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The Variable Subdomain of *Escherichia coli* SecA Functions To Regulate SecA ATPase Activity and ADP Release

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Bacterial SecA proteins can be categorized by the presence or absence of a variable subdomain (VAR) located within nucleotide-binding domain II of the SecA DEAD motor. Here we show that VAR is dispensable for SecA function, since the VAR deletion mutant secAΔ519–547 displayed a wild-type rate of cellular growth and protein export. Loss or gain of VAR is extremely rare in the history of bacterial evolution, indicating that it appears to contribute to secA function within the relevant species in their natural environments. VAR removal also results in additional secA phenotypes: azide resistance (Azir) and suppression of signal sequence defects (PrID). The SecAΔ(519–547) protein was found to be modestly hyperactive for SecA ATPase activities and displayed an accelerated rate of ADP release, consistent with the biochemical basis of azide resistance. Based on our findings, we discuss models whereby VAR allosterically regulates SecA DEAD motor function at SecYEG.

Bacterial protein transport is primarily facilitated by the Sec pathway, which consists of a protein-conducting channel, the SecYEG complex, and its associated SecA ATPase (reviewed in reference 14). SecA binds preprotein substrates and SecYEG, and these interactions activate its translocation ATPase activity (32). SecA ATPase activity drives the conformational cycles of SecA-bound SecYEG that facilitate the stepwise movement of preproteins through the channel (55). Whether SecYEG functions as a monomer or dimer or as a higher-order structure has been a matter of considerable controversy (see reference 28 and references contained within), similar to the state of affairs regarding the functional oligomeric state of SecA protein (reviewed in reference 47).

The SecA protomer is comprised of six conserved domains arranged within two substructures (see Fig. 1) (reviewed in reference 44). The N-domain substructure consists of a DEAD ATPase motor of the helicase superfamily II, which is comprised of two nucleotide-binding domains, NBD-I and NBD-II, along with the preprotein-binding domain (PPXD), which is attached to NBD-I. The C-domain substructure is comprised of the remaining three domains, the helical scaffold domain (HSD), the helical wing domain (HWD), and the carboxyl-terminal linker domain (CTL). The former two domains contribute to SecA organizational architecture, while CTL is involved in SecB and lipid binding. SecA binds to both the signal peptide and mature regions of preproteins at domain-domain interfaces. Signal peptide binding occurs largely at the PPXD-HSD interface, while mature regions of the preprotein bind at an expandable groove located between NBD-II and PPXD that has been termed the SecA “clamp” (1, 18, 58). Preproteins can be targeted to SecA either cotranslationally or posttranslationally, often utilizing the export-specific SecB chaperone in the process (reviewed in reference 46). SecA has also been shown to interact with ribosomes in order to improve the protein-targeting step (23). SecA is able to target preproteins to the translocon through its high-affinity association with SecYEG protein (21). SecA monomer appears to interact with two SecYEG protomers, with one protomer possibly serving as a SecA receptor, while the other SecYEG copy encompasses the active channel (10, 37, 42, 59). The two-helix finger subdomain of HSD has been proposed to act as the translocation ratchet portion of the SecA nanomotor based on its location at the entry of the protein-conducting channel in the X-ray structure of the *Thermotoga maritima* SecA-SecYEG complex (59). Given the complex domain organization of SecA and its multiple interaction partners, SecA conformational dynamics and its allosteric regulation lie at the heart of the SecA-dependent protein translocation mechanism. In particular, further studies are needed to uncover and understand the various signaling pathways that occur between the DEAD motor and SecA’s other domains as well as the additional signaling events that take place between SecA and SecYEG protein.

