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Escherichia coli SecA Helicase Activity Is Not Required *in Vivo* for Efficient Protein Translocation or Autogenous Regulation*

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SecA is an essential ATP-driven motor protein that binds to preproteins and the translocon to promote protein translocation across the eubacterial plasma membrane. *Escherichia coli* SecA contains seven conserved motifs characteristic of superfamily II of DNA and RNA helicases, and it has been shown previously to possess RNA helicase activity. SecA has also been shown to be an autogenous repressor that binds to its translation initiation region on *secM-secA* mRNA, thereby blocking and dissociating 30 S ribosomal subunits. Here we show that SecA is an ATP-dependent helicase that unwinds a mimic of the repressor helix of *secM-secA* mRNA. Mutational analysis of the seven conserved helicase motifs in SecA allowed us to identify mutants that uncouple SecA-dependent protein translocation activity from its helicase activity. Helicase-defective *secA* mutants displayed normal protein translocation activity and autogenous repression of *secA in vivo*. Our studies indicate that SecA helicase activity is nonessential and does not appear to be necessary for efficient protein secretion and *secA* autoregulation.

Protein translocation across the bacterial plasma membrane has been studied intensively during the past decade (for recent reviews, see Refs. 1 and 2). Current thinking indicates that certain membrane proteins are delivered to the translocon cotranslationally via SRP and its receptor FtsY, while other membrane and secretory proteins rely on interactions with cytoplasmic chaperones such as SecB as well as SecA protein for their membrane targeting (3–7). The translocon itself consists of integral membrane proteins SecYEG and SecDFyajC and the peripheral membrane protein, SecA ATPase. SecYE is the presumed preprotein channel and SecA receptor (8–11). While SecG and SecDFyajC are not essential under all conditions, they greatly enhance the rate of protein translocation by regulating SecA membrane cycling (12–14). SecA is central to protein translocation, since it binds to presecretory and certain membrane proteins, and it acts as a motor protein to drive protein translocation at the translocon (see references in Ref. 1). One current model proposes that SecA undergoes ATP-driven cycles of insertion and retraction at SecYE, thereby promoting the stepwise translocation of proteins (15–17). Another model posits that protein translocation takes place from SecA that is permanently inserted into the plasma membrane (18).

By catalyzing perhaps the first committed step in the protein translocation cycle, SecA occupies a pivotal position in this pathway. It is the only component of the Sec machinery that is known to be regulated by the protein secretion status of the cell. Inhibition of protein secretion by either genetic or biochemical means results in an ~10-fold increase in the rate of *secA* translation (19–21). *secA* is the second gene in the *secM-secA* operon, and its translation is coupled to that of *secM* (22). Analysis of this system has revealed that ribosomes translating the distal portion of *secM* are needed to disrupt the repressor helix (helix II) that normally occludes the *secA* Shine-Dalgarno site (23, 24). SecM, a nonessential periplasmic protein, has been found recently to regulate *secA* expression by a mechanism involving a coupling of its translation and secretion (25, 26). In particular, a translation pause site distal in *secM* that controls helix II formation has been demonstrated, and the duration of the pause was regulated by the rate of SecM secretion and translocon activity within the cell (27). This mechanism explains how *secA* translation rate is controlled by the protein secretion status of the cell with SecM export serving as the secretion monitor. In addition to this elegant system of regulation, it has been shown previously that SecA protein itself acts as an autogenous repressor during excess protein secretion capacity by binding to and blocking its translation initiation region (28). SecA can also prevent its translation by specifically dissociating a 30 S $tRNA^{fMet}$-*secM-secA* RNA initiation complex (29). However, the role that SecA repressor activity may play in regulating the *secM* translation pause-release system remains unexplored.

Little attention has been paid to the report by Koonin and Gorbalenya (30) that SecA protein may belong to the superfamily II of DNA and RNA helicases and that this activity could be important for *secA* autoregulation. By alignment of the SecA protein sequence with a number of known helicases, the authors were able to identify the seven conserved helicase motifs within SecA. Indeed, SecA has been reported to possess ATP-dependent helicase activity utilizing an RNA duplex from hepatitis C virus (31).

In the present study, we have further characterized SecA helicase activity and explored its potential role in autoregulation. In particular, we felt that characterization of SecA helicase activity utilizing the more natural substrate, *secM-secA* RNA, was important. Our results confirm those published previously that SecA is an ATP-dependent helicase. Furthermore, we report the effect of mutations in the seven conserved helicase motifs on SecA's various biochemical activities. This approach allowed us to uncouple SecA-dependent protein translocation activity from its helicase activity and show that the

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latter activity is nonessential for efficient protein secretion and *secA* autoregulation.

EXPERIMENTAL PROCEDURES

Strains, Media, Enzymes, and Chemicals—BL21.14 (pT7secA2) and BL21.19 (pT7secA2), which overproduce SecA protein, have been described previously (32). BL26 (λDE3) (BL21 Δ(*argF-lac*)U169 (λDE3)) and BL26.1 (BL21 Δ(*argF-lac*)U169 *recA::CAT* (λDE3)) are BL21 (λDE3) derivatives (33) that were constructed by P1 transduction. Plasmid pLG552.1 is a derivative of pLG552, a pSC101 plasmid that carries a 13.5-kilobase pair DNA fragment containing the *secM-secA* operon (34). It was modified by *in vivo* recombination to contain Φ(*secA-lacZ*)*f181*(Hyb) (35), which was verified by PCR analysis using appropriate primers to regions of *secA* and *lacZ*. A plasmid that overproduced SecA with a C-terminal His_6 tag under T7 promoter control, pT7secA-his, was constructed as follows: (i) the *kan* gene from pET-29b (Novagen) was removed using *Alw*NI and *Dra*III and replaced with the *bla* gene from pBluescript SK+ (Stratagene) to give pET29V; (ii) the *secA* gene from pT7secA2 was PCR[1]-amplified using primers 5′-TTTTCATATGCTAATCAAATTG-3′ and 5′-GGACTCGAGTTGCAGGCGGCCATGGCACTGCTTG-3′; (iii) this PCR product was cleaved with *Nde*I and *Xho*I and cloned into pET29V that had been similarly cleaved; and (iv) to avoid sequence errors due to PCR amplification, an *Nco*I DNA fragment comprising nearly the entire *secA* gene was isolated from pT7secA2 and used to replace the newly constructed *secA* plasmid, resulting in pT7secA-his. pT7secA-his was verified by DNA sequence analysis, *in vivo* complementation of the *secA* defect of BL21.19 at 42 °C, and Western blot analysis using a Ni^{2+}-horseradish peroxidase conjugate (Qiagen). pT7secA-D209N-his was made by replacing the *Nco*I fragment of pT7secA-his with that from pT7secA-D209N (32). This plasmid was unable to complement BL21.19 at 42 °C, although it overproduced SecA-D209N-his as verified by Western blot analysis with Ni^{2+}-horseradish peroxidase conjugate. All other *secA* mutants were constructed by site-directed mutagenesis of pT7secA-his utilizing a QuikChange™ kit (Stratagene) and the appropriate oligonucleotides (Integrated DNA Technologies). All mutations were confirmed by DNA sequence analysis at the University of Pennsylvania DNA Sequencing Facility. DNA restriction enzymes and T4 DNA ligase and polynucleotide kinase were obtained from New England Biolabs, while *Pfu* DNA polymerase and T3 RNA polymerase were from Stratagene. LB broth (Sigma) and M63 minimal medium have been described previously (36). Unless specified otherwise, most other chemicals and proteins were from Sigma or an equivalent supplier and were laboratory grade or better.

