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Induction and Relaxation Dynamics of the Regulatory Network Controlling the Type III Secretion System encoded within *Salmonella* Pathogenicity Island 1

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Summary

Bacterial pathogenesis requires the precise spatial and temporal control of gene expression, the dynamics of which are controlled by regulatory networks. A network encoded within *Salmonella* Pathogenicity Island 1 controls the expression of a type III protein secretion system involved in the invasion of host cells. The dynamics of this network are measured in single cells using promoter-green fluorescent protein (gfp) reporters and flow cytometry. During induction, there is a temporal order of gene expression, with transcriptional inputs turning on first, followed by structure, and effector genes. The promoters show varying stochastic properties, where graded inputs are converted into all-or-none and hybrid responses. The relaxation dynamics are measured by shifting cells from inducing into non-inducing conditions and measuring the fluorescence decay. The gfp expressed from promoters controlling the transcriptional inputs (*hilC* and *hilD*) and structural genes (*prgH*) decay exponentially with a characteristic time of 50–55 minutes. In contrast, the gfp expressed from a promoter controlling the expression of effectors (*sicA*) persists for 110 ± 9 minutes. This promoter is controlled by a genetic circuit formed by a transcription factor (InvF), chaperone (SicA) and secreted protein (SipC) that regulates effector expression in response to the secretion capacity of the cell. A mathematical model of this circuit demonstrates that the delay is due to a split positive feedback loop. This model is tested in a $\Delta sicA$ knockout where *sicA* is complemented with and without the feedback loop. The delay is eliminated when the feedback loop is deleted. Further, a robustness analysis of the model predicts that the delay time can be tuned by changing the affinity of SicA:InvF multimers to an operator in the *sicA* promoter. This prediction is used to construct a targeted library, which contains mutants with both longer and shorter delays. This combination of theory and experiments provides a platform to predict how genetic perturbations lead to changes in the global dynamics of a regulatory network.

Keywords

bistability; cascade; genetic circuit; invasion; stochastic

Introduction

Type III secretion is a virulence mechanism shared by many gram negative pathogens (Galan and Collmer, 1999; Cornelis, 2006). It consists of a needle-like structure that crosses both the inner and outer bacterial membranes and extends ~50nm from the cell surface (Marlovits et al., 2004). It functions to translocate effector proteins from the bacterial cytoplasm to the host cell, where they hijack signalling processes and reorganize actin to facilitate endocytosis (Galan and Zhou, 2000). The type III secretion system (T3SS) is a complex molecular machine requiring more than 40 structural, effector, and chaperone genes, most of which are encoded together on a genomic island (Groisman and Ochman, 1996). An internal regulatory network controls when and where each of the genes is expressed (Lucas and Lee, 2000).

Salmonella enterica contains two T3SSs that facilitate different stages of an infection. The needle encoded in *Salmonella* Pathogenicity Island 1 (SPI-1) can be induced in culture and is required for bacteria to invade mammalian cells (Jones et al., 1994; Clark et al., 1994). Once in the intracellular environment, SPI-1 is strongly repressed and SPI-2 is activated, which encodes a different type III apparatus and new effectors (Eriksson *et al.*, 2003). Additional effectors exist at other genomic locations and in the *spv* operon on a virulence plasmid (Eichelberg and Galan, 1999; Matsui et al., 2001).

The network is organized such that environmental and cell state regulators converge on three transcription factors (HilC, HilD, and HilA), whose regulation is highly interconnected (Figure 1) (Bajaj et al., 1996; Lucas and Lee, 2001; Schechter and Lee, 2001; Olekhovich et al., 2002; Boddicker et al., 2003). These transcription factors form a commitment circuit and differentially control the transcription of SPI-1 operons. HilA is required for the transcription of the *prg* operon, whose genes form the inner membrane ring and needle shaft (Kubori et al., 1998; Lostroh and Lee, 2001; Sukhan et al., 2001). All three *hil* transcription factors induce the long *invFGEABCspaMNOPQRSsicAsipBCDAiacPsicPsptP* operon, which encodes the outer membrane ring and functional components, chaperones, translocators, and effectors (Kubori et al., 1998; Lostroh et al., 2000; Sukhan et al., 2001). Two upstream promoters control the *inv* operon, which are activated by HilC/D and HilA respectively (Eichelberg and Galan, 1999; Lostroh et al. 2000; Akbar et al., 2003). In addition, there is an internal promoter upstream of the *sicA* gene, whose activity is controlled by the InvF transcription factor and SicA chaperone (Darwin and Miller, 1999).

The SPI-1 network contains several regulatory motifs whose dynamics have been previously characterized (Alon, 2006). The network is centered on a four-tiered regulatory cascade (Lucas and Lee, 2000). Transcriptional cascades have been shown to alter the timing and stochastic properties of gene expression (Pedraza and van Oudenaarden, 2005; Hooshangi et al., 2005; Rosenfeld et al., 2005). A coherent feedforward motif occurs when an input activates an intermediate regulator and together they turn on a downstream process (*e.g.*, HilD activates HilC and together they activate HilA) (Shen-Orr *et al.*, 2002). These motifs can filter short pulses of input and can delay the activation and relaxation of the circuit (Mangan et al., 2003; Kalir et al., 2005). The SPI-1 network contains several such interlocked feedforward motifs (Ellermeier et al., 2005).

Several promoters also have interesting architectures that can convert the signalling properties of a pathway. The *hila*, *invF*, and *prgH* promoters contain multiple cooperative binding sites

(Lostroh and Lee, 2001). In addition, the activator binding sites of the *hilA* promoter can also be bound by repressor in a mutually exclusive manner (Schechter and Lee, 2001; Schechter et al., 2003). These properties have been shown previously to result in all-or-none activation, where individual cells in a population are either fully expressing a gene or not expressing it at all (Rossi et al., 2000; Bigger et al., 2001). The *inv* operon is also controlled by multiple promoters, which can act to integrate the properties of different input signals (Lostroh, et al. 2000; Akbar et al., 2003).