X-ray structures of SecA proteins from different bacterial species display a common protomer fold comprised of the six conserved domains described above and an additional variable subdomain (VAR), which has no known function and is present in only certain bacterial species. Here we report the first functional analysis of the *Escherichia coli* SecA VAR region, utilizing a combined phylogenetic, genetic, and biochemical approach. Our results indicate that, while VAR is dispensable for *E. coli* secA function within the laboratory, it appears to be important for secA function within the relevant bacterial species in their natural environments. Biochemical studies show that VAR plays a role in regulating SecA ATPase activity and
Designed on the Agilent (Santa Clara, CA) website, and all mutations were made by the QuikChange method (Agilent Technologies) utilizing oligonucleotide primers (Integrated DNA Technologies) and verified by DNA sequence analysis (University of Pennsylvania DNA Sequencing Facility). Plasmid-borne secA function was assessed by measuring plating efficiencies as follows: BL21.19 or BL21.20 containing a given pT7secA plasmid was grown overnight in LB medium supplemented with appropriate antibiotics at 30°C, when serial dilutions were prepared and plated on duplicate LB antibiotic-containing plates, which were incubated at either the restrictive or permissive temperature (42°C or 30°C, respectively) for 24 h; the ratio of colony counts on the 42°C versus 30°C plates was defined as the plating efficiency of a given strain. The secAΔ(519–547) allele was recombined into the chromosomal secA gene of MC4100 as follows. First, a HpaI deletion derivative of pT7secAΔ(519–547), pΔHpaI-secAΔ(519–547), was constructed by standard cloning procedures that removed the 5′ end of the lacI gene, the T7 promoter, and the 5′ end of the secA gene through codon 6. Next, this plasmid was transformed into BA13 [MC4100 secA13(Δ) supF lacZ102–547], and a purified transformant was grown on LB medium supplemented with ampicillin (Amp; 100 μg ml⁻¹) at 30°C and selected for temperature-resistant recombinants by plating the culture on LB-Amp plates at 42°C. A P1 lysate was grown on the pool of temperature-resistant colonies and used to transduce MC4100 lec::Tn10 to leucine prototrophy. Most leu+ transductants were found to be azide resistant and ampicillin sensitive, consistent with the successful introduction of the secAΔ(519–547) allele into the chromosomal secA locus. The presence of the secAΔ(519–547) allele was verified by performing DNA sequence analysis of the relevant portion of the chromosomal secA gene, which was amplified by PCR utilizing appropriate oligonucleotide primers. MC4100 secAΔ(519–547) [ApmalE-lacZ72-47(Hyb)], MC4100 malF-lacZ102(Hyb), and MC4100 secAΔ(519–547) malF-lacZ102(Hyb) were constructed in this study by P1 transduction. MC4100

Materials and Methods

Materials. LB (Miller) broth and agar were obtained from EMD Chemicals and Difco, respectively. [¹⁵S]methionine (1,000 Ci/mmol) was purchased from PerkinElmer. 7-Diethylamino-3-(((2-maleimidyl)ethyl)amino)carbonyl)coumarin (MDCC) was acquired from Invitrogen. Protease inhibitor cocktail was obtained from Sigma-Aldrich. Other chemicals were obtained from Sigma-Aldrich or comparable suppliers and were of reagent quality or better.

E. coli strains and plasmids. MC4100 [ApmalE-lacZ72-47(Hyb)] has been described previously (2). DHB7640 contains the secAVAR mutations. pCDF-BAD-phoA contains the T7 promoter-driven secA gene fused to a C-terminal His tag in PET29b (Novagen), and it was used to construct all of the secA VAR mutations. pCDF-BAD-phoA contains the E. coli phoA gene under the control of the araBAD promoter and araC regulator with a replication origin derived from CloDF13 along with a streptomycin-spectinomycin resistance gene and has been described recently (20). Plasmid mutations were made by the QuikChange method (Agilent Technologies) utilizing oligonucleotide primers (Integrated DNA Technologies) designed on the Agilent (Santa Clara, CA) website, and all mutations were verified by DNA sequence analysis (University of Pennsylvania DNA Sequencing Facility). Plasmid-borne secA function was assessed by measuring plating efficiencies as follows: BL21.19 or BL21.20 containing a given pT7secA plasmid was grown overnight in LB medium supplemented with appropriate antibiotics at 30°C, when serial dilutions were prepared and plated on duplicate LB antibiotic-containing plates, which were incubated at either the restrictive or permissive temperature (42°C or 30°C, respectively) for 24 h; the ratio of colony counts on the 42°C versus 30°C plates was defined as the plating efficiency of a given strain. The secAΔ(519–547) allele was recombined into the chromosomal secA gene of MC4100 as follows. First, a HpaI deletion derivative of pT7secAΔ(519–547), pΔHpaI-secAΔ(519–547), was constructed by standard cloning procedures that removed the 5′ end of the lacI gene, the T7 promoter, and the 5′ end of the secA gene through codon 6. Next, this plasmid was transformed into BA13 [MC4100 secA13(Δ) supF lacZ102–547], and a purified transformant was grown on LB medium supplemented with ampicillin (Amp; 100 μg ml⁻¹) at 30°C and selected for temperature-resistant recombinants by plating the culture on LB-Amp plates at 42°C. A P1 lysate was grown on the pool of temperature-resistant colonies and used to transduce MC4100 lec::Tn10 to leucine prototrophy. Most leu+ transductants were found to be azide resistant and ampicillin sensitive, consistent with the successful introduction of the secAΔ(519–547) allele into the chromosomal secA locus. The presence of the secAΔ(519–547) allele was verified by performing DNA sequence analysis of the relevant portion of the chromosomal secA gene, which was amplified by PCR utilizing appropriate oligonucleotide primers. MC4100 secAΔ(519–547) [ApmalE-lacZ72-47(Hyb)], MC4100 malF-lacZ102(Hyb), and MC4100 secAΔ(519–547) malF-lacZ102(Hyb) were constructed in this study by P1 transduction. MC4100

