differently, and thus require different forms of regulation. In Caulobacter, SidA and DidA suppressor mutations in ftsW and ftsI exhibit small cell phenotypes indicating that these divisome components perform rate-limiting steps in division. The concept of a rate-limiting step is difficult to imagine when considering a multifunctional division machine that is itself poorly understood. In strict temporal terms, the rate-limiting step could control the onset of division and dictate its timing within the cell cycle, or it could determine the rate at which constriction proceeds once division is triggered. In biochemical terms, there could be a rate limiting enzymatic step within a linear biosynthetic pathway, for instance the translocation of cell wall precursors could be rate-limiting for the synthesis of the septum. Alternatively, a rate-limiting step could serve as a checkpoint that activates several downstream functions in parallel. Energetically, rate-limiting and force-generating steps are frequently related (Brenner and Eisenberg, 1986; Smith et al., 2001), although this is by no means a theoretical requirement. Whichever model or combination thereof is most correct, small cell alleles have been observed in E. coli, with gain-of-function mutations in the FtsZ-associated membrane tether FtsA (Geissler et al., 2007), and in B. subtilis, with N-terminal truncations in FtsL (Bramkamp et al., 2006). Intriguingly, most known regulators of division in E. coli target the Z-ring while in B. subtilis, FtsL has been implicated in mediating division inhibition in response to both YnaA and replication stress (Goranov et al., 2005; Kawai and Ogasawara, 2006). Much additional research will be required to precisely define rate-limiting steps as they
pertain to bacterial cell division. However, it is generally possible that the most critical steps in division are performed by different divisome components or complexes in different species. Although *Caulobacter* and the Gram-positives may have converged upon the inhibition of septum synthesis following DNA damage, under the aforementioned divisome assembly checkpoint model, SidA and DidA could instead target FtsW/FtsI/FtsN activities relating to Z-ring constriction.

In addition to the properties of the divisome itself, several lifecycle and evolutionary distinctions could explain the targeting of different divisome components between the α- and γ-proteobacteria. In *Caulobacter*, the Z-ring is assembled in stalked cells, a cell cycle stage when the nucleoid still occupies the mid-cell (Kelly et al., 1998). Z-ring formation at a similar stage is prevented in *E. coli* by the nucleoid-associated division inhibitor SlmA (Bernhardt and de Boer, 2005). Therefore, a mixed population of *Caulobacter* cells contains a higher percentage of Z-rings compared to *E. coli*, and the inhibition of a later stage in division might be less temporally and energetically costly than the complete dis-assembly and reassembly of Z-rings and divisomes. Alternatively, the choice of a divisome target could reflect the type of DNA damage most frequently encountered by that organism. For instance, if *Caulobacter* evolved during transient episodes of minimal DNA damage, it might be desirable to momentarily prevent divisome activity while leaving it fully assembled and ready to divide once the damage is removed. Alternatively, the inhibition of Z-rings in *E. coli* could reflect a history of more severe DNA damaging agents, during which the complete disassembly of Z-rings more robustly prevented any chance of mistakenly attempting division.

A deeper understanding of these evolutionary questions will require the identification and characterization of additional cell division inhibitors in other bacterial clades. Unfortunately, if the *Caulobacter* inhibitors are any indication, such genes are not always easy to find. Unlike SulA and the Gram-positive inhibitors, SidA and DidA are representative of a growing class of
small proteins which have been understudied as a result of incomplete or inaccurate genome annotations (reviewed in (Hobbs et al., 2011)). Indeed, both genes were mis-annotated in Caulobacter, and their homologs in other α-proteobacteria were often completely unannotated. Directed informatic and biochemical approaches are proving useful in the search for small proteins, and intriguingly, many in E. coli are single-pass transmembrane proteins that play important regulatory roles (Fontaine et al., 2011; Hemm et al., 2010; Hemm et al., 2008). As the repertoire of damage-induced inhibitors grows, it may be informative to determine the activity of each in heterologous species and the consequences of substituting one for another. If some inhibitors are broadly effective, it could be telling to determine consequences of substituting one for another. This type of experimental strategy could be used to support evolutionary and functional hypotheses regarding the selection of regulatory targets within the divisome.