To study the effect that the *secA* helicase mutations had on protein secretion and *secA* autoregulation, the following strains were constructed. BL26 (λDE3) *leu::Tn5* Φ(*secA-lacZ*)*f181*(Hyb) *recA*::CAT carrying the appropriate pT7secA-his plasmid was constructed by P1 transduction utilizing the appropriate donor strains. The azide sensitivity, super sensitivity, or resistance of the resulting strain was scored to confirm the presence of the appropriate *secA* allele. MCH2 is a derivative of MC4100 containing *secA-S458C leu::Tn10* and was constructed as follows. An *Eco*RI-*Xho*I DNA fragment containing the *secA-S458C* allele was isolated from pT7secA-S458C-his and subcloned into pBluescript II SK. The resulting plasmid was transformed into BG13.1 (*secA13(Am) supD43,74 sueA sueC trp(Am) tsx relA rpsL* Δ*lac leu::Tn10*). A derivative of this strain that recombined the *secA-S458C* allele into the chromosomal *secA* locus and had lost the *secA13(Am)* allele was selected by plating for recombinants that could grow at 42 °C and that also tested as being resistant to 2 mM sodium azide. This isolate was used to introduce the *secA-S458C* allele into MC4100 by P1 transduction, utilizing its linkage to *leu::Tn10*. The presence of the *secA-S458C* allele in MCH2 was confirmed by DNA sequence analysis after PCR amplification and cloning of an appropriate DNA fragment into pGEM-T (Promega).

Protein Purification—Laboratory glassware was treated by baking at 180 °C for 12 h, and wherever possible, solutions were treated with 0.1% (v/v) diethylpyrocarbonate for at least 12 h to avoid any nuclease activity. SecA proteins were overproduced and purified from BL21.19 (pT7secA-his) derivatives by affinity chromatography on nickel columns (Novagen), and they were stored in 25 mM Tris-HCl, pH 7.5, 25 mM KCl, 1 mM $Mg(OAc)_2$, 1 mM dithiothreitol, 10% (v/v) glycerol at −80 °C. Protein purity was determined by SDS-PAGE and staining with Coomassie Brilliant Blue. Prolonged incubation of RNA with purified SecA protein did not give any evidence of contaminating nuclease activity as determined by PAGE analysis.

Preparation of RNA Substrate—The *secM-secA* RNA fragments RS294 and RS170 were prepared by *in vitro* transcription of *secM-secA* DNA fragments with T3 RNA polymerase as described previously (29). For the filter-binding assay, RNA was labeled by adding [^{32}P]UTP (Amersham Pharmacia Biotech) to the *in vitro* transcription reaction. DNA oligonucleotides were end-labeled with T4 polynucleotide kinase using [γ-^{32}P]ATP (Amersham Pharmacia Biotech). DNA2s consisted of the following sequence 5′-TCGCCCTCAAACGCCCCGCATTTT-3′ (where the underlined nucleotides are complementary to the helix II region of *secM-secA* RNA (23)). 6 pmol of DNA oligonucleotide and RNA transcript were annealed in a 1:1 ratio in 50 μl of 25 mM Tris-HCl, pH 7.5, 25 mM KCl, 1 mM EDTA by heating to 98 °C for 2 min followed by gradual cooling over 3 h to room temperature and overnight incubation at 4 °C. The duplex was purified on native 12% polyacrylamide gels containing 89 mM Tris borate, pH 8.3, 2 mM EDTA (37). After autoradiography, the duplex band was excised from the gel, crushed, and extracted with 0.4 M NaOAc, pH 5.5, 1 mM EDTA, and precipitated with ethanol. The pellet was resuspended in 25 mM Tris-HCl, pH 7.5, 25 mM KCl, 1 mM $Mg(OAc)_2$ and stored frozen until needed.

Helicase Assays—Reaction mixtures (20 μl) contained H buffer (10 mM Tris-HCl, pH 7.5, 25 mM KCl, 1 mM $Mg(OAc)_2$, 10 mM dithiothreitol, 100 μg/ml BSA, and 20 units of RNase Block (Stratagene)), 3 fmol (150 pM) of DNA-RNA duplex, and 1 mM ATP unless otherwise indicated. Reactions were initiated by the addition of the indicated amount of SecA protein. After incubation at 37 °C for the indicated time period, reactions were stopped by the addition of 4 μl of loading buffer (1.2% SDS, 10 mM EDTA, 40% glycerol, 0.1% bromphenol blue, 0.1% xylene cyanol, 250 μg/ml proteinase K) and incubated for 5 min on ice. As controls, one sample lacked any SecA protein (total duplex), while another contained DNA-RNA duplex that was heated at 95 °C for 2 min, followed by rapid chilling on ice (total single-stranded DNA). Samples were separated on 12% polyacrylamide gels in 89 mM Tris borate, pH 8.3, 2 mM EDTA (37). Gels were dried, and the radioactivity in bands was quantified using a Storm PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). The data were evaluated using ImageQuant software (Molecular Dynamics). The radioactivity of each band was normalized for the total radioactivity added to each lane. The intensity of each band was expressed as the percentage of total duplex or total single-stranded DNA.

ATPase Assays—Unless indicated otherwise, ATPase assays were performed as described previously (32). Reaction mixtures (25 μl) contained 40 μg/ml SecA protein, 25 μg/ml IMV from CK1801.4 (for membrane and translocation ATPase activity) (21), 30 μg/ml chimeric preprotein composed of *E. coli* alkaline phosphatase signal sequence fused to staphylococcal nuclease with K97C and W140H substitutions (38) (diluted 50-fold from 6 M urea, 1 mM EDTA, 50 mM Tris-HCl, pH 9.2) (for translocation ATPase activity), 4 mM ATP, 0.5 mg/ml BSA, 50 mM HEPES-KOH, pH 7.0, 30 mM KCl, 30 mM NH_4Cl, 1 mM dithiothreitol, and 5 mM $Mg(OAc)_2$. 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 − (ATPase activity in the presence of IMV + endogenous ATPase activity); translocation ATPase activity = ATPase activity in the presence of preprotein, IMV, and SecA − (ATPase in the presence of preprotein and IMV + membrane ATPase activity).

RNA-binding Assays—SecA-bound RNA was determined using a Bio-Rad filtration apparatus providing support for 12 filters of 13-mm diameter. Filters were presoaked in TKMD-BSA (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM $Mg(OAc)_2$, 1 mM dithiothreitol, 100 μg/ml BSA) at 4 °C, and a sandwich was made by positioning the nitrocellulose filter (HA, pore size 0.45 μm, Millipore Corp.) on top of a DEAE filter (pore size 0.45 μm, Schleicher & Schuell), which were placed on the frits of the apparatus. The former filter retains RNA-protein complexes, while the latter one retains free RNA. Reaction mixtures (100 μl) contained 0.5 μM SecA protein and 0.58 fM (3.4×10^6 cpm/fmol) RS170 RNA in TKMD-BSA. They were filtered by gentle suction, rinsed twice with 0.5 ml of TKMD-BSA, dried, and subjected to liquid scintillation counting. ^{32}P-labeled RNA bound in the absence of SecA protein was less than 2.5% of that bound in the presence of SecA protein and subtracted in each case. The sum of bound and free RNA was at least 90% of the total input RNA.

Data Analysis—Analysis of the data from the helicase, ATPase, and

[1] The abbreviations used are: PCR, polymerase chain reaction; AMP-PNP, 5′-adenylylimidodiphosphate; BSA, bovine serum albumin; IMV, inverted membrane vesicles; IPTG, isopropyl-thio-β-D-galactopyranoside; NBD, nucleotide-binding domain; PAGE, polyacrylamide gel electrophoresis.

