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SpoIIIE achieves directional DNA translocation through allosteric regulation of ATPase activity by an accessory domain*

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*Running title: Gamma modulates ATPase activity of motor domain

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Background: ATP-dependent bacterial chromosome transporters mediate substrate recognition through an accessory, DNA-interacting domain.

Results: DNA-interacting domain regulates ATPase activity of the motor domain in a sequence-specific manner.

Conclusion: The DNA-interacting domains mediate directional transport through allosteric regulation of the motor domains.

Significance: Coordinated communication between accessory domains is a critical component of directional activation of DNA transport.

ABSTRACT

Bacterial chromosome segregation utilizes highly conserved directional translocases of the SpoIIIE/FtsK family. These proteins employ an accessory DNA-binding domain (gamma) to dictate directionality of DNA transport. It remains unclear how the interaction of gamma with specific recognition sequences coordinates directional DNA translocation. We demonstrate that the gamma domain of SpoIIIE inhibits ATPase activity of the motor domain in the absence of DNA, but stimulates ATPase activity through sequence-specific DNA recognition. Further, we observe that communication between gamma subunits is necessary for both regulatory roles. Consistent with these findings, the gamma domain is necessary for robust DNA

transport along the length of the chromosome *in vivo*. Together our data reveal that directional activation involves allosteric regulation of ATP turnover through coordinated action of gamma domains. Thus, we propose a coordinated stimulation model in which gamma-gamma communication is required to translate DNA sequence information from each gamma to its respective motor domain.

In bacteria, the RecA-like SpoIIIE/FtsK family of translocases transports large DNA molecules between spatially separated compartments (1). These ring-shaped ATPases play vital roles in processes such as chromosome segregation, viral DNA packaging, and conjugation. Many such ATPases recognize their substrates through accessory domains. For example, the N-terminal domain of ClpX is necessary for substrate recognition and has a separate fold from the large AAA+ domain, which forms a hexameric ring and hydrolyses ATP to power substrate unfolding in the absence of the N domain (2). Cytosolic dynein mediates microtubule binding via the microtubule-binding domain, which is separated from the six force-generating AAA+ domains via a long coiled-coil stalk (3). SpoIIIE/FtsK proteins are involved in chromosome segregation and their sequence specific DNA interaction is conferred through an accessory domain called gamma (γ) (4-9). Several studies have implicated the DNA-interaction domain in conferring correct directionality to DNA translocation (10-13). Yet, the mechanism coordinating DNA interaction

and motor activity has remained unclear. Here, using SpoIIIE from *Bacillus subtilis*, we demonstrate that communication between gamma domains of SpoIIIE allosterically modulates ATPase activity in a sequence specific fashion, providing a new understanding of the molecular mechanism behind the critical step of directional activation.

Two components are required for directional DNA transport; the highly conserved accessory DNA-interaction gamma domain and an asymmetric, overrepresented eight-nucleotide sequence motif recognized by gamma (5,6,12,14). The asymmetric directionality motifs, SRS (SpoIIIE Recognition Sequence) in *B. subtilis* and the KOPS (FtsK Orienting/Polarizing Sequence) in *E. coli*, are involved in directing motion of the translocase during chromosome segregation (11,12,14-16). *In vivo*, SpoIIIE and FtsK translocate DNA in the direction of the 85% of the SRS and KOPS that are skewed towards the terminus (permissive orientation), while the rest are pointing towards the origin (non-permissive orientation) (6,11).

Previous work suggested that SpoIIIE continuously recognizes all SRS to maintain directionality during active translocation (11). This recognition model, leaves open the question of how SpoIIIE successfully segregates DNA in the face of ~15% of the SRS that are found in the non-permissive orientation *in vivo*. A recent study, which builds on the recognition model, proposed a sliding/hopping search mechanism for SRS identification, but did not resolve the question left open by the recognition model (13). In contrast, studies with FtsK suggest that directional assembly and loading only occurs at KOPS. Once FtsK is translocating on DNA, FtsK ignores subsequent KOPS regardless of their orientation (10,17). The loading model informs upon how FtsK is able to deal with the non-permissive KOPS sequences, but does not address the extreme fidelity of directionality in the presence of only 85% skew of permissive KOPS on the chromosome. Furthermore, in this model it is unclear why frequency of directionality sequences is so high throughout the chromosomes of bacteria as divergent as *B. subtilis* and *E. coli*. Most critically, the mechanistic link between DNA sequence

recognition and directional movement remains unresolved by these previous studies.

Here we provide new insight into the mechanism behind directional activation of the ATP turnover that powers DNA transport. We show that the interactions between SpoIIIE gamma domains are required to modulate levels of ATP turnover by the motor domain, inhibiting turnover in the absence of substrate and activating turnover through sequence specific recognition of SRS in the permissive orientation. These data lead us to propose a coordinated activation mechanism by demonstrating how permissive recognition sequences can establish directional transport, while non-permissive sequences remain unnoticed by the translocase. Further, we find evidence that contacts between gamma subunits are required to properly relay sequence specificity information from gamma to the motor domain. Given the strong sequence and functional conservation within the SpoIIIE/FtsK family, these proteins likely employ a similar mechanism to convert directional information into chromosome translocation activity.

EXPERIMENTAL PROCEDURES

Bacterial growth conditions

All *B. subtilis* strains were derived from laboratory prototrophic strain PY79 (18). Transformation of *B. subtilis* was performed with double-stranded PCR fragments, *B. subtilis* genomic DNA or linearized plasmid (19). Synchronized sporulation was induced by resuspension in minimal media at OD600 0.6 at 37 °C as described (20). All plasmids were propagated in *E. coli* strain *DH5α*, which was grown and transformed as described (21).

Cloning and in vivo Fluorescence Microscopy

Point mutations in pJB103 (22) were introduced using the QuickChange Method (Stratagene). For two-color DNA transport assay, long-flanking PCR was performed to insert P_{liq}-cfp (tet) into *yhdGH* (90°), *ykcC* (116°), *ykoA* (129°), *ylaE* (132°), *ylaA* (138°), *ylqB* (142°), *ymzD* (152°), *xynB* (162°), and *pelB* (174°). All clones were propagated in *E. coli* DH5α cells and verified by sequencing. Fluorescence

microscopy was performed as previously described (18,19,21,23).

Computational analyses

Gamma domain of SpoIIIE was modeled onto the gamma domain of FtsK (2VE9.pdb) using Swiss Model Repository. Gamma subunit interface region was identified using PyMol. Alignment of gamma domains from SpoIIIE (NP_389562.2), FtsK (YP_489162.1 and ZP_14647434.1) was performed using ClustalW Multiple Sequence Alignment.

Protein expression and purification

Expression and purification of soluble SpoIIIE variants was performed using pJB103 in *E. coli* DH5 α as described (22). Protein was purified using NiNTA gravity flow column followed by HiTrap Desalting column (GE Healthcare). Purification buffers used were same as described (22) with the addition of 2 mM MgCl₂ to all buffers and 1 mM ATP in lysis and wash buffers. Storage conditions were as described (22) in 10% glycerol.

ATPase assays

ATPase activity was measured as described (24). ATPase rates were calculated using: ATPase rate = $-(dA_{340}/dt) \times (1/K_{\text{path}}) \times (1/\text{mol of ATPase})$; where 'ATPase rate' is calculated for ATP/minute; ' dA_{340}/dt ' is expressed in OD/min; and ' K_{path} ' is the molar absorption coefficient for NADH at a given optical pathlength. dsDNA fragments used for ATPase activity and DNA binding studies were of 3 types: 3xSRS, 3xrevSRS, and random. The sequence of 3 overlapping permissive SRS is 5'GAGCAGGGAGCAGGGAGCAGGG (11), the sequence of 3 overlapping nonpermissive SRS is 5'CCCTGCTCCCTGCTCCCTGCTC, and the sequence of "random" sequence is 5'AGACAGAGACGATTTTATTCAA.

DNA Binding studies

EMSA of SpoIIIE-DNA binding was performed as described (25). Fluorescence anisotropy measurements were performed as described (26). Binding buffers used: 8 mM Tris pH 8.0, 60 mM NaCl, 12% glycerol, 0.08 mM EDTA, 4 mM MgCl₂, 2 mM DTT, 1 mM AMP-PNP, 0.1mg/mL BSA. EMSA performed with 4 nM

42 bp dsDNA fragments. Fluorescence polarization performed with 10 nM fluorescein-labeled DNA.

