A Novel Class of *secA* Alleles That Exert a Signal-Sequence-Dependent Effect on Protein Export in *Escherichia coli*

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ABSTRACT

The murine plasminogen activator inhibitor 2 (PAI2) signal sequence inefficiently promotes the export of *E. coli* alkaline phosphatase (AP). High-level expression of PAI2::AP chimeric proteins from the arabinose P_{BAD} promoter is toxic and confers an Ara^S phenotype. Most Ara^R suppressors map to *secA*, as determined by sequencing 21 independent alleles. Mutations occur throughout the gene, including both nucleotide binding domains (NBDI and NBDII) and the putative signal sequence binding domain (SSBD). Using *malE* and *phoA* signal sequence mutants, we showed that the vast majority of these *secA* suppressors exhibit weak Sec phenotypes. Eight of these *secA* mutations were further characterized in detail. Phenotypically, these eight suppressors can be divided into three groups, each localized to one domain of SecA. Most mutations allow near-normal levels of wild-type preprotein export, but they enhance the secretion defect conferred by signal sequence mutations. Interestingly, one group exerts a selective effect on the export of PAI2::AP when compared to that of AP. In conclusion, this novel class of *secA* mutations, selected as suppressors of a toxic signal sequence, differs from the classical *secA (prlD)* mutations, selected as suppressors of defective signal sequences, although both types of mutations affect signal sequence recognition.

SINCE the development of the signal sequence model (Blobel and Dobberstein 1975), protein translocation across the plasma membrane has been characterized both genetically and biochemically in *Escherichia coli* (Schatz and Beckwith 1990; Danese and Silhavy 1998; Driessen *et al.* 1998, 2001). In prokaryotes, periplasmic and outer membrane proteins are synthesized in the cytoplasm and translocated post-translationally. The signal sequence of secreted proteins functions both as a targeting signal and as a recognition signal during export. Signal sequences are composed of three domains: a positively charged N-terminal region, a hydrophobic core, and a polar C-terminal region containing the leader peptidase cleavage site. The precursors of exported proteins are maintained in a translocation-competent conformation by chaperones such as SecB (Collier *et al.* 1988), GroE (Lecker *et al.* 1989), or DnaK (Wild *et al.* 1996). At the membrane, they bind the homodimeric ATPase SecA (PrlD) and the heterotrimeric SecYEG (PrlAGH), the integral membrane complex that constitutes the core of the translocase. SecYEG also promotes the insertion and determines the topology of integral membrane proteins (Prinz *et al.* 1998).

Precursor translocation across the membrane is an energy-requiring process driven by ATP hydrolysis and by the proton motive force (Chen and Tai 1986; Geller *et al.* 1986; Schiebel *et al.* 1991). Translocation is initiated by the ATP-dependent co-insertion of SecA and the preprotein at SecYEG. Interaction of SecA and SecYEG stimulates the ATPase activity of SecA, which leads to the dissociation of SecA from the preprotein. During this process, SecA undergoes large conformational changes (Economou and Wickner 1994; Kim *et al.* 1994). After this initial stage, the proton motive force can drive preprotein translocation to completion (Driessen and Wickner 1991). Electron microscopic analysis of both the *Bacillus subtilis* and *E. coli* SecYE complexes indicates that they form a large channel with a quasi-pentameric structure, at least in octyl-glucoside detergent (Meyer *et al.* 1999; Manting *et al.* 2000). However, two other studies suggest that the functional state of SecYEG is either monomeric (Yahr and Wickner 2000) or dimeric (Bessonneau *et al.* 2002). SecY and SecE are encoded by essential genes and promote both SecA-dependent protein translocation into proteoliposomes (Akimaru *et al.* 1991) and integral membrane protein insertion mediated by the signal recognition particle (Newitt and Bernstein 1998). The latter process is also SecA dependent (Qi and Bernstein 1999), although initial membrane insertion can occur *in vitro* in the absence of SecA (Scotti *et al.* 1999). SecG is a nonessential component of the translocase (Nishiyama *et al.* 1994), which stimulates translocation both *in vivo* and *in vitro* (Nishiyama *et al.* 1993; Bost and Belin 1995; Hanada *et al.* 1996). SecG undergoes an inversion of its membrane topology coupled with SecA insertion-deinsertion cycles (Nishiyama *et al.* 1996). Two other integral membrane proteins, SecD and SecF, are required for efficient translocation (Arkowitz

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and Wickner 1994; Pogliano and Beckwith 1994). However, despite the fact that these two proteins can be isolated together with SecYEG (Duong and Wickner 1997), their precise function during translocation remains poorly understood.

Early genetic selections led to the identification of most *sec* genes and gave rise to two kinds of mutations in most of these genes (Schatz and Beckwith 1990). Mutations conferring a Sec phenotype have a generalized secretion defect. Suppressors with a Prl phenotype are more subtle mutants that have lost the ability to discriminate against inefficient signal sequences and that even allow export of proteins lacking a recognizable signal sequence (Derman *et al.* 1993). These observations led to the hypothesis that the translocase has a signal sequence proofreading activity (Osborne and Silhavy 1993). Prl mutations have been isolated in *secA*, *secY*, *secE*, and *secG* (Schatz and Beckwith 1990; Bost and Belin 1997), suggesting that each protein contributes somehow to the signal sequence recognition function of the translocase.

In this study, we describe the isolation and characterization of a novel class of *secA* alleles. These mutants were isolated as suppressors of the murine PAI2 signal sequence, which is toxic when expressed at high levels. Most suppressors map to *secA*, *secY*, and *secG*, supporting the notion that the toxicity of PAI2::AP fusion proteins is due to a defective interaction between the PAI2 signal sequence and the translocase (Bost and Belin 1995; Bost *et al.* 2000). Since many suppressors map to *secA*, we reasoned that their molecular defect could help us understand the function of SecA during an early step of the translocation process. Unlike *secG* suppressors, which are mostly localized to three contiguous codons (the central $T_{41}L_{42}F_{43}$ domain), *secA* mutations are distributed throughout the first two-thirds of the gene. Like *secG* suppressors, all *secA* mutants exhibit a weak Sec phenotype. Most of these suppressors have a different effect on the activity of several wild-type and mutated signal sequences, and one group has a selective effect on PAI2::AP export. Thus, these mutants, unlike classical *sec* mutants, do not confer a strong and generalized secretion defect. In conclusion, this class of suppressors defines a weak Sec phenotype that appears opposite to that of *prl* mutants in the context of mutated or inefficient signal sequences, suggesting that they are affected mainly in the signal sequence recognition activity of SecA.

MATERIALS AND METHODS

Reagents: Liquid and solid media were prepared as described (Miller 1992). Antibiotics were used at the following concentrations: kanamycin, 40 μg/ml; ampicillin, 200 μg/ml; and tetracycline, 15 μg/ml. 5-Bromo-4-chloro-3 indolyl-phosphate (XP) was purchased from DCL (Oxford, CT) and used at 40 μg/ml. Rabbit antisera against MalE (MBP)-DegP, RbsB (RBP), OmpA, and SecA were kind gifts from J. Beckwith, M. Ehrmann, and. W. Wickner. Anti-MalE serum was purchased from New England Biolabs (Beverly, MA).

Bacterial strains: All strains are described in Table 1 or were constructed by phage P1-mediated transduction or by transformation (Miller 1992); the *secA* gene cotransduces with the *leu*::Tn*10* transposon of LMG194 with a frequency of 40%. All studies have been performed with chromosomal *secA* alleles. The *secA (prlD)* strains were kindly provided by T. Silhavy. In the absence of registered *sec* allele numbers, they are referred to as *secA (prlD2)*, *secA (prlD5)*, and *secA (prlD21)* in the text and as *prlD2*, *prlD5*, and *prlD21* in the figures, using the published *prl* numbers (Fikes and Bassford 1989; Huie and Silhavy 1995).