TABLE I
In vivo characterization of secA helicase mutants

Strain	Motif	Complementation	Growth on azide (mM)					
			0.5	1	1.5	2	3	Azide sensitivity
Wild type		+	+	+	+/−	−	−	Azi^s
D209N	II	−						
S213C	II	+	+	−	−	−	−	Azi^{ss}
T391A,T393A	III	−						
T393C	III	−						
S458C	IV	+	+	+	+/−	+/−	−	Azi^R
S462A	IV	+	+	+	+	+/−	+/−	Azi^R
A498C	V	+	+	+	+	−	−	Azi^s
M506C	V	+	+	+	+	+	+/−	Azi^R
G508S	V	+	−	−	−	−	−	Azi^{ss}
Q570A	VI	−						
R577A	VI	−						

Strains were BL21.19 with the indicated allele on pT7secA-his. Complementation was assessed by the ability of the indicated strain to grow well and form single colonies on LB plates containing 100 μg/ml ampicillin after overnight incubation at 42 °C. Azide sensitivity, supersensitivity, or resistance was assessed similarly to complementation, except plates also contained the indicated concentration of sodium azide. +, +/−, or − indicate good growth and single colony formation, poor growth and single colony formation, or no growth and single colony formation, respectively.

RNA-binding assays utilized Prism 3.0 software (GraphPad Software, Inc.).

RNA Folding—RNA folding was performed using the online version of mFold 3.0 software (available on the World Wide Web at mfold.wustl.edu/~folder/rna/form3.cgi) by Michael Zuker (Washington University School of Medicine). The energy calculation for annealing of oligonucleotides was performed using RNAstructure 3.5 software.

RESULTS

Characterization of SecA Helicase Activity—We found that SecA purified by our standard procedure often contained contaminating nuclease activity. In order to circumvent this problem, we constructed a SecA variant with a C-terminal hexahistidine tag, SecA-his, which generally lacked any discernible nuclease activity when purified on a nickel column (see "Experimental Procedures" for details). The SecA-his protein exhibited normal activity as accessed by *in vivo* complementation of the *secA(Am) supF(Ts)* mutant strain BL21.19 at 42 °C as well as analysis of its ATPase activities (Table I, Fig. 1). In studying SecA helicase activity, we wanted to employ the portion of *secM-secA* mRNA that binds SecA protein with high affinity and acts as a target for *secA* autorepression. We have previously demonstrated the existence of three RNA helices within the translationally regulated region of *secM-secA* mRNA (helix I, II, and III; see Fig. 2) (24). Helix II was shown to sequester the *secA* Shine-Dalgarno sequence and was necessary for proper *secA* repression (23). In addition, SecA's footprint was shown to overlap with the *secA* ribosome-binding site, thereby blocking translation initiation and facilitating autogenous regulation (29). In order to prepare a putative physiological substrate mimic to study SecA helicase activity, we synthesized and purified a 290-nucleotide RNA molecule, RS290, whose structure and high affinity SecA binding activity have been characterized previously (24, 29). A ^{32}P-labeled DNA oligonucleotide, DNA2s, was prepared in order to hybridize within the repressor helix (helix II) region of RS290 (Fig. 2). The duplex formed should contain 14 base pairs flanked by single-stranded DNA tails of five nucleotides each. The stability calculated for this duplex is −16.8 kcal/mol, or a melting temperature of 62.4 °C. Appropriate annealing conditions were found for duplex formation, and the duplex was purified on a native polyacrylamide gel (see "Experimental Procedures" for details). The helicase assay consisted of monitoring the release of DNA2s from the duplex by PAGE. SecA-his protein promoted the release of DNA2s from the duplex in a time- and ATP-dependent manner (Fig. 3). Neither ADP nor the nonhydrolyzable ATP analog, AMP-PNP, were active in the reaction. In addition, the helicase activity was inhibited by the addition of affinity-purified antibody against SecA (data not shown), strongly suggesting that SecA was responsible for the observed helicase activity. This inference received additional support by the isolation of helicase-defective SecA mutant proteins (see below). The helicase activity followed a first order reaction with a half-life of ~11 min (Fig. 3*C*), which is exceptionally slow for a typical helicase. This result may be due to a missing co-factor in the helicase reaction, or alternatively, it could be that our nucleic acid substrate is not optimally configured for SecA helicase activity.

Construction of secA Helicase Mutants and Their in Vivo Function—To continue to test the hypothesis that SecA belongs to helicase superfamily II, we constructed a series of mutations within the proposed helicase motifs II–VI of *secA* (Fig. 4) (30). Specific amino acid residues were chosen based on sequence alignments with other helicases of this superfamily as well as published observations regarding the effect that such alterations had on the ATPase and/or helicase activities of these proteins (reviewed in Ref. 39). Helicase motifs I and II coincide with the Walker A and B motifs, respectively, that facilitate nucleotide binding. Motif III is potentially involved in coupling ATP hydrolysis to unwinding activity (40–42), while in Rep helicase it might be involved in binding to single-stranded DNA (43). Motif IV appears to be important for ATP binding in certain helicases such as UvrD (44). Mutations in motif V impair helicase activity but not ATP hydrolysis of UvrB (45). Several mutations in motif VI abolish both ATPase and helicase activity (45–47).

Our primary goal was to construct mutants that would be active in the ATPase and protein translocase functions of SecA while disrupting its helicase activity. This would allow us to determine whether SecA helicase activity was essential *in vivo* (*e.g.* to unwind messenger RNA for membrane and secretory proteins during co-translational secretion) and to test its involvement in *secA* auto-regulation. For our studies here, we utilized the well characterized SecA protein with an alteration in motif II, SecA-D209N-his, which allowed us to test the importance of SecA ATPase activity for its helicase activity. Previously, we showed that mutations within the Walker A or B motif of the high affinity nucleotide binding domain of SecA resulted in a nonfunctional SecA protein that displayed diminished nucleotide-binding activity and lacked appreciable trans-

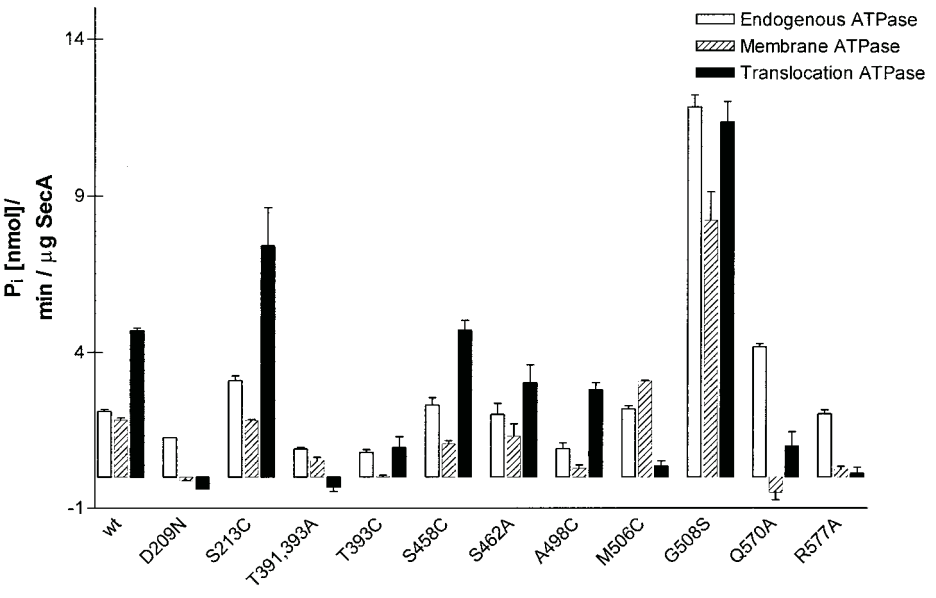

FIG. 1. **ATPase activities of SecA proteins.** SecA ATPase assays were performed in duplicate at 36 °C, and the data are an average of two independent experiments.