RESULTS:

The gamma domain modulates DNA-dependent ATPase activity of the motor domain

Single molecule studies demonstrated that SpoIIIE and SpoIIIE $\Delta\gamma$ have nearly identical translocation rates, but SpoIIIE $\Delta\gamma$ frequently reverses and it remains unclear what is the primary cause of these reversals (11). First, we asked whether the gamma domain has any effect on the ATPase activity of the protein. We purified soluble SpoIIIE and SpoIIIE $\Delta\gamma$, which were truncated for the four N-terminal transmembrane domains. Consistent with previous work (22), we found that the ATPase activity of SpoIIIE was substrate dependent (Fig. 1a). Strikingly, we found that the DNA-stimulated ATPase activity was nearly two orders of magnitude higher than previously observed (13,22). SpoIIIE exhibited a basal rate of 15 ATP/sec/monomer and a maximal rate of 800 ATP/sec/monomer in the presence of DNA (fragmented *B. subtilis* genomic DNA), while the previous studies were performed with a protein whose maximal activity was only 10 ATP/sec/monomer. To our knowledge this is the first time that the ATPase rate of SpoIIIE measured *in vitro* has been sufficiently high to support the necessary DNA transport *in vivo*, estimated to be ~100 ATP/sec/monomer. This estimate is derived from the following: a SpoIIIE hexamer transports ~1.5 MB of DNA across the septum (27,28), 2 bp of DNA are moved per ATP turned over (29,30), and it takes 20 min to complete chromosome transport (23). Indeed, the DNA-stimulated ATPase activity of SpoIIIE that we observed *in vitro* is 800 ATP/sec/molecule, which is 8 times higher than the calculated minimal rate, suggesting that the purified protein displays physiologically relevant activity.

Supercoiled pUC19 stimulated the same ATPase rate of SpoIIIE as genomic DNA (data not shown). To confirm the specificity of the activity, we measured the ATPase activity of a Walker A mutant, SpoIIIE^{K475A}. As expected the basal rate was reduced from wild type and was

not stimulated by the addition of DNA (data not shown).

The ATPase activity of SpoIIIE reflects a composite of many inputs such as DNA-sensing, oligomerization around DNA, and DNA translocation. Additionally, work on FtsK suggests that SpoIIIE/FtsK exhibit cooperative substrate binding and ATP turnover (30), consistent with our results showing apparent sigmoidal dependency of ATPase activity on DNA.

Next, we compared ATPase activity of SpoIIIE to that of SpoIIIE^{Δγ} and made two intriguing observations. First, deletion of the gamma domain abolished DNA-dependent ATPase stimulation. Second, the basal ATPase rate of SpoIIIE^{Δγ} was elevated five fold (to 80 ATP/sec/monomer) over that of SpoIIIE (Fig. 1b). These findings differ from an early observation that FtsK^{Δγ} is a robust ATPase (28), but concentration differences between the two assays readily explain this difference. *In vivo*, the local concentration of SpoIIIE during chromosome transport is predicted to be 200-800 nM. Our studies were performed with 400 nM SpoIIIE^{Δγ}, while ATPase activity for FtsK^{Δγ} was measured at 20 μM, which is significantly higher than the physiological concentration. Our data highlight two previously unknown roles for the gamma domain: 1) activation of ATPase activity generated by the motor domain in the presence of DNA and 2) inhibition of ATPase activity in the absence of DNA.

Structural studies have indicated that the RecA-like motor domain forms a hexameric ring around the DNA with an extensive DNA-motor domain interface (13,28). Since the nucleotide-binding pocket of SpoIIIE/FtsK is at the interface of adjacent motor domains, it has been suggested that nucleotide binding is closely linked to oligomerization. Thus, these observations postulated that the RecA-like motor domain senses DNA for both oligomerization and stimulation of ATP hydrolysis. Yet, our findings clearly demonstrate that the spatially separate gamma domain senses DNA and stimulates ATPase activity of the motor domain.

A simple model to explain the inhibition we observe would be that the gamma domain affects the affinity of SpoIIIE for ATP. To address this, we asked if increasing ATP

concentration could rescue ATPase activity of SpoIIIE^{Δγ}. Titrating in ATP, we compared ATPase activity of SpoIIIE and SpoIIIE^{Δγ} and observed that the apparent K_m stayed the same, 0.26 ± 0.12 mM and 0.27 ± 0.11 mM respectively (Fig. 1c). These data confirm that deletion of the gamma domain is not altering the intrinsic affinity for ATP. Instead, we find that the V_{max} has changed, suggesting that the inhibitory role of the gamma domain may be more complex than simple inhibition of ATPase activity.

The gamma domain activates DNA-dependent ATPase activity through sensing of the recognition motif in a permissive orientation

We wondered whether the role of gamma in DNA-stimulated ATPase activity of SpoIIIE is separable or linked to its role in directionality. The substrate-dependent ATPase activity measured in Fig. 1a did not distinguish between stimulation resulting from DNA binding and that resulting from processive transport. Therefore we needed a way to measure ATPase activity that is associated primarily with the DNA sensory step and not that derived from continuous translocation. To do this, we designed short dsDNA fragments with the SRS sequence at the end. These fragments are long enough to accommodate assembly of SpoIIIE on DNA but such that after recognition of the SRS the protein is not provided with any DNA for active translocation.

We utilized three different classes of dsDNA fragments: 1) 3 overlapping SRSs in a permissive orientation (3xSRS), 2) 3xSRS in a non-permissive orientation (3xrevSRS), and 3) random fragments where the GC-rich SRS motif is replaced with a random AT-rich sequence to avoid possible recognition of degenerate SRS-like sequences (Fig. 2a). We compared ATPase activity of SpoIIIE when stimulated by these dsDNA fragments. All DNA substrates were used at a base pair concentration of 20 μM, which stimulated maximal ATPase activity in the genomic DNA titration (Fig. 1b). 3xSRS dsDNA fragment stimulated comparable ATPase activity to *B. subtilis* genomic DNA (Fig. 2b). In contrast, both random and 3xrevSRS dsDNA fragments did not stimulate significant ATP turnover above the basal rate

(Fig. 2b), demonstrating that the gamma domain specifically stimulates high ATP turnover in response to SRS. 3xrevSRS does not provide sufficient DNA for SpoIIIE to bind in the opposite orientation. Our data demonstrate that when SpoIIIE does bind to 3xrevSRS, it does not recognize the non-permissive SRS and inhibition of ATPase activity is not relieved.

We show that permissive SRS stimulates ATPase activity to more than 20 fold above the stimulation by random or non-permissive SRS (Fig. 2b). A recent study observed a potential (1.6 fold) stimulation by SRS DNA, although in this case the assay did not uncouple activity from DNA sensing and translocation (13). Our data demonstrates that the gamma domain stimulates high ATPase activity when encountering recognition sequences in a permissive orientation and does not stimulate ATPase activity when encountering either non-specific DNA or recognition sequences in a non-permissive orientation.

We next asked if, once activated by permissive SRS, the gamma domain actively inhibits ATPase activity of SpoIIIE during translocation when it encounters recognition sequences in a non-permissive orientation. Previous structural studies combined with the loading model suggest that since a trimer of gamma domains is required to recognize the entire 8-nucleotide SRS/KOPS sequence and the linker between the motor and the gamma domains is relatively short, that linker length may geometrically constrain the orientation of the gamma domain with respect to the motor (10,28). To address this directly, we measured ATPase activity in the presence of dsDNA fragments with permissive SRS followed by non-permissive SRS. We found that, once activated, SpoIIIE ignores the so-called non-permissive SRS. Therefore, our work supports the structural hypothesis. Similar ATPase rates were stimulated by fragments with permissive SRS as by those where the permissive SRS were followed by non-permissive SRS: 3xSRS-3xrevSRS and 3xSRS-1xrevSRS dsDNA fragments stimulated comparable ATPase activity as 3xSRS dsDNA; and 1xSRS-3xrevSRS dsDNA fragment stimulated comparable ATPase activity as 1xSRS dsDNA

(Fig. 2c). These data demonstrate that during translocation the gamma domain couples ATPase activity to sensing of recognition sequences in a permissive orientation. Once ATPase activity is stimulated, the gamma domain does not inhibit ATPase activity when encountering the specificity sequence in a non-permissive orientation. Thus, a translocating SpoIIIE/FtsK protein is insensitive to the non-permissive recognition sequences found throughout bacterial chromosomes. This observation explains how a member of this family of proteins can successfully transport DNA in the face of ~15% non-permissive recognition sequences, found throughout bacterial chromosomes.

We confirmed that the ATPase activity we were measuring was primarily a result of SpoIIIE assembly and sequence recognition by determining the minimal DNA length required for SpoIIIE assembly and V_{max} ATPase stimulation. Based on modeling onto the FtsK crystal structure (10,28), soluble SpoIIIE is predicted to protect 24 bp of DNA (the motor domain protects ~18 bp and the gamma trimer protects 8 bp, which is the size of the recognition sequence). We compared ATPase activity of SpoIIIE when stimulated by dsDNA fragments of different sizes, namely: 22 bp (accommodating only the 3xSRS sequence), 42 bp, 61 bp, and 80 bp (Fig. 2b). SpoIIIE exhibited length-dependent ATPase activity up to 61bp DNA fragments, which we determined is the minimal dsDNA fragment length that stimulates maximal ATPase activity. Similar to other DNA-binding ATPases, such as the RSC complex (31), SpoIIIE exhibits maximal ATPase activity when stimulated by a DNA fragment that is significantly longer than its minimal DNA binding length.