Plasmid construction: Plasmids pBAD72K, pBADhBK, and pBADhBS are pBAD24 derivatives (Bost and Belin 1995; Guzman *et al.* 1995; Bost *et al.* 2000). p*secA*$^+$ is a derivative of pBE2 (Kim *et al.* 1994) lacking both the promoter of the *secA* operon and most of the *gene X* (≡*secM*) coding sequence; *secA* expression is driven from the tetracycline promoter of pACYC184. Briefly, a 1.6-kbp *Mlu*I/Klenow fragment from pBE2 encoding the 3′ part of *secA* was first subcloned in the *Sma*I site of pBS-KS. The resulting vector was digested with *Bss*HII and *Eco*RI and ligated to a 1.1-kbp *Bss*HII fragment of pBE2 encoding the promotorless 5′ terminus of *secA*. At that step, one of the *Bss*HII sites of the insert was not ligated to the *Eco*RI site of the vector. The ends of the linear intermediate were filled in with the Klenow fragment of DNA PolI and ligated prior to transformation. From the resulting plasmid, a *Xho*I/*Xba*I/Klenow fragment containing the entire *secA* coding sequence was cloned into pACYC184 digested with *Sal*I/Klenow and *Eco*RV to generate p*secA*$^+$. SecA expression was confirmed by the fact that p*secA*$^+$, like pBE2, confers a dominant Ara^S phenotype to all the Ara^R *secA* strains. Plasmids containing the mutant *secA* alleles were constructed with the primers described in Table 2 as follows: p*secA303*, a PCR fragment (primers A3UP/A4DON) was digested with *Pvu*I/*Sph*I and cloned in p*secA*$^+$; p*secA307–310*, a PCR fragment (primers A5UP/A8DON) was digested with *Sph*I/*Bss*HII and cloned in p*secA*$^+$; p*secA311–312*, a PCR fragment (primers A7UP/A10DON) was digested with *Bss*HII/*Bgl*II and cloned in p*secA*$^+$. p*secA314*, a PCR fragment (primers A9UP/A12DON) was digested with *Bgl*II/*Kpn*I and cloned in p*secA*$^+$. p*secA315–319*, a PCR fragment (primers A11UP/A16DON) was digested with *Kpn*I/*Sna*BI and cloned in p*secA*$^+$. All p*secA* mutant plasmids confer an Ara^R phenotype to the KK1 strain.

Measurements of doubling times: Saturated cultures grown overnight from single colonies in Luria broth (LB) medium were diluted 1/50 in M63 medium containing 0.2% glucose and 40 μg/ml leucine and grown overnight. Cells were collected by centrifugation, washed once in M63 medium, and diluted 1/50 in M63 medium supplemented with 1% LB medium and either 0.2% glucose or 0.2% maltose; under these conditions, growth of *secA*$^+$ cells is sufficiently rapid to measure the effect of *secA* alleles exhibiting a mild-to-strong Sec phenotype. For the comparison of *secA*$^+$ and *secA (prlD)* strains, LB was not added to the minimal media. Growth was followed by measuring the absorbance at 600 nm with an open chamber spectrophotometer (S250, Secomam).

Isolation of suppressors of PAI2::AP toxicity: Cells expressing the PAI2::AP or the hB::AP chimeric proteins from the arabinose P_{BAD} promoter are unable to form colonies on plates containing arabinose. Spontaneous and UV-induced suppressors occur at a frequency of 10^{-7} and 10^{-6}, respectively (Bost and Belin 1995; Bost *et al.* 2000). To ensure that suppressor strains retain the capacity to export the chimeric protein, the alkaline phosphatase (AP) substrate XP was included in the plates, and only dark blue colonies were further characterized; white or pale blue colonies represent about one-half of the total suppressors. Of a total of 205 mutants (105 spontaneous), 59 carried plasmid-encoded mutations, and 67 had chromo-

TABLE 1

***E. coli* K12 strains**

Strain	Genotype	Reference
MC1000	F⁻ *araD139* Δ(*ara-leu*)$_{7696}$ Δ*lacX74 rpsL150 galU galK thi*	Casabadan and Cohen (1980)
DHB3	MC1000 *malF*Δ*3 phoA*Δ(*Pvu*II) *phoR*	Boyd *et al.* (1987)
MC4100	F⁻ *araD139 relA1 thi rpsL150 flbB5301* Δ(*argF-lac*) *U169 deoC7 ptsF25 rbsR*	Casabadan (1976)
MM1	MC4100 *malE10-1*	Bedouelle *et al.* (1980)
MM2	MC4100 *malE14-1*	Bedouelle *et al.* (1980)
MM4	MC4100 *malE18-1*	Bedouelle *et al.* (1980)
MM5	MC4100 *malE19-1*	Bedouelle *et al.* (1980)
LMG194	MC1000 Δ(*ara*)$_{714}$ *leu::*Tn*10 phoA*Δ(*Pvu*II)	Guzman *et al.* (1995)
Mph56	MC1000 *phoR phoA73*	Michaelis *et al.* (1986)
KK1	DHB3 pBADhBK	This study
KK3	MC4100 *malE14-1* Δ(*ara*)$_{714}$ pBADhBS	This study

somally encoded *pcnB* mutations or synthesized reduced levels of the chimeric protein. The remaining 79 were mapped by P1 transduction, and 35 suppressors mapped near or in *secA* (11 spontaneous and 24 UV induced); the localization of 8 *secY* and 7 *secG* suppressors was confirmed by sequencing (Bost and Belin 1995; Bost *et al.* 2000). The remaining mutations map to *rpoA* (5), *ydeA* (2), and other yet-uncharacterized loci.

Alkaline phosphatase assay: AP activity was measured by determining the rate of *p*-nitro-phenyl-phosphate (Sigma, St. Louis) hydrolysis and was normalized to the A_{600nm} of the cell suspension (Bost and Belin 1995).

Pulse-labeling and immunoprecipitation: Cell cultures were grown and pulse labeled with [^{35}S]methionine (IS-103, Hartmann, Braunschweig, Germany) at 37° as described previously (Bost and Belin 1995). For the analysis of MalE, cultures were induced with 0.2% maltose for 60 min before the labeling. Quantification was performed by scanning the gels with a Molecular Dynamics (Sunnyvale, CA) phosphorimager, using the ImageQuant version 3.22 software.

Single-strand conformation analysis and sequencing: Primers were designed to cover all of the *secA* coding sequence with eight overlapping PCR fragments of ~400 bp (Table 2). The SecA primers were labeled with [γ-^{32}P]ATP and T4-polynucleotide kinase for 30 min at 37°. Reactions were stopped by heating for 5 min at 95°. Labeled and unlabeled primers were mixed at a ratio of 1:5 and used to amplify the chromosomal *secA* sequences (Russo *et al.* 1993). PCR products were boiled for 20 min, chilled on ice, and loaded on mutation detection enhancement 10% glycerol gels (AT Biochem, Malvern, PA). Gels were run for 18 hr at 40 W at 4° and at room temperature for all tested PCR fragments, dried, and exposed to XAR films (Kodak). PCR-amplified products were sequenced with a PCR sequencing kit (Amersham, Buckinghamshire, UK).