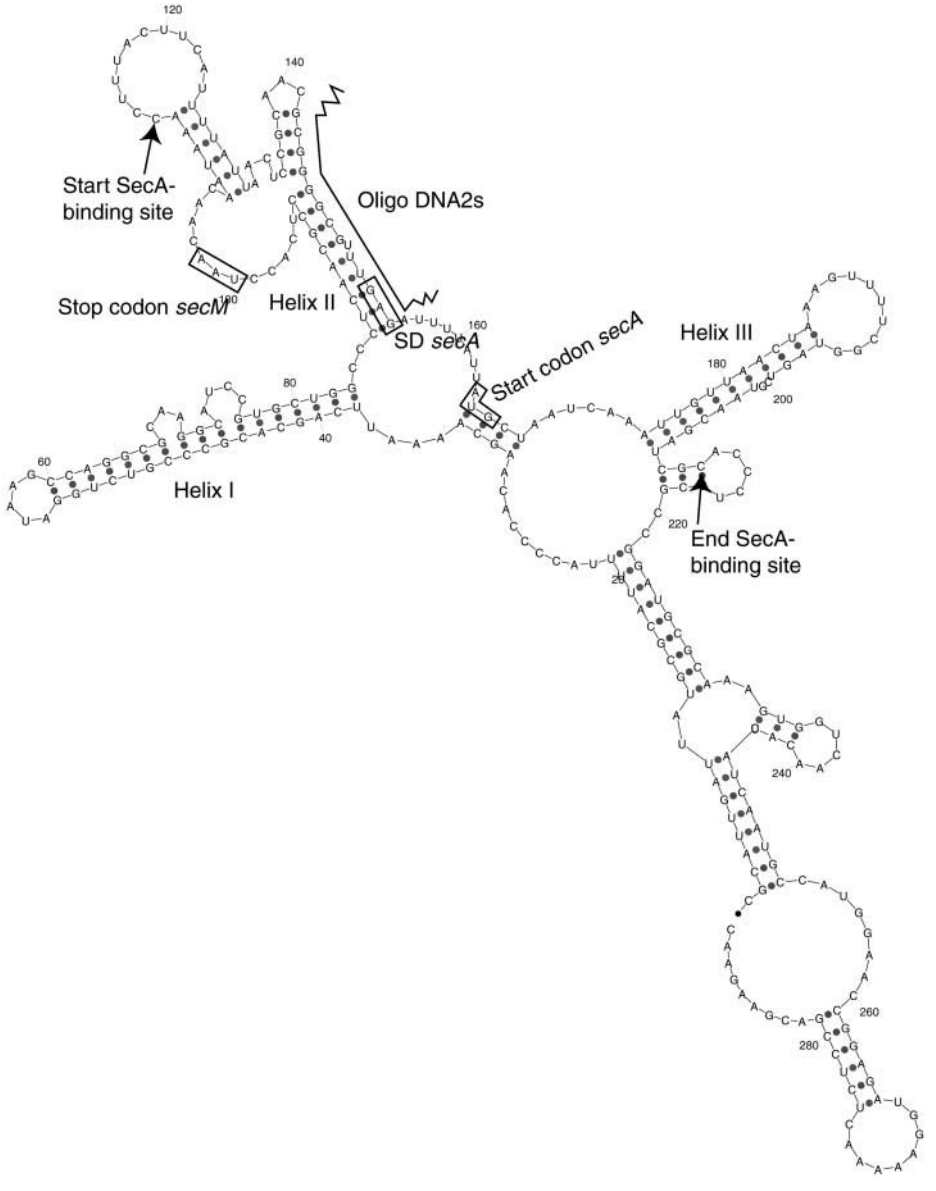

FIG. 2. **Proposed secondary structure of RS294 RNA and location of annealed DNA2s.** The secondary structure was calculated using mFold and is in agreement with experimental results (23, 24). The termination codon of *secM* and the Shine-Dalgarno sequence and initiation codon of *secA* are *boxed*, and the binding site for SecA protein (nucleotides 113–211) is indicated by *arrows*.

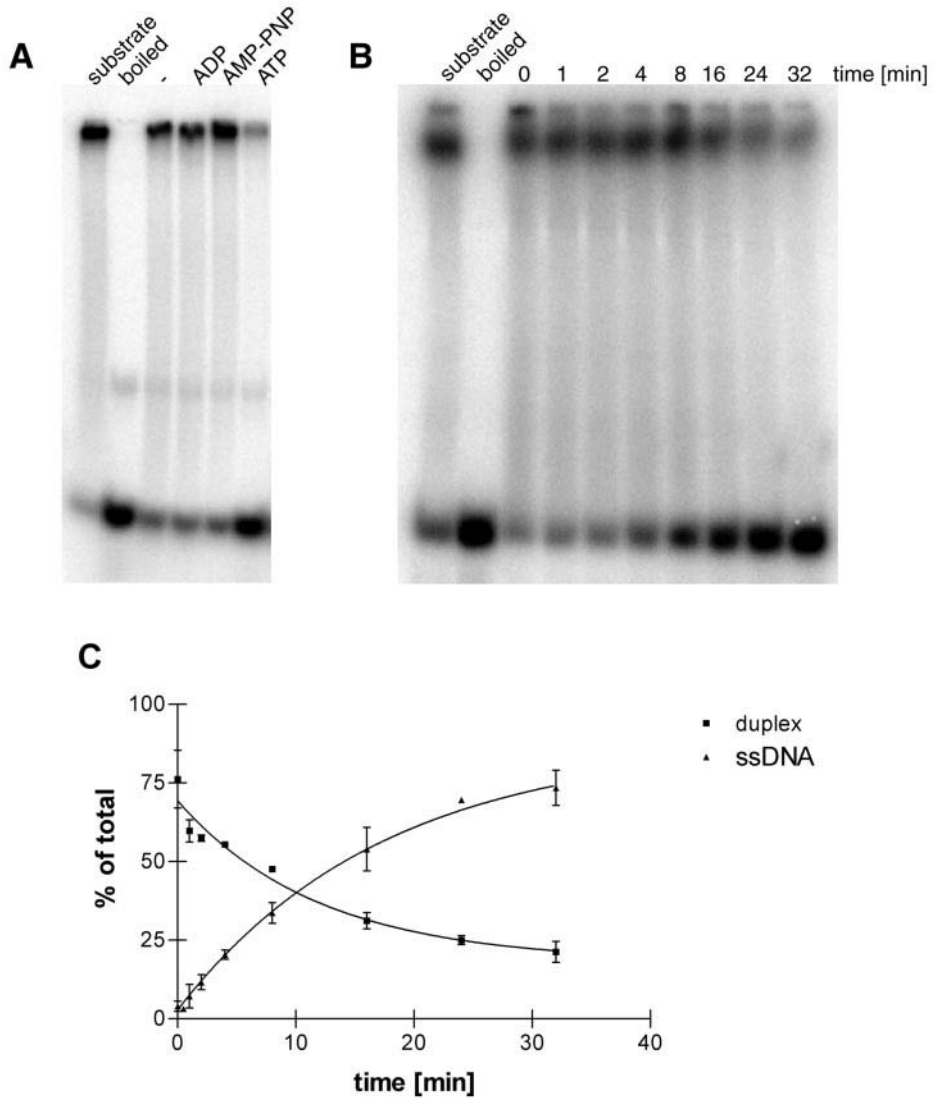

FIG. 3. **ATP-dependent helicase activity of SecA protein.** A strand displacement assay was performed to measure helicase activity of SecA protein as described under "Experimental Procedures." *A*, reactions in H buffer contained 150 pM DNA-RNA duplex, 25 nM SecA protein, and the indicated nucleoside phosphate. After incubation at 37 °C for 32 min, reactions were quenched with loading buffer and analyzed by PAGE. ADP and ATP were at 1 mM, while AMP-PNP was at 10 mM. Substrate and boiled samples lacked SecA protein, and the latter sample was heated at 95 °C for 2 min, followed by rapid chilling on ice. *B*, time dependence of the reaction; *C*, its quantification after PhosphorImager scanning and analysis of data.