Lastly, we asked if a higher density of SRS results in a greater stimulation of ATPase activity. We performed DNA titrations with dsDNA fragments of different lengths. The apparent K_m for gDNA was 6.5 μ M base pairs, while the apparent K_m values for 42 bp, 61 bp, and 80 bp 3xSRS dsDNA fragments were 5.1 μ M, 1.8 μ M, and 1.3 μ M, respectively (Fig. 2d). Shorter templates have a higher ratio of SRS to non-SRS DNA than genomic DNA, which suggests that the search time necessary to find

SRS in gDNA is longer than in the dsDNA fragments where the SRS are more readily available. Thus, to achieve comparable ATPase activity, SpoIIIE requires a higher concentration of the gDNA substrate than the dsDNA fragments. These results also suggest that the rate-limiting step in DNA transport by SpoIIIE is translocation rather than DNA sensing, since short DNA templates stimulate ATPase activity to a higher level than long ones.

The gamma domain contributes to DNA binding

In vitro studies with FtsK showed that, in isolation, gamma preferentially binds to the KOPS over random DNA (12,16). Given the newly determined role of the gamma domain, we set out to determine if the gamma-DNA interactions result in a relay of ATP-stimulating information to the motor domain. First, we applied a solution-based assay, fluorescence polarization, to determine whether the gamma domain confers sequence-specificity. We measured the fluorescence anisotropy of fluorescein (f) end-labeled 30bp dsDNA fragments in the presence of SpoIIIE. A protein titration showed that the apparent K_d of SpoIIIE for f-2xSRS dsDNA fragments is 14.7 times lower than the apparent K_d of SpoIIIE $^{\Delta\gamma}$ (Fig. 3a, Table 1). Our findings demonstrate that the gamma domain significantly contributes to DNA-binding of soluble SpoIIIE.

To independently confirm these results, we tested whether unlabeled 2xSRS dsDNA fragments competitively inhibit SpoIIIE binding to f-2xSRS dsDNA fragments by looking at two different concentrations of SpoIIIE, 152 nM and 305 nM. Competition experiments with both concentrations of SpoIIIE yielded the same K_i for 2xSRS dsDNA fragments (Fig. 3b, data not shown), establishing that unlabeled DNA is a competitive inhibitor of end-labeled DNA. Performing a similar competition experiment with SpoIIIE $^{\Delta\gamma}$ demonstrated that its K_i was 30 times higher than that of SpoIIIE (Fig. 3c, Table 1). This data confirms that the gamma domain has a considerable contribution to DNA binding of SpoIIIE. In the context of this experiment, we noted that K_i values of unlabeled 2xSRS fragments are lower than the K_d values for f-2xSRS fragments, suggesting that SpoIIIE binds

less tightly to labeled than unlabeled DNA. Thus, all binding results were compared either only between labeled or between unlabeled substrates. Importantly, the trends observed in the DNA binding studies were consistent between measurements made by direct binding to fluorescein-labeled template or competitive unbinding with unlabeled template.

Next we wanted to determine whether deleting the gamma domain impairs the ability of SpoIIIE to oligomerize on DNA. To this end, we performed electrophoretic mobility shift assays (EMSA) with 42 bp 3xSRS dsDNA fragments. Each of the tested SpoIIIE variants showed a number of shifted DNA species with the slowest migrating species accumulating proportionally to protein concentration (Fig. 4a). This slowest migrating species accumulated with both SpoIIIE and SpoIIIE $^{\Delta\gamma}$, though a higher concentration of SpoIIIE $^{\Delta\gamma}$ was required to achieve similar accumulation. In contrast, the non-translocating variant SpoIIIE V429M (previously called SpoIIIE36) (32) does not form this species. Structural studies have demonstrated that both SpoIIIE and FtsK $^{\Delta\gamma}$ oligomerize into hexameric rings both in the absence and presence of DNA (13,28). Additionally, we have confirmed that both SpoIIIE and SpoIIIE $^{\Delta\gamma}$ oligomerize into a higher molecular weight complex by size exclusion chromatography, while the *in vivo* non-translocating variant SpoIIIE V429M does not (data not shown). Given that both SpoIIIE and SpoIIIE $^{\Delta\gamma}$ are able to support *in vivo* function, we suggest that only the translocation competent proteins form the slowest migrating band observed by EMSA, while absence of the slowest migrating species correlates with non-translocation. We quantified the disappearance of free DNA in the EMSA to determine if deleting the gamma domain results in a DNA-binding defect of the motor protein. The apparent K_d of SpoIIIE variants for DNA indicated that SpoIIIE binds DNA six fold tighter than SpoIIIE $^{\Delta\gamma}$ (Fig. 4b). Our data demonstrate that deleting the gamma domain does not prevent the RecA-like motor domain from oligomerizing on DNA into the same species as wild type. The EMSA observations also support our earlier finding that the gamma domain contributes to DNA binding of SpoIIIE.

Computational and biochemical studies of FtsK KOPS-dependent oligomerization and ATPase activity suggested cooperative kinetics (30), so we wanted to know if deleting the gamma domain affects the way in which SpoIIIE binds DNA. Using our EMSA data, we calculated that the Hill coefficients for SpoIIIE and SpoIIIE^{Δγ} are 2.31 and 2.34, respectively, indicating that deletion of the gamma domain does not impair the mode of DNA binding by SpoIIIE. This means that the gamma domain significantly contributes to the affinity for DNA, but that its absence does not prevent SpoIIIE from hexamerizing on DNA.

The gamma domain confers SRS-specific binding

Studies of FtsK suggested that the gamma domain mediates directional assembly of the protein on the DNA through its interactions with the KOPS (17). We asked whether gamma facilitates binding specificity and if specific binding stimulates ATPase activity. To answer these questions, we measured ATPase activity of SpoIIIE when stimulated by 1 μM and 2 μM of 80bp 3xSRS dsDNA fragments, while titrating in increasing concentrations of 80 bp random dsDNA fragment. At both dsDNA concentrations, the K_i of random DNA was 14 times higher than the apparent K_m of SpoIIIE for 80bp 3xSRS dsDNA fragments (Fig. 4c).

ATPase activity studies showed that the gamma domain activates ATPase activity in a sequence specific manner, while DNA binding studies demonstrated that deletion of the gamma domain lowers the affinity of the soluble translocase for DNA. We hypothesized that the gamma domain confers DNA-binding sequence specificity. To this end, we compared affinity of SpoIIIE for f-30 bp 2xSRS with f-30 bp random dsDNA fragments by fluorescence polarization. The results show that SpoIIIE prefers SRS-containing DNA nearly by a factor of 3 (Fig. 3a, Table 1). We also measured the K_i of unlabeled 2xSRS and random dsDNA fragments when titrating away SpoIIIE from fluorescein-labeled 2xSRS dsDNA fragments (Fig. 3b). The apparent K_i of SpoIIIE is 7.6 times lower for 2xSRS than it is for random dsDNA fragments (Table 1).

Additionally, we demonstrated that deleting the gamma domain abolishes SRS specificity. We compared fluorescence anisotropy of f-2xSRS dsDNA fragments to f-random dsDNA fragments when bound by SpoIIIE^{Δγ}. The affinity of SpoIIIE^{Δγ} for 2xSRS and random DNA was nearly identical (Fig. 3a, Table 1). The same trend was observed by titrating increasing concentrations of unlabeled 2xSRS or random dsDNA fragments into a reaction where SpoIIIE^{Δγ} was bound to f-2xSRS dsDNA fragments (Fig. 3c). Our findings demonstrate that that gamma domain mediates tighter binding of SpoIIIE to DNA containing the recognition sequence, which is translated into sequence-specificity of ATPase stimulation.

Together, we show that the deletion of the gamma domain abrogates the protein's ability to selectively identify and bind to recognition sequences. The binding and ATPase data demonstrate that the gamma domain promotes selective binding of SpoIIIE to SRS over non-SRS containing DNA, which is translated into ATPase stimulation.

Perturbing the putative gamma interface uncouples DNA sensing from SRS recognition by conferring tighter DNA-binding

We wondered whether communication is strictly within a single subunit from gamma to the motor domain or whether inter-subunit communication between adjacent gamma domains relays information regarding directional motion. A crystal structure of FtsK gamma domains revealed that a trimer of gamma domains is necessary to recognize all 8 nucleotides of the KOPS (10). Thus, we predicted that perturbing the gamma subunit interface would impair the ability of SpoIIIE to sense SRS. Making use of the 42% identity between the gamma domains (Fig. 5a), we threaded the sequence of the SpoIIIE gamma (Fig. 5b) onto the structure of FtsK gamma from *P. aeruginosa* (10). The threaded model allowed us to identify highly conserved R745 and R756 (Fig. 5a), which fall in the putative subunit interface. We mutated these residues to probe whether communication between adjacent gamma domains contributes to sequence recognition. We made two substitutions in position 745, removing the positive charge (A)

and reversing the charge (E), and one substitution in position 756, reversing the charge (E).