TABLE 2

Sequence of the *secA* primers

Sequence 5′ → 3′	Base no.[a]	Name
TTCTTTCGCAATGGCACC	526-543	A1UP
TTTTCCATCTCCGGTTCC	931-914	A2DON
TTTTCGGTAGTCGTAACG	847-864	A3UP
GTAAGTGATGTCAGCTGC	1349-1331	A4DON
CGCTGTTTGAATTCCTTGG	1255-1273	A5UP
AGAGACTCCCCTTCATCC	1714-1697	A6DON
GGTGAACCTGACCGAACG	1634-1651	A7UP
ACAACGACGGTATCCAGC	2056-2039	A8DON
CTGATACCGAAGCTTTCG	2002-2019	A9UP
TTTCAATTTGCTCTGCGG	2427-2410	A10DON
TACAGATATTGTGCTCGG	2351-2368	A11UP
AGTTCGTTACGCTGGGA	2818-2802	A12DON
GGAATATGATGACGTGGC	2759-2776	A13UP
CCCTGACGCAGATAGTCC	3184-3167	A14DON
CGTCACTTCGAGAAAGGC	3099-3116	A15UP
CCTACCGCAATTTGCAGC	3612-3595	A16DON

[a] The numbering is based on the *secA* sequence (AN = M20791); the AUG start codon is at 822.

Western blot and quantification: Triplicate cultures were grown for 2 hr at 37°. Cells (1 ml, A_{600nm} = 0.3) were centrifuged for 2 min at 13,000 rpm, resuspended in 100 μl of SDS sample buffer, diluted 1/100, and boiled for 5 min. Lysates (20 μl) were loaded on 10% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred onto a nitrocellulose membrane (Protran BA85, Schleicher & Schuell, Keene, NH), with a semidry transfer apparatus (Biorad, Richmond, CA). SecA was detected with an enhanced chemiluminescence kit (Amersham) using a rabbit anti-SecA serum followed by a goat anti-rabbit IgG coupled to peroxidase. Membranes were exposed to XAR films. Quantification was performed by scanning non-saturated films with an Arcuss II scanner (Agfa), using the ImageQuant version 3.22 software. For calibration, each gel was loaded with triplicates of four different concentrations of the *secA*$^+$ sample.

RESULTS

Rationale of the genetic selection: The selection used to isolate mutants in the translocation machinery genes is described in materials and methods. Briefly, two different PAI2::AP fusions were cloned in derivatives of pBAD24, an inducible expression vector containing the promoter of the *araBAD* operon (Guzman *et al.* 1995). The eukaryotic signal sequence promotes the export of these chimeric proteins, albeit inefficiently. Upon arabinose induction, high-level expression of the PAI2::AP fusions is toxic probably because of a defective and prolonged interaction between the eukaryotic signal sequence and the translocase. Thus, wild-type cells expressing these

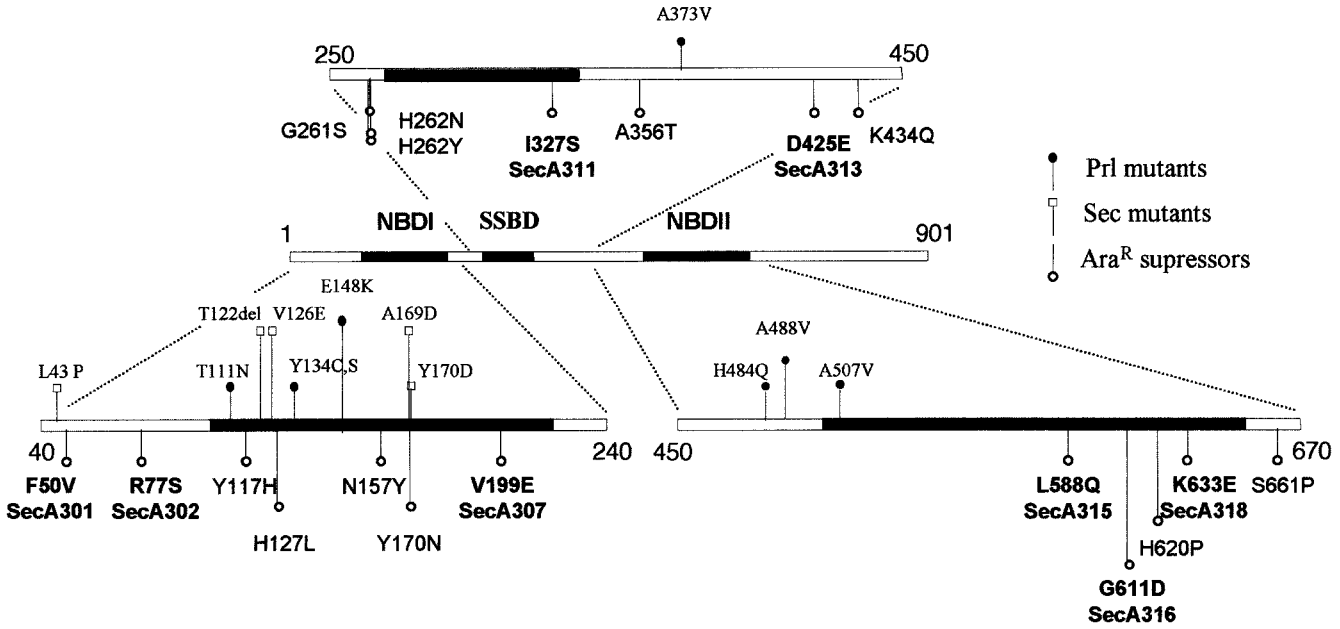

FIGURE 1.—Localization of the Ara^R SecA substitutions. The entire SecA protein is shown in the middle line, where three known interacting domains are indicated: NBDI, SSBD, and NBDII. These domains and flanking regions are enlarged in the top and bottom lines. Previously identified Sec (SCHMIDT *et al.* 1988) and Prl (FIKES and BASSFORD 1989; HUIE and SILHAVY 1995) substitutions in SecA are indicated above the line in the one-letter code with the position of the affected amino acids. Mutated residues identified in this work are shown below the lines; the eight alleles studied in detail are indicated by boldface type. *secA312* and *secA317* have been isolated twice. *SecA315* is a double mutant, and the S458F substitution is not indicated because the L588Q substitution expressed on a plasmid alone was sufficient to confer the Ara^R phenotype.

fusions are unable to form colonies on arabinose-containing plates (Ara^S phenotype). Spontaneous and UV-induced Ara^R suppressors have been isolated, and most mutations suppress both toxic fusions (BOST and BELIN 1995; BOST *et al.* 2000). Approximately one-half of the Ara^R suppressors formed blue colonies in the presence of the AP substrate XP and therefore retain the capacity to export PAI2::AP, at least to a certain extent. This second condition was imposed in the selection of Ara^R suppressors to avoid the isolation of *sec* mutants with a generalized secretion defect. Most Ara^R suppressors mapped in or near *secA*, while several others mapped to *secY* and *secG*. So far no mutations have been detected in *secD* or *secF*, suggesting that the chimeric proteins are toxic because they affect an early step of the translocation process. The *secA* alleles were recessive to *secA*$^+$ expressed from a multicopy plasmid (KIM *et al.* 1994; data not shown). The high prevalence of *secA* suppressors could be explained by a combination of the following facts: (i) SecA plays a key role in the export step blocked by the PAI2::AP fusion; (ii) SecA acts upstream of the affected step; (iii) *secA* is the largest *sec* gene; or (iv) one or more mutational hot spots are in the *secA* gene. We thus characterized these *secA* alleles.

DNA sequence analysis: Since *secA* is a large gene, we first mapped the mutations by single-strand conformation analysis, a highly sensitive technique that can detect most single-base substitutions (ORITA *et al.* 1989). For each mutant, eight overlapping PCR fragments were analyzed on nondenaturing gels (data not shown). Among the 26 mutants analyzed, we detected 18 single and one double mutation; only 2 of the single mutations occurred twice (Figure 1). Five mutations could not be mapped by this technique and were not further characterized. For the purpose of clarity, the sequenced alleles were numbered from *secA301* to *secA319* according to the position of the mutations along the gene (Figure 1). None of the sequenced mutations have been described previously and they all affect well-conserved amino acids in bacterial and chloroplastic *secA* homologs, as determined with the ProDom database (http://protein.inra.fr/prodom.html). To ensure that the detected mutations are responsible for the Ara^R phenotype, we constructed plasmids expressing several of these *secA* alleles. All tested plasmids (p*secA303*, *-307–312*, and *-314–319*) were dominant over chromosomal *secA*$^+$, conferring the expected Ara^R phenotype (data not shown).

