JOURNAL OF BACTERIOLOGY, June 1997, p. 4003–4012 Vol. 179, No. 12
0021-9193/97/$04.00+0

Comparative Characterization of SecA from the α-Subclass Purple Bacterium *Rhodobacter capsulatus* and *Escherichia coli* Reveals Differences in Membrane and Precursor Specificity

REGINA HELDE,[1] BEATE WIESELER,[1] ELMAR WACHTER,[2] ANNETTE NEUBÜSER,[3] HEDDA K. HOFFSCHULTE,[1] THOMAS HENGELAGE,[1] KARL-LUDWIG SCHIMZ,[4] ROSEMARY A. STUART,[2] AND MATTHIAS MÜLLER[1]*

Adolf Butenandt Institut für Physikalische Biochemie[1] und Physiologische Chemie,[2] Ludwig-Maximilians-Universität München, D-80336 Munich, Institut für Organische Chemie und Biochemie, Universität Freiburg, D-79104 Freiburg,[3] and Institut für Biotechnologie, Forschungszentrum Jülich, D-52425 Jülich,[4] Germany

Received 10 February 1997/Accepted 14 April 1997

We have cloned the *secA* gene of the α-subclass purple bacterium *Rhodobacter capsulatus*, a close relative to the mitochondrial ancestor, and purified the protein after expression in *Escherichia coli*. *R. capsulatus* SecA contains 904 amino acids with 53% identity to *E. coli* and 54% identity to *Caulobacter crescentus* SecA. In contrast to the nearly equal partitioning of *E. coli* SecA between the cytosol and plasma membrane, *R. capsulatus* SecA is recovered predominantly from the membrane fraction. A SecA-deficient, cell-free synthesis-translocation system prepared from *R. capsulatus* is used to demonstrate translocation activity of the purified *R. capsulatus* SecA. This translocation activity is then compared to that of the *E. coli* counterpart by using various precursor proteins and inside-out membrane vesicles prepared from both bacteria. We find a preference of the *R. capsulatus* SecA for the homologous membrane vesicles whereas *E. coli* SecA is active with either type of membrane. Furthermore, the two SecA proteins clearly select between distinct precursor proteins. In addition, we show here for the first time that a bacterial *c*-type cytochrome utilizes the canonical, Sec-dependent export pathway.

The export of periplasmic and outer membrane proteins across the plasma membrane of the gram-negative bacterium *Escherichia coli* involves a distinct set of Sec proteins. According to the current model (19, 65), precursor proteins, which are synthesized on free ribosomes, interact with cytosolic chaperones such as SecB. SecB mediates targeting of the precursor (17, 22, 53) to membrane-attached SecA (24). The so-called preprotein translocase, consisting of the peripheral part, SecA, and the integral membrane proteins SecY/E/G and SecD/F, mediates the ensuing translocation across the bacterial plasma membrane. SecA is an ATPase which couples the hydrolysis of ATP to protein translocation and thereby plays a pivotal role in Sec protein-dependent protein transport.

SecA has so far been detected only in eubacteria and in bacterial descendants like chloroplasts. In bacteria, a defect in SecA is lethal, and studies with conditional *secA* mutants revealed a SecA requirement for the export of all periplasmic and outer membrane proteins of *E. coli* tested thus far (42). The SecA protein, which is believed to function as a homodimer (4, 18), possesses two essential nucleotide binding domains (termed NBD1 and -2), each one consisting of classical Walker A and B boxes. NBD1 is a high-affinity ATP binding site located in the NH_2-terminal domain while the low-affinity NBD2 has been localized to the beginning of the COOH-terminal half of the SecA protein (38). Cross-linking experiments have revealed the occurrence of a precursor binding site downstream of NBD1 (29). A putative SecB binding site has also recently been mapped to the COOH-terminal 70 amino acids of SecA (10).

Wild-type SecA partitions roughly equally between cytosol-ribosomes and the plasma membrane of *E. coli* (11, 34), with about one-third of the membrane-associated SecA being found integrated into the lipid bilayer. A complete removal of SecA from membranes therefore requires the use of detergents (63). Furthermore, SecA was shown to penetrate into model membranes (9, 60), and anionic phospholipids were found to influence its ATPase and translocation activity (25, 33, 35). In addition, a 30-kDa, protease-resistant fragment of SecA can be recovered from plasma membrane vesicles under conditions of ongoing protein translocation and overproduction of SecD/F (20, 21). Insertion of SecA into the plasma membrane is a cyclic event. ATP binding to the NH_2-terminal, high-affinity nucleotide binding domain leads to a conformational change (51) with the subsequent insertion of parts of the molecule into the membrane (44). SecA thereby becomes accessible from the periplasmic side of the plasma membrane (1, 28). ATP hydrolysis at NBD1 leads to a release of SecA from the membrane (9, 21, 62). Insertion-deinsertion is accompanied by a stepwise (48, 59) translocation of precursor proteins (21), a process which somehow involves ATP hydrolysis at NBD2.

Comparatively little is known about the molecular details by which SecA recognizes precursor proteins. This process involves the signal sequence (14) and depends on its extreme NH_2-terminal, positively charged amino acids (3). The latter results were obtained with a single preprotein, and their general applicability therefore remains to be demonstrated. Different from the model outlined above (65), which ascribes the major function of SecA to its membrane-attached form, increasing evidence is accumulating that an initial interaction of SecA with precursor proteins can occur in the cytosol preceding the actual targeting step (12, 26, 32). A mediating role of

* Corresponding author. Mailing address: Institut für Physikalische Biochemie, Universität München, Schillerstrasse 44, D-80336 Munich, Germany. Phone: 49-89-5996-442. Fax: 49-89-5996-483. E-mail: muller@pbm.med.uni-muenchen.de.