Helicase Motifs		I		Ia		II		III		IV		V		IV	
ERCC6	(533-950)	EMGLGKTI	30	IVCPTTVMH	63	VILDEGHK	20	IILSGSPM	180	LLFSQSRQ	45	LTTRVGGLGVNLTGAN	15	QARERAWR	
NS3	(204-466)	PTGSGKST	14	VLNPSVAAT	52	IICDECHS	24	VLATATPP	37	LIFCHSKK	35	VSTDALMTGFTGDFDS	35	QRRGRTGR	
eIF4A	(76-365)	QSGTGKTA	20	VLAPTRELA	65	FVLDEADE	23	VLLSATMP	57	VIFINTRR	42	ITTDLLARGIDVQQVS	15	HRIGRGGR	
UvrB	(40-544)	VTGSGKTL	14	VLAPNKTLA	271	LVVDESHV	47	IYVSATPG	53	LVTTLTKR	43	VGINLLKEGLDMPEVS	21	QTIGRAAR	
DbpA	(23-311)	KTGSGKTA	20	VLCPTRELA	64	LVMDEADR	23	LLFSATWP	56	VVFCNTKK	43	VATDVAARGLDIKSLE	16	HRIGRTAR	
RecQ	(50-328)	PTGGGKSL	14	VVSPLISLM	64	LAVDEAHC	27	MALTATAD	54	IIYCNSRA	43	VATVAFGMGINKPNVR	16	QETGRAGR	
RecG	(297-587)	DVGSGKTL	17	LMAPTELLA	63	VIIDEQHR	23	LIMTATPI	59	TLIEESEL	49	VATTVIEVGVDVPNAS	17	QLRGRVGR	
Consensus		oGoGKT		+LAPTR		+++DEAH		+++SATPP		++F+oo+o		+xTo++ooG+o+xo+o		QR+GRxGR	
SecA	(103-577)	RTGEGKTL	18	VVTVNDYLA	69	ALVDEVDS	174	AGMTGTAD	61	ISIEKSEL	38	IATNMAGRGTDIVLGG	52	QLRGRSGR	
						D209N S213C		T391,393A T393C		S458C S462A		A498C M506C G508S		Q570A R577A	

FIG. 4. **Mutagenesis of helicase motifs within SecA.** Alignment of conserved motifs in helicases of superfamily II. The sequences of ERCC6 (human putative helicase, involved in Cockayne's syndrome), NS3 (hepatitis C virus RNA helicase), eIF4A (eukaryotic translation initiation factor 4A), UvrB (*E. coli* DNA helicase for the repair of UV-damaged DNA), DbpA (DEAD box protein A, an *E. coli* RNA helicase), RecQ (*E. coli* DNA helicase for DNA repair), and RecG (*E. coli* DNA helicase for recombination) were from the Swiss-Prot data bank. The consensus is derived from the entire set of superfamily II helicases (68). *x*, any amino acid residue, *o*, charged or polar residue, +, hydrophobic residue. Mutations introduced into SecA are shown *below* the corresponding motif, where the altered residue is depicted in *boldface type*.

location ATPase and *in vitro* protein translocation activities (32). Such proteins were also defective in SecA membrane cycling, since they remained stuck in their membrane-inserted state due to a defect in ATP hydrolysis (16). In addition, from an ongoing cysteine-scanning mutagenesis project, we had previously constructed a number of SecA mutant proteins with alterations within motif III (SecA-T393C-his), motif IV (SecA-A458C-his), and motif V (SecA-M506C-his), as well as ones with alterations adjacent to motif II (SecA-S213C-his) and motif V (SecA-A498C-his). Additionally, we constructed secA mutations within motifs III, IV, V, and VI. The core region of motif III (SAT) is fairly conserved within SecA (TGT). Accordingly, we created a SecA variant with two alterations within this region, SecA-T391A,T393A-his. For motif IV, we created SecA-S462A-his. For motif V, we constructed SecA-G508S-his that altered a fairly conserved Gly residue. Finally, for motif VI, which is rather well conserved in SecA, we altered the two conserved residues at the beginning and end of this motif, giving rise to SecA-Q570A-his and SecA-R577A-his.

To test whether the mutations in the proposed helicase mo-

tifs of SecA altered its function *in vivo*, we performed complementation studies utilizing BL21.19 at 42 °C (Table I). These tests were performed utilizing glucose minimal medium, since under these conditions the plasmid-borne copy of *secA* is expressed at chromosomal levels (48), ensuring that the mutant proteins must be reasonably active in order to allow for normal cell growth. Only SecA proteins with mutations in motif IV and motif V were able to complement BL21.19 under these conditions. Additionally, SecA-S213C-his and SecA-A498C-his, which contain substitutions adjacent to motif II and V, respectively, were also functional in this assay.

Characterization of ATPase, Helicase, and RNA Binding Activity of SecA Proteins—The mutant SecA proteins were purified, and their ATPase activities were quantified. SecA protein has three distinct ATPase activities: a basal activity in solution (endogenous ATPase), a membrane-stimulated activity (membrane ATPase), and a membrane- and preprotein-dependent activity (translocation ATPase) (49). In general, there was a good correlation between the membrane and translocation ATPase activities of the mutant proteins and their functionality *in vivo*. Proteins that were nonfunctional *in vivo* generally showed sharply decreased membrane and translocation ATPase activities, while those proteins that were functional *in vivo* had activities that were more similar to wild-type SecA-his (Fig. 1 and Table I). SecA proteins with alterations adjacent to or within motif V proved to be an exception to this pattern. SecA-A498C-his displayed very low membrane ATPase activity, while SecA-M506C-his had surprisingly low translocation ATPase activity given its functional nature. Additional studies of this latter protein should be useful for elucidation of the coupling between the preprotein translocation and ATPase cycles of SecA. Finally, SecA-G508S-his was hyperactive for all three SecA-dependent ATPase activities, and it displayed a more open conformation as assessed by susceptibility to trypsinolysis when compared with wild-type SecA-his or the other mutant proteins (data not shown). Superactive SecA mutants with similarly high ATPase activities have been reported also by Ito's group, and they were shown to be capable of suppressing a variety of defects in *secY*, *secE*, and *secG* mutants (50, 51).