Five mutations are located close to or within a region that encodes the domain of SecA defined by crosslinking as a putative signal-sequence-binding domain (SSBD; KIMURA *et al.* 1991). To our knowledge, only two chromosomal mutations, *secA (prlD5)* (A373V) and *secA283* (H309Y), have been previously described near this region (FIKES and BASSFORD 1989; MATSUMOTO *et al.* 2000). *secA* site-directed mutations encoding substitutions at Y326 provided evidence that this region is critical in controlling SecA-preprotein interaction (KOURTZ and OLIVER 2000). Five mutations are located in a re-

TABLE 3

Mal phenotypes of selected *secA* alleles

		secA$^+$	*secA1*	*secA2*	*secA7*	*secA11*	*secA13*	*secA15*	*secA16*	*secA18*
malE10-1	L10P	+++++	+++++	+++++	+++++	−	+	+	+	++
malE14-1	A14E	+++++	++++	+++	+++	−	+	+	++	+
malE18-1	M18R	+++++	++++	++++	++++	−	+	+	++	+++
malE19-1	M19R	+++++	+++	+++++	+++	−	+	±	±	++++

Each *secA* allele was transduced into the indicated *malE* strains; the substitutions in the MalE signal sequence are indicated by the one-letter code. The Mal phenotypes were tested at 37° on McConkey maltose (*malE10-1, -14-1,* and *-19-1*), tetrazolium maltose (*malE14-1* and *-19-1*), and minimal maltose (*malE18-1*) plates. The capacity to utilize maltose of the *malE secA*$^+$ strains shows the following order: *malE10-1* ≥ *malE14-1* > *malE19-1* > *malE18-1* (Bedouelle *et al.* 1980). For clarity, the Mal phenotype of *secA*$^+$ in each *malE* strain has been arbitrarily set to 5+. Decreasing maltose utilization as compared to the *secA*$^+$ isogenic strain is designated progressively as ++++, +++, ++, +, ± and −. In the *malE14-1* and *malE19-1* strains, all *secA* (*prlD*) alleles used in this study were scored as >5+.

gion encoding the nucleotide binding domain I (NBDI) domain, where most *sec* mutations map. Four single mutations and one double mutation are located in and around the region encoding the NBDII domain. The remaining mutations encode two substitutions in the N-terminal part of SecA and two between SSBD and NBDII.

The effect of *secA* suppressors on maltose binding protein (*malE*) signal sequence mutations defines three classes of mutants: Two types of mutations have been described in *sec* genes: Sec mutations, with a generalized defect that decreases protein export, and Prl mutations, which improve export mediated by defective signal sequences. Some Prl mutations have a selective effect depending on the signal sequence mutation, and mutations like *secE (prlG1)* and *secA (prlD21)* also reduce the kinetics of export of wild-type preproteins (Stader *et al.* 1989; Huie and Silhavy 1995). Since all of the *secG* suppressors isolated in the same selection have a weak Sec phenotype (Bost and Belin 1995; Bost *et al.* 2000), it was interesting to determine whether the *secA* suppressors are also Sec mutations. The phenotype of the *secA* alleles was first investigated in the *malE14-1* strain, which carries a signal sequence mutation in preMalE that interferes with its export (Bedouelle *et al.* 1980). The Mal phenotype of this strain can be scored on McConkey maltose indicator plates, where improved (Prl phenotype) or decreased (Sec phenotype) MalE export can be easily monitored. All *secA* suppressors exhibited a Sec phenotype and none showed a Prl phenotype (data not shown). In addition, the phenotype of the 26 *secA* alleles in this strain showed that we could qualitatively differentiate them according to the strength of their secretion defect.

To further characterize the *secA* suppressors, we decided to concentrate our efforts on a set of suppressors in which all mutated regions of *secA* are represented. *secA301* and *secA302* affect the N-terminal region, *secA307* affects the Walker B motif in NBDI, *secA311* affects the SSBD domain and showed the strongest Sec phenotype, *secA313* affects a residue located between SSBD and NBDII, and *secA315*, *-316*, and *-318* affect NBDII. We asked whether a similar Sec phenotype could be observed in other *malE* signal sequence mutant strains and whether the strength of this phenotype could help classify the *secA* alleles. Three other *malE* signal sequence mutations (*malE10-1*, *malE18-1*, and *malE19-1*) were used in addition to *malE14-1*; the mutations are described in Table 3. All of these signal sequence mutations exert a detectable effect on MalE export that ranges from a 70% (*malE10-1*) to a 96% (*malE18-1*) export defect, as defined by pulse-labeling experiments (Bedouelle *et al.* 1980 and our unpublished results). The Sec phenotypes of the selected *secA* alleles in the four different *malE* strains are shown in Table 3. The amino acid substitutions in SecA can be separated into three classes: (i) Those that confer a very weak Sec phenotype are located in the N-terminal region and in NBDI; (ii) those with an intermediate Sec phenotype localize in and around NBDII; and (iii) the strongest Sec phenotype observed among all tested *secA* alleles was that of *secA311*, the only suppressor isolated so far that alters the SSBD domain. It appears striking that the three classes of mutations localize to defined regions of SecA and are not randomly distributed along the protein.

The analysis shown in Table 3 is based on color or size of colonies and is therefore only qualitative. To provide a more quantitative assay, we measured the growth rates in maltose or glucose minimal medium of *malE14-1* strains carrying each of the *secA* alleles. Indeed, in strains carrying this *malE* signal sequence mutation, growth in maltose as the only carbon source is dependent on the amount of periplasmic MalE (Bedouelle *et al.* 1980). Thus, a Sec mutation should confer a slower growth rate and a Prl mutation should confer a faster growth rate. The different growth rates, expressed as the doubling times, are shown in Figure 2. The doubling times in glucose minimal medium were equivalent to those of *secA*$^+$ for all *secA* mutant strains, indicating that growth is not significantly affected by these *secA* mutations. In contrast, growth in maltose minimal medium showed

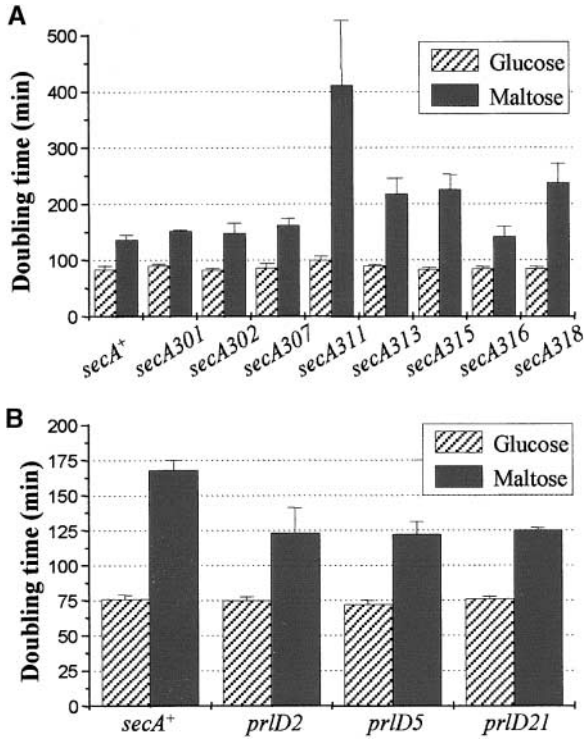

FIGURE 2.—Doubling time of strains carrying different *secA* alleles in minimal media with either maltose or glucose as the carbon source. Cells were grown at 37° in minimal media with either glucose or maltose as described in MATERIALS AND METHODS. Growth rates, which depend on sugar import, are expressed as doubling times. All alleles were transduced into the KK3 strain, which contains the *malE14-1* signal sequence mutation. Each bar graph represents the average result from at least three independent cultures; error bars indicate the SD. (A) Ara^R *secA* mutants are compared to the $secA^+$ isogenic strain. The significance of the results was evaluated with the Mann-Whitney nonparametric test. For growth in maltose minimal medium, the *P* value was 0.004 for *secA313*, *-315*, and *-318*. (B) *secA (prlD)* mutants were compared to the $secA^+$ isogenic strain; for growth in maltose minimal medium, the *P* value was 0.03 for the three *secA (prlD)* strains.