Positive feedback loops can amplify expression and introduce irreversibility into a switch (Becskei et al., 2001; Xiong and Ferrell, 2003; Ozbudak et al., 2004). The commitment circuit formed by HilC/D/A contains several positive feedback loops (Olekhovich and Kadner, 2002; de Keersmaecker, et al., 2005). Another positive feedback loop is present in a SPI-1 circuit that upregulates the expression of effector proteins in response to the capacity of the cell to export proteins (Figure 1) (Darwin and Miller, 1999, 2000, 2001). At the core of the circuit is the InvF transcription factor, which is only active when bound to the SicA chaperone. Prior to the completion of functional needles, SipC binds to and sequesters SicA (Tucker and Galan, 2000). Once the needle is functional, SipC is exported and SicA is free to bind InvF. The InvF:SicA complex then activates the *sicA* promoter, thereby upregulating the expression of additional chaperones and effectors, including some that are encoded outside of SPI-1 (Eichelberg and Galan, 1999; Darwin and Miller, 2001). This circuit forms a “split” positive feedback loop, where SicA is internal to the loop, but InvF is encoded outside of the loop.

The cascade, feedforward/feedback motifs, and promoter architectures collectively determine the dynamics of the regulatory pathway. To characterize these dynamics, we construct plasmid-based reporters with transcriptional fusions between SPI-1 promoters and green fluorescent protein (gfp). Cells are shifted from non-inducing to inducing conditions and the activation dynamics are measured in single cells using flow cytometry. These experiments reveal a temporal ordering in the activation of each promoter and a conversion between graded and all-or-none responses. After induction, the cells are shifted into non-inducing conditions and the decay of fluorescence is measured. There is a pronounced delay in the decay of gfp expressed from the promoter controlling effector expression (*sicA*). In contrast, the gfp from the other SPI-1 promoters decays rapidly and exponentially. Genetic mutants and a mathematical model are used to demonstrate that the delay is due to the split positive feedback loop formed by InvF and SicA. The model is used to predict how the delay can be varied by changing a biochemical parameter. This prediction is confirmed by applying random mutagenesis to an operator and measuring the change in the relaxation delay. Together, the experiments and theory provide a quantitative framework to study the dynamics of this virulence pathway.

Results

Activation Dynamics of the SPI-1 Regulatory Network

A series of reporter plasmids is constructed, consisting of transcriptional fusions between four SPI-1 promoters and green fluorescent protein (Materials and Methods). The *hilC*, *hilD*, *prgH*, and *sicA* promoters were chosen to be representative of the transcriptional inputs, the T3SS structural genes, and effectors, respectively. These plasmids are used in parallel growth experiments (Kalir et al., 2001) in *Salmonella typhimurium* SL1344 to measure the network dynamics in response to shifts in environmental conditions.

The SPI-1 genes are induced when *Salmonella* enters stationary phase in Luria-Bertani (LB) broth (Lee and Falkow, 1990; Lundberg et al., 1999). This induction is dependent on the concentration of NaCl. SPI-1 is not induced in 0.85 M NaCl (L broth) and is maximally induced in 3 M NaCl (inducing medium, IM). To induce the SPI-1 genes, *Salmonella* cultures are grown overnight in L-broth and then diluted into IM (Materials and Methods). As the culture grows,

aliquots are taken every half-hour and expression is stopped by adding kanamycin and putting the cells on ice. The samples are then analyzed for fluorescence using flow cytometry. Single cell experiments are critical when only a subset of the population is active, as has been observed previously for SPI-1 genes (Bumann, 2002; Hautefort et al., 2003).

The average gated fluorescence is shown in Figure 2 as a function of cell density (OD_{600}). As the cell density increases, the *hilD* and then the *hilC* transcriptional inputs turn on first. Next, the *prgH* promoter is activated, which controls the expression of structural components of the TTSS. This delay is consistent with the transcriptional cascade and feedforward loops, which can act to implement an input threshold that must be reached before the downstream promoters are activated (Alon, 2006). Finally, the *sicA* promoter is induced, which controls the expression of chaperones, effectors, and translocators. Similar transcriptional ordering has been observed during the assembly of the flagella basal body in *E. coli* (Kalir et al., 2001) and *Caulobacter crescentus* (Laub et al., 2000).

Different promoters in the SPI-1 network also generate different stochastic effects (Figure 3). Both transcriptional inputs (*HilC* and *HilD*) follow a graded induction, where the entire population uniformly turns on expression under inducing conditions. In contrast, the *prgH* promoter, which controls structural genes, is activated in an all-or-none manner, where individuals in the population are either fully expressing this operon or not expressing it at all (Novick and Weiner, 1957). The fraction of the population expressing *prg* genes increases as the population is induced. The architectures of the *hilA* and *prgH* promoters contain motifs that have been previously shown to be able to convert a graded input into an all-or-none output (Schechter and Lee, 2001; Rossi et al., 2000; Bigger et al., 2001). The *sicA* promoter, which controls the expression of effectors, has both graded and all-or-none properties. Only a fraction of the population turns on, but that population increases in fluorescence 200-fold as cells are induced.

Relaxation Dynamics of the SPI-1 Regulatory Network

The regulatory motifs present in the SPI-1 network could also impact the inactivation of the network when the input signal is removed (Alon, 2006). In particular, the presence of positive feedback loops and feedforward motifs can impact the relaxation time (Becskei et al., 2001; Kalir et al., 2005). Thus, experiments were performed to determine how the network relaxes when cells are shifted into an environment where SPI-1 is repressed. After SPI-1 is fully induced, an aliquot of bacteria is washed and rediluted into non-inducing L-broth and time points are taken every half hour (Materials and Methods). As before, the fluorescence in individual cells is analyzed by flow cytometry.

After dilution, the fluorescent reporters expressed from the *hilC*, *hilD*, and *prgH* promoters decay rapidly, whereas expression from the *sicA* promoter persists (Figure 3). The *gfp* expressed from the *hilC* and *hilD* promoters decays with a half-life of 55 ± 6 and 50 ± 14 minutes, respectively (Figure 4). The *gfp* expressed from the *prgH* promoter, which controls the expression of structural genes, also degrades with a half-life of 50 ± 11 minutes. Because a stable variant of green fluorescent protein is used, the primary contributor to fluorescence decay is dilution due to cell division. Indeed, for all three promoters, the decay fits a simple exponential model, which appears as a linear fit on a semi-log plot.

In contrast, the *sicA* promoter exhibits approximately a one hour delay, with a half-life of 110 ± 9 minutes (Figure 4B). In contrast to the upstream promoters, the decay does not fit an exponential model, implying that it is following non-linear kinetics, as would be expected from a positive feedback loop. It is noteworthy that the maximum fluorescence from the *hilC* and *sicA* promoters are nearly identical prior to dilution, thus eliminating any potential non-linear effects due to fluorescence as the source of the delay.