SecB in the binding of SecA to its precursor substrate is suggested by previous findings (24, 26) but has not yet rigorously been proven.

We have started to analyze protein export in *Rhodobacter capsulatus* (57, 58, 66, 67), which belongs to the group of α-subclass purple bacteria. Based on a 16S rRNA sequence analysis (68), these bacteria are closely related to the ancestral prokaryote that, according to the endosymbiont theory, gave rise to mitochondria. We have now cloned, isolated, and characterized SecA of *R. capsulatus*. The existence of a homologous in vitro system (58, 66) to study the translocation activity of SecA enabled us to directly compare SecA of *R. capsulatus* with that of *E. coli*. It thereby became evident that the activity of a distinct SecA species depends not only on the target membranes but also exquisitely on the precursor to be translocated.

MATERIALS AND METHODS

Molecular cloning techniques. Chromosomal DNA was prepared from *R. capsulatus* according to the method in reference 23. For amplification of *secA* by PCR, about 200 ng of chromosomal DNA was reacted with 0.16 mM deoxynucleoside triphosphates, 1 U of *Taq* DNA polymerase (Promega), and 1 μM (each) the following primers: 5′-*GCATGCTCTAGA*CCA$^{C}_{T}$GA$^{A}_{G}$GCI$^{A}_{G}$TIGA$^{A}_{G}$GCIAA$^{A}_{G}$GA-3′ and 5′-*CGAGCTCCTGCAG*ATITCIGTICCIC$^{G}_{T}$ICCIGCCAT-3′, which both possessed 5′ extensions (italic letters) containing suitable cleavage sites (underlined sections). Standard procedures (46) were employed for mini-scale preparations of plasmid DNA, digestion of DNA with restriction enzymes, agarose gel electrophoresis of DNA, ligation of DNA fragments obtained by electroelution from agarose gels into vector DNA, transformation of competent *E. coli* cells, screening of recombinant hosts by colony hybridization, and Southern blotting. Large-scale preparations of plasmid DNA were obtained with Qiagen kits (Qiagen, Hilden, Germany). Labeled *secA* probes were prepared by PCR by use of a digoxigenin labeling mixture (Boehringer, Mannheim, Germany).

Isolation and sequencing of the *R. capsulatus secA* gene. A mini-gene bank was prepared by resolving restricted chromosomal DNA of *R. capsulatus* by agarose gel electrophoresis, electroelution of fragments that hybridized to a labeled *secA* probe, and ligation into vector pGEM-3Z (Promega). Both strands of the *secA* gene were sequenced by the dideoxy chain termination method (47) with the deaza-dGTP version of the Sequenase kit (Amersham) with appropriate subclones of the *Pst*I and the *Eco*RI fragments in pGEM-3Z (see Results). The oligodeoxynucleotide primers used were either complementary to the SP6 and T7 promoter sites of the vector or derived from internal *secA* sequence sections.

Subcloning of *R. capsulatus secA*. To obtain a full-length clone of *R. capsulatus secA*, an *Eco*RI subclone containing the 5′ end of the gene was cut first with *Sal*I and subsequently with *Hin*cII. A *Pst*I subclone bearing the major 3′ part of *secA* was cleaved with *Pst*I and *Sal*I. Both fragments were simultaneously ligated into pGEM-3Z linearized with *Pst*I and *Sma*I, yielding plasmid pRHSecA1. In pRHSecA1, the *secA* gene is flanked by 144 nucleotides on the 5′ end and approximately 400 nucleotides on the 3′ side. To obtain plasmid pRHSecA3, pRHSecA1 was cleaved with *Sac*I and *Nae*I, leaving 144 nucleotides upstream and 31 nucleotides less than in pRHSecA1 downstream of *secA*. The *Sac*I-*Nae*I fragment was ligated into pTrc99A (7) after opening it with *Sac*I and *Sma*I. Plasmid pRHSecA2 was constructed in a similar manner as pRHSecA1 except that the *Eco*RI fragment was first cut with *Sac*II, blunt ended with T4 DNA polymerase, and then cleaved with *Sal*I. The *Pst*I fragment was treated as described for the construction of pRHSecA1. Both fragments were then cloned into pGEM-3Z cut with *Pst*I and *Sma*I. In contrast to pRHSecA1, plasmid pRHSecA2 contains only 9 nucleotides 5′ of *secA*. For the construction of pRHSecA4, the *secA*-containing insert of pRHSecA2 was excised with *Kpn*I and *Nae*I and the *Kpn*I end was filled in with T4 DNA polymerase and ligated into pKK223-3 (6) cleaved with *Sma*I. Positive clones were examined by restriction analysis. In addition, the occurrence of the *Sal*I site of the composite *secA* gene in plasmids pRHSecA1 and pRHSecA2 was confirmed by sequencing.