The SOS-independent regulation of didA

The SOS system has long served as the lone example of a bacterial regulatory response to DNA damage. However, the recent discovery of the di-adenylate cyclase DisA in B. subtilis demonstrated that bacteria can in fact respond to damage with multiple recognition elements and signaling pathways (Bejerano-Sagie et al., 2006). Furthermore, the modulation of the transcriptional activities of DnaA in response to replication stress indicates that in B. subtilis, several overlapping modes of regulation can address the downstream consequences of DNA damage in addition to the lesions themselves (Goranov et al., 2005). In E. coli, many anecdotal reports have suggested the existence of SOS-independent responses that help to prevent cell division following damage, although mechanistic insights are lacking. I identified didA, which is to our knowledge the first SOS-independent, damage-induced cell division inhibitor.

How is didA regulated if not by the SOS regulators RecA and LexA? I initially observed DidA induction following exposure to the DNA damaging agent mitomycin C, an alkylating agent that causes a variety of lesions arising from single-strand adducts, double-strand crosslinks
and the intermediate structures resulting from the repair of each lesion. DidA induction could therefore be triggered by any of these structures or by indirect consequences of an MMC treatment, such as replicative or oxidative stress. I am currently investigating the mechanisms of DidA regulation, and our preliminary results have uncovered genetic networks involved in the transcriptional induction of DidA and the stationary phase-specific induction of a phage-like system that likely mediates horizontal gene transfer and induces DidA indirectly.

**DriD is required for DidA induction**

To better understand the signals responsible for DidA induction, I monitored a DidA reporter following a variety of stresses, including DNA damaging agents, replication inhibitors, antibiotics and other physical stressors. Strikingly, DidA was induced exclusively by DNA damaging agents suggesting that its SOS-independent signal is likely derived from a damaged DNA structure (Fig 4.2). I next sought to determine whether DidA was induced by the SOS signal (ssDNA), albeit with different regulatory machinery, or by a distinct damaged structure. DidA and SidA reporter strains were treated with a panel of DNA damaging agents, each with a
Figure 4.2. *didA* is induced specifically by DNA damaging agents. Western blot of a strain expressing *didA* from its native promoter tagged at its C-terminus with the 3xFLAG epitope treated with mitomycin C (MMC), hydroxyurea (HU), cephalixin (ceph), novobiocin (nov), ultraviolet light (UV), ethanol (EtOH), high salt (NaCl), hydrogen peroxide (H₂O₂), kanamycin (kan), oxytetracycline (tet), and chloramphenicol (chlor). Where indicated, cells were treated at high (hi) and low (lo) doses, and blots were probed with α-FLAG (Modell and Laub, unpublished).

different mechanism of action. Indeed, DidA and SidA showed differential induction levels with maximal DidA induction occurring following exposure to zeocin which causes double-strand breaks (Fig. 4.3). Within the eukaryotic DNA damage response, there are proteins that specifically recognize double-strand breaks, and it is tempting to speculate that DidA could participate in a similar network in *Caulobacter*.

![Figure 4.3](image)

*Figure 4.3. sidA and didA are differentially regulated. Western blots of strains expressing gfp from the sidA or didA promoter treated with the DNA damaging agents as indicated at low and high doses were probed with α-GFP (Modell and Laub, unpublished).*