Reconstitution of *sicA* with and without Positive Feedback

There are two regulatory mechanisms that together lead to the persistence in effector expression. First, the *sicA* promoter is activated by SicA:InvF multimers (Darwin and Miller, 2000). This forms a split positive feedback loop because InvF is encoded upstream of the *sicA* promoter and outside of the loop (Figures 1 and 6). Positive feedback loops can lead to a delay in the deactivation of a circuit. A second mechanism is continued leaky secretion due to a delay in the loss of functional T3SS structures from the cells. Leaky secretion continues to allow free SicA to be available to bind InvF. It is expected that both leaky secretion and a feedback loop are required for the effector expression to persist.

To assess the role of the feedback loop on the delay, a Δ *sicA* knockout was obtained, which eliminates the feedback loop (Darwin and Miller, 2000). In this knockout, expression from the *sicA* promoter is completely abolished (Figure 5B, PBAD30) (Materials and Methods). The feedback loop can be reconstituted by introducing the *sicA* promoter and *sicA* gene on a plasmid (Figure 5A, *P30.psicA.sicA*). This plasmid is based on the p15a origin of replication and is co-transformed with the colE1-based *sicA* reporter plasmid. The reconstituted feedback loop recovers wild-type expression from the *sicA* promoter (Figure 5B). The delay when the network relaxes is slightly longer (165 ± 22 minutes) than the wild-type feedback loop, which may be the result of reconstituting the loop on a multi-copy (p15a origin) plasmid. However, the maximum fluorescence observed from the *sicA* reporter does not increase significantly.

A second plasmid was designed to complement the expression of *sicA*, but eliminate the positive feedback loop. To complement *sicA*, a plasmid was constructed that contains the *sicA* gene under the control of an arabinose-inducible promoter (Figure 5A, *PBAD30.sicA*). Here, *sicA* is no longer under the control of the *sicA* promoter, and thus the feedback loop has been broken, but the network can still be activated by adding arabinose to the media. The network is induced by growing cells in the presence of arabinose and the relaxation is measured by shifting cells into media lacking arabinose (Materials and Methods). An advantage of using the arabinose-inducible system is that this strain retains the arabinose utilization operon. Thus, the addition of arabinose produces a pulse of expression that reaches a peak 60 minutes after induction and is completely off 140 minutes after induction (not shown). This eliminates any potential positive feedback effects that could occur from the inducible system itself. The pulse of activity is uniform throughout the cell population.

Constructing the arabinose-inducible plasmid required variation of the ribosome binding site (rbs) controlling *sicA* expression. Initially, a strong rbs was used (part BBa_B0030 from <http://parts.mit.edu>). This construct produced a constitutively active variant, where in the absence of arabinose, sufficient SicA was expressed through leaky transcription to turn on the *sicA* promoter on the reporter plasmid (not shown). A weak rbs was then tested (BBa_B0033), which produced the opposite problem, where even in the presence of arabinose, too little SicA was expressed and the network did not turn on. Finally, an intermediate rbs (BBa_B0032) was used, which was just right. In the absence of arabinose, there is only a slight shift in the fluorescence recorded from *psicA_gfp* reporter (Figure 5B). When $1.3 \mu\text{M}$ of arabinose is added to the inducing media, the expression from the *sicA* promoter reporter reaches the same fluorescence as wild-type.

Growth induction and dilution experiments were performed to assay the impact of the positive feedback loop on the relaxation of expression from the *sicA* promoter. The arabinose inducer is added to the media after 150 minutes (Materials and Methods). This roughly mimics an alternative regulatory structure where *sicA* is regulated by a cascade with InvF as an upstream regulator, as opposed to one containing a positive feedback loop (Figure 6, Topology 2). After the network is fully induced, the cells are diluted into L broth lacking arabinose and the relaxation dynamics were measured. While the inducible construct achieves the same level of

activation during induction (Figure 5B), the fluorescence rapidly decays after cells are shifted into media lacking the inducer. The delay fits an exponential model with a half-life of 51 ± 5 minutes, which is equivalent to what is observed for the *hilC*, *hilD*, and *prgH* promoters (Figure 7). The delay returns when the full feedback loop is reconstituted on the plasmid. Together, these experiments demonstrate that the split positive feedback loop is required for the observed relaxation delay.

Mathematical Model of the SicA:InvF Genetic Circuit

A simple mathematical model of the SicA:InvF positive feedback loop is constructed to quantify the observed relaxation dynamics. A schematic of the circuit and biochemical parameters is shown in Figure 6. The model is abstracted to describe a genetic circuit containing three proteins. The transcription factor X (InvF) is activated when bound by the chaperone Y (SicA). The chaperone also binds to the effector protein to be secreted Z (SipC). The formation of protein complexes is assumed to be fast with respect to transcription and translation. This leads to a set of three ordinary differential equations that capture the change in the total number of each protein (X_T , Y_T , and Z_T) in the cell:

$$\frac{dX_T}{dt} = \alpha_x k_0 - \gamma_x (X_T - C_{XY}) - \gamma_{XY} C_{XY} \quad (1)$$

$$\frac{dY_T}{dt} = \alpha_y (\eta k_0 + k_1 \frac{C_{XY}}{K + C_{XY}}) - \gamma_y (Y_T - C_{XY} - C_{YZ}) - \gamma_{XY} C_{XY} - \gamma_{yz} C_{YZ} \quad (2)$$

$$\frac{dZ_T}{dt} = \alpha_z (\eta k_0 + k_1 \frac{C_{XY}}{K + C_{XY}}) - \gamma_z (Z_T - C_{YZ}) - \gamma_{yz} C_{YZ} - k_z C_{YZ} \quad (3)$$

where the parameters are defined in Table 1 and shown in Figure 6. The number of X:Y and Y:Z complexes is given by the relationships $C_{XY} = K_{XY} X_0 Y_0$ and $C_{YZ} = K_{YZ} Y_0 Z_0$, where K_{XY} and K_{YZ} are equilibrium constants and X_0 , Y_0 , and Z_0 are the number of unbound proteins. In addition, there are three conservation relationships for the total number of each protein: $X_T = X_0(K_{XY} Y_0 + 1)$, $Y_T = Y_0(K_{XY} X_0 + K_{YZ} Z_0 + 1)$, and $Z_T = Z_0(K_{YZ} Y_0 + 1)$. Here, we assume that X (InvF) and Y (SicA) forms a 1:1 complex. It has been proposed that SicA could interact with InvF as dimmers (Darwin and Miller, 2002). If this turns out to be the case, then bistability could be introduced into the model, although even then, the bistability occurs in a very narrow slice of parameter space due to the split nature of the feedback loop (not shown).