Whereas structural studies showed that three gamma domains are required for interaction with an entire SRS, it was not clear whether stimulation of ATPase activity requires communication between gamma subunit or whether activation was limited to the gamma and motor domains of the same subunit. Thus we tested whether perturbing the gamma subunit interface would affect ATPase activity. We found that the ATPase activity of SpoIII^E^{R745A}, SpoIII^E^{R745E} and SpoIII^E^{R756E} was not stimulated by the addition of gDNA and that basal rate of all of these variants was elevated at least 2 fold above wild type (Fig. 5c). Yet, ATPase activity of all three variants was stimulated ~two fold by 80 bp dsDNA fragments. SpoIII^E^{R745A} and SpoIII^E^{R756E} displayed no sequence specificity for SRS fragments, while SpoIII^E^{R745E} exhibited only a mild preference (1.2 fold) for SRS fragments (Fig. 5c). These data suggest that perturbing the subunit interface of the gamma domain results in impaired inhibition of basal ATPase rate and a loss of sequence recognition.

We wanted to exclude the possibility that, in the absence of DNA, charge repulsion between gamma domains could account for the low wild type basal rate of ATPase activity. Thus, to confirm that the elevated basal rate of SpoIII^E^{Δγ} was not an electrostatic artifact, we performed ATP titrations with SpoIII^E^{R745A} and SpoIII^E^{R745E} (Fig. 6a). Both of these variants exhibited similar affinity for ATPase wild type ($K_m = 0.26 \pm 0.12$ mM) and SpoIII^E^{Δγ} ($K_m = 0.27 \pm 0.11$ mM), K_m of 0.37 ± 0.16 mM and 0.39 ± 0.13 mM for SpoIII^E^{R745A} and SpoIII^E^{R745E} respectively. Our data demonstrate that the deletion or subtle mutations within the gamma domain do not alter the ability of the RecA-like motor domain to bind and oligomerize around nucleotide.

The ATPase assays with short DNA fragments and SpoIII^E suggest that the rate-limiting step in transport is translocation rather than DNA sensing. As expected, we see that these variants display a similar, but exaggerated, trend as observed for wild type (Fig. 2d). Since the interface variants are stimulated to a lower

ATPase rate by longer substrates (primarily translocation on DNA) than by shorter substrates (primarily DNA binding), we hypothesized that interface impaired gamma domains result in tighter DNA binding of the full-length protein. This hypothesis was confirmed by EMSA and fluorescence polarization. EMSA studies found that both SpoIII^E^{R745A} and SpoIII^E^{R745E} maintain the accumulation of the slowest-migrating species (Fig. 6b), suggesting they stimulate at least the same level of assembly on DNA as SpoIII^E. In fact, fluorescence anisotropy measurements showed that both interface variants had higher DNA-affinity than SpoIII^E (Fig. 6c/d). In agreement with ATPase results, SpoIII^E^{R745E} exhibited mild sequence specificity, while SpoIII^E^{R745A} had none (Table 2). The tighter binding of both interface variants may provide a possible explanation why longer DNA fragments stimulate lower ATPase activity than shorter fragments, since the primary contribution to the ATPase activity measured for short fragments is from the SpoIII^E-DNA binding event, while for long fragments it is from translocation.

If the differences between ATPase stimulation by gDNA and dsDNA fragments were due to different DNA binding affinities rather than translocation rates, then we would expect an increase in ATPase activity when adding short dsDNA fragments to interface variants that are translocating on gDNA. Increasing the dsDNA fragment concentration resulted in increased stimulation of ATPase activity in both SpoIII^E^{R745A} and SpoIII^E^{R745E} (Fig. 5d/e). Our data demonstrate that gamma-gamma contacts are important for maintaining an appropriate DNA affinity to mediate sequence recognition without disrupting the ability to transmit information and translocate DNA. Damaging these inter-subunit contacts results in non-specific DNA binding that may be too tight, thereby causing impaired sequence recognition and hindering processive DNA translocation. Thus, gamma subunit coordination is necessary to maintain a high sequence specific ATPase activity and to inhibit ATPase activity in the absence of DNA, preventing futile rounds of ATP turnover.

The gamma domain is necessary for robust DNA transport

Our data demonstrate that the gamma domain plays an important role in ATPase stimulation of sequence-recognition. Therefore we predicted that the defect exhibited by SpoIIIE^{Δγ} is not limited to the terminal region, but manifests along the entire length of the chromosome.

To address this, we took advantage of a previously defined two-color fluorescence DNA transport assay in sporulating *B. subtilis* cells (23) (Fig. 7a). In this assay we assess the apparent transport rate of a *cfp* reporter integrated at positions 90°, 117°, 129°, 132°, 138°, 142°, 152°, 162°, and the terminus at 174° (on a circular 360° chromosome) (Fig. 7b). We integrated an *yfp* reporter near the chromosome origin, which is anchored in the forespore prior to asymmetric septation. Both *yfp* and *cfp* reporters were expressed under an early forespore-specific transcription factor, which is activated upon the completion of septation. Thus, detection of YFP serves as a measure of initiation of sporulation and detection of CFP indicates that a specific region of the chromosome has been transported into the forespore (Fig. 7d).

We measured the ratio of CFP-positive to YFP-positive cells in a large population of cells in the early stages of sporulation. In this assay, SpoIIIE transports the entire forespore chromosome across the septum in approximately 20 min (23) (Fig. 7b). Transport by SpoIIIE is too rapid for the assay to fully resolve the transport of different loci along the length the chromosome. In contrast, SpoIIIE^{Δγ} has an overall much lower apparent rate of DNA transport with loci distinguishable along the length of the chromosome (Fig. 7c). SpoIIIE^{Δγ} transports an origin-proximal site (90°) at the same apparent rate as SpoIIIE, but the apparent rate of DNA transport of origin-distal sites declines significantly in cells with SpoIIIE^{Δγ}. Only 20% of the population successfully transports *cfp* at 138° and barely 5% of the population completes transport of the terminus (174°). The latter require over 3 hours to do so (Fig. 7c). These data reveal that deletion of the gamma domain results in a translocation defect that becomes progressively worse along the

length of the chromosome. Our findings demonstrate that the defect of SpoIIIE^{Δγ} is not limited to the terminus region but is rather manifested along the entire length of the translocated chromosome. Additionally, our findings show that DNA-stimulated ATPase activity is not essential to support some chromosome transport. The elevated basal rate of SpoIIIE^{Δγ} (80 ATP/sec/monomer, which is close to the minimal theoretical ATPase rate of 100 ATP/sec/monomer) is sufficient to support significant transport, even if the rate of transport is severely impaired.

DISCUSSION

We set out to understand how the DNA-interaction domain of SpoIIIE/FtsK proteins can provide both specificity for DNA recognition and the ability to maintain processive translocation. Our findings provide insight into the molecular mechanism of coordinated stimulation that underlies the critical step of directional activation (Fig. 7e). Our results demonstrate that the gamma domain modulates ATPase activity of the motor in two ways: 1) inhibition of ATPase activity in the absence of DNA or in the presence of DNA but in the absence of the recognition sequence and 2) activation of ATPase activity upon sensing the recognition motif in a permissive orientation (Fig. 1b, 2b). Titration of ATP revealed that the $V_{\max}$ of SpoIIIE^{Δγ} was much lower than the $V_{\max}$ of SpoIIIE, while the K_m remained unchanged (Fig. 1c). Thus, without gamma, the motor domain is incapable of achieving high DNA-stimulated ATP turnover. Since the gamma domain is a spatially separate domain from the RecA-like ATPase domain, our findings reveal that the gamma domain functions as an allosteric regulator of ATPase activity of the motor domain through DNA sensing.

Furthermore, we illustrate that the gamma domain regulates ATPase activity via sequence specific interactions defined first at the stage of sequence recognition following initial loading onto the DNA and later during directional translocation. First, we showed that the gamma domain confers higher affinity for SRS-containing DNA (Fig. 3a). Second, the gamma domain modulates directionality by stimulating ATPase activity only when it

encounters a permissive recognition motif (Fig. 2b). Previous experiments suggested that when directional translocases encounter non-permissive sequences, they reverse direction (11,14). Our data demonstrates that when confronted with non-permissive recognition sequences, SpoIIIE is insensitive to them (Fig. 2c), which agrees with the geometric constraints potentially imposed by the short linker between the motor and gamma domains (10). This explains how a translocase can successfully maintain directional DNA transport despite encountering recognition sequences orientated opposite to the direction of translocation. We suggest that the necessity to maintain a robust ATPase rate, which we conclude is needed to support rapid transport (Fig. 7b/c), accounts for the strict conservation of the directionality motifs throughout the chromosomes of organisms that employ member of the FtsK/SpoIIIE family to mediate chromosomal segregation. Additionally, since the gamma domain inhibits ATPase activity in the absence of recognition sequences, we suggest that ATP hydrolysis is not part of the sequence search as described independently (13). We propose that sensing a permissive recognition sequence triggers the ATPase activity through allosteric transmission of sequence information to the motor domain.

A closer look at the mechanism of sequence-specificity revealed that coordination is not limited to the gamma and motor domains within the same subunit, but that gamma-gamma contacts between subunits are important to maintain sequence specificity (Fig. 5, Fig. 6). Threading the sequence of the SpoIIIE gamma domain onto the FtsK gamma domain structure (10) allowed us to identify two conserved arginines (R745 and R756) that protrude into the gamma-gamma subunit interface (Fig. 5b). The functional groups of both arginines are in close proximity ($\sim 3\text{\AA}$) from charged residues of adjacent subunits. Whereas the backbone of R745 is within $4\text{\AA}$ of the DNA, R756 is more than $10\text{\AA}$ away from the DNA. We show that mutating either of these arginines results in an almost identical loss of sequence specificity and a significant decrease in DNA-dependent ATPase stimulation (Fig. 5c), demonstrating that both of these residues act on the same level of

gamma domain function and that an intact gamma-gamma interface is vital for gamma functionality.