Purification of Sec proteins. SecA was purified from *E. coli* XL 1-Blue transformed with plasmid pMKL180, which is a pUC19 derivative containing *E. coli secA* under the control of the *lac* promoter (kindly provided by M. Klose and R. Freudl). Cells were grown at 37°C in Luria-Bertani (LB) medium containing 0.5% (wt/vol) glucose, 100 μg of ampicillin ml^{-1}, and 20 μg of tetracycline ml^{-1}. The last was important to maintain the *lacI*^{q}-bearing F′ episome, thereby repressing overproduction of SecA in the host. A batch culture of 6 liters was inoculated at a 1:50 ratio with a starter culture grown overnight. When cultures had reached an A_{578} of 0.5, cells were harvested, washed twice with glucose-free LB medium, and resuspended to the original concentration in fresh medium containing 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After another 3 h of growth, the bacteria were harvested, washed with buffer A1 (50 mM Tris-HCl [pH 7.5], 1 mM EDTA) containing 0.5 mM phenylmethylsulfonyl fluoride, and frozen in liquid N_2. For breakage, the thawed bacteria were resuspended in the same buffer at a 1:1 (wt/vol) ratio, passed four times through a French pressure cell at 8,000 lb/in^2, and centrifuged at 30,000 × g_{av} for 30 min at 4°C. The supernatant was subsequently freed from membranes by ultracentrifugation for 2.5 h at 4°C at 180,000 × g_{av} and stored frozen. It was applied at 2 ml h^{-1} to a HiLoad 26/10 Q-Sepharose column (Pharmacia) previously equilibrated with buffer A1. The column was washed with 80 ml of buffer A1, and the following, discontinuous KCl gradient was applied by use of buffer B1 (buffer A1 containing 0.7 M KCl): most of the bound proteins were eluted by 300 ml of a linear gradient ranging from 0 to 34% buffer B1. After a 30-ml step of 34% buffer B1, remaining proteins were eluted within a 360-ml gradient ranging from 34 to 55% buffer B1. SecA, determined by immunoblotting with mouse anti-SecA antibodies (53), started to elute at approximately 38% buffer B1. The majority of it was collected in three fractions of 12 ml each. These fractions were lyophilized and redissolved in buffer A2 [buffer A1 containing 50 mM KCl, 15% (wt/vol) $(NH_4)_2SO_4$, 0.5 mg of leupeptin (Boehringer) ml^{-1}, and 2 mg of aprotinin ml^{-1} (Boehringer)]. An equivalent of one-third of each fraction was chromatographed on a phenyl-Superose HR 5/5 column (Pharmacia) preequilibrated with buffer A2. The column was washed with 15 ml of buffer A2 and developed with a discontinuous gradient prepared from buffers A2 and B2 [buffer A2 without $(NH_4)_2SO_4$], which consisted of 20 ml of 0 to 60% buffer B2, 8 ml of 60% buffer B2, and 16 ml of 60 to 100% buffer B2. SecA was eluted between 60 and 75% buffer B2. Fractions containing the highest amount of SecA were essentially pure except for one contaminating 42-kDa protein which by immunodetection turned out to be elongation factor EF-Tu. Appropriate fractions were pooled, concentrated about 10-fold with Centricon 10 microconcentrators (Amicon), and buffer exchanged against buffer C [50 mM triethanolamine (TEA)-CH_3COO, 50 mM KCH_3COO, 5 mM $Mg(CH_3COO)_2$]. The purification of SecB from *E. coli* has been described elsewhere (26).

The purification of SecA from *R. capsulatus* followed the protocol described for the *E. coli* protein with the following modifications. *E. coli* BL21 transformed with pRHSecA4 was grown in LB medium containing 0.5% glucose and 150 μg of ampicillin ml^{-1}. A 4-liter batch culture lacking glucose was inoculated with the harvested cells of a 200-ml starter culture grown overnight. IPTG was added to 1 mM, and cells were grown for 6 h, at which time they had reached an A_{578} of 1.5. Wash buffer was 50 mM TEA-CH_3COO; cells were broken in 50 mM TEA-CH_3COO–60 mM KCH_3COO–0.5 mM EDTA (pH 8)–1 mM dithiothreitol (DTT) containing 0.5 mg of Pefabloc ml^{-1} (Boehringer). One-third of the supernatant obtained by ultracentrifugation was applied at 1 ml min^{-1} to the Q-Sepharose column equilibrated with buffer A3 (50 mM TEA-CH_3COO, 60 mM KCl, 0.5 mM EDTA [pH 8], 1 mM DTT). The column was washed with 30 ml of buffer A3 and then eluted at 2 ml min^{-1} with a discontinuous KCl gradient consisting of 600 ml of 0 to 35% buffer B3 (buffer A3 containing 1 M KCl) followed by 50 ml of 35 to 100% buffer B3 and 50 ml of 100% buffer B3. *R. capsulatus* SecA eluted within four fractions of 10 ml each at about 28% buffer B3. These fractions were pooled, concentrated to 300 μl by ultrafiltration with Centricon 10 microconcentrators, applied to a HiLoad 26/60 Superdex 200 (Pharmacia) column, and eluted at 0.25 ml min^{-1} with 50 mM TEA-CH_3COO–60 mM KCH_3COO–0.5 mM EDTA (pH 8)–1 mM DTT. For in vitro assays, 2-ml fractions containing *R. capsulatus* SecA were concentrated 10-fold.

Preparation of cell extracts and membrane vesicles for in vitro protein synthesis. High-speed supernatants (S-135s) of cell homogenates were prepared by published procedures (40, 53) from the *E. coli secA*(Ts) strain MM66 (43) and *R. capsulatus* 37b4 (66). The preparations of gradient-purified inside-out plasma membrane vesicles (INV) from *E. coli* MRE600 (39) and of intracytoplasmic membranes (ICM) from *R. capsulatus* 37b4 (66) have been described elsewhere. Urea extraction of INV was carried out by adding 4 volumes of 7.5 M urea dissolved in 50 mM TEA-CH_3COO (pH 7.5) containing 250 mM sucrose to an aliquot of INV and incubating for 60 min on ice. The membranes were then collected by centrifugation through a 750 mM sucrose cushion prepared in 50 mM TEA-CH_3COO (pH 7.5) in the Beckman Airfuge. Low-salt extraction of ICM has been described elsewhere (66).