FIG 1 (A) X-ray structures of SecA proteins of (i) B. subtilis (Protein Data Bank [PDB] accession code 1M6N) (25) and (ii) T. maritima (PDB accession code 3JUX) (58) that lack the VAR subdomain. SecA domains are colored as follows: NBD-I, dark blue; NBD-II, light blue; PPXD, orange; HSD, green; HWD, light green; CTL (where present), red. (B) X-ray structures of SecA proteins of (i) Mycobacterium tuberculosis (PDB accession code 1NL3) (49), (ii) Escherichia coli (PDB accession code 2FSF) (43), and (iii) Thermus thermophilus (PDB accession code 2IPC) (56) that contain a VAR subdomain. Coloring is as follows: VAR, pink; NBD-II, light blue; other domains, gray.

Effects of secA VAR mutations on ADP release kinetics, which explains the Azi7 and PrlD phenotypes of a strain deleted for VAR.
malle41-1 derivatives that contain the secA, azi-4, or prlD5 alleles of secA have been described previously (45), while MC4100 malle41-1 secAD (519–547) was constructed for this study by P1 transduction.

Cell growth, protein and IMV purification, and enzyme assays. Strains were grown in LB supplemented with appropriate antibiotics (ampicillin at 100 μg/ml, chloramphenicol at 25 μg/ml, and streptomycin at 50 μg/ml) at 30°C, 37°C, or 42°C as indicated. β-Galactosidase assays were performed as described by Miller (35). His-tagged SecA proteins were overproduced as described previously (13), and they were purified utilizing a HisBind resin column (Novagen) according to the manufacturer’s protocol. The protein concentration was determined using the Bradford assay (Bio-Rad) with bovine serum albumin as the standard. Inverted membrane vesicles (IMV) from E. coli CK1801.4 were made and treated with 6 M urea in order to inactivate SecA function as described previously (9). The chimeric preprotein PSN, composed of the E. coli alkaline phosphatase signal peptide and the mature portion of staphylococcal nuclease, with K97C and W140H substitutions, was prepared as described previously (7). SecA ATPase activities were determined by the Malachite green method (31), utilizing modifications described previously (36). ATPase activity was calculated using the following formulas: endogenous ATPase activity = ATPase activity in the presence of SecA − ATPase activity in the absence of SecA; membrane ATPase activity = ATPase activity in the presence of SecA and IMV − endogenous ATPase activity; translocation ATPase activity = ATPase activity in the presence of SecA, IMV, and preprotein − membrane ATPase activity.

Stopped-flow ADP release kinetics. Stopped-flow experiments to measure the rate of ADP dissociation from SecA were performed on a KinTek Corp. SF-2001 stopped-flow instrument (Austin, TX). ADP release was assayed using E. coli ParM labeled with 7-diethylamino-3-(((2-maleimidyl)ethyl)-amino)carbonyl)coumarin (MDCC-ParM) as a fluorescent reporter (MDCC-ParM), as described previously (30). The experiments were carried out in buffer containing 50 mM HEPES-KOH (pH 7.5), 30 mM KCl, and 10 mM Mg(OAc)2, at 20°C. SecA (4 μM) was preincubated with 2 μM ADP for 15 min and mixed rapidly with an equal volume of 30 μM MDCC-ParM. Changes in MDCC-ParM fluorescence on binding ADP were measured by excitation at 436 nm and monitoring emission above 450 nm (cutoff filter; Corion LL-450 F) over time up to 1,200 s. A calibration curve relating MDCC-ParM fluorescence to ADP concentration was generated by mixing 30 μM MDCC-ParM with increasing concentrations of ADP (0, 1, 2, and 4 μM). Background fluorescence (initial signal at 0.7 s) was subtracted from the traces. Averaged data from two or more traces were divided by the slope of the calibration curve to determine the molar amount of ADP released during the reaction. The dissociation rate constant was determined from a double-exponential function, where the first, fast phase measures MDCC-ParM binding free ADP already in solution and the second, slower phase measures MDCC-ParM binding ADP after it is released from SecA.