Our biochemical analysis of SpoIIIE^{R745A} and SpoIIIE^{R745E} reveals that the severely decreased ATPase stimulation and a loss of sequence specificity are likely the result of higher DNA affinity exhibited by both variants (Fig. 5, Fig. 6). Our findings indicate the importance of the gamma-gamma interface in sequence recognition and DNA binding. Cattoni et al. present data that suggest that gamma-gamma perturbations do not significantly impact levels of ATP turnover and of DNA binding affinity (13). These data are in contradiction with both our work and structural studies, which propose that oligomerization of three gamma domains is necessary for sequence-specific binding (10).

We propose that, upon sensing of a permissive recognition sequence, gamma undergoes a conformational change, which translates information to the motor domain as well as to the adjacent gammas, setting up an information relay such that each consecutive gamma domain will activate its own motor domain. Since directionality sequences are found $\sim 12\text{ kB}$ throughout bacterial chromosomes (16), ATPase re-stimulation must be necessary throughout transport. These results point to the question of whether and how ATPase activity decays throughout translocation.

SpoIIIE ^{$\Delta\gamma$} allows us to examine how a sequence-insensitive translocase is able to transport DNA, pointing to the importance of an accessory domain like gamma. In fact, the *in vivo* transport study revealed a delay in chromosome transport that is manifested along the entire chromosome. We suggest that this delay is caused at least partly by the inability of SpoIIIE ^{$\Delta\gamma$} to modulate ATPase activity. Our work excludes neither the loading model (10,17,30) nor the recently published sliding/hopping search model (13). Both models leave open the question of how the ATPase state of the motor domain is switched from “off” to “on”. Our data provides a mechanistic context in which to understand the switch.

The strongest molecular evidence for the loading model comes from single-molecule translocation studies, which showed that in a

non-hydrolyzing state (in the presence of ADP or AMP-PNP), FtsK preferentially binds to KOPS, which determines the direction of translocation. Yet, this same study noted that in a hydrolyzing state (in the presence of ATP), KOPS recognition is suppressed and the protein binds anywhere (17), which supports the findings of the recently proposed sliding/hopping search model (13). Since in vivo the concentration of ATP is >3mM (33), members of the SpoIIIE/FtsK family primarily exist in a hydrolyzing state. Given the findings of Lee et al. and the present study, this allows SpoIIIE/FtsK to oligomerize to DNA even in the absence of a recognition sequence and upon translocation past a permissive SRS/KOPS, the gamma domain stimulates robust ATPase activity and rapid transport.

Our work rules out the recognition model because once bound to DNA, SpoIIIE is not affected by non-permissive SRS. Yet, our work unites the loading and sliding/hopping search models (11,13,17) because regardless of the mode of oligomerization (loading directly only DNA or oligomerizing prior to DNA binding, respectively), we show that the coordination between subunits of the gamma domain is necessary to support sequence-specific activation of the motor domain that is required for robust directional chromosome transport (Fig. 7). We propose that the gamma domain allows SpoIIIE/FtsK proteins to maintain a low basal ATPase rate prior to their recruitment to the trapped chromosome, preventing futile rounds of ATP hydrolysis. Upon recruitment, the translocase binds to the DNA, which brings the gamma domain in contact with DNA. Initially the protein exhibits a low ATPase rate and presumably translocates DNA slowly, but a coordinated encounter with a permissive recognition sequence results in gamma-mediated stimulation of a high ATPase rate and robust translocation.

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FIGURE LEGENDS:

Figure 1: The γ domain modulates ATPase activity of SpoIIIE. a) Soluble SpoIIIE exhibits DNA-dependent ATPase activity. ATP turnover was measured using a NADH⁺-coupled ATPase assay. The basal rate is 15 and the maximal rate is 800 ATP/sec/molecule. Fits modeled using Hill form of Michaelis-Menten equation: $15+m1*m0^m3/(m2+m0^m3)$; where $m1$ is V_{max} , $m2$ is K_m , and $m3$ is the Hill coefficient. b) Deletion of the γ domain results in an elevated basal rate at 80 ATP/sec/molecule, which is not stimulated by DNA. c) ATPase activity of ATP concentration titration for SpoIIIE and SpoIIIE $\Delta\gamma$ in the presence of 20 μ M gDNA. Inset shows curve at lower ATP concentrations. Fits modeled using substrate activation form of Michaelis-Menten equation (34): $m1 / (1+m2/m0) * ((1+m3*m0/m4) / (1+m0/m4))$; where $m1$ is the V_{max} , $m2$ is the K_m , $m3$ is the efficiency of product formation and $m4$ is the activation constant.

Figure 2: The γ domain confers sequence specific ATPase stimulation. a) dsDNA fragments (3xSRS, 3xrevSRS, random) were used to measure ATPase activity that is primarily generated by initial DNA binding and sequence recognition. b) The γ domain only stimulates ATPase activity when binding to permissive SRS. On x-axis: 0 = no DNA; 22, 42, 61, 80 = length of dsDNA fragment in base pairs; and g = gDNA. This data is presented in semi-log format (y-axis is log scale). c) SpoIIIE is insensitive to non-permissive SRS. ATPase experiments suggested that 3xrevSRS dsDNA fragments do not stimulate ATPase activity above basal rate. To confirm, we compared ATPase stimulation by dsDNA fragments that contain SRSs followed by revSRS. The 64 bp 3xSRS-3xrevSRS (cartoon depicted) and 50 bp 3xSRS-1xSRS stimulated similar ATPase activity as 61 bp 3xSRS, while 50 bp 1xSRS-3xrevSRS stimulated similar ATPase activity as 61 bp 1xSRS. On x-axis: 1 = no DNA, 2 = gDNA, 3 = 64bp 3xSRS-3xrevSRS, 4 = 50bp 3xSRS-1xrevSRS, 5 = 50bp 1xSRS-3xrevSRS, 6 = 61bp 1xSRS. Fits modeled using Hill form of Michaelis-Menten equation: $15+m1*m0^m3 / (m2+m0^m3)$; where $m1$ is V_{max} , $m2$ is K_m , and

$m3$ is the Hill coefficient. d) K_m values for 3xSRS fragments are lower than for gDNA. ATPase activity measured during DNA titrations of 42 bp, 61 bp, and 80 bp 3xSRS dsDNA fragments. The x-axis was scaled to emphasize the differences in V_{max} , which somewhat obscures the fact that all dependencies presented here sigmoidal. This data is presented in linear format (y-axis is linear scale). Data represented as mean $\pm$ SEM.

Figure 3: The γ domain exhibits higher DNA affinity for SRS-containing DNA. a) The γ domain confers DNA binding and sequence specificity. Fluorescence polarization studies of SpoIIIE and SpoIIIE $\Delta\gamma$ with SRS and random DNA templates. 10 nM of f-labeled 2xSRS 30 bp dsDNA was used as binding template. Binding data was modeled using Hill form of the Michaelis-Menten equation: $m1*m0^m3/(m2+m0^m3)$; where $m1$ is V_{max} , $m2$ is K_d , and $m3$ is the Hill coefficient. b) SpoIIIE preferentially binds to SRS over random DNA. After incubating 152 nM of SpoIIIE with 10 nM of 30 bp f-2xSRS dsDNA fragment, we titrated in increasing concentrations of unlabeled 30 bp 2xSRS or random DNA. SpoIIIE binds tighter to 2xSRS than to random by a factor for 7.6. K_i of 2xSRS is 27.8 ± 15.8 nM, while random is 211.5 ± 41.1 nM. c) SpoIIIE $\Delta\gamma$ does not exhibit sequence specificity in binding. After incubating 1550 nM of SpoIIIE $\Delta\gamma$ with 10 nM of 30 bp fluorescein-2xSRS dsDNA fragment, we titrated in increasing concentrations of unlabeled 30 bp 2xSRS or random DNA. SpoIIIE $\Delta\gamma$ binds SRS and random sequences with the affinity. K_i of 2xSRS is 856.3 ± 93.3 nM, while random is 876.0 ± 131.7 nM. K_i values were calculated after modeling data using equation: $K_i=[I]_{50}/([L]_{50}/K_d+[P]_0/K_d+1)$ where $[I]_{50}$ denotes the concentration of the free inhibitor at 50% inhibition, $[L]_{50}$ is the concentration of the free labeled ligand at 50% inhibition, $[P]_0$ is the concentration of the free protein at 0% inhibition, and K_d is the dissociation constant of the protein-ligand complex. Data represented as mean $\pm$ SEM.