We tested the secretion-proficient *secA* mutants for their sensitivity, supersensitivity, or resistance to sodium azide, an inhibitor of SecA membrane and translocation ATPase activities (21, 53), since mutations within SecA's nucleotide-binding domains often affect this property (52, 54). This test was done under conditions where only the mutant form of SecA protein was produced (the *secA13(Am) supF(Ts)* pT7secA-his-bearing strain at 42 °C). Five out of six of the secretion-proficient *secA* mutants displayed some alteration in their sensitivity profile to sodium azide (Table I). The *secA-G508S-his* mutant was supersensitive to azide, while the *secA-S213C-his* mutant was slightly more azide-sensitive. The *secA-S458C-his*, *secA-S462A-his*, and *secA-M506C-his* strains displayed increasingly higher levels of azide resistance.

Helicase assays were performed on the mutant SecA proteins. Most mutant proteins, even those that exhibited *in vivo* function and more normal ATPase activities, were clearly defective in helicase activity (Fig. 5*A*). SecA-T391A,T393A-his, SecA-S458C-his, SecA-A498C-his, SecA-M506C-his, and SecA-Q570A-his were comparable with SecA-D209N-his, which is defective in a critical residue in the Walker B motif, lacks membrane and translocation ATPase activity (Fig. 1), and can be taken as a classic helicase negative mutant protein based on analysis of other systems. In addition, similar results were obtained with these proteins when ATP was omitted in the assay (data not shown). In contrast, SecA-S213C-his and SecA-S462A-his exhibited essentially normal levels of helicase activity in a time-dependent assay (Fig. 5*B*). Since three of our mutant proteins, SecA-T393C-his, SecA-G508S-his, and SecA-R577A-his, showed a reduction in the total counts recovered in our assay system (Fig. 5*A*), we performed additional assays with these proteins and confirmed that they were essentially negative for helicase activity (Fig. 5*C*). The decrease in total counts recovered in this case was most likely due to contaminating levels of a nuclease or phosphatase in these SecA preparations.

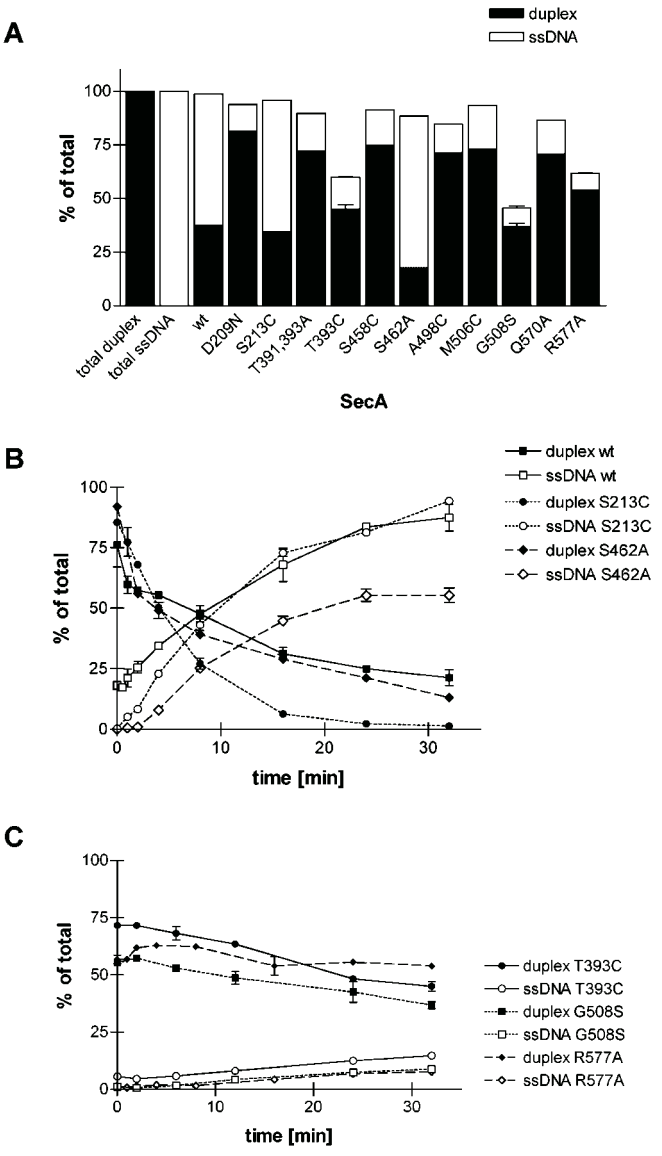

FIG. 5. **Helicase activities of SecA mutant proteins.** *A*, helicase reactions were performed for the indicated SecA proteins in the presence of 1 mM ATP at 37 °C for 32 min. Total duplex and total single-stranded DNA correspond to "substrate" and "boiled" controls in Fig. 3. *B* and *C*, time-dependent helicase reactions for the indicated SecA proteins. Reactions, controls, and data analysis were similar to Fig. 3. Each value represents at least two independent measurements.

One reason that the mutant SecA proteins could be defective in helicase activity is that they might be defective in binding to the RNA substrate. Accordingly, we performed filter-binding assays to measure the RNA binding activity of the mutant proteins. The helicase-defective SecA proteins displayed higher RNA binding activity than either their helicase-proficient or wild-type SecA-his counterparts (Fig. 6). SecA-D209N-his and

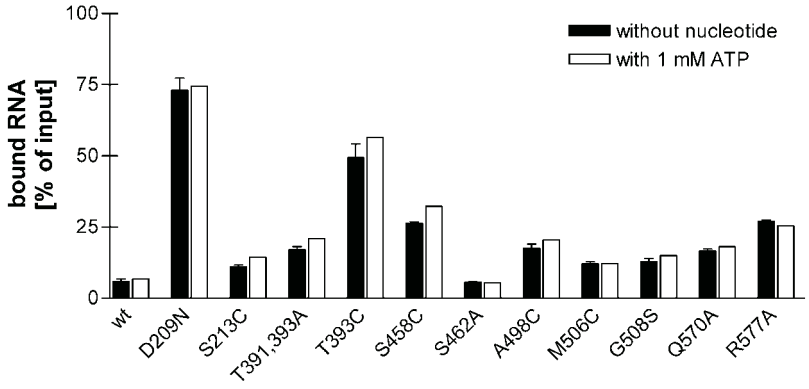

FIG. 6. **SecA helicase-defective proteins have higher RNA binding activity.** RNA binding activity of SecA protein was assessed by a filter-binding assay as described under "Experimental Procedures." Each value represents at least two independent measurements.

SecA-T393C-his showed the highest RNA binding activity, but the other mutant proteins had at least double the RNA binding activity of the helicase-proficient proteins, SecA-S213C-his and SecA-S462A-his. Therefore, it is clear that a defect in RNA binding was not responsible for the helicase defects that we found. Furthermore, the observation that similar results were obtained in the presence or absence of ATP indicates that helicase activity is not an important factor in promoting SecA release from its RNA substrate in this assay.