To solve the equations, the network is initially induced by setting $k_0 = 1.0$ transcripts/min (Materials and Methods). Once the network reaches steady-state, the shift to non-inducing conditions is simulated by stopping transcription from the upstream promoter ($k_0 = 0$). The relaxation of the network is then recorded. The presence of the positive feedback loop yields the observed delay (Figure 7). Importantly, the activity of the feedback loop requires the continued secretion from the TTSS ($k_z > 0$). When there is no secretion, the delay is eliminated (as the secretion rate constant $k_z \rightarrow 0$ in Figure 8).

When *sicA* is knocked out of the genome and reconstituted on the P_{BAD} inducible plasmid, this produces a circuit architecture resembling a cascade (Figure 6, Topology 2). The feedback loop encoded in the *sicA* promoter is not present and it is predicted that this would reduce the observed delay. To simulate the P_{BAD} data, the first term of Equation 2 is changed to $\alpha_y k_0$, but otherwise the equations remain the same. This has the effect of decreasing the delay, which is observed in the experiments (Figure 7).

Robustness and Evolvability of the SicA:InvF Genetic Circuit

The mathematical model is used to perform a robustness analysis for the circuit (Voigt et al., 2005). Starting from the set of nominal parameter values (Table 1), each parameter is independently varied through a physiologically relevant range. For each parameter set, Equations 1–3 are solved and the secretion-dependent induction and the time scale for the delay in deactivation are measured (Figure 8).

Some combinations of parameters result in a circuit that can no longer be induced when secretion becomes active. To keep track of this, the expression from the *sicA* promoter is recorded for cells that are secreting (upper green lines) and compared to the expression when cells are not secreting (lower red lines). When both of these curves are high or low, this indicates that the circuit is either always on or always off, independent of secretion. The difference between these curves indicates the degree to which the circuit can be induced by secretion. Some of the parameters are robust, such as the degradation rate of free SicA (γ_Y) (Figure 8). In contrast, other parameters only have narrow regions that result in functional circuits, including the degradation rate of SicA:SipC complexes (γ_{YZ}), which needs to be slow.

The delay in the deactivation dynamics of each parameter set is also recorded. The timescale is defined as the time required for half of the maximum fluorescence to degrade ($\tau_{1/2}$). Some of the parameters that are relatively robust, such as the expression rate of SipC (a_Z), have almost no effect on the delay (Figure 8). The only parameter that has a significant effect on the delay is the binding constant (K) for InvF:SicA binding to the internal *sicA* promoter. By varying this parameter over a physiologically relevant range, the model predicts that the delay can be varied from one hour (where the delay to feedback is eliminated) to four hours and still produce a secretion-inducible circuit. In essence, this parameter could act as an evolutionary “tuning knob” where mutations could control how long protein expression persists after the network is deactivated.

To test this prediction of the model, the binding constant K was varied experimentally by mutating the known SicA:InvF operator in the *sicA* promoter (Darwin and Miller, 2001). Targeted random mutagenesis was used to vary the sequence of the operator on the plasmid containing the reconstituted feedback loop (Figure 5A, P30.psicA.sicA) in the Δ *sicA* strain (Materials and Methods). The relaxation dynamics of seven sequenced mutants were measured (Figure 9A). A remarkable diversity of relaxation times was obtained in this small library. The fastest mutant has a $\tau_{1/2} = 78 \pm 17$ minutes, which is only slightly slower than for the *hilC*, *hilD*, *prgH*, and PBAD.sicA decay times (Figure 9B). Interestingly, mutants with slower decay times were also obtained, with the slowest being $\tau_{1/2} = 210 \pm 33$ minutes. Each relaxation trajectory was fit with the model to obtain the predicted binding constant K (Figure 9A). It is intriguing that such a large variety of delay times could be obtained from such a small mutant library.

Discussion

Pathogens interact with their hosts through the timed release of multiple protein and chemical effectors. This timing has to be carefully controlled to reflect the specific requirements of particular microenvironments encountered during an infection. Complex molecular machines, such as the type III secretion system, also require regulation to coordinate their self-assembly and function. Here, we have experimentally characterized the induction and relaxation dynamics of the regulatory network controlling *Salmonella typhimurium* T3SS encoded within SPI-1. This network contains a series of integrated genetic circuits that control the temporal and stochastic properties of gene expression when the secretion system is induced. In addition, a split positive feedback loop is shown to affect the relaxation dynamics, where effector expression persists after cells are shifted into non-inducing conditions.

Upon induction, the SPI-1 promoters are activated in a manner that is consistent with the self-assembly of the needle. The overall dynamics are very similar to that observed for flagella (Kalir et al., 2001), despite significant divergence with the SPI-1 operon structure and regulatory interactions. In addition, when induced, only a fraction of bacteria turn on the SPI-1 genes (Bumann, 2002; Hautefort et al., 2003; Boddicker and Jones, 2004). Single cell experiments are critical to characterize the dynamics of networks that are only active in a subset of the population (Suel et al., 2006).

Other *Salmonella* genes have been shown to be induced within a subpopulation. For example, intracellular bacteria differ in their induction of SPI-2 and other virulence genes (Hautefort et al., 2003; Detweiler et al., 2003; Drecktrah et al., 2006). Stochastic gene expression may lead to different physiological roles; for example, individuals within a single host cell have been observed to bifurcate into a static or rapid growth state (Abshire and Neidhardt, 1993). Population heterogeneity has been observed for other intracellular pathogens, such as the presence of both static and motile *Mycobacteria* phenotypes within a single host cell (Stamm et al., 2003). The stochastic control of virulence genes enables individuals in a population to probabilistically vary their physiological behaviour at each stage of an infection (Wolf and Arkin, 2003).