RESULTS

Phylogenetic analysis of the SecA VAR region. X-ray structures of SecA protein from a variety of bacteria display a common protomer fold containing six conserved domains (25, 43, 49, 56) (Fig. 1A). In addition, the SecA proteins of some bacterial species contain a VAR subdomain of NBD-II with no known function (Fig. 1B). SecA proteins missing VAR lack the relevant amino acid sequence, which is normally flanked by highly conserved regions of SecA (see phylogenetic analysis below for additional details). VAR comprises a simple helix-loop–helix fold, as detected by nuclear magnetic resonance (NMR) spectroscopy of E. coli SecA (29), although the numbers of α-helices and turns differ somewhat for the three examples given in Fig. 1B. We conducted a phylogenetic study of VAR by utilizing a previously described tree of 350 bacterial species, representing all cultivated divisions. Wu et al. (57) created the tree based on a concatenation of 31 broadly conserved genes in order to better define phylogenetic relationships among the major bacterial groupings. E. coli SecA residues 517 to 548, which comprise the VAR region, were used to query for the presence or absence of VAR within the relevant bacterial SecA sequences within PubMed. A total of 117 bacterial species were found to lack VAR and contained only a short linker sequence in its stead (an average of 2.1 ± 1.0 residues), while 233 bacterial species were found to have a VAR region whose length was usually rather uniform within a particular division (averaging 48.9 ± 27.4 residues, ranging from 17 to 142). In gammaproteobacteria, for example, the length of VAR ranged from 28 to 32 residues. While Chloroflexi species showed a loss of VAR within their division, all five remaining VAR regions sampled were invariant in length. In divisions that showed substantial diversity of VAR length (i.e., differences > 20%), this diversity was usually consistent with one or two evolutionary events yielding major changes in VAR length, with other events yielding minor changes. For example, in actinobacteria the length of VAR ranged from 43 to 47 residues, except for the clade of Egerthella lenta and Cryptobacterium curatum, which had VAR regions of 56 and 61 residues, respectively. A pattern similar to that seen with the actinobacteria was also observed for members of the Alphaproteobacteria and Deltaproteobacteria divisions.

A parsimony analysis suggested that the presence of VAR within SecA protein represents the ancestral state, as organisms in the most basal division of Deinococcus-Thermus contain this region (see Fig. S1 in the supplemental material). The phylogenetic analysis also revealed that the losses and gains of VAR have been remarkably infrequent—an unexpected observation if VAR was readily dispensable for secA function and did not contribute in some manner to the overall fitness of a given bacterial species. With the exception of members of the Firmicutes division, members of the Bacteria have shown only six losses and one gain of the VAR region in the entire history of bacterial evolution. We note that the evolutionary transition between the presence and absence of VAR in most divisions has been less frequent than the most difficult ecological transitions in bacterial history, for example, changes between the adaptations to saline and nonsaline habitats (33). This observation is even more striking when one considers the frequency of horizontal gene transfer between bacteria. The Firmicutes division did not show a similar constraint, as it has undergone six losses throughout its history—the same number as seen in the rest of the tree. It should be interesting to investigate why such transitions have occurred at a much higher rate within the Firmicutes division than among the remainder of the bacterial divisions.