In vitro synthesis and translocation into membrane vesicles. Precursor proteins were expressed in vitro with pGAH317 encoding Skp (55); plasmid pDMB encoding OmpA under the control of the T7 RNA polymerase promoter (7a); plasmid pC2P2.71 encoding *cycA* of *Rhodobacter sphaeroides*, i.e., cytochrome c_2 (67); and plasmid pBSRB-1 encoding porin of *Rhodopseudomonas blastica* (31). In vitro transcription-translation in *E. coli* S-135s was performed as described elsewhere (40, 53) (both variations were tested with each S-135 and plasmid for optimal synthesis activity). Cell-free protein synthesis with an S-135 prepared from *R. capsulatus* was carried out for 45 min at 32°C according to the method in reference 39 with the omission of NH_4CH_3COO and EDTA. Expression of *lac*-promoter-dependent genes (*cycA* and porin) was accomplished by the addition of 0.5 mM cyclic AMP and 0.4 mM IPTG. Membrane vesicles and Sec proteins were either present during synthesis or added after 30 min of incubation. In the latter case, ATP, DTT, creatine phosphate, and creatine phosphokinase were readded with the membranes. Translation products were either directly precipitated with 1 volume of 10% trichloroacetic acid (TCA) or first incubated with 1 volume of 1-mg/ml proteinase K for 20 to 30 min at 25°C.

Miscellaneous methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography were performed as described elsewhere (40). Separation of precursor and mature forms of cytochrome c_2 required SDS-urea gels (2). Immunoblotting has been detailed elsewhere (67). Antibodies

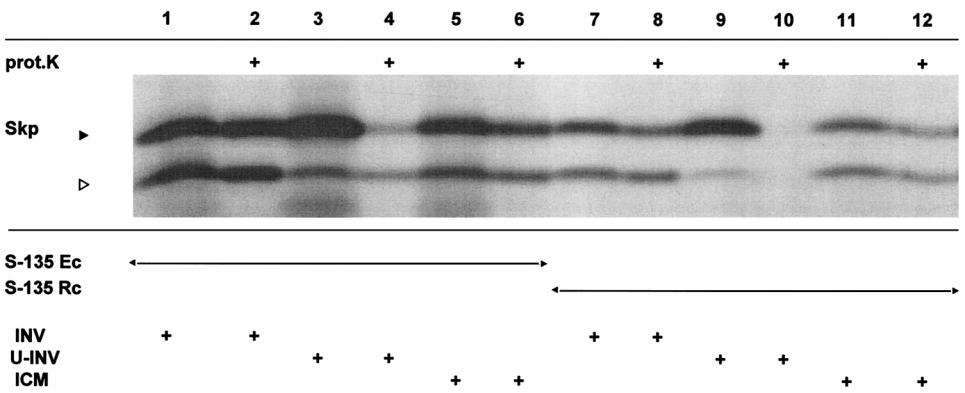

FIG. 1. Localization of a SecA-like activity in a cell-free export system of *R. capsulatus*. Shown is the cell-free synthesis of precursor and mature forms (closed and open arrowheads, respectively) of Skp, a periplasmic protein from *E. coli*. Plasmid pGAH317 containing the *skp* gene was expressed bye a cell extract (S-135) prepared either from the *E. coli secA*(Ts) amber mutant MM66 (S-135 Ec) or from *R. capsulatus* (S-135 Rc). Inside-out plasma membrane vesicles from *E. coli* (INV) and *R. capsulatus* (ICM) were present during synthesis as specified. U-INV, INV pretreated with 6 M urea; prot.K, proteinase K. Cell-free translation products labeled with [^{35}S]methionine were resolved by SDS-PAGE and visualized by fluorography. Only the relevant portion of the SDS gel is shown.

against *Bacillus subtilis* SecA were raised in female rabbits (chinchilla bastard) by applying 300 μg of pure SecA initially and 200 μg for each booster injection. *B. subtilis* SecA was purified essentially as described elsewhere (49) with preparative SDS-PAGE as the final purification step.

Nucleotide sequence accession number. The nucleotide sequence of the *R. capsulatus secA* gene has been deposited with the EMBL database under the accession no. X89411 RCSecA.

RESULTS

Search for a SecA-like activity in a cell-free export system of *R. capsulatus*. Throughout these studies, SecA was assayed via its translocation-stimulating, rather than ATP-hydrolyzing, activity. For this purpose, we used cell-free synthesis-export systems which consisted of high-speed supernatants (S-135s) prepared from *E. coli* and *R. capsulatus* cell homogenates, as well as of plasma membrane vesicles (INV, designating inside-out vesicles from *E. coli*, and ICM, from *R. capsulatus*). Cell-free translation products were precipitated with TCA, separated by SDS-PAGE, and visualized by fluorography. Translocation assays are always depicted in pairs (cf. Fig. 1), one showing conversion of precursor to mature form by the membranes (i.e., processing) and the cognate reaction indicating the degree of translocation of precursor and mature forms into the vesicles as reflected by their resistance towards proteinase K.