Within the SPI-1 regulatory network, there is a genetic circuit that links the expression of effectors with the secretion state of the cell (Darwin and Miller, 2001). A unique aspect of this circuit is that the positive feedback loop is split (Topology 1, Figure 6). One can imagine two alternate network topologies. Each topology can be secretion-inducible, but they differ in the way the network relaxes. First, both the chaperone and transcription factor could be encoded upstream of the internal promoter (Topology 2, Figure 6). This would produce a cascade, as is often found in regulatory networks (Shen-Orr et al., 2002). This topology produces a significantly shorter relaxation delay and it is difficult to increase the delay by changing the model parameters. This topology is roughly simulated when the *sicA* gene is placed under inducible control, in which case the network was similarly induced, but the delay was almost eliminated (Figure 7).

The SicA:InvF circuit, including the split feedback loop, is conserved in *Yersinia enterocolitica* (YsaE, SycB and YspC are homologous to InvF, SicA, and SipC) (Walker et al., 2004). Interestingly, *Shigella flexneri* also has homologues to these proteins (MxiE, IpgC, and IpaC); however, the genomic organization and the resulting genetic circuit differ considerably (Marvis et al., 2002). In this circuit, the IpgC:MxiE complexes activate outside promoters controlling effector expression, but they do not upregulate their own production (Gall et al., 2005). Therefore, there is no positive feedback loop and the circuit resembles a simple cascade (Topology 2). It will be interesting to compare the induction and relaxation dynamics of the *Salmonella* circuit with those encoded in *Yersinia* and *Shigella*.

A third topology is also possible, where both the transcription factor and the chaperone are downstream of the internal promoter (Topology 3, Figure 6). This forms a complete positive feedback loop, where all of required genes are internal to the loop. If there is higher-order cooperativity in the non-linear term, this topology can produce a bistable switch, which is difficult to deactivate once it is turned on (Becskei et al., 2001; Xiong and Ferrell, 2003; Ozbudak et al., 2004). A similar topology is present in the regulatory network controlling the expression of flagella (Chilcott and Hughes, 2000). Within the network, a genetic circuit controls the expression of flagellin, which forms the filament. After the basal body is constructed, the sigma factor FliA (analogous to X) activates its own expression and an anti-sigma factor FlgM (analogous to Y), which is secreted. After the filament is constructed, FlgM accumulates in the cytoplasm and deactivates the positive feedback loop by binding to FliA. The irreversibility intrinsic to this topology with respect to the input signal enables the circuit

to continue the formation of filament until the flagellum is fully constructed once the pathway is initiated. This irreversibility would be disadvantageous in the case of SPI-1 because the effectors are temporally induced to promote invasion, after which their expression needs to be downregulated.

The SicA:InvF split feedback loop and the resulting relaxation delay may have a physiological role in controlling a physiological bifurcation that occurs immediately post-invasion. After invading a macrophage, *Salmonella* either persists and grows in the intracellular environment or it rapidly induces apoptosis within an hour (Lundberg *et al.*, 1999; Monack *et al.*, 2001). Rapid apoptosis is dependent on the SipB effector (transcribed from the *sicA* promoter) and caspase-1 (Hersh *et al.*, 1999; Monack *et al.*, 2000). Transcription from the *sicA* promoter has been shown to persist during the first hours after invasion (Boddicker and Jones, 2004). The inability to turn off SipB expression post-invasion (via a Δlon mutant) has been shown to increase the probability of apoptosis (Takaya *et al.*, 2005). The length of effector expression post-invasion could alter the ratio of persistent to apoptotic cells, and ultimately the speed of pathogenesis. The SicA:InvF operator in the *sicA* promoter could act as an evolutionary tuning knob by which the probability of inducing apoptosis could be controlled. This hypothesis could be tested by perturbing the strength of the feedback loop and measuring the fraction of infected macrophages that enter an apoptotic state.

The combination of theory with experiments provides a quantitative platform for understanding the dynamics of regulatory networks. These tools enable more refined methods to interrogate networks by perturbing their underlying biochemical mechanisms. Here, this approach is applied to the regulatory network controlling SPI-1 in two ways. First, we were able to rewire the network to convert a split positive feedback loop into a cascade. Second, mutations were made to a transcription binding site. In both cases, the model was used both to predict the effect of making these genetic changes and then to quantitatively frame the resulting change in dynamics. This represents a powerful approach to perturb and understand how sets of biochemical interactions lead to the observed complex global dynamics.

Methods and Methods

Plasmids and Strains

The reporter plasmids were constructed based on the pPROTet Cm^r system (ColE1 ori) available from Clontech/BD. The SPI-1 promoters were cloned from *Salmonella enterica* Typhimurium SL1344 genomic DNA, transcriptionally fused with green fluorescent protein (*gfpmut3*) using SOEing PCR, and ligated into pPRO using the XhoI/XbaI restriction sites. A large upstream region associated with each promoter was cloned: *hilD* (−312), *hilC* (−432), *prgH* (−317), *sicA* (−166). The *hilC* region contains two promoters in series. The 5'-UTR was included to the start codon of each respective gene. SVM687, a *sicA*:aphT mutant of SL1344, was used for all complementation experiments (Darwin and Miller, 2000). A plasmid encoding *psicA:sicA* was cloned from SL1344 genomic DNA and ligated into pBAD30 using and XhoI site introduced upstream of the pBAD promoter together with the XbaI site in the multiple cloning site. An inducible *sicA* construct was made by cloning the *sicA* gene with an optimized RBS (TCACACAGGAAG, Bba_B0030 from parts.mit.edu) from SL1344 genomic DNA and ligating into the KpnI and XbaI sites of pBAD30.

Salmonella Growth Curves

All ODs were measured using a Cary 50 Bio spectrophotometer. Bacteria transformed with a reporter plasmid were grown overnight (~13 hours) in 5ml L broth (Difco, Lennox) supplemented with 34.4 µg/ml chloramphenicol. The overnight cultures were then diluted 1:500 into 5ml fresh L-broth and grown 150 minutes at 37°C to OD₆₀₀ ~0.15. A 2 ml aliquot

is then added to 50 ml of inducing media (7g/L NaCl added to Difco Luria Bertani, Miller) in a non-baffled 250 ml flask. The cultures were grown in a shaker at 37°C at 160 rpm. During growth, 500 µl aliquots were taken every 30 min and the OD₆₀₀ was measured, the cells were spun down, resuspended in 200 µl PBS with 2 mg/ml kanamycin and put on ice to stop gfp expression. For the dilution experiments, cells were grown to maximum induction of SPI-1 (OD₆₀₀ ~2.05). A 500 µl aliquot was then taken, spun down, washed with fresh L broth, and then diluted 1:100 into 50 ml of fresh L broth containing chloramphenicol. Aliquots are taken every 30 min and the sample was spun down and resuspended in PBS as before.