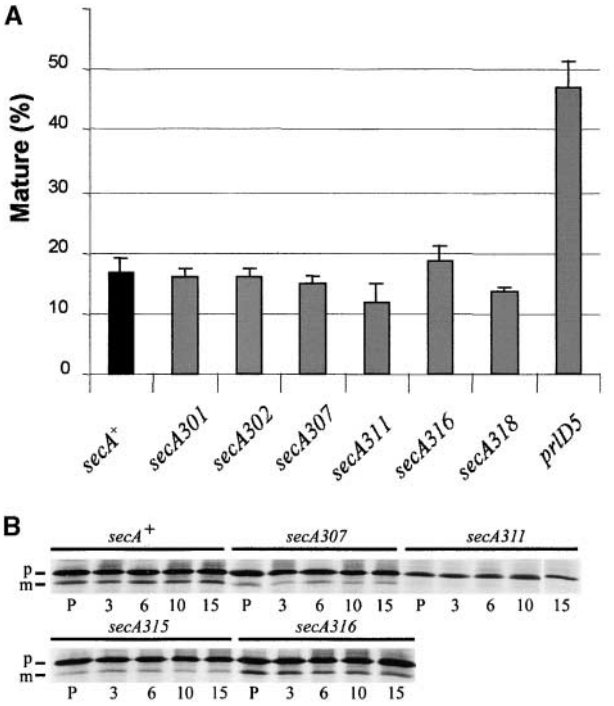

FIGURE 3.—Effect of the *secA* alleles on MalE export, determined by the efficiency of signal sequence cleavage. All *secA* alleles were transduced into the KK3 strain bearing the *malE14-1* mutation. (A) Cells were pulse labeled for 30 sec and chased for 12 min. Lysates were immunoprecipitated with anti-MalE antibodies. The amounts of precursor and mature MalE were measured, and the relative amount of mature protein is indicated. Each bar represents the average of three independent cultures; error bars indicate the SD. (B) Time course of preMalE export. Cells were pulse labeled for 30 sec and chased for the indicated times. p, preMalE; m, mature MalE.

the same trend as that observed on maltose indicator plates (Figure 2A). The *secA* alleles could be grouped in three categories: *secA311* had the strongest effect, with an almost threefold increase in doubling time (scored as "−" in Table 3); *secA313*, *-315*, and *-318* conferred an intermediate but significant increase in doubling time (scored as "+" in Table 3); and the four remaining alleles had only a marginal effect on doubling time (scored "++" to "++++" in Table 3). The McConkey maltose indicator plates therefore appear to be the most sensitive assay to detect subtle differences in the amount of periplasmic MalE.

We have also determined growth rates with three Prl mutations in *secA*: *secA (prlD2)*, *secA (prlD5)* (FIKES and BASSFORD 1989), and *secA (prlD21)* (HUIE and SILHAVY 1995). The first two *secA (prlD)* mutations were selected as suppressors of *malE14-1* on maltose indicator plates. As expected, growth rate in maltose minimal medium was faster with the *secA (prlD)* strains than with the $secA^+$ strain (Figure 2B).

The two assays described above, fermentation on maltose indicator plates and growth in maltose minimal medium, both reflect the steady-state level of periplasmic MalE. To directly assay preMalE export, we measured the efficiency of signal sequence cleavage in pulse-chase labeling experiments (Figure 3). These experiments were performed with strains carrying the *malE14-1* mutation, since the amount of mature MalE produced in a $secA^+$ strain is low but sufficient to accurately measure an export defect conferred by the *secA* alleles. In the first set of experiments, cells were labeled for 30 sec and chased for 12 min. A small but significant decrease in the relative amount of mature MalE was observed with *secA311* and *secA318*, which were scored as − and + in Table 3; similar results were observed with *secA313* and *-315*, which are similar to *secA318* (data not shown). No significant difference was observed with the other four strains (scored as ++ to ++++ in Table 3). In contrast, the amount of mature MalE was increased more than twofold by the *secA (prlD5)* mutation.

We also performed a series of pulse-chase experiments to determine whether export of the MalE14-1 protein occurs with similar kinetics in *secA*$^+$ and *secA* mutant strains (Figure 3B). In *secA*$^+$ cells, 5–6% of the protein synthesized during the 30-sec pulse was converted into mature MalE, and there was no significant increase during the chase period. The difference between this value and that shown in Figure 3A reflects the instability of cytoplasmic preMalE (Pogliano and Beckwith 1993). The strongest difference was observed with *secA311*, where only 1–2% of mature MalE was produced during the pulse, with no detectable increase during the 15-min chase. In addition, we consistently observed that the total amount of MalE synthesized in a strain carrying the *secA311* allele was reduced two- to fivefold. The most likely explanation of this observation is that the added effects of the *secA311* and *malE14-1* mutations result in too little MalE in the periplasm to fully induce the maltose regulon. This difference probably also explains the drastic effect of *secA311* on maltose fermentation (Table 3) and on growth in minimal maltose medium (Figure 2). With *secA307* and *-315*, there was an approximately twofold reduction in the amount of mature MalE. Surprisingly, the effect of *secA316* on preMalE signal sequence cleavage was smaller than that of *secA307* and *-315*, although its effect on indicator plates was more severe than that of *secA307*. It is possible that these *secA* alleles also affect other components of the maltose import system, such as integration of MalF and MalG into the membrane. If this is the case, the good correlation observed between phenotypes on maltose indicator plates and growth rates in minimal maltose medium may not completely extend to the export of the MalE14-1 protein.

In conclusion, these experiments showed that none of the *secA* suppressors confers a Prl phenotype and that *secA311*, the only mutation affecting the SSBD domain, showed the strongest effect with all tested *malE* signal sequence mutations.

The effect of *secA* suppressors on an AP signal sequence mutation: To provide an independent quantitative assay of protein export in strains carrying the Ara^R *secA* alleles, we used the Mph56 strain that carries the *phoA73* mutation altering the signal sequence of AP. The *phoA* gene is constitutively expressed in this strain and the L14Q substitution results in a 70% decrease in AP export (Michaelis *et al.* 1986). On plates containing the PhoA substrate XP, the secretion phenotype can be scored by the blue color intensity of the colonies. When compared to the *secA*$^+$ strain, all strains carrying the Ara^R *secA* alleles formed more pale colonies, indicative of a Sec phenotype. In contrast, a strain carrying the *secA (prlD5)* allele formed darker colonies, as expected (data not shown). To quantify the secretion defect conferred by the Ara^R *secA* alleles, we measured the steady-state level of AP activity in derivatives of the Mph56 strain (Figure 4A). The same general trend described in Table 3 was also observed with the *phoA73* signal sequence mutation. The *secA311* allele had a strong defect resulting in a 10- to 20-fold reduction in AP activity. The *secA313*, *-315*, and *-316* alleles had an intermediate defect, resulting in a 3- to 5-fold reduction in AP activity. The remaining mutants had a weaker defect, resulting in a 2-fold reduction in AP activity. It should be noted that *secA318*, which had an effect similar to that of *secA301*, *-302*, and *-307* with *phoA73*, exhibited the most variable effects with the different *malE* signal sequence mutations (Table 3). In contrast, the three *secA (prlD)* alleles tested improved export mediated by the *phoA73* signal sequence mutation (Figure 4D).