Genetic analysis of secA var mutants. We next performed a genetic analysis of VAR in order to assess its importance for E. coli SecA function. In order to determine if specific residues or regions of VAR were functionally relevant, alanine substitutions at both conserved (Trp519, Lys538, Trp541, His545) and nonconserved residues were constructed (based on the Gammaproteobacteria amino acid sequence alignment of VAR) along with several deletions, including a complete removal of VAR (Fig. 2). The secA mutant alleles were constructed in pT7secA-his, and their in vivo functions were assessed by measuring plating efficiency for strains whose chromosomal secA expression is conditionally temperature sensitive and whose plasmid-encoded secA is moderately overexpressed (−8-fold for BL21.19 hosts) or expressed at a normal level (for BL21.20 hosts) (see Materials and Methods) (11). Furthermore, the in vivo stability of the mutant SecA proteins was assessed.
by Western blot analysis of cultures that were shifted to the restrictive temperature. We found that most alanine substitution mutants displayed normal (~1.0) plating efficiencies similar to that seen with the wild-type secA in either host strain. The exception was the secAW541A strain, whose plating efficiency was relatively normal for BL21.19 (pT7secAW541A) but was ~10^{-6} for BL21.20 (pT7secAW541A). Western blot analysis indicated the presence of a smaller amount of SecAW541A protein compared to wild-type SecA in the latter case, and a pro-OmpA precursor was found to accumulate as well, indicative of a substantial protein secretion defect (Fig. 3A). In the former case, although SecAW541A was overproduced and some of this protein was functional, as determined by the observed plating efficiency, a substantial fraction of it was insoluble and aggregated, as determined by flotation sedimentation analysis of the fractionated cell lysate (Fig. 3B and data not shown). Examination of the E. coli SecA NMR structure suggested that stacking interactions of Trp541 with Trp519 and His545 might be important for the structural stabilization of the VAR subdomain (see Fig. S2 in the supplemental material). We also found that VAR could be functionally deleted, since BL21.19 [pT7secAΔ(519–547)] and BL21.20 [pT7secAΔ(519–547)] had relatively high plating efficiencies (0.9 ± 0.18 and 0.5 ± 0.11, respectively). The mutant SecAΔ(519–547) protein was not effectively overproduced in the former strain, although it was produced normally in the latter case, suggesting that VAR helps to stabilize SecA protein during its overproduction (Fig. 3B). Furthermore, when we created a strain with the secAΔ(519–547) deletion crossed into the sole secA copy at its normal chromosomal locus, MC4100 secAΔ(519–547) grew normally and gave normal colony sizes on LB plates at all temperatures tested (22°C, 30°C, 37°C, and 42°C). However, we did find that certain VAR deletions abolished secA function: the secAΔ518–547 and secAΔ519–548 mutants had plating efficiencies of less than 10^{-5} even in the BL21.19 host where the relevant mutant SecA proteins were overproduced (Fig. 3B). The latter result suggests that Ser518 and Val548 are critical for SecA function or, alternatively, that these two residues contribute structurally to essential regions flanking VAR. In that regard, we note that VAR is sandwiched between the helicase V and VI motifs that encompass SecA residues 499 to 513 and 568 to 577, respectively, which have been shown to be important for SecA DEAD motor function (29). Based on these findings, we conclude that VAR is dispensable for SecA essential function, although when VAR is normally present, certain amino acid residues within the sequence (e.g., Trp541) appear to be important for SecA stability and/or function.

In order to assess the importance of the VAR domain for SecA-dependent protein export, we measured protein secretion rates in the secAΔ(519–547) mutant by three different methods. First, we employed pulse-labeling of exponentially growing cultures with

![FIG 2 Genetic analysis of VAR. The E. coli SecA VAR amino acid sequence from 517 to 548 is shown along with positions of single alanine substitutions (downward arrows) or deletion mutations (black bars).](http://jb.asm.org/)

![FIG 3 (A) BL21.20 or (B) BL21.19 strains containing pT7secA (WT) or the indicated secA allele were grown in LB supplemented with appropriate antibiotics to an A_600 of 0.2, when they were shifted to 42°C for an additional 2 h. Cultures were normalized by dilution with LB to an A_600 of 0.8, and equivalent cell volumes were harvested by sedimentation and processed for SDS-PAGE and Western blotting with SecA and OmpA antisera as described previously (11). Protein concentrations were measured by a Bradford assay (Bio-Rad), and equivalent amounts of total protein were loaded onto SDS-PAGE gels. MC4100 has no plasmid and produces wild-type SecA levels. M denotes the lane containing molecular mass markers.)](http://jb.asm.org/)
[35S]methionine followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of particular secretory proteins in order to physiologically assess protein secretion with minimal perturbation to the system. We detected a high rate of protein secretion in MC4100 secAΔ(519–547) based on the absence of any detectable precursors of maltose (Mal)-binding protein or OmpA during a short pulse-labeling experiment (Fig. 4A). Pretreatment of MC4100 with the known SecA inhibitor sodium azide was utilized to establish a protein secretion block for control purposes (41). Second, we measured the kinetics of induction and accumulation of alkaline phosphatase activity in the MC4100 secAΔ(519–547) mutant containing the pCDF-BAD-phoA plasmid (Fig. 4B). Alkaline phosphatase, which requires SecA and SecYE for its secretion, needs to achieve a periplasmic location in order to be enzymatically active in this case (34). We observed a similar rate of accumulation of araBAD-dependent alkaline phosphatase activity in MC4100 secAΔ(519–547) (pCDF-BAD-phoA) compared to its isogenic parent MC4100 (pCDF-BAD-phoA). Third, we compared β-galactosidase activity levels in MC4100 and MC4100 secAΔ(519–547) derivatives containing either the malE-lacZ72-47(Hyb) or malf-lacZ102(Hyb) fusion (Fig. 4C). The latter two gene fusions have been utilized extensively as sensitive reporters for the detection of minor defects in protein secretion or membrane protein biogenesis, respectively (17, 40, 54). We found that MC4100 secAΔ(519–547) [Amale-lacZ72-47(Hyb)] had β-galactosidase activity that was 4-fold higher than that of its MC4100 [Amale-lacZ72-47(Hyb)] parent strain, while MC4100 secAΔ(519–547) malf-lacZ102(Hyb) and MC4100 malf-lacZ102(Hyb) had essentially identical β-galactosidase activities. In the former case, the two strains containing the malf-lacZ72-47(Hyb) fusion showed similar levels of maltose sensitivity when they where grown on maltose minimal plates (data not shown), conditions that cause toxic translocon jamming by the MalE-LacZ chimera (40). We conclude that the secA mutant lacking the VAR region has robust protein secretion activity under normal physiological conditions. However, the results of our latter assay suggest that the SecAΔ(519–547) protein is modestly less active for protein secretion when the protein export machinery is otherwise compromised.