Effect of Helicase Mutations on Protein Secretion and secA Autoregulation—We studied the effect that our novel *secA* alleles had on *secA* autoregulation and protein secretion. It was previously hypothesized that SecA helicase activity may be responsible for *secA* autorepression by unwinding an RNA secondary structure that is needed for efficient *secA* translation initiation (30). According to this model, a protein export block would titrate SecA away from its mRNA in favor of preprotein or translocon binding, thereby allowing accumulation of the translationally active mRNA species and *secA* derepression. Previously, we have shown that SecA autorepressor activity can be assayed by overproduction of a plasmid-encoded SecA protein that resulted in superrepression of a *secA-lacZ* reporter (20, 23). For this purpose, we constructed a low copy number, pBR322-compatible plasmid, pLG552.1, that carries a *secA-lacZ* translational fusion (see "Experimental Procedures" for details). The fusion was correctly regulated, since β-galactosidase activity was 15-fold higher in a secretion-defective *secD1(Cs)* (pLG552.1) strain grown at 23 °C compared with its secretion-proficient sec^+ (pLG552.1) counterpart (data not shown). We then transformed the different pT7secA-his plasmids into BL26.1 (pLG552.1) and measured the ability of the mutant SecA protein to superrepress the *secA-lacZ* reporter. Induction of these strains with IPTG also allowed us to modulate the level of SecA overproduction from ~8-fold (before induction) to 35-fold (after induction) above the wild-type level (data not shown). BL26.1 (pLG552.1, pT7secA-his) had greater than a 2-fold reduction in β-galactosidase activity compared with BL26.1 (pLG552.1, pBR322), and this activity decreased further after IPTG induction (Fig. 7). Most secretion-proficient *secA* mutants had β-galactosidase activities that were similar to the wild-type strain, indicating that they were active in SecA-dependent superrepression activity. *secA-M506C-his* and *secA-G508S-his* showed somewhat less superrepression. There was no correlation between helicase and superrepression activities (*e.g.* compare *secA-S213C-his* and *secA-S462A*-his that have normal helicase activity with *secA-S458C-his* and *secA-A498C-his* that lack helicase activity), suggesting that SecA helicase activity was not required for SecA autorepressor activity. Finally, strains with secretion-defective *secA* alleles had very high β-galactosidase activities, particularly after IPTG induction. This pattern is reminiscent of our previous work, where secretion-defective *secA* alleles caused dominant protein secretion defects and *secA* derepression due to subunit mixing and "poisoning" of the SecA homodimer (55).

Since the foregoing analysis of helicase-defective *secA* alleles made use of strains that were merodiploid for *secA*, our results could have been affected by the presence of minor amounts of wild-type SecA protein. Therefore, we also performed studies in *secA* haploid strains for those alleles that were secretion-proficient but helicase-defective. We constructed strains that carried the appropriate pT7secA-his plasmid along with a *secA-lacZ* fusion at the chromosomal *secA* locus (see "Experimental Procedures" for details). An analysis of *secA* regulation and protein secretion in these strains was performed utilizing pulse labeling methods as well as sodium azide to block protein secretion and assess its effect on *secA* derepression. Our results show that the helicase-defective *secA* mutants *secA-S458C-his*, *secA-A498C-his*, and *secA-M508S-his* were repressed similarly to the wild type and that they underwent normal *secA* derepression after an azide-induced block in protein secretion (Fig. 8*A*). However, we noticed that for all of the strains containing the His-tagged SecA, whether wild-type or mutant, there was a reduced rate of OmpA secretion compared with the wild type (compare *lane 1* with *lanes 2–5*). This result is consistent with our previous report that mutations at the extreme C terminus of SecA can reduce the rate of secretion of SecB-dependent preproteins (56). It has been shown previously that this region of SecA contains the SecB binding site (57). Furthermore, the reduced rate of OmpA secretion in this case did not appear to cause *secA* derepression, consistent with our observation that *secA* repression is normal in a *secB* null mutant (58).

In order to eliminate the complication that the His tag had on our study, we crossed the *secA-S458C* allele into the chromosome of the wild-type strain MC4100, giving rise to MCH2 (see "Experimental Procedures" for details), and we repeated our analysis of *secA* regulation and protein secretion. The results show that this helicase-negative mutant displayed normal *secA* regulation and a rapid rate of secretion for OmpA and maltose-binding protein (Fig. 8*B*). While MCH2 was azide-resistant, the addition of azide to 3 mM prior to the pulse labeling resulted in some inhibition of protein secretion and a level of *secA* derepression that was comparable with the wild-type strain. Taken together, our results indicate that SecA helicase activity plays no apparent role in *secA* autoregulation or efficient protein secretion.

DISCUSSION

All known ATP-dependent helicases have conserved stretches of amino acids comprising motifs that allow classification of these enzymes into superfamilies. As ATPases, they also contain the Walker A and B motifs important for coordination and hydrolysis of the nucleotide during their enzymatic

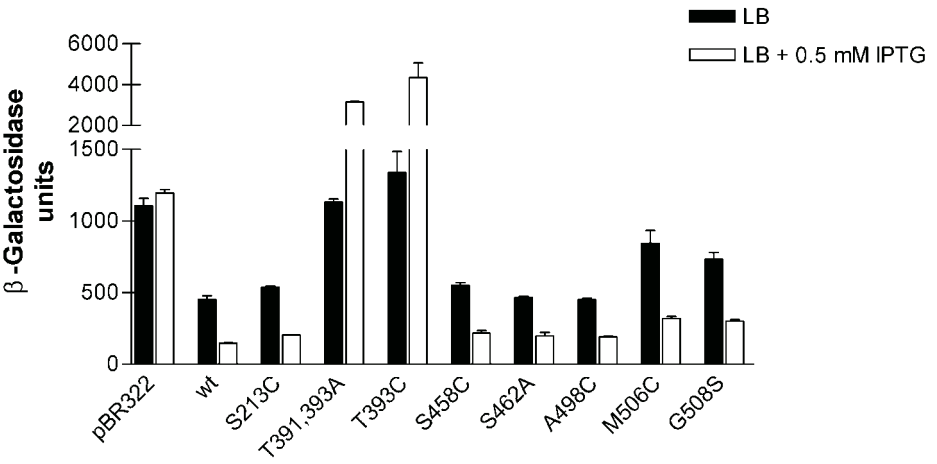

FIG. 7. **SecA helicase-defective strains superrepress a *secA-lacZ* reporter.** BL26.1 (pLG552.1) containing pBR322 or the indicated pT7secA-his derivative was grown in LB broth containing 20 μg/ml kanamycin and 100 μg/ml ampicillin at 37 °C. At early logarithmic phase growth, cultures were split, and one portion was induced with IPTG at 0.5 mM. After an additional 2 h of growth, cultures were placed on ice, and β-galactosidase assays were performed at 30 °C as described by Miller (36). Each value represents at least four independent measurements.

cycle. As reviewed recently (39) and shown in crystal structures for yeast IF4A (59), PcrA (60), hepatitis C virus NS3 RNA helicase (61), Rep helicase (43), UvrB (62), and a DExx box helicase (63), most of the proposed helicase motifs are located in proximity to the nucleotide-binding cleft, where they are able to regulate the nucleic acid binding and unwinding cycle in accordance with the nucleotide-bound state of the enzyme. SecA appears to be no exception to this rule, since the seven proposed helicase motifs (30) appear to reside in either the high affinity (NBD-I, motifs I, Ia, II, and III) or low affinity nucleotide-binding domains (NBD-II, motifs IV, V, and IV) of SecA (32). It has been shown previously that NBD-I controls interaction with the preprotein and the membrane along with SecA membrane cycling (16, 64, 65). Recent studies suggest that NBD-II carries out a regulatory role in SecA function (51, 66, 67). Mutations within this domain have been isolated that result in superactive forms of SecA protein that greatly stimulate the ATPase activity of NBD-I and globally suppress secretion defects of *secY*, *secE*, and *secG* mutants (51, 66). Another group proposed a regulatory link between NBD-I and NBD-II. Mutations in the consensus helicase motifs V and VI in NBD-II deregulated SecA's ATPase activity (67). The authors suggest that NBD-II optimizes the ATPase activity of NBD-I and controls ADP release.