Figure 4: The γ domain contribution to DNA binding is separable from sequence specificity. a) Both SpoIIIE and SpoIIIE $\Delta\gamma$ accumulate a slow-migrating species. DNA binding of

SpoIIIE, SpoIIIE^{Δγ}, and SpoIIIE^{V429M} was observed using EMSA on 4 nM of 42bp 3xSRS dsDNA fragments. b) Quantification of the free DNA in EMSA shows that SpoIIIE has a K_d of 63.2 ± 11.7 nM, while SpoIIIE^{Δγ} has a K_d of 204.5 ± 37.6 nM for 3xSRS fragments. EMSAs were performed with 4 nM 42 bp 3xSRS dsDNA fragments. c) SpoIIIE preferentially binds to SRS. ATPase activity stimulated by either 2 μM or 1 μM base pairs of 80 bp 3xSRS dsDNA fragments while titrating in 80 bp random dsDNA fragments. K_i values were calculated after modeling data using equation: $K_i = [I]_{50} / ([L]_{50} / K_d + [P]_0 / K_d + 1)$ where $[I]_{50}$ denotes the concentration of the free inhibitor at 50% inhibition, $[L]_{50}$ is the concentration of the free ligand at 50% inhibition, $[P]_0$ is the concentration of the free protein at 0% inhibition, and K_d is the dissociation constant of the protein-ligand complex. Data represented as mean±SEM.

Figure 5: Mutations in the putative γ subunit interface uncouple DNA binding from SRS recognition. a) Sequence alignment of γ domains from FtsK in *E. coli*, FtsK in *P. aeruginosa*, and SpoIIIE in *B. subtilis*. Arrow points to conserved Arg (Arg745 in SpoIIIE). SpoIIIE sequence numbering is based on revised sequence (YP_007533637.1). b) Cartoon of threaded structure of trimer of SpoIIIE γ domains onto γ domain structure from FtsK in *P. aeruginosa* (2VE9.pdb). Arg745 is shown in black. c) SpoIIIE^{R745A} and SpoIIIE^{R745E} exhibit low stimulation of ATPase activity by gDNA, but 80 bp dsDNA fragments stimulate higher ATPase activity. All DNA templates were provided at 20 μM base pair concentration. d) Shorter templates stimulate higher ATPase activity of SpoIIIE^{R745A} and e) SpoIIIE^{R745E} than longer templates. An ATPase assay was initiated in the presence of 10 μM base-pairs of gDNA, before 80 bp dsDNA fragments (3xSRS or random) were titrated in. On x-axis for parts d and e: 1 = no DNA; 2 = gDNA, no addition; 3 = gDNA, 20 μM of 80 bp dsDNA fragment; 4 = gDNA, 40 μM of 80 bp dsDNA fragment; 5 = gDNA, 100 μM of 80 bp dsDNA fragment. Data represented as mean±SEM.

Figure 6: Perturbing the γ-γ interfaces results in tighter DNA binding and loss of

sequence specificity. a) ATPase activity of ATP concentration titration for SpoIIIE^{R745A} and SpoIIIE^{R745E} compared to SpoIIIE and SpoIIIE^{Δγ} in the presence of 20 μM gDNA. The inset curve zooms in to the lower concentrations, which are included in the full graph. Fits modeled using Hill form of the Michaelis-Menten equation: $m1 * m0^m3 / (m2 + m0^m3)$; where $m1$ is V_{max} , $m2$ is K_m , and $m3$ is the Hill coefficient. SpoIIIE and SpoIIIE^{Δγ} curves are the same as shown in Fig. 1c. b) SpoIIIE^{R745A} and SpoIIIE^{R745E} maintain the prevalence of the slowest migrating species by EMSA. EMSAs were performed with 4 nM of 42 bp 3xSRS dsDNA fragments. c) SpoIIIE^{R745A} binds DNA in a sequence unspecific manner. Fluorescence anisotropy measurements with 10 nM of 30 bp 2xSRS and random dsDNA fragments show the same binding curves. Additionally, SpoIIIE^{R745A} binds DNA twice as tightly as SpoIIIE. d) SpoIIIE^{R745E} exhibits mild sequence-specificity in DNA binding. Fluorescence anisotropy measurements were performed with 10 nM of 30 bp 2xSRS and random dsDNA fragments. Additionally, SpoIIIE^{R745E} binds DNA twice as tightly as SpoIIIE. Binding data was modeled using Hill form of the Michaelis-Menten equation: $m1 * m0^m3 / (m2 + m0^m3)$; where $m1$ is V_{max} , $m2$ is K_d , and $m3$ is the Hill coefficient. Data represented as mean±SEM.

Figure 7: Coordinated activity of γ domains is important for maintaining robust chromosome transport in vivo. a) Transport of several loci on the *B. subtilis* chromosome were analyzed using a quantitative two-color fluorescence microscopy assay (23). The genes for both of these proteins were placed under P_{spoIIQ} promoter, which is turned on immediately after septation by a forespore specific transcription factor (σ^F). To identify cells that have completed septation, the *yfp* reporter was placed near the origin, which is found inside the forespore prior to septation. The *cfp* reporter was placed at different loci that were trapped in mother cell, which allowed us to track when the marked loci were transported into the forespore. b) The apparent rate of transport by SpoIIIE of all chromosomal loci is essentially indistinguishable. >1500 cells were analyzed for each strain at each time point. Inset depicts

circular *B. subtilis* chromosome with dotted lines designating 3 MB of the chromosome that is trapped in the mother cell and *cfp*-marked loci on right arm of the chromosome indicated. c) SpoIIIE^{Δγ} exhibits a severe translocation defect along the entire length of the chromosome. At three hours after induction of sporulation, only 5% of cells are able to complete chromosome transport. d) Sample images taken of strains containing *cfp* integrated at 174° locus. Transport of this locus by cells with SpoIIIE and SpoIIIE^{Δγ} was monitored over time. Images depict transport at 3 hours after sporulation synchronization. e) Schematic representation of the coordinated stimulation model. Three types of DNA sequence are depicted: random, permissive SRS, and non-permissive SRS. Once bound to DNA, a SpoIIIE hexamer requires coordination between the gamma domains to sense the identity of the DNA sequence. If the sequence is random, the gamma domains will inhibit ATP turnover by the motor domains, keeping the motor domain in an ATPase “off” state. In this state, SpoIIIE translocates slowly along the DNA. Upon encounter with a permissive SRS, gamma domain coordination is necessary to sense the SRS and activate robust ATPase activity of the motor domain. This ATPase “on” state results in rapid and processive translocation along the DNA. A non-permissive SRS is not recognized by the gamma domain and, thus, does not result in an effect of the ATPase state of the motor domain. Mutations in the gamma-gamma subunit interface abrogate the ability of the gamma domains to communicate with one another and, thus, prevent SpoIIIE from sensing specific sequences. Thus, a gamma interface variant neither inhibits nor stimulates ATPase activity, and it translocates at the same rate regardless of the DNA sequence it encounters.

TABLES:**Table 1: DNA-binding of SpoIIIIE and SpoIIIIE^{Δγ}**

Fluorescein-labeled template*	Titration in	SpoIIIIE §	SpoIIIIE^{Δγ} §
2xSRS	SpoIIIIE	$K_d = 127.4 \pm 5.8$	$K_d = 1867 \pm 85$
Random	SpoIIIIE	$K_d = 365.8 \pm 58.1$	$K_d = 1813 \pm 108$
2xSRS	Unlabeled 2xSRS**	$K_i = 27.8 \pm 15.8$	$K_i = 856.3 \pm 93.3$
2xSRS	Unlabeled Random***	$K_i = 211.5 \pm 41.1$	$K_i = 876.0 \pm 131.7$

* 10nM of fluorescein-labeled template was used in each experiment

** 152nM of SpoIIIIE was used in each experiment

*** 1550nM of SpoIIIIE^{Δγ} was used in each experiment

§ nM units for all K_d and K_i values

Table 2: DNA-binding of SpoIIIIE^{R745A} and SpoIIIIE^{R745E}

Fluorescein-labeled template*	Titration in	SpoIIIIE^{R745A} §	SpoIIIIE^{R745E} §
2xSRS	SpoIIIIE	$K_d = 66.0 \pm 6.5$	$K_d = 73.9 \pm 7.1$
Random	SpoIIIIE	$K_d = 68.3 \pm 5.1$	$K_d = 159.5 \pm 18.3$