Biochemical studies of SecA oligomerization, SecA-SecYEG binding, and SecA ATPase activities. VAR is present at the interprotomer interface of the E. coli SecA dimer structure (see Fig. S3A in the supplemental material), where it is thought to be involved in dimer stabilization, based on symmetrical contacts between Ala524, Glu527, Asn528, Gln530, and Lys538 as well as asymmetrical contacts between the alpha (Trp519 and Pro529) and beta (Ala525 and Leu526) SecA protomers (43). To determine whether VAR contributes appreciably to stabilizing the soluble SecA dimer, we assessed the monomer-dimer equilibrium of the SecAΔ(519–547) protein by size exclusion chromatography and static light scattering. We found that the SecAΔ(519–547) protein had a monomer-dimer equilibrium constant similar to that of the wild-type SecA (Kd ~ 1 μM) (see Fig. S3B in the supplemental material), indicating that VAR does not appear to appreciably stabilize the solution-state SecA dimer. During this analysis, it was necessary to include glycerol in our buffers in order to prevent a modest level of SecAΔ(519–547) protein aggregation, unlike the level seen with wild-type SecA, again suggesting that VAR has some effect on SecA protein folding and stability. By comparison, deletion of amino-terminal residues 2 to 11 of SecA results in
greater than a 100- to 1,000-fold dimerization defect, depending on the salt concentration utilized (11). Recent work from our laboratory also suggests that the solution-state E. coli SecA dimer is structurally similar to the Bacillus subtilis SecA dimer described by Hunt et al. (12, 25) (S. M. Auclair, D. B. Oliver, and I. Mukerji, unpublished data). When VAR was modeled onto the latter structure, we found that this subdomain is not proximal to the SecA dimer interface (see Fig. S3C in the supplemental material).

In vivo studies of SecA membrane topology indicate that portions of VAR are proximal to the SecYEG channel based on extrinsic labeling of VAR residues 518 and 530 (26). Furthermore, both of these residues can also be cross-linked to the SecY protein by site-specific in vivo photo-cross-linking methods (10). In order to assess whether removal of VAR affects SecA association with SecYEG in vivo, we subjected strains BL21.20 [pT7secA(519–547)] and BL21.20 [pT7secAΔ(519–547)] to subcellular fractionation under conditions where lipidic SecA is effectively removed from the membrane fraction whereas SecA association with SecYEG is preserved (15). When the P300 membrane fraction was treated with 0.2 M sodium carbonate (pH 11.5) and reisolated, we found equivalent levels of SecA protein present in the P300P fraction of both strains (see Fig. S4 in the supplemental material). This result indicates that SecAΔ(519–547) protein lacking VAR is strongly anchored to SecYEG, consistent with the multidomain contacts observed between these two proteins both in vitro and in vivo (10, 59).