In *E. coli*, SecA is found distributed about equally between the cytosol and the plasma membrane. We have recently reported that, upon depletion of the soluble SecA fraction, in vitro translocation of precursors like pOmpA and pSkp becomes dependent on the amount of membrane-associated SecA (22). Figure 1 depicts this situation for Skp, a periplasmic protein of *E. coli*. When synthesized by an S-135 of an *E. coli secA*(Ts) amber mutant grown at the restrictive temperature, translocation of pSkp into INV is drastically diminished upon treatment of the INV with 6 M urea (U-INV) (lanes 1 to 4). Urea treatment has been shown to remove most (15) but not all (63) membrane-associated SecA. Intriguingly, the residual translocation of pSkp into U-INV is almost completely lost after synthesis of pSkp by an S-135 prepared from *R. capsulatus* (compare lanes 3 and 4 with lanes 9 and 10), while translocation occurred normally into SecA-containing INV under these conditions (lanes 7 and 8).

The failure of pSkp to translocate into U-INV when synthesized by an *R. capsulatus* S-135 can be interpreted in two ways: either the *R. capsulatus* S-135 does not contain sufficient soluble SecA or the SecA of *R. capsulatus*, though present, cannot productively interact with the *E. coli* membrane vesicles. Another possibility, namely, that urea-extracted INV are inactive per se and therefore not reactivatable by SecA alone, is ruled out by findings described below. Even if synthesized by the seemingly SecA-lacking S-135 of *R. capsulatus*, pSkp was efficiently translocated when U-INV were replaced by the *R. capsulatus* ICM (Fig. 1, lanes 11 and 12). This finding suggested a SecA-like activity being associated with these membranes, provided that the S-135 used was in fact devoid of active SecA.

Cloning, sequencing, and purification of SecA of *R. capsulatus*. In order to be able to study the properties of the presumed SecA of *R. capsulatus* in more detail, we cloned its gene to allow its subsequent overexpression and purification of the protein. This was done by PCR-mediated amplification of a piece of genomic *R. capsulatus* DNA hybridizing to two degenerate oligonucleotides which had been derived from the most conserved sequence sections of known *secA* genes. A PCR product of the expected size of about 500 bp was thus obtained, and it had a nucleotide sequence homologous, but not identical, to *E. coli secA*. This 500-bp polynucleotide was then used as a hybridization probe to screen restricted genomic DNA of *R. capsulatus* following separation by agarose gel electrophoresis. A 3-kb *Pst*I fragment of *R. capsulatus* DNA was thereby identified, ligated into the vector pGEM-3Z, and subsequently isolated by colony hybridization. Sequencing of the plasmid DNA prepared from one of two reactive clones revealed the presence of 2,564 bp of a *secA* open reading frame, including the 3′ end of the gene on the *Pst*I fragment. By using a SecA probe derived from the beginning of the *Pst*I fragment, the missing 5′ end of *secA* was localized to a 3.5-kb *Eco*RI fragment of *R. capsulatus* DNA. This *Eco*RI fragment was isolated and cloned in an identical fashion. To obtain the full-length clone of *R. capsulatus secA*, the isolated *Eco*RI and *Pst*I fragments were cleaved at an overlapping *Sal*I site and simultaneously ligated into vector pGEM-3Z, yielding plasmid pRHSecA1. Sequence analysis confirmed that pRHSecA1 contained the entire *secA* gene of *R. capsulatus.*

Figure 2 depicts the derived sequence of the 904 amino acids of the *R. capsulatus secA* gene. Alignment with the published SecA sequences of *Caulobacter crescentus* (27), *E. coli* (50), *B. subtilis* (45), and the red alga *Antithamnion* sp. (61) revealed the highest degree of identity (54%) with *C. crescentus*, another member of the α-subclass purple group of gram-negative bacteria. The *R. capsulatus* SecA sequence has 53% identity of