Differential effect on PAI2::AP and wild-type AP export: Most *secA* suppressors conferred only a weak secretion defect on the export of mutated MalE signal sequences (Table 3 and Figure 3). This is probably due to the fact that the Ara^R suppressors were screened for forming blue colonies on selective plates containing XP and thus are still able to export the PAI2::AP fusions. The mechanism of suppression by *secG* mutations was proposed to result from a selective reduction in the export kinetics of PAI2::AP, without affecting that of wild-type AP (Bost and Belin 1995; Bost *et al.* 2000). PAI2, like ovalbumin, lacks a leader peptidase cleavage site at the end of its signal sequence. Thus, we cannot analyze the export of the chimeric proteins by pulse-chase labeling and determination of cleavage efficiency.

We therefore determined the effect of the *secA* alleles on the export of wild-type AP (Figure 4B) and PAI2::AP (Figure 4E) by measuring the amount of AP activity after 10 min of induction with arabinose. For most *secA* suppressors, the accumulation of active PAI2::AP at this early time after induction was less than that measured in the *secA*$^+$ strain (Figure 4E). Interestingly, the PAI2::AP export defect was again stronger with *secA311* than with the other alleles. In contrast, accumulation of active AP was slightly higher [*secA (prlD5)*] or equal in strains carrying a *secA (prlD)* mutation when compared to the *secA*$^+$ isogenic strain (Figure 4F). The situation was somewhat different for wild-type AP export: Five of the eight *secA* alleles tested conferred only a slight reduction in export, and only *secA311* showed a twofold reduction in wild-type AP export (Figure 4B). A similar slight reduction in wild-type AP export was also detected with the *secA (prlD)* alleles (Figure 4C).

When PAI2::AP and wild-type AP export were compared, the *secA* alleles showed three different behaviors. Surprisingly, *secA301* and *secA307* had a slightly stronger effect on wild-type AP export than on PAI2::AP export (P value = 0.08). Mutants *secA302* and *secA311* have the same effect on both proteins. Finally, the four mutants that localize to NBDII had a weaker effect on wild-type AP (P value = 0.08) and thus appear selective for the chimeric protein. Thus, the *secA* suppressors of the toxic murine signal sequence appear to have a more complex

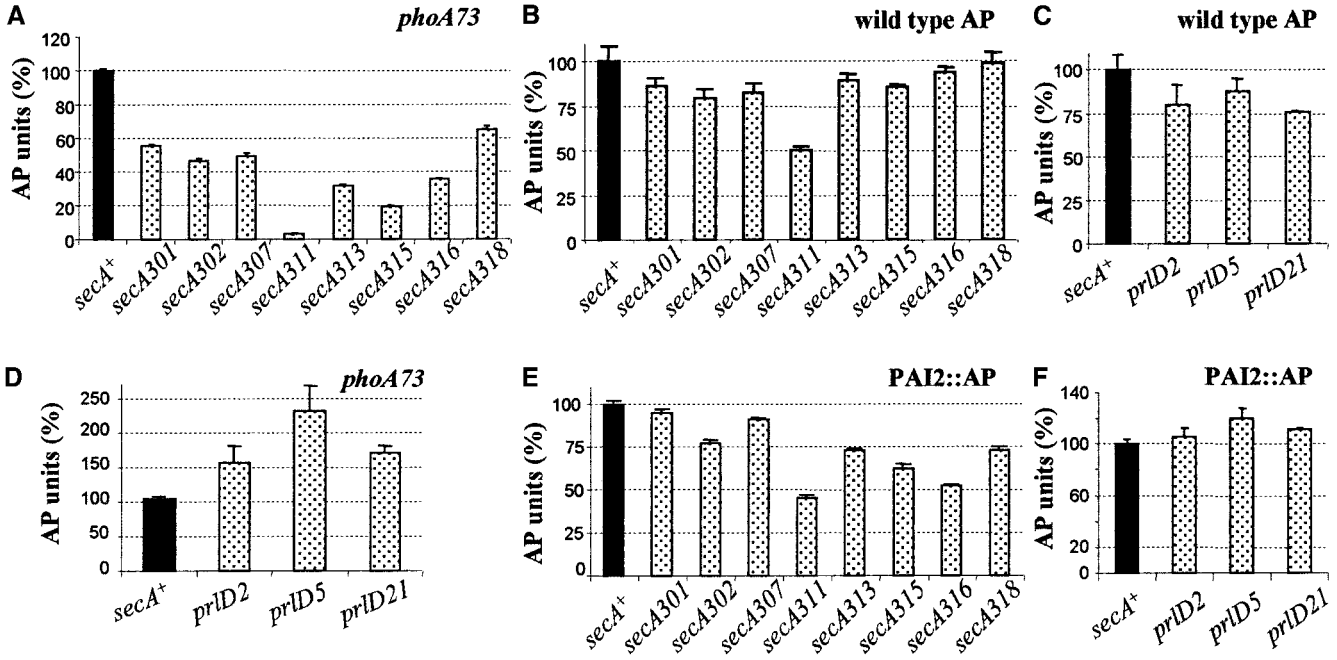

FIGURE 4.—Effect of *secA* alleles on the export of alkaline phosphatase derivatives. Cells were grown at 37° to an A_{600nm} of 0.2 in LB medium and induced with 0.2% arabinose for 10 min. Cells were assayed for AP activity. Each bar represents the average from three independent cultures; error bars indicate the SD. In A–F, the activity determined with the *secA*$^+$ strain was taken as 100% and used to normalize the values measured with the *secA* mutant strains. (A and D) Effect of *secA* alleles on export of the *phoA73* mutant; all *secA* alleles were transduced into the Mph56 strain; cultures were not induced with arabinose. (B and C) The strains are derivatives of the DHB3 (B) or MM2 (C) strains, carry the pDB3 plasmid, and express wild-type AP. (E and F) The strains are derivatives of the KK1 strain, carry the pBADhBK plasmid, and express a PAI2::AP fusion. The significance of the results was evaluated with the Mann-Whitney nonparametric test. (A) For PhoA73, *P* values were ≤0.08 for all *secA* alleles. (D) *P* values were ≤0.08 for all *secA (prlD)* alleles. (B) For wild-type AP, the *P* value was 0.08 for *secA302*, *secA311*, and *secA315*, when compared to *secA*$^+$. (C) The results were not significantly different, except for *secA (prlD21)* (*P* value was 0.08). (E) For PAI2::AP, *P* values were 0.08 for all mutant alleles, when compared to *secA*$^+$. (F) The *P* value was 0.08 for *secA (prlD5)* and *secA (prlD21)*. When PAI2::AP and wild-type AP were compared in B and E, the *P* value was 0.08 for all mutant alleles except *secA302* and *secA311*.

phenotype than that of the *secG* suppressors (BOST and BELIN 1995; BOST *et al.* 2000), and suppression is not necessarily achieved by a selective reduction in PAI2::AP export.

Export of wild-type proteins is not drastically affected by the *secA* suppressors: Since the Sec phenotype of the *secA* suppressors was defined in strains carrying *malE* and *phoA* signal sequence mutations (BEDOUELLE *et al.* 1980), the observed phenotypes could reflect interactions that are particularly defective with mutated signal sequences. Indeed, seven *secA* suppressors exerted only a minor effect on wild-type AP export (Figure 4B). It was therefore interesting to determine whether a secretion defect could be observed with other wild-type preproteins. Short pulse-labelings and immunoprecipitations were performed to quantify the export of RbsB, DegP, OmpA, and MalE; export efficiency was determined by measuring the extent of signal sequence cleavage calculated from the relative amounts of precursor and mature proteins (Figure 5). The strongest secretion defect was observed with *secA311*, although a more than twofold increase of the amount of uncleaved precursor was observed only with RbsB and DegP. A similar effect was also observed for RbsB with *secA302*, *secA315*, and *secA316* and for DegP and OmpA with *secA316*. Finally, practically no export defect was observed for the four remaining mutants. The effects observed with wild-type MalE are too small to produce an altered phenotype on maltose indicator plates.