Finally, given the physical linkage between VAR and NBD-II, we examined whether this subdomain plays any role in regulating SecA ATPase activity. SecA possesses a basal ATPase activity (endogenous ATPase) that is modestly activated upon binding to SecYEG (membrane ATPase), and the latter activity is stimulated further upon preprotein binding and translocation (translocation ATPase) (32). We found that the SecAΔ(519–547) protein displayed modestly elevated levels for all three SecA-dependent ATPase activities (Fig. 5). However, its membrane ATPase activity was elevated the most, suggesting that the loss of VAR affects the activation step that occurs when SecA associates with SecYEG (38).

We have previously demonstrated that SecA proteins purified from azide-resistant (Azir) and signal sequence suppressor (PrlD) strains also possess higher levels of membrane ATPase activities (48). Accordingly, we tested whether the secAΔ(519–547) mutant displayed azide-resistant and signal sequence suppressor phenotypes. Remarkably, BL21.19 [pT7secAΔ(519–547)] was found to be azide resistant, since it showed normal colony sizes and numbers on LB-Amp plates containing 2 mM sodium azide after streaking and incubation at 37°C for 16 h (data not shown). In contrast, BL21.19 [pT7secA] was found to be azide sensitive, while the prlD5-containing strain BL21.19 [pT7secA-A373V] was used as an azide-resistant control in this case (24). MC4100 secAΔ(519–547) was also found to be azide resistant on LB plates containing 1 mM sodium azide after streaking and incubation at 37°C for 16 h, while its MC4100 parent was azide sensitive. The secAΔ(519–547) mutant was also found to suppress the malE14-1 signal sequence defect for maltose-binding protein secretion (3) based on its Mal+ phenotype on a maltose tetrazolium plate (Fig. 6). The somewhat darker pink coloration observed in the latter case indicated that suppression was not as strong as with the known prlD5 allele (16). Thus, we conclude that VAR removal from SecA confers Azir and PrlD properties. Furthermore, we tested a number of our alanine substitution mutants in this manner, and they also displayed increased azide resistance, although the level was not as pronounced as that seen with the secAΔ(519–547) mutant (data not shown).

We have previously demonstrated that Azir’ and PrlD SecA proteins have higher ADP release rates (48), thus counteracting the effect of sodium azide, which inhibits ADP release from SecYEG-bound SecA (4, 15). In order to determine if the VAR deletion has an effect on ADP-bound SecA similar to those of the azi and prlD mutations, we directly measured the rate of ADP release from SecAΔ(519–547) by using the ADP biosensor MDCC-ParM, whose fluorescence increases on binding ADP (30). In a stopped-flow experiment, ADP release from SecA was measured after mixing the ADP-bound protein with a molar excess of MDCC-ParM. We included in vivo and in vitro data for SecA and SecAΔ(519–547) proteins (Fig. 5).

The kinetic data indicated that ADP dissociates at least 10-fold faster from SecAΔ(519–547) than from wild-type SecA (k2 = 0.07
proves SecA folding and stability and that the cellular growth and for rapid protein secretion under normal condi-

tions. In this regard, we also note that the secAΔ(519–547) mutant may not be as efficient for protein secretion under normal conditions. However, our data also suggested that VAR modestly improves SecA folding and stability and that the secAΔ(519–547) mutant may not be as efficient for protein secretion under conditions where protein secretion physiology is otherwise compromised. Furthermore, we found that VAR removal results in Azi1 and PrlD phenotypes and that the secAΔ(519–547) protein is modestly hyperactive for SecA ATPase activities, due at least in part to an accelerated rate of ADP release.

All of our data are consistent with VAR playing a role in modulating SecA DEAD motor function—particularly during its association with the SecYEG channel. The constellation of genetic and biochemical properties noted above point to a mechanism whereby VAR directly or indirectly contributes to a regulatory function within the SecYEG-bound SecA complex. The SecA monomer makes distinct interactions with two SecYEG protomers: one bound by the DEAD motor, and the other bound primarily by PPXD and HSD that serves as the active protein-conducting channel (10, 37, 42, 59). Deletion of VAR apparently somehow “loosens” the association between the two halves of the DEAD motor, NBD-I and NBD-II, allowing an accelerated release of ADP and higher ATPase activity from the SecYEG-bound SecA complex (38). This altered step represents the biochemical basis for azide resistance (4, 15), and it is also tied into the signal peptide proofreading activity of the SecA-SecYEG complex (24). Loss of VAR in this context hyperactivates SecA-dependent membrane ATPase activity and removes a level of substrate proofreading activity that normally improves translocon fidelity and perhaps efficiency as well.