* 10nM of fluorescein-labeled template was used in each experiment

§ nM units for all K_d values

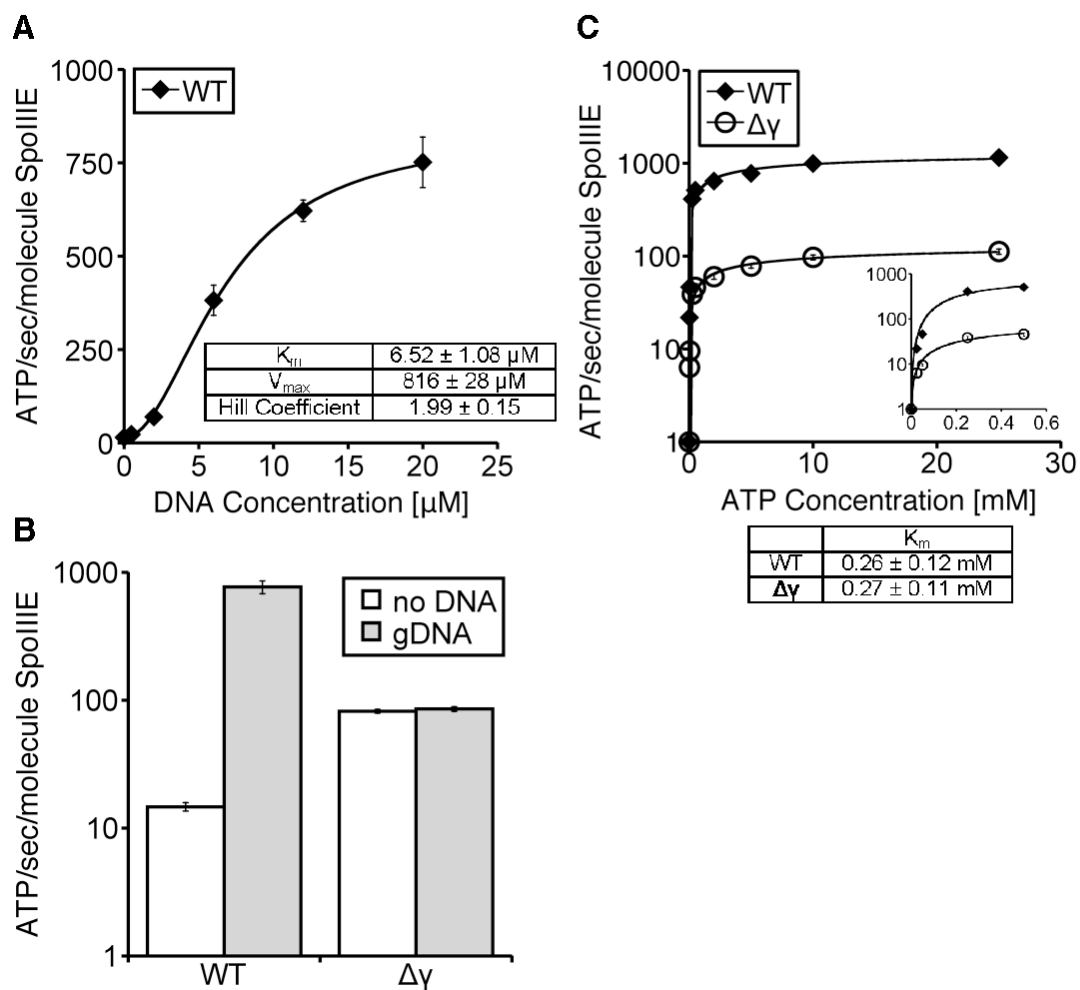
Figure 1

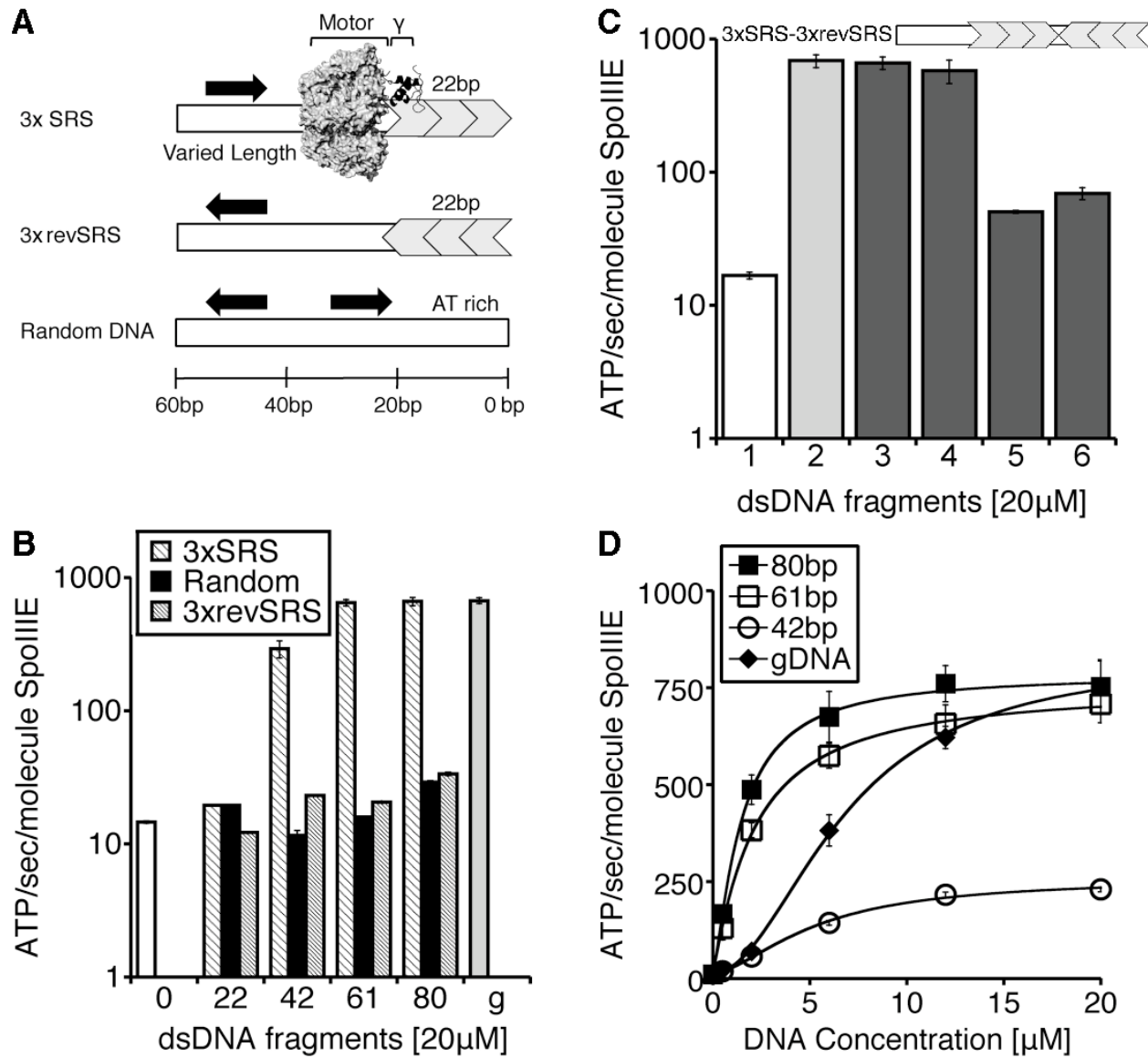
Figure 2

Figure 3

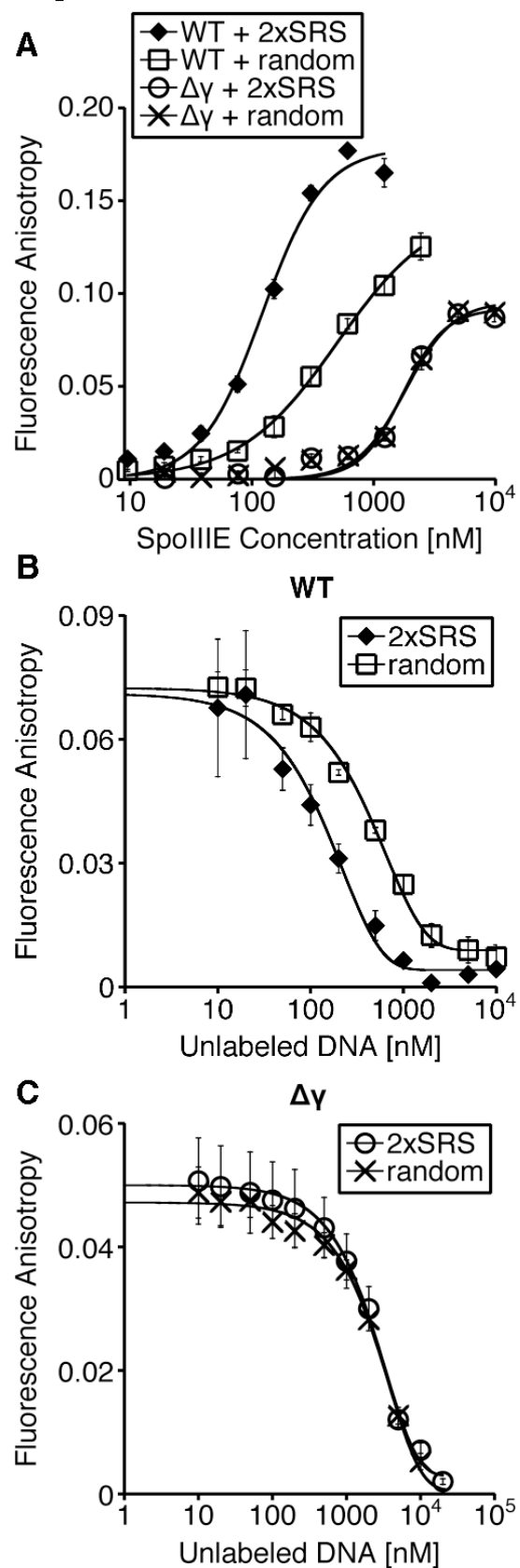


Figure 4

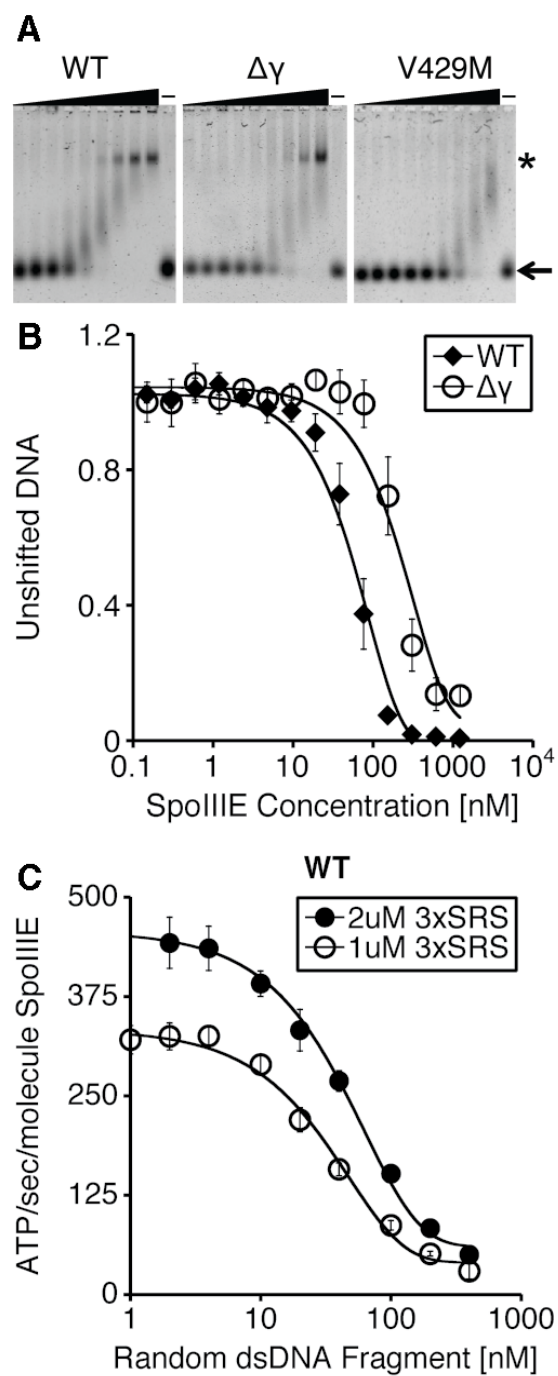


Figure 5**A**

E.coli	1260	--G-- EE LDPL ED CAV Q VFTE KR ASIS SV Q R FRIGYN	1295
P.aeruginosa	738	GS CE S EDDPLYDEAV RF VTES RE ASIS AV Q R KLIGYN	776