Some Prl mutations decrease the kinetics of export of wild-type proteins. For instance, the *secA (prlD21)* allele confers a slight secretion defect on MalE and LamB (HUIE and SILHAVY 1995). A similar effect was observed with RbsB and DegP, but not with OmpA (Figure 5). The two other *secA (prlD)* alleles did not significantly affect the export of these three wild-type proteins.

SecA expression is translationally autoregulated (NAKATOGAWA and ITO 2001), and its expression can reach up to 20-fold its normal level in case of severe secretion defects caused (i) by the jamming of the Sec machinery with LacZ fusions or (ii) by Sec mutations in *secA*, *secD*, *secE*, or *secY* (RIGGS *et al.* 1988; ROLLO and OLIVER 1988). Thus, the SecA steady-state level provides an indirect way to assess the overall export capacity. SecA levels in exponentially growing cells were quantified by Western blot for each suppressor strain. The data in Figure 6A

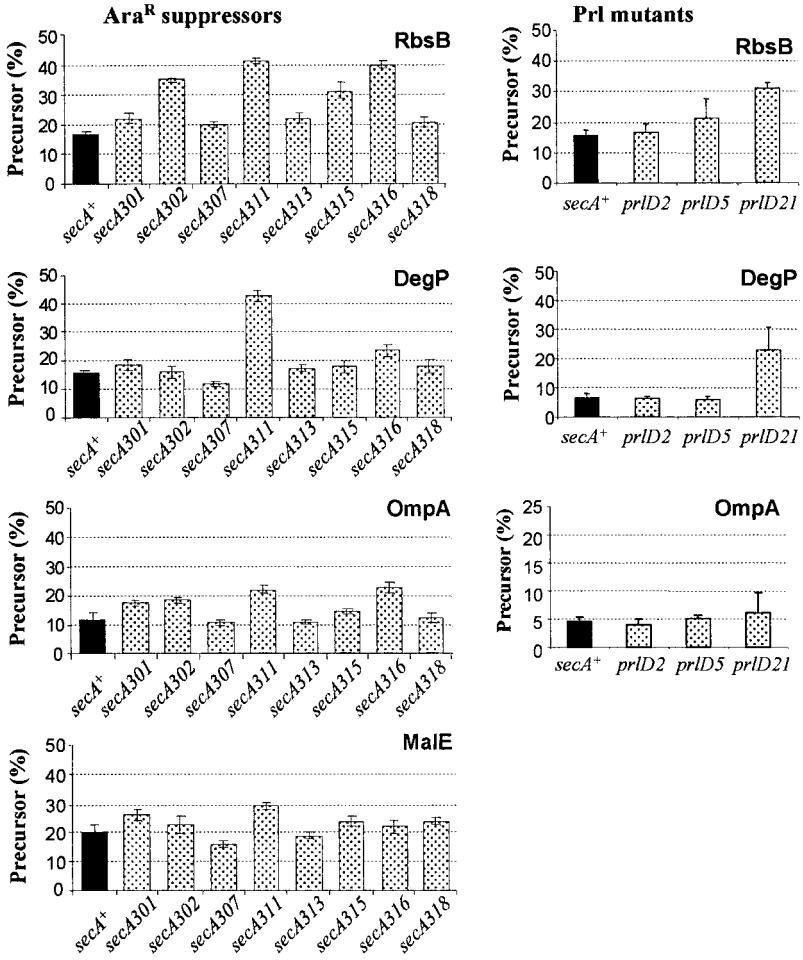

FIGURE 5.—Effect of the *secA* alleles on wild-type preprotein export, determined by the efficiency of signal sequence cleavage. All *secA* alleles were transduced into the KK1 strain. Cells were pulse labeled for 30 sec at 37°. Lysates were immunoprecipitated with anti-RbsB, anti-MalE-DegP, and anti-OmpA antibodies. For each protein analyzed, the precursor and the mature form were measured, and the relative amount of precursor is indicated. Each bar graph represents the average from three independent cultures; error bars indicate the SD.

show a less than twofold increase in SecA expression in seven of the strains, which therefore exhibit only a very weak overall secretion defect. It is interesting to note that *secA313* showed a nearly twofold increase in SecA level, although this allele had little effect on the export of five wild-type proteins (Figure 4B and Figure 5). In the *secA307* strain, we detected a 50% increase in SecA level, even though this allele slightly accelerated DegP and MalE export (Figure 5). With *secA311*, which had the strongest defect in all previous assays, the level of SecA expression was increased threefold. As previously reported (HUIE and SILHAVY 1995), *secA (prlD21)* showed a threefold increase in SecA level and the other *secA (prlD)* alleles had only a marginal effect (Figure 6B).

DISCUSSION

The *secA* suppressors affect three different domains of the protein: We describe here the characterization of a novel class of *secA* alleles. Since only 2 of 21 independent mutations affect the same site, this collection is not yet saturated for all possible suppressor sites. The 19 substitutions (Figure 1) span a large part of SecA, from the N-terminal region to NBDII, but none was found in the C-terminal 240 residues. A large collection of *secA* suppressors of the cold-sensitive *secY205* allele has been described (MATSUMOTO *et al.* 2000). Since the 51 mutations map to 40 different sites, this collection is saturated only slightly more than our collection, and the total number of sites that could be identified by the two selections is of the same order. Nevertheless, there is no overlap between these two sets of *secA* alleles, although the Y117H substitution reported here is adjacent to the A116V and L118Q ones. In contrast, two of the *secY205* suppressors are identical to *secA (prlD3)* and *secA (prlD5)*, and four are different substitutions at residues affected by *secA (prlD)* mutations.

Using *malE* and *phoA* signal sequence mutations, we observed that all of our *secA* alleles enhance their export defect and therefore confer a weak Sec phenotype. The relative strengths of their phenotypes appear to define three classes of alleles: the N-terminal and NBDI mutants (class I), the SSBD mutant (class II), and the last four mutants (class III). This distribution is purely operational at this stage, since *secA318* behaves as a class III allele in three *malE* strains, but as a class I allele in strains carrying the *malE18* or *phoA73* mutations. With the help of J. Hunt (Columbia University, New York), we

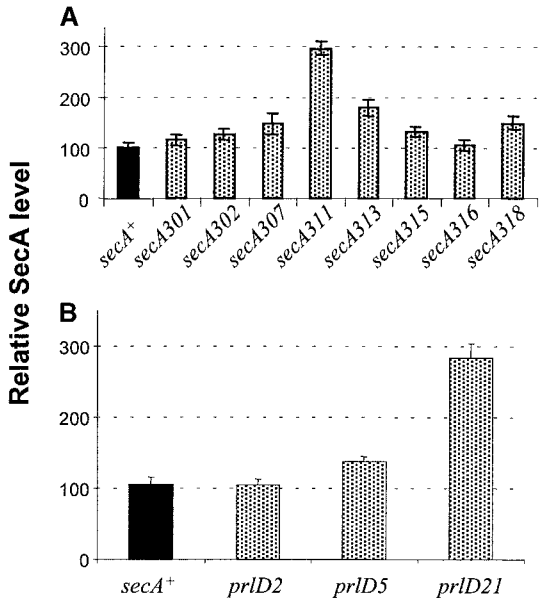

FIGURE 6.—Level of SecA expression in the different suppressor strains. (A) Ara^R suppressors. (B) Prl mutants. All *secA* alleles were transduced into the KK1 strain. Exponentially growing cells were lysed and extracts were subjected to 10% SDS-PAGE. Samples were diluted according to the A_{600nm} of the cultures and each lane contained the same amount of cell equivalents (0.3 mA_{600nm}). SecA was detected by chemiluminescence and quantification was performed by scanning nonsaturated films with an Arcuss II scanner (Agfa) and by using the ImageQuant version 3.22 software. Each bar represents the mean of three independent cultures; error bars indicate the SD.

could calculate the spatial distribution of these mutant sites in the *B. subtilis* SecA crystal structure. Our initial conclusion was confirmed by the spatial distribution of the sites. Indeed, all 19 substituted residues identified in Figure 1 are clustered into three folded domains of SecA: *secA301-307* and *secA319* in the first domain, *secA308–312* in the second domain, and *secA313–318* in the third domain (J. F. HUNT, S. WEINKAUF, D. B. OLIVER and J. DEISENHOFER, personal communication).