We conclude that SecA is an ATP-dependent RNA helicase, as hypothesized by Koonin and Gorbalenya (30) and as first demonstrated by Park *et al*. (31). Our observations that helicase activity could only be observed in the presence of ATP, and not ADP or AMP-PNP, along with our demonstration that nonconservative mutations within motifs II–VI eliminated helicase activity strongly suggest that SecA is a member of helicase superfamily II. While SecA helicase activity obeyed first order kinetics, the reaction rate was exceptionally slow. Potential explanations for this observation include the following: (i) the low helicase activity is an evolutionary remnant derived from an ancestral protein that had a more robust helicase activity, (ii) the substrate assayed here was nonphysiological, or (iii) a protein or other cofactor necessary for stimulating SecA helicase activity was lacking under our assay conditions. In this regard, we note that our substrate was complex and contained many RNA helices whose possible dissociation by SecA was not monitored. An examination of a simpler RNA substrate containing only the repressor helix region could help to rule in or out this second possibility.

Our studies succeeded in their primary goal of dissociating the protein translocation and helicase activities of SecA protein. Therefore, while mutations in all seven motifs abolished SecA helicase activity, those in motifs IV and V still allowed SecA-dependent protein translocation activity. These latter results are not due to the irrelevance of such sequences in protein translocation, since mutations in motif IV have been isolated previously that abolished SecA-dependent protein translocation activity (*e.g.* SecA-R509Q (32)). Rather, it seems plausible

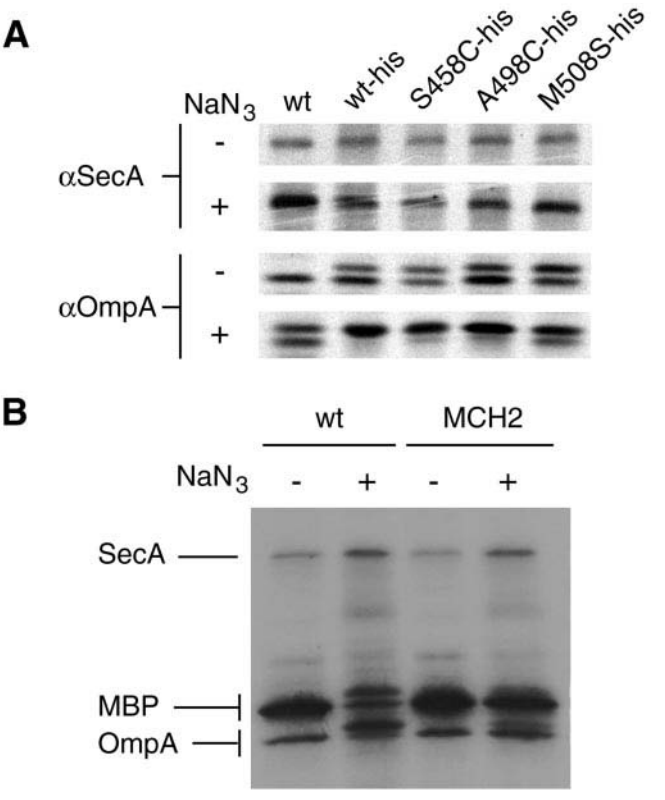

FIG. 8. **SecA helicase-defective strains show normal *secA* autoregulation and protein secretion.** Strains were grown in M63 minimal medium containing 0.4% maltose, 0.4% glycerol, 20 μg/ml each of 18 amino acids (lacking methionine and cysteine), 100 μg/ml ampicillin, where necessary, at 37 °C until midlogarithmic phase. Where indicated, sodium azide was added to 3 mM for 5 min prior to labeling. This level was sufficient to inhibit protein secretion of even azide-resistant *secA* mutants. A 0.5-ml aliquot of each culture was pulse-labeled with 10 μCi of Tran^{35}S-label (>1000 Ci/mmol; ICN) for 1 min, followed by the addition of an equal volume of ice-cold 10% trichloroacetic acid. SecA, maltose-binding protein, and OmpA were immunoprecipitated and analyzed by SDS-PAGE and autoradiography as described previously (21). *A*, BL26 (λDE3) *leu::Tn5* Φ(*secA-lacZ*)*f181*(Hyb) *recA*::CAT carrying pT7secA2 (wild type), pT7secA-his (wild type-his), or the indicated allele on pT7secA-his. αSecA and αOmpA indicate immunoprecipitation with SecA and OmpA antisera, respectively. *B*, MC4100 *leu::Tn10* (wild type) and MCH2. The positions of SecA and the precursor and mature forms of maltose-binding protein (*MBP*) and OmpA are shown.

that residues around motifs IV and V are involved in coupling the nucleotide-binding cycle to the movement of effector domains that drive the SecA helicase and translocase motors. Two partially overlapping sets of residues can be envisioned for a bifunctional protein such as SecA, where certain residues would only affect one function (*e.g.* helicase but not translocase or *vice versa*), while others would affect both.

Two important conclusions reached in our studies are that SecA helicase activity does not appear to be required for either efficient protein secretion or *secA* autoregulation *in vivo*. A role of SecA helicase activity for efficient protein secretion could have involved, for example, removing secondary or tertiary structure from messenger RNA of proteins that are being secreted co-translationally or promoting release of translocon-bound ribosomes after protein translocation is complete. While our results do not directly exclude these functions *per se*, they do suggest that they are not rate-limiting for rapid protein export kinetics. An important caveat here is that we recognize that our helicase-negative mutants may still possess a low level of helicase activity that we could not detect (*e.g.* 5%) and that this level may be sufficient to promote such functions *in vivo*. Clearly, more careful assessment of this possibility will require additional studies beyond the scope of the present work.

We found no support for the attractive speculation that SecA helicase activity may play a role in *secA* autoregulation (30). Our helicase-defective *secA* mutants were as proficient as the wild-type strain for *secA* repression, and they could be derepressed normally by a block in protein secretion. Again, we cannot exclude a low level of leakiness of our helicase-defective mutants in these experiments. However, if this were the case, it seems improbable that all of the helicase-defective mutants would have behaved similarly regarding *secA* auto-regulation. Therefore, we consider this alternative to be unlikely. Furthermore, recent work on *secA* regulation does not support the original model posited by Koonin and Gorbalenya (30). Rather than facilitating *secA* translation, an RNA secondary structure (*i.e.* the repressor helix) occludes the *secA* Shine-Dalgarno sequence and inhibits *secA* translation initiation (23, 24). Translational pausing within the distal portion of *secM* is needed to facilitate transient opening of the repressor helix, thereby allowing *secA* translation initiation (27). The duration of pausing appears to be controlled by SecM protein secretion in a co-translational fashion. Factors that inhibited SecM secretion, such as a poorly active signal peptide or defective translocon, lengthened the duration of the translational pause and resulted in *secA* derepression.

In addition to this elegant system for regulating *secA* translation during conditions of limiting protein secretion, we have previously described two mechanisms that allow for *secA* repression during protein secretion-proficient conditions. SecA was shown to bind to *secM-secA* RNA around its translation initiation site, thereby blocking binding of the 30 S ribosomal subunit (29). In addition, by an as yet undefined mechanism, SecA protein was able to dissociate a newly formed initiation complex consisting of the 30 S ribosomal subunit, $tRNA^{fMet}$, and *secM-secA* RNA (29). This latter type of repression would appear to be redundant, and it may involve, for example, use of SecA helicase activity to release a based-paired structure between the *secA* Shine-Dalgarno sequence and the 3′-end of 16 S ribosomal RNA. This possibility can now be directly assessed utilizing the SecA proteins from this study. Clearly pinpointing the physiological role of SecA helicase activity will require additional studies along several lines of investigation.

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