B.subtilis	716	---H SE VTDELYDEAV EL IVGM QT AS VS ML QR RRFRIGYT	752

E.coli	1296	RA AR LI E Q ME AC GV SE Q GH NG RE VL AE PF FD --	1329
P.aeruginosa	777	RA AR LI E AM E MA GV VP M NT NG S RE VL AE AF VR D	811
B.subtilis	753	RA AR LI E AM E RG V VG P YE G SK P RE VL LS KE---	

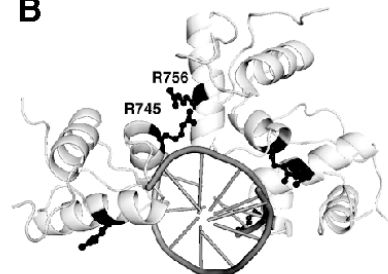
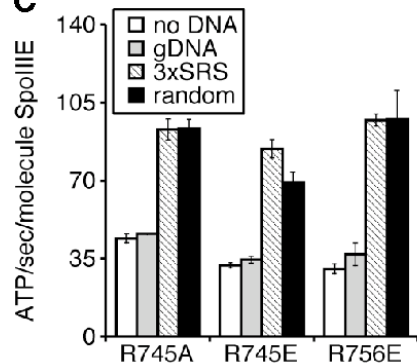
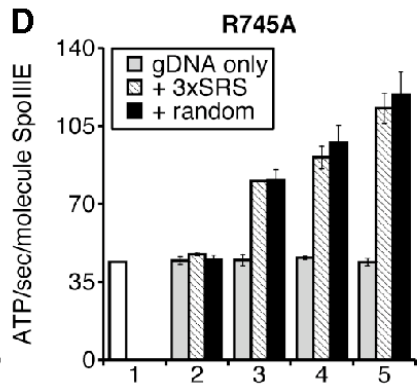
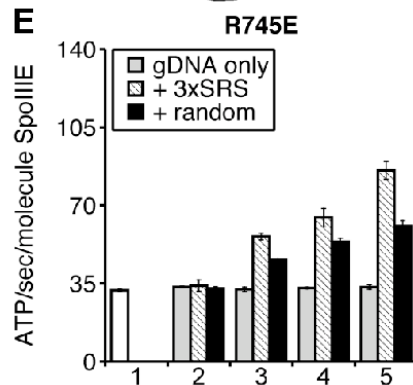
B**C****D****E**

Figure 6

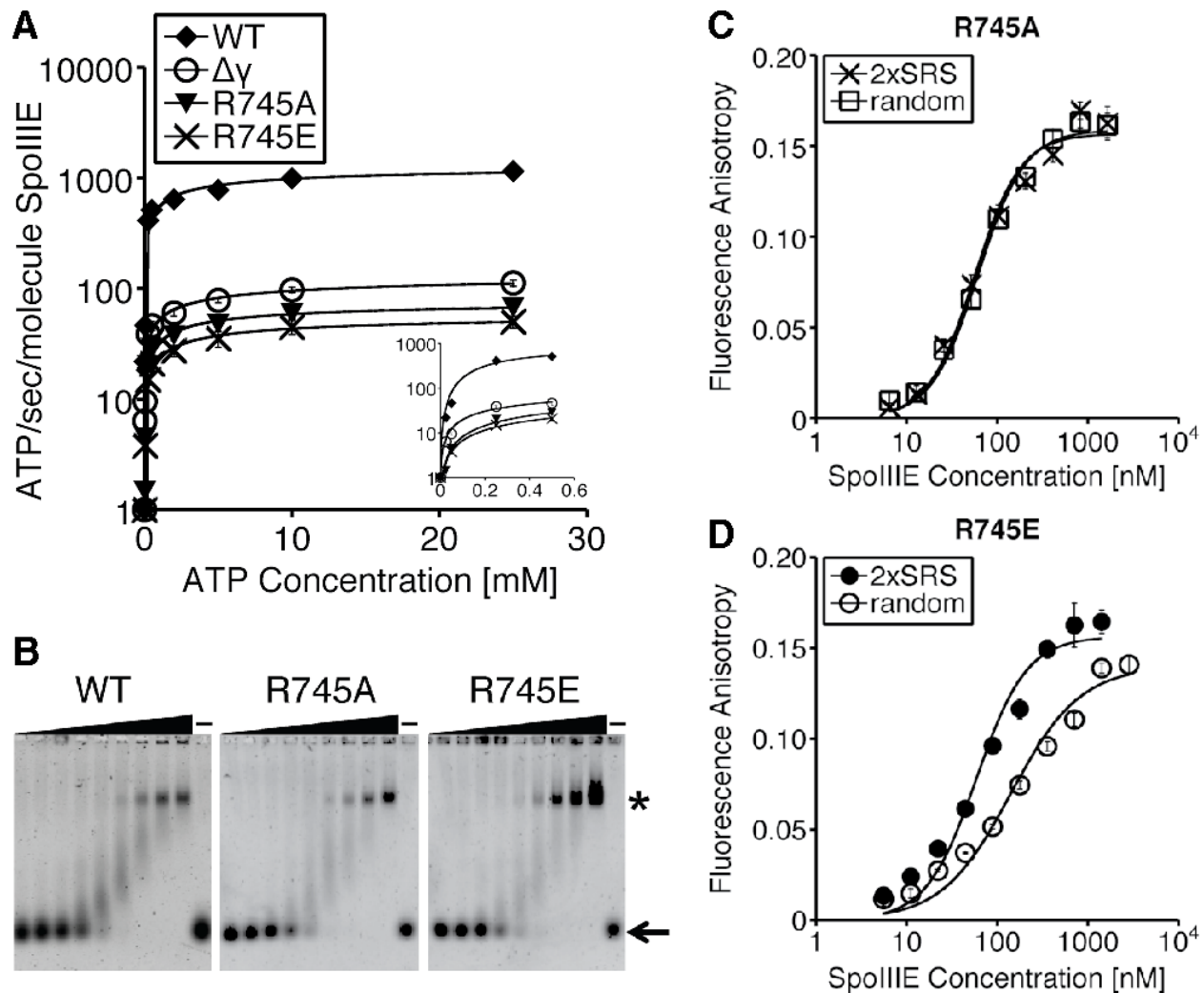


Figure 7