Test for Plasmid Loss

To test for potential plasmid loss effects, 500 µl samples were taken from the last time point after the shift to non-inducing conditions. The samples were serially diluted 1:10⁶, 20 µl of which was spread on a plate with no antibiotics and one containing chloramphenicol. The plates were grown overnight and colonies were counted. The *sicA* promoter reporter produced 16 ± 5 colonies on the plate lacking antibiotics and 14 ± 5 on the plate with Cm. The *hilC* promoter produced 25 ± 9 colonies on the plate lacking antibiotics and 24 ± 8 on the plate with Cm. The data represents eight replicates on different days. This demonstrates that the delay is not due to a change in plasmid stability.

Complementation Assays

Cultures were handled as described in the reporter assay with the following exceptions. Binary plasmid systems were grown in media supplemented with 34.4 µg/ml Cm and 100 µg/ml Amp. Overnight cultures were diluted 1:100 into fresh L broth. After 150 minutes, a 3 ml aliquot was added to 30 ml inducing media. Cultures were induced after 150 minutes with 60 µl of 100mg/ml arabinose, leading to a final concentration of 1.33 mM arabinose. During growth, 600 µl aliquots were taken hourly. Three hours post-arabinose induction, a 600 µl aliquot is taken, spun down, washed with fresh L broth and diluted 1:50 into 30 ml fresh L broth containing appropriate antibiotics. Post-dilution aliquots were taken hourly.

SicA Promoter Library Construction

A small library was constructed of the SicA:InvF binding site in the *sicA* promoter. In addition to this library, the SicA:InvF binding site in the *sicA* promoter was changed to the one from the *sopE* promoter. The *psopE* binding site mutant and library of *psicA* binding sites were constructed using the phosphorylated primers aaGGaCtTTTTTTGAAaGTTCACTAACCACCGTCGGGGTTTAATAACTGC (for the *psopE* site) and nnGGnCtTTTTTTGAAnGTTCACTAACCACCGTCGGGGTTTAATAACTGC (for the library) and a reverse primer TGAATACATCGCTACTGCCTTACGCGGCTC. The mutagenesis was carried out according to Finnzyme's Phusion Site-Directed Mutagenesis protocol with no alterations. The primers were used to amplify the *psicA:sicA* plasmid using Hot Start Phusion DNA polymerase and each resulting PCR product was ligated and transformed into *E. coli* strain DH10B and plated on ampicillin selective plates. Sequencing confirmed that the *invF* binding site of *psicA* was replaced with that of *psopE*. Seven colonies from the library plate were chosen and sequencing revealed each plasmid had a unique SicA:InvF binding site of *psicA*. These seven plasmids were cotransformed with the *psicA:GFP* plasmid into Δ *sicA* strain SVM687.

Flow Cytometry

Flow cytometry data was obtained on a BD FACSCalibur system as part of the UCSF core facility. Each bacterial dataset consists of at least 30,000 cells. The data was gated by forward

and side scatter to observe bacteria-sized particles. Data analysis was performed using the WINMDI and FlowJo programs.

Solution of Model Equations

Numerical solutions were obtained for the system of ordinary differential equations (ODEs) (Equations 1–3) using MATLAB. The solution of these differential equations also depends on three algebraic equations for the total number of molecules (X_T , Y_T , and Z_T) as well as two that describe the equilibrium relationships for the formation of dimers. First, a physically relevant analytic solution is obtained for the 6th order polynomial that results from the algebraic equations. Then, the non-linear ODEs are numerically solved using a multi-step stiff solver (ode15s). The simulations were started with no secretion ($k_Z = 0$) and then shifted to $k_Z = 0.1$ at time T_1 . During this time, the external promoter was activated ($k_0 = 1.0$). At T_2 , the external promoter was turned off ($k_0 = 0$) and the relaxation dynamics were recorded. The times T_1 and T_2 are selected so that the system has reached steady-state prior to each event.

To model the expression of green fluorescent protein from the *sicA* promoter reporter, an additional differential equation was added to the model: $\frac{dg}{dt} = a_g \left(\frac{C_{XY}}{K + C_{XY}} \right) - \gamma_g g$, where a_g and γ_g are the expression and degradation rate of gfp. The degradation rate was set to 0.022 to reflect degradation due to cell division. All of the fluorescence data was fit in normalized form, so the expression rate was arbitrarily set to 1.0. The model with this equation was solved in the same manner as described above.

Parameter Fitting

The parameters were either fixed to estimated values using data from the literature or were fit using the experimental data presented in this paper (Table 1). The parameters that were fit were determined by identifying values that minimize the collective L_1 distance between the

normalized experimental and model data sets, or $\chi = \frac{1}{N} \sum_{i=1}^N |GFP_{\text{exp}}^{\text{norm}}(t_i) - GFP_{\text{model}}^{\text{norm}}(t_i)|$, taken at time points t_i and for each data set, including time series data from both the P30.*psicA.sicA* and the PBAD30.*sicA* data (Figure 7). The dynamics of the GFP fluorescence are normalized by dividing by its initial value at time T_2 (for the model) and $t = 0$ hours (for the experiment time series). A combination of manual fitting and automated simulated annealing resulted in a parameter set that fit the P30.*psicA.sicA* and PBAD30.*sicA* experimental data with L_1 distances of 0.0186 and 0.0181, respectively. Using a similar procedure, the affinity of the InvF:*SicA* complex to each mutant promoter (Figure 9A) was determined by identifying the affinity (K) that minimized the L_1 distance between the respective mutant time series data and the resulting model solution, while keeping all other parameters of the model constrained. For fitting the mutant data, only the K for *sicA* expression was varied because the mutants are on a plasmid and only affect that gene (*SipC* expression uses the data fit for the wild-type). Using the standard deviations of the experimental time series data as upper and lower bounds, the standard deviations of the affinities for each mutant promoter were also determined, according to the same fitting procedure.

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Figure 1.