To identify the genes responsible for the transcriptional induction of *didA*, I constructed a strain in which the promoter of *didA* is fused to the chromogenic reporter LacZ (Fig. 4.4). When cells harboring this construct were plated in the presence of MMC at a low concentration, colonies still formed slowly and appeared blue owing to the MMC-induced expression of the DidA reporter. I next mutagenized this strain with the Tn5 transposon and isolated colonies that were unable to induce DidA and thus appeared white. Such strains contained transposon insertions in either the *P_{didA}-lacZ* reporter or an un-annotated transcription factor which we named *driD*. I constructed a clean, in-frame deletion of *driD* and confirmed that DidA was no longer induced following an MMC treatment (Fig. 4.5A). I additionally observed that DriD protein levels remain unchanged during DidA induction suggesting that its activity is regulated post-translationally following DNA damage (Fig. 4.5B).
Figure 4.4. Screening for transcriptional regulators of *didA*. A strain expressing *lacZ* from the *didA* promoter was used to screen for *didA* regulators. Repressors are identified as blue colonies on plates containing no MMC while inducers are identified as white colonies on plates containing MMC (Modell and Laub, unpublished).

Figure 4.5. DriD is activated post-translationally to induce *didA*. (A) Western blot of wild-type and Δ*driD* cells expressing *didA*-3xF from its native locus. (B) Western blot of cells expressing 3xF-*driD* or *driD*-3xF as the only copy of *driD* and *didA*-3xF from its native promoter. Cells in A and B were treated with MMC for 1 hour. Blots were probed with α-FLAG. (Modell and Laub, unpublished).

With the identification of *driD*, we have begun to unravel the genetic network responsible for DidA induction, but many questions remain. Does DriD directly participate in *didA* transcription, or does it recognize a damaged DNA structure? Are double-strand break ends responsible for DriD activation or is there another damaged structure that is enriched during a zeocin treatment? How is DriD activated post-translationally and what genes other than DidA are contained within its regulon? ChIP-PCR and ChIP-seq, in conjunction with a site-specific double-strand break generation system recently developed by our lab, will allow the
characterization of DriD binding sites within the genome, whether they are damaged sites, promoters or both. If DriD indeed recognizes DNA damage directly, gel-shift assays with an assortment of DNA structures should help to home in on the specific DNA damage signal. Finally, microarrays and additional genetic screens will uncover other components of the DriD regulatory network and perhaps shed light on the mechanisms of its post-translational activation.

The identification of a gene transfer agent (GTA)

Using the same $P_{didA}$-lacZ reporter, I performed a screen for transcriptional repressors of $didA$ (Fig. 4.4). Cells harboring this construct form white colonies when plated in the absence of MMC. We conjectured that just as $\Delta lexA$ cells constitutively transcribe SOS genes in the absence of DNA damage, transposon insertions in a $didA$ repressor should constitutively transcribe the lacZ reporter resulting in blue colonies. I isolated blue colonies and mapped the transposon insertions sites, many of which were within genes participating in DNA synthesis and repair which likely increase basal levels of DNA damage. However, several insertions mapped to an un-annotated transcription factor which we named sprD. Surprisingly, cells lacking sprD only showed $didA$ induction when grown into late stationary phase, whereas wild-type cells showed no detectable $didA$ induction in any growth phase (Fig. 4.6A). I next tagged SprD with the 3xFLAG epitope and found that SprD is expressed at very low levels during exponential phase but then accumulates significantly at higher ODs consistent with its ability to repress $didA$ during stationary phase in wild-type cells (Fig. 4.6B). To better understand the role of SprD, I performed a microarray analysis comparing $\Delta sprD$ and wild-type cells grown into stationary phase. Strikingly, $\Delta sprD$ cells showed the dramatic induction of a $\sim$15 gene operon that bears homology to a locus encoding a GTA in the $\alpha$-proteobacterium $R.~capsulatus$ (Leung et al., 2010). GTAs are phage-like particles with capsids that are too small to carry their own genomes (Fig. 4.7; reviewed in (Lang et al., 2012)). Instead they are thought to incorporate random fragments of the host genome into their capsids and then mediate horizontal gene transfer events between the same or closely related species. We believe this genome fragmentation step, which
likely produces double-strand breaks, generates the signal causing \textit{didA} induction in stationary phase cells. In support of this model, cells lacking the GTA locus no longer significantly induce the \textit{didA} reporter construct when grown into late stationary phase (Fig. 4.8A). Furthermore, cells lacking both \textit{sprD} and \textit{driD} no longer induce \textit{didA} during stationary phase (Fig. 4.8B), indicating that the DriD regulatory machinery lies downstream from the GTA induced by the \textit{sprD} deletion.