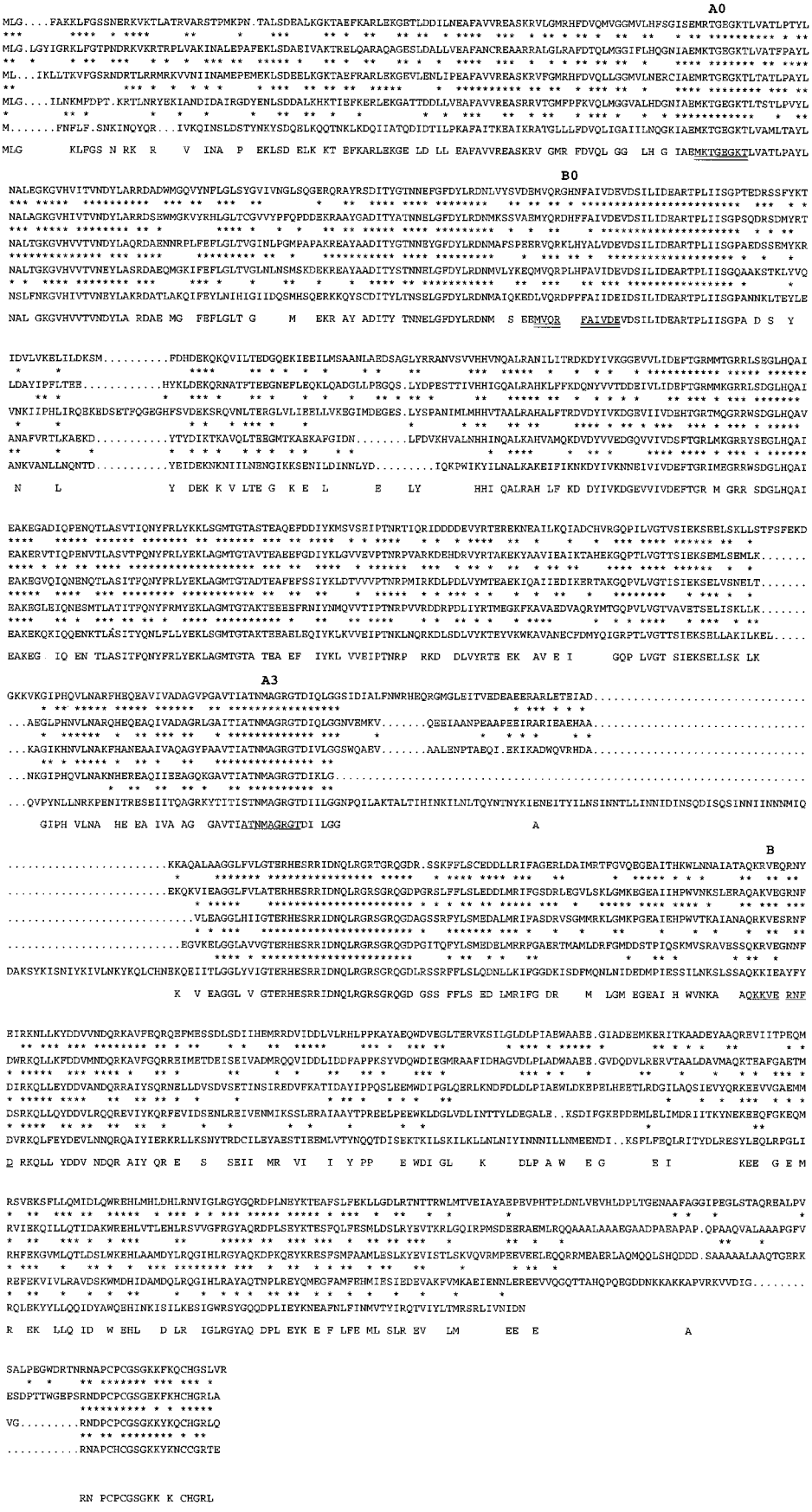

FIG. 2. Alignment of the amino acid sequence of SecA from *R. capsulatus* with those from various other organisms. The amino acid sequence of SecA from *R. capsulatus* (RC) is compared to those from *C. crescentus* (CC), *E. coli* (EC), *B. subtilis* (BS), and *Antithamnion* sp. (AS). The alignment was performed with the Multalin program (13) optimized by hand. The asterisks indicate identical residues in the sequences above and below. ++ is a consensus sequence obtained when at least three of the five sequences showed identical amino acids. NBD1 (consisting of Walker boxes A0 and B0 according to reference 38) is depicted by double underlining; NBD2 (boxes A3 and B) is depicted by single underlining.

amino acids with that of the γ-subclass purple bacterium *E. coli*, 48% with that of a gram-positive organism (*B. subtilis*), and 40% with the SecA of the red alga. Regions of highest sequence identity are those found around the two nucleotide binding sites (NBD1 and NBD2) and the extreme COOH terminus.

For expression in *E. coli*, *R. capsulatus secA* was subcloned under both the inducible *trc* promoter of vector pTrc99A (plasmid pRHSecA3) and the *tac* promoter of vector pKK233-3 (pRHSecA4). In the experiment whose results are depicted in Fig. 3, cells of *E. coli* XL 1-Blue were transformed with either the *secA*-containing pRHSecA3 or the vector pTrc99A and grown in the absence or presence of the inducer IPTG, respectively. Whole-cell proteins were collected by TCA precipitation, resolved by SDS-PAGE, and immunoblotted with antibodies raised against *B. subtilis* SecA. The antibodies detected a protein of around 100 kDa in all samples, which therefore must be the *E. coli* SecA of the host strain. In contrast, a protein with a slightly lower electrophoretic mobility was detected only in cells containing plasmid pRHSecA3 and only after induction by IPTG (lane 1). Due to its selective expression by IPTG, this SecA cross-reacting protein must be the pRHSecA3-encoded *R. capsulatus* SecA. Incidentally, it was not recognized by antibodies raised against SecA of *E. coli* (not shown). Attempts to purify active *R. capsulatus* SecA in *E. coli* XL 1-Blue failed. This was most likely due to cleavage of the first 9 amino acids occurring under these conditions, as revealed by partial NH_2-terminal amino acid sequence analysis (not shown). Active *R. capsulatus* SecA could, however, be obtained from *E. coli* BL21, which is deficient in the Lon and OmpT proteases. Since OmpT cuts at the center of paired basic amino acids (52), it is likely that the NH_2 terminus of the recombinant *R. capsulatus* SecA is sensitive to cleavage by OmpT between R and K at positions 9 and 10 (Fig. 2).

For purification of the *R. capsulatus* SecA protein, *E. coli* BL21 was freshly transformed with pRHSecA4 and induced with 1 mM IPTG for 6 h. After breakage of cells in a French press, an S-150 was prepared and subsequently fractionated by anion-exchange chromatography on Q-Sepharose with a discontinuous KCl gradient. Fractions containing the highest amounts of *R. capsulatus* SecA, as determined by immunoblotting, were pooled, concentrated by ultrafiltration, and applied to a Superdex 200 gel filtration column. As illustrated in Fig. 4, this method yielded a rather complete separation of the two SecA cross-reactive species. A rough estimation of the elution volumes suggested that the (larger) *R. capsulatus* protein behaved like a dimer on the Superdex column whereas the *E. coli* SecA eluting first apparently formed higher-molecular-weight aggregates under these conditions. When a fraction consisting exclusively of the presumed *R. capsulatus* SecA was subjected to Edman degradation, the following partial NH_2-terminal amino acid sequence was obtained: MLGLGYIGxKLF. This sequence is identical to that predicted from the nucleotide sequence (Fig. 2), proving that this protein is in fact *R. capsulatus* SecA.