With mutated signal sequences, the *secA* Ara^R suppressors have a phenotype opposite to that of *secA (prlD)* mutants: Two types of mutations have been isolated in most genes encoding components of the protein translocation machinery: Sec mutations and Prl mutations. Suppressors of the toxic PAI2 signal sequence represent a third class of *secA* mutations. Since Ara^R and Prl mutations suppress inefficient signal sequences, they were systematically compared.

In contrast with the small increase observed with *secA (prlD5)*, the Ara^R alleles do not improve the inefficient export mediated by this mammalian signal sequence. The three *secA (prlD)* alleles tested confer the same Ara^S phenotype as the *secA*$^+$ strain. Moreover, the irreversibility of this lethal phenotype, measured after induction of the toxic chimeric protein, was more pronounced with the *secA (prlD)* alleles when compared to a *secA*$^+$ strain (data not shown). Most importantly, the *secA* Ara^R suppressors decrease export mediated by mutated *malE* and *phoA* signal sequences, which are suppressed to various extents by *secA (prlD)* alleles. Although the strength of the enhanced Sec phenotype conferred by our *secA* alleles depends on the signal sequence tested, export was more drastically affected with mutated signal sequences than with their wild-type counterparts. Thus, these *secA* alleles appear to have an increased specificity that preferentially excludes weak signal sequences, while the *secA (prlD)* alleles improve their export activity.

With wild-type preproteins, the situation is more complex. A weak Sec phenotype can be observed with conditional-lethal *sec* mutants grown at the permissive temperature (RIGGS *et al.* 1988), with some *sec (prl)* alleles, including *secA (prlD21)* (SAKO and IINO 1988; STADER *et al.* 1989; HUIE and SILHAVY 1995), and with several of our suppressors. While this is probably often the result of a reduced activity of the mutated protein, an enhanced discrimination of signal sequences would have the same effect. If the signal sequence discrimination function is altered, we may also identify wild-type signal sequences that are more rapidly exported in the *secA* suppressor strains. This is indeed what was observed with *secA307* and wild-type DegP and MalE.

Taken together, our results suggest that the *secA* Ara^R suppressors alter the same function as the *secA (prlD)* mutations, *i.e.*, the interaction of SecA with signal sequences. With mutated or inefficient signal sequences, these alleles display an opposite phenotype. The variability observed with different signal sequences appears to be an inherent property of the export process, considering that the translocase must process a highly degenerate set of signal sequences and that SecA is a multifunctional protein that participates in all known steps of export. At this stage, it is difficult to make predictions concerning the type of interaction(s) between the signal sequence and different domains of SecA.

Mechanism of suppression of the PAI2 signal sequence: We observed three different behaviors for the export of PAI2::AP and wild-type AP in the *secA* strains, suggesting that there are at least three different ways to suppress the toxicity of PAI2::AP. First, mutations affecting the putative SSBD motif are predicted to generally decrease the affinity of SecA for signal sequences. Although we did not directly measure the affinity of SecA311 (I327S) for signal sequences, the phenotypes of the *secA311* mutation are fully compatible with this interpretation. Furthermore, substitutions at Y326, which affects the adjacent residue, strongly affect the SecA-preprotein interaction (KOURTZ and OLIVER 2000). By reducing protein export mediated by nearly all the signal sequences tested, including the chimeric protein, this type of mutation probably prevents translocation jamming by PAI2::AP. Second, mutations like *secA313*, *sec3A15*, *secA316*, and *secA318* show a behavior similar

to that of *secG* suppressors, since they selectively slow down PAI2::AP export when compared to that of wild-type AP. Third, mutations affecting the N-terminal region are somewhat surprising, since they show a stronger defect in the export of wild-type AP when compared to that of PAI2::AP and have only a very weak Sec phenotype. These mutations could make the translocase less susceptible to blockage by PAI2::AP, perhaps by altering the dynamic interaction(s) of SecYEG with SecA (Economou and Wickner 1994; Nishiyama *et al.* 1996).

Are the nucleotide binding domains of SecA involved in signal sequence recognition? It has been proposed that the main function affected in Prl mutants is signal sequence recognition, suggesting that this recognition provides a proofreading activity to the translocase (Osborne and Silhavy 1993). In the case of the SecYEG complex, the Prl phenotype is associated with structural changes affecting the dynamic interaction of the translocase subunits. Indeed, Prl mutations in *secY* and *secE* (i) cause a "general relaxation" of the translocase and (ii) stabilize SecA at the SecYEG complex (Nouwen *et al.* 1996; van der Wolk *et al.* 1998; Duong and Wickner 1999). The abolition of *secA (prlD)* suppression in a *secG* null strain extends the notion of a dynamic interaction to SecA (Ramamurthy *et al.* 1998). The distribution of the *secA* Ara^R and *secA (prlD)* mutations shows that the SSBD and both NBDI and NBDII motifs contribute to the recognition of signal sequences. The fact that some mutations affect SSBD was expected, since this domain crosslinks to the OmpA signal sequence (Kimura *et al.* 1991). However, most of our mutations affect either NBDI or NBDII. This may be surprising since these regions are thought to be involved in the binding and hydrolysis of ATP and therefore in the catalytic activity of SecA during translocation. Our results suggest that the nucleotide binding domains of SecA also function during signal sequence recognition and thus contribute to the postulated proofreading activity of the translocase. A similar conclusion was reached by Huie and Silhavy (1995) on the basis of the phenotypes of *secA (prlD)* alleles.

The concept of proofreading requires a "stop and go" energy-dependent process, as is the case for protein synthesis (Thompson *et al.* 1986). After ATP binding and insertion of the preprotein in the membrane, a SecA-SecYEG bound state, the stop and go process could depend on the ATPase activity of SecA. The interaction of SecYEG with the preprotein and with SecA would set the timer for ATP hydrolysis and release of SecA. A longer interaction would be expected to favor the release of a low-affinity signal sequence from the translocase. In contrast, a shorter interaction would be less discriminative. Signal peptides modulate the ATPase activity of SecA in a complex manner (Wang *et al.* 2000; Triplett *et al.* 2001). The observation that several SecA (PrlD) proteins have an increased membrane-associated ATPase activity strongly supports this model (Schmidt *et al.* 2000). A faster recognition step could also lead to overall faster translocation. Indeed, the *secY (prlA4)* mutation influences the rate of translocation (Nouwen *et al.* 1996; van der Wolk *et al.* 1998) probably by accelerating SecA deinsertion (Nishiyama *et al.* 1999). The *secA* mutations described here, together with *secA (prlD)* mutations, provide useful tools for dissecting the molecular mechanism of signal sequence recognition during protein export.

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Note added in proof: The structure of SecA was recently published (J. F. Hunt, S. Weinkauf, L. Henry, J. J. Fak, P. McNicholas *et al.*, 2002, Nucleotide control of interdomain interactions in the conformational reaction cycle of SecA. Science **297:** 2018–2026).

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