![Figure 4.6](image1.png)

\textbf{Figure 4.6.} SprD represses \textit{didA} during stationary phase. (A) Wild-type and \textit{ΔsprD} cells expressing \textit{lacZ} from the \textit{didA} promoter were grown from mid-exponential phase into stationary phase. (B) Cells expressing \textit{sprD-3xF} as the only copy of \textit{sprD} were grown from mid-exponential into stationary phase. Samples were taken at the ODs indicated for western blot and probed with \textit{α-LacZ} (A) and \textit{α-FLAG} (B) (Modell and Laub, unpublished).

![Figure 4.7](image2.png)

\textbf{Figure 4.7.} Gene transfer agents (GTAs). The red chromosomal region denotes the GTA genome. When induced, most GTA particles incorporate random fragments of the host genome (blue). Occasionally, a GTA particle will incorporate a fragment of its own genome (red), but the capsid head is too small to fit the GTA genome in its entirety. GTAs can inject their contents into recipient cells where they can be incorporated into the host genome (green) by recombination (adapted from (Lang et al., 2012)).
AsprD AsprD

AsprD/AdriD

WT

AGTA

OD: 0.8 1.0 1.1 0.8 1.0 1.1

- 4 DidA-3xF

Pdd-acZ

Figure 4.8. GTA production in stationary phase induces didA. (A) In a \( \Delta sprD, P_{didA-didA-3xF} \) background, cells harboring or lacking the GTA genomic region were grown into stationary phase. Samples were taken for western blot and probed with \( \alpha \)-FLAG. (B) In a \( \Delta sprD, P_{didA-lacZ} \) background, cells harboring or lacking \( driD \) were grown into stationary phase. Samples were taken at the indicated ODs for western blot and probed with \( \alpha \)-LacZ (Modell and Laub, unpublished).

As with \( driD \), many questions remain regarding \( sprD \) and regulation of the GTA locus. Several GTAs have been identified in other \( \alpha \)-proteobacteria which are no longer capable of performing gene transfer (Lang et al., 2012). I am currently attempting to transfer antibiotic resistance mutations and gene cassettes from \( \Delta sprD \) donors which overexpress the GTA locus to determine if the \( Caulobacter \) GTA has retained its function. I am also individually mutating the genes within the GTA locus to determine which is responsible for generating the signal involved in didA induction. Finally, our work with SprD has generated a number of new questions regarding stationary phase in \( Caulobacter \). Given that cells lacking \( sprD \) only induce didA during late stationary phase, what is the stationary phase-specific signal responsible for the induction of the GTA and didA? GTAs in other species can be regulated by quorum-sensing, although such a system has not been found in \( Caulobacter \). It is also unclear whether there are conditions that allow GTA induction during stationary phase in cells with functional SprD. I am currently devising genetic screens that will allow the dissection of the stationary phase pathways that control SprD activity.

Concluding Remarks

The identification and characterization of \( sidA \) and didA have highlighted considerable diversity within the regulation of bacterial division. One of the best ways to understand a system is to perturb it, and through our work on division inhibitors, we have developed tools and ideas...
that should help clarify the molecular mechanisms of division. Our genetic screens have opened
doors in novel and unexpected areas of *Caulobacter* biology, and future work on the SOS-
independent transcriptional response to DNA damage and the regulation of a gene transfer agent
will further illuminate the richness of regulation that constitutes the bacterial DNA damage
response.
References