Purified *R. capsulatus* SecA possesses translocation activity when assayed with its homologous membranes. In order to examine whether the purified *R. capsulatus* SecA was active in translocating precursors across the plasma membrane, it was tested in a cell-free system consisting of U-INV and an S-135 of *R. capsulatus* synthesizing pSkp. As described above (Fig. 1), almost no translocated material could be detected under these conditions (Fig. 5, lanes 1 and 2). Whereas purified *E. coli* SecA markedly stimulated translocation of pSkp into U-INV (lanes 5 and 6), the purified *R. capsulatus* SecA showed only marginal activity (lanes 3 and 4). However, the *R. capsulatus* SecA was active when U-INV were replaced by membrane vesicles of *R. capsulatus*. As described previously (66), *R. capsulatus* ICM lose their translocation activity when extracted at low ionic strength (washed ICM [wICM]; compare Fig. 5, lanes 7 and 8, with Fig. 1, lanes 11 and 12). Translocation into wICM is restored by adding the low-salt extract back (Fig. 5, lanes 9 and 10). Similarly, not only *E. coli* SecA but also the recombinant *R. capsulatus* SecA very efficiently stimulated translocation of pSkp into wICM (Fig. 5, lanes 11 to 14). This clearly indicates that purified *R. capsulatus* SecA possesses translocation activity.

Although the same amounts of *R. capsulatus* SecA were tested against the SecA-depleted U-INV from *E. coli* and the wICM from *R. capsulatus* (cf. lanes 3 to 6 and 11 to 14), activity was found almost exclusively with the homologous membranes (wICM). In contrast, the *E. coli* protein exhibited activity towards both kinds of membranes. This was not due to a limiting concentration of *R. capsulatus* SecA, because, in this experiment, at least twice as much *R. capsulatus* SecA protein as *E. coli* SecA had been used. These results therefore demonstrate that the *R. capsulatus* SecA cannot efficiently interact with *E. coli* membranes.

The distribution of SecA among subcellular fractions varies between *E. coli* and *R. capsulatus*. According to Fig. 5 (lanes 7 to 14), essentially no translocation of pSkp into wICM occurred unless purified SecA (or the low-salt extract of ICM) was provided. These results support the conjecture that the *R. capsulatus* S-135 used for in vitro synthesis of pSkp does not contain significant amounts of soluble SecA. In addition, they suggest that an active component of the low-salt extract of ICM must be SecA. These findings were experimentally corroborated by determining via immunoblotting the SecA content of equivalent amounts of various subfractions used in the in vitro assays (Fig. 6). Whereas *E. coli* SecA was about evenly

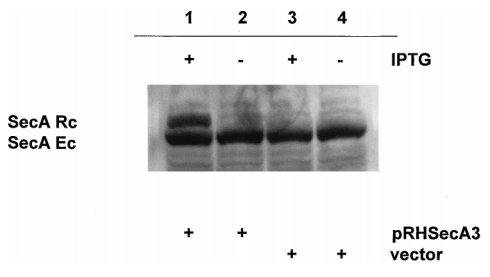

FIG. 3. Expression of *R. capsulatus* SecA in *E. coli*. Cells of *E. coli* XL 1-Blue transformed either with plasmid pRHSecA3 or with vector pTrc99A were grown in LB medium supplemented with either 0.5% glucose or 1 mM IPTG as indicated. After 3 h of growth, cells were pelleted from a 1-ml aliquot of each culture and proteins were precipitated with TCA, resolved by SDS-PAGE, and immunoblotted with polyclonal antiserum raised against *B. subtilis* SecA. The positions of SecA from *R. capsulatus* (SecA Rc) and *E. coli* (SecA Ec) are indicated.

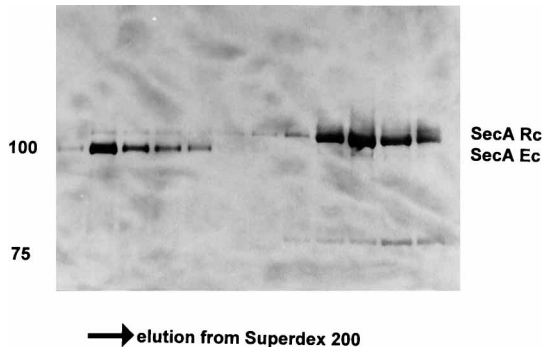

FIG. 4. Purification of *R. capsulatus* SecA by gel filtration. Fractions eluted from Q-Sepharose and containing the highest amounts of *R. capsulatus* SecA, as analyzed by immunoblotting, were pooled, concentrated by ultrafiltration, and applied to a Superdex 200 column. Two-milliliter fractions were collected, and 50 μl of each was subjected to SDS-PAGE and probed with *B. subtilis* anti-SecA antibodies after blotting onto a nitrocellulose membrane. The SecA proteins of *E. coli* and *R. capsulatus* are marked as in Fig. 3. The approximate migration of marker proteins (molecular mass in kilodaltons) on SDS-PAGE is indicated to the left.

distributed between the S-135 and INV (lanes 7 and 8), reflecting the in vivo situation (11), only trace amounts of *R. capsulatus* SecA could be recovered from the homologous S-135 (lane 1). Considerably more SecA was found in the ICM fraction (lane 2).