Previous work has shown that the SecA DEAD motor is allosterically regulated by both the PPXD and HSD modules that coordinate the SecA ATPase cycle with preprotein binding and release cycles that promote protein translocation at SecYEG (reviewed in reference 43). The intermolecular regulator of ATP hydrolysis element IRA1 (equivalent to the two-helix finger subdomain of HSD) negatively regulates SecA DEAD motor ATPase activity, while IRA2 (equivalent to NBD-II) acts as a positive regulator (50). Within the DEAD motor, residues located at the NBD-I–NBD-II interface and within the six helicase motifs are critical for controlling ATPase activity. DEAD motor function relies on a disorder-order folding transition of the inner portion of NBD-II that underlies its ability to productively interact with NBD-I, the intrinsic ATPase domain (29). However, unlike the previously described mutations that affect SecA DEAD motor function directly and lie at this critical interface, VAR is an extrinsic subdomain of NBD-II that likely exerts its effect by a more indirect, allosteric mechanism—perhaps akin to those of the PPXD and HSD modules. In this regard, we also note that the structural context of the Azi1 and PrlD phenotypes resulting from VAR deletion is also different from that of azi and prlD mutations that have been described previously (24, 41), which are single amino acid substitutions that lie at domain-domain interfaces within the SecA structure (25).

One attractive model for VAR action posits that it normally affects the disorder-order folding transition that lies at the heart of the IRA2 regulatory mechanism. In this regard, we note that
NBD-II residues 564 to 579 adjacent to VAR, including helicase motif VI, were found to be mobile by NMR spectroscopy of soluble SecA (8). It is possible that VAR contributes to stabilization of this nearby motif, leading to the stabilization of the SecA ADP-bound state and a repressive effect, while its absence has a destabilizing effect, consistent with the observed increased rate of ADP release and higher SecA ATPase activities. Alternatively, VAR could act through the other adjacent helicase V motif that encompasses SecA residues 499 to 513. VAR may also directly act at SecYEG, given our recent in vivo studies indicating that portions of this element become proximal to the SecY channel based on their ability to be extrinsically labeled or photo-cross-linked to SecY itself (10, 26). These studies suggest that VAR is repositioned from the location seen in X-ray and NMR structures of soluble SecA to a position proximal to the SecY channel, where it could directly interact with SecY and exert the requisite effects on SecA DEAD motor function (18, 39, 43). Loss of VAR at this stage of translocase assembly leads to the more complex Azi and PrlD phenotypes and their associated biochemical phenotypes: accelerated ADP release and higher levels of membrane and translocation ATPase activities. Finally, our data suggest that VAR action is not mediated by a direct contribution to SecA dimerization unless an alternative dimer with VAR at its interface forms during SecA association with SecYEG.

Our phylogenetic analysis showed that VAR is present in SecA proteins from two-thirds of bacterial species sampled, wherein it often displays a conserved residue length according to division or at least subdivision. The rarity in the gain or loss of this element during all of bacterial history suggests that the VAR subdomain plays an important role in optimizing SecA function and, consequently, in the overall fitness of a particular species, given SecA’s essential role in secretome biogenesis. Presumably, SecA proteins from those species that lack VAR have optimized NBD-II-regulatory function in some manner that no longer requires this element. In the case of members of the Firmicutes, such alterations appear to have introduced unusual plasticity that allows a more flexible gain or loss of the VAR element with time. The difficulty of eliminating or altering the VAR region during evolution may at first seem at odds with our data for the E. coli system. However, it is important to note that, once SecA function was substantially reduced, further changes would generally be eliminated if they produced even a minor decrease in growth or survival rates, particularly in less-than-optimal environments outside the ideal laboratory environment (51). Furthermore, VAR function would tend to be relatively species specific, given the idiosyncratic nature of the interaction surfaces of SecA and SecYEG proteins as well as their requirement for particular concentrations of phospholipids in order to function well within the membrane environment of a particular species (19, 22). Such species-specific protein-protein and protein-lipid requirements would be expected to severely limit the success of horizontal gene transfer events in this case: a conclusion that is supported by our phylogenetic studies as well as studies of hybrid SecA-SecY systems from particular bacterial species (53).

In conclusion, we found that VAR is a conserved but nonessential subdomain of NBD-II that appears to be important in regulating SecYEG-bound SecA ATPase activity (at least in part) at the ADP release step. Further investigation of this complex system is required in order to increase our structural and mechanistic understanding of how VAR regulates this multisubunit enzyme complex during its chemomechanical cycle of protein translocation.

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