The regulatory network encoded within *Salmonella* Pathogenicity Island 1 (SPI-1) is shown. The SPI-1 T3SS is induced by a number of environmental and cell state signals, such as osmolarity, Mg^{2+} , and extracellular phosphate, which differentially affect the regulators *hilD*, *hilC*, and *hilA* (Ellermeier and Slauch, 2007). These transcription factors co-regulate each other and control different operons internal to SPI-1. Only *HilA* regulates the expression of genes forming the inner membrane ring and shaft (*prg*), but all three converge at two promoters controlling a long operon that begins with the *invF* gene. There is a promoter internal to this operon that is activated by *InvF* when bound to the *SicA* chaperone (Darwin and Miller, 1999, 2000, 2001). The chaperone is sequestered by the *SipC* protein until it is exported, after which *SicA* can bind to *InvF*, leading to the upregulation of additional effector proteins. The colors correspond to the temporal data shown in Figure 2. The cryo EM structure of the needle complex is shown (Marlovits et al., 2004).

Figure 2.

The SPI-1 promoters are induced in a temporal order. The *hilD* (orange), *hilC* (red), *prgH* (purple) and *sicA* (blue) promoters are transcriptionally fused to green fluorescent protein and introduced into cells on a plasmid. Cells are grown in non-inducing media (L Broth) and diluted into inducing media and fluorescence is measured in single cells as a function of density (OD_{600}). The average gated fluorescence of the population is calculated for the fraction of the population that turn on at the end of the induction experiment. The average is then normalized by the maximum (Kalir et al., 2001). The data represents four replicate experiments performed on different days for each promoter. Each data point is an aliquot from these 16 growth experiments. The lines are drawn for each promoter using a polynomial averaging algorithm. The dashed line is drawn as a reference for when the promoter reaches 20% of its maximum.

Figure 3. SPI-1 promoters follow graded, all-or-none, or hybrid induction dynamics. Gated cytometry data is shown for the *hilC*, *hilD*, *prgH*, and *sicA* promoters transcriptionally fused to green fluorescent protein. Cells are grown in non-inducing media (L broth with 0.9 M NaCl) and then shifted into inducing media (LB broth with 3 M NaCl) (Materials and Methods). Data is shown for each promoter pre-induction (first row), at intermediate induction (second row), and fully induced (third row). The OD₆₀₀ of these samples are: *hilC* (0.13, 0.80, 1.9), *hilD* (0.11, 0.75, 1.8), *prgH* (0.12, 1.5, 2.06), and *sicA* (0.12, 1.4, 2.07). Both the *hilC* and *hilD* promoters have a graded response to the shift in condition, where the entire population is induced uniformly. In contrast, the *prgH* promoter has an all-or-none response and the *sicA* promoter has a hybrid response with features of both all-or-none and graded dynamics. After the cells are fully induced, they are shifted back into non-inducing conditions. Fluorescence from the *hilD*, *hilC*, and *prgH* promoters decays rapidly to the pre-induced level. However, expression from the *sicA* promoter persists strongly. After dilution into L Broth, time points are shown after 50 and 140 minutes. The dashed lines are drawn as a guide for the eye.

Figure 4.

Expression from the *sicA* promoter persists after cells are shifted into non-inducing conditions. Cells are grown in inducing media (3M NaCl LB Broth) until the network is fully activated after 400 minutes (Materials and Methods). Cells are then shifted into non-inducing L media (0.3M NaCl LB Broth) and grown for 140 minutes. The decay of fluorescence is shown after the cells are shifted into non-inducing conditions ($t = 0$ min). The fluorescence values are normalized by the maximum fluorescence prior to dilution. On a semi-log plot, exponential decay appears as a straight line, as is the case for the *hilC* (orange circles), *hilD* (red +), and *prgH* (pink triangles) promoters. There is a significant delay in the decay of the *sicA* promoter (white diamonds). The straight lines are the best fit to an exponential equation, as determined using linear regression algorithm. It is noteworthy that the *sicA* and *hilC* promoters have almost equal fluorescence prior to the shift to non-inducing conditions. Each data point represents the average of four experiments performed on different days and the standard deviation of these experiments is shown as error bars.

Figure 5.

An inducible *sicA* plasmid was constructed and tested for complementation in a Δ *sicA* knockout strain. **(A)** To reconstitute the feedback loop, the *sicA* promoter and gene were cloned together from the *Salmonella* genome and *ligated* into PBAD30 at an introduced *XhoI* site that eliminates the *araC* gene and *P_{BAD}* promoter (top). To create an inducible system without the feedback loop, the *sicA* gene was cloned into the PBAD30 plasmid (bottom). Different ribosome binding sites were tested to obtain an inducible construct. **(B)** Cytometry data is shown for the Δ *sicA* knockout containing the *psicA_gfp* reporter on a ColE1 plasmid. Data is shown for (bottom to top) 2, 4, 6, and 8 hours after shifting cells into inducing conditions. No activity from the *sicA* promoter is observed when Δ *sicA* cells carry the empty PBAD30 plasmid as a control (top left). When the *sicA* gene is driven by the wild-type *sicA* promoter (P30.*psicA.sicA*), induction is recovered (bottom left). When the *sicA* gene is fused to an arabinose-inducible promoter (PBAD30.*sicA*) the *sicA* promoter is induced in the presence of 1.33 mM arabinose (bottom right), but not in the absence of arabinose (top right).

Figure 6.

A generalized schematic of the secretion-control circuit is shown for the topology that appears in *Salmonella* and two alternative topologies (2 and 3). Topology 1 shows the split positive feedback loop motif, where the transcription factor x (InvF) appears external of the feedback loop, but the activating chaperone y (SicA) is internal to the loop. The parameters used in the model are shown (Equations 1–3 and Table 1). Topology 2 is a simple cascade, where both the transcription factor and chaperone are outside of the internal promoter, thus eliminating any feedback. This topology is roughly recreated when *sicA* is placed under inducible control and inducer is added at the time when *invF* is expressed (Figures 5A and 7). Topology 3 is a complete feedback loop, where all of the necessary components are internal to the loop.

Figure 7.

The persistence of expression from the *sicA* promoter is eliminated by a disruption of the positive feedback loop. Data is shown for the Δ *sicA* deletion mutant, where the *sicA* gene is complemented either by an inducible construct (PBAD30.*sicA*, Figure 5A) or under the control of the *sicA* promoter (P30.*psicA.sicA*). Cells are grown in inducing conditions and then shifted into non-inducing conditions (time = 0 minutes) and the decay in fluorescence is measured. When the *sicA* promoter is under the control of an inducible promoter, the total fluorescence from the reporter reaches approximately the same maximum (Figure 5B), but the delay is eliminated (triangles). When the feedback loop is reconstituted on the plasmid, the delay is recovered (squares). The lines represent the fit to the model (Equations 1–3 and Table 1). Each data point is the average of six experiments performed on different days and the error bars represent the standard deviation. The data is normalized by the maximum fluorescence under inducing conditions.