When amounts of both purified SecAs identical to those subjected to immunoblotting (lanes 5 and 6) were stained with Coomassie blue (lanes 9 and 10), it became evident that no major difference in the cross-reactivity of both SecA species towards the *B. subtilis* antibodies existed. Therefore, the SecA contents of the various subfractions from *E. coli* and *R. capsulatus* can directly be estimated from the blot shown in Fig. 6. Accordingly, the cell-free system of *R. capsulatus* contains considerably less SecA than that of *E. coli*. Furthermore, a substantial portion of *R. capsulatus* SecA indeed is removed from ICM by low-salt washing (compare lanes 2 and 3) and recovered from the low-salt extract (lane 4).

Cytochrome c_2 biogenesis involves a SecA-dependent protein transport step. As previously reported (66), translocation of precytochrome c_2 into ICM is nearly completely abolished when the membranes are extracted with a buffer of low ionic strength (cf. Fig. 7A, lanes 1 to 4) but can be restored by the low-salt extract (lanes 5 and 6). Low-salt extraction had been shown to inhibit translocation of precursor proteins into *E. coli* INV by removal of the F_1-ATPase (40). Although translocation of precytochrome c_2 had been found to strongly depend on a transmembrane H^+ gradient (66), experimental evidence was provided suggesting that the impairment of translocation into ICM upon low-salt extraction was not, or at least not exclusively, due to a deenergization of the membranes (66). We show here that purified SecA of *R. capsulatus* efficiently replaces the translocation-stimulating activity of the low-salt extract with precytochrome c_2 (Fig. 7A, lanes 7 and 8). We therefore conclude that the predominant translocation-stimulating component of the low-salt extract of ICM is SecA. In addition, these results demonstrate that a bacterial *c*-type cytochrome utilizes the general Sec-dependent pathway of export. This finding is not trivial in view of the noncanonical import pathway of cytochrome *c* into mitochondria (36) and the involvement of ATP-binding cassette transporters in the biogenesis of bacterial *c*-type cytochromes (see reference 56).

The SecAs from *R. capsulatus* and *E. coli* exhibit different precursor specificities. Interestingly, SecA of *E. coli* did not stimulate translocation of precytochrome c_2 into wICM to the same extent as did the *R. capsulatus* counterpart (Fig. 7A, lanes 7 to 10) although it was added in a larger quantity than the SecA of *R. capsulatus* (same amounts as those shown in Fig. 6, lanes 5 and 6). Thus, SecA of *R. capsulatus* translocated precytochrome c_2 more efficiently across the *R. capsulatus* plasma membrane than did SecA of *E. coli*. This preference for the *R. capsulatus* SecA was, however, seen only with precytochrome c_2. With pre-OmpA of *E. coli* and preporin of *Rhodopseudomonas blastica*, SecA of *E. coli* turned out to be more active with wICM than did *R. capsulatus* SecA (Fig. 7B, lanes 1 to 8), irrespective of the amount of *R. capsulatus* SecA. This difference in activity was also seen when wICM were replaced by unwashed ICM (lanes 9 to 14). The comparison of the translocation activities of *E. coli* and *R. capsulatus* SecA therefore reveals discrimination of both SecAs not only between membranes but also between precursor proteins.

DISCUSSION

Identification of *secA* in α-subclass purple bacteria. SecA has now been identified in two species of α-subclass purple bacteria, *C. crescentus* (27) and *R. capsulatus* (in this report). Since these bacteria are closely related to mitochondrial ancestors, there has been speculation as to what degree the Sec machinery has been conserved during the evolution of mitochondria. However, the recently completed sequencing of the yeast genome revealed no evidence for the existence of bacte-

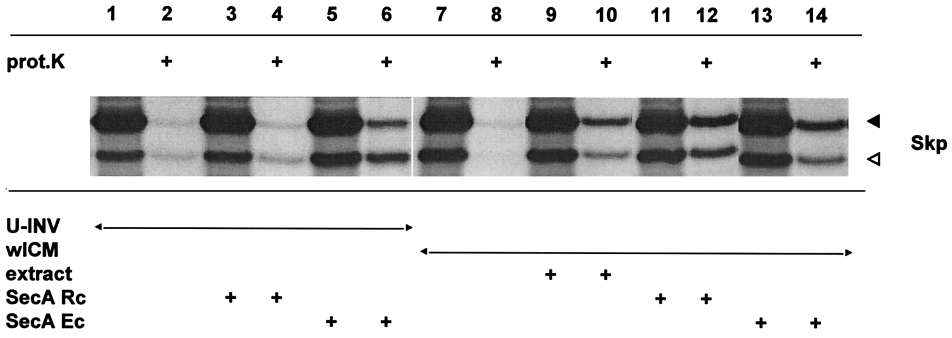

FIG. 5. Purified *R. capsulatus* SecA exhibits membrane-selective translocation activity. Skp was synthesized in vitro with an S-135 prepared from *R. capsulatus*. The additions indicated at the bottom of the figure were made posttranslationally (see Materials and Methods). Low-salt extraction of ICM yielded washed membranes (wICM) and the low-salt extract (extract) thereof. For further details, see the legend to Fig. 1. Note that, in this experiment, the amount of *E. coli* SecA added was less than 10% of what was used in the experiments depicted in Fig. 7.