Figure 8.

A robustness analysis of the model is performed. Each parameter is independently varied from the nominal set (Table 1). For each set of parameters, Equations 1–3 are solved and three values are recorded from the simulation: the maximum fluorescence without secretion (top graph, red line), the maximum fluorescence with secretion (top graph, green line), and the half-life for the decay of fluorescence ($\tau_{1/2}$, bottom graph). Very few of the parameters have the capacity to affect the decay half-life without resulting in a circuit that cannot be induced by secretion. Note that when there is no secretion ($k_Z = 0$), the positive feedback loop is broken and the delay is eliminated. The units for the parameters are provided in Table 1.

	SicA:InvF	-35	$\tau_{1/2}$ (min)	K (nM)
wt sicA	ATTGGGCGTTTTTTGAATGTTTC		162 ± 22	32 ± 14
wt sopE	AAAGGACTTTTTTTTGAAAGTTTC		165 ± 8	35 ± 10
library	ANNNGNCTTTTTTTGAANGTTTC			
A.1	AAAGGACTTTTTTTGAATGTTTC		210 ± 33	2 ± 8
A.2	ATTGGACTTTTTTTGAATGTTTC		190 ± 20	18 ± 8
A.3	AATGGGCTTTTTTTGAATGTTTC		173 ± 26	32 ± 18
A.4	AGGGGACTTTTTTTGAAAGTTTC		143 ± 20	48 ± 9
A.5	AGAGGTCTTTTTTTGAACGTTTC		137 ± 13	56 ± 10
A.6	AGAGGACTTTTTTTGAACGTTTC		132 ± 8	58 ± 6
A.7	ACGGGGCTTTTTTTGAACGTTTC		78 ± 17	146 ± 81

Figure 9. Mutations to the SicA:InvF binding site in the internal promoter vary the strength of the positive feedback loop, resulting in different relaxation times. (A) The -50 to -30 region of the *sicA* promoter is shown, which includes the previously identified SicA:InvF binding site (Darwin and Miller, 2001). For each sequence, the resulting empirical half-life ($\tau_{1/2}$) is shown in addition to the binding constant (K) derived from fitting the mutant data to the model. The errors are reported as the standard deviation of six experiments performed on different days. When the

binding site from the *sicA* promoter is mutated to the *sopE* site, there is no change in the observed half-life. A small library was then created, where the differences between the *sicA* and *sopE* operators were randomly mutated (library). The resulting mutants have a significant diversity of decay times (A.1 to A.7). **(B)** The decay data is shown for three representative mutants. The wild-type *sicA* promoter (top black line) and the PBAD30.*sicA* (lower black line) data are shown as a reference (Figure 7). The mutant with the longest decay time (A.1, red triangles) and two with the fastest decay times (A.6, blue diamonds and A.7, red squares) are shown. The error bars are the standard deviation from six experiments performed on different days.

Table 1

Model Parameters

Name	Description	Value	Units ^b	Notes
k_0	transcription rate of external promoter	1	trans min ⁻¹	
k_1	transcription rate of internal promoter	22	trans min ⁻¹	<i>a</i>
η	efficiency of transcriptional read-through	0.01		<i>c</i>
α_x	X proteins produced per transcript	12	prot trans ⁻¹	<i>a</i>
α_y	Y proteins produced per transcript	14	prot trans ⁻¹	<i>a</i>
α_z	Z proteins produced per transcript	90	prot trans ⁻¹	<i>d</i>
γ_x	degradation rate of X and XY complexes	0.022	min ⁻¹	<i>e</i>
γ_y	degradation rate of Y	0.046	min ⁻¹	<i>af</i>
γ_z	degradation rate of Z	0.139	min ⁻¹	<i>g</i>
γ_{zy}	degradation rate of ZY complexes	0.022	min ⁻¹	<i>gh</i>
K_{xy}	equilibrium dissociation constant for XY dimers	1700	nM	<i>ai</i>
K_{yz}	equilibrium dissociation constant for YZ dimers	28	nM	<i>ji</i>
k_z	secretion rate of Z	0.1	min ⁻¹	<i>k</i>
K	equilibrium dissociation constant of XY to the internal promoter	33	nM	<i>i</i>

^a These data were fit to the experimental data shown in Figure 7 (Materials and Methods). The remainder was fixed to values estimated from the literature.

^b Abbreviations: prot/proteins, molec/molecules, trans/transcript.

^c This parameter captures the probability that an mRNA transcribed from the upstream promoter also encodes the *sicA* gene. The two genes are encoded 25kb apart.

^d The production rate is set to reach a steady-state of 6000–10,000 SipC proteins per cell. The intracellular concentration of SipA has been measured to be 6000 ± 3000 molecules per cell (Shlumberger et al., 2005).

^e Corresponds to a ~32 minute in vivo half-life. SicA does not affect the stability of InvF in vivo (Darwin and Miller, 2001).

^f The degradation rate of SicA is set to a half-life of 15 minutes.

^g In the absence of chaperone, the SipC protein degrades rapidly in vivo. This rate has been shown to range between 2 and 10 minutes *in vivo* in *Salmonella* (Tucker and Galan, 2000) and *Shigella* (Menard et al., 1994). Here, the rate is chosen to yield a half-life of 5 minutes.

^h The SipC protein is stabilized when bound to SicA (Tucker and Galan, 2000). In the presence of SicA, the in vivo half-life of SipC has been measured to be ~30 minutes. The monomeric form of SipC interacts with SipB, greatly increasing its degradation rate. Dilution is the primary mode of degradation, so this half-life reflects the cell division time. For the analogous system in *Shigella*, IpaC degrades in the cytoplasm with a half-life of ~5 minutes, but is stabilized when bound to IpgC (>20 minutes) (Menard et al., 1994).

ⁱ Assumes an intracellular volume of 10^{-15} liters. The units are converted from concentration to molecules for Equations 1–3.

^j The SipC:SicA binding affinity has been measured (Osiecki et al., 2001).

^k The rate of IpaC (homologous to SipC) secretion has directly been measured to be 0.17 min^{-1} in *Yersinia* (Enninga et al., 2005). The secretion rate of SipA was similarly measured and found to be 0.06 min^{-1} (Shlumberger et al., 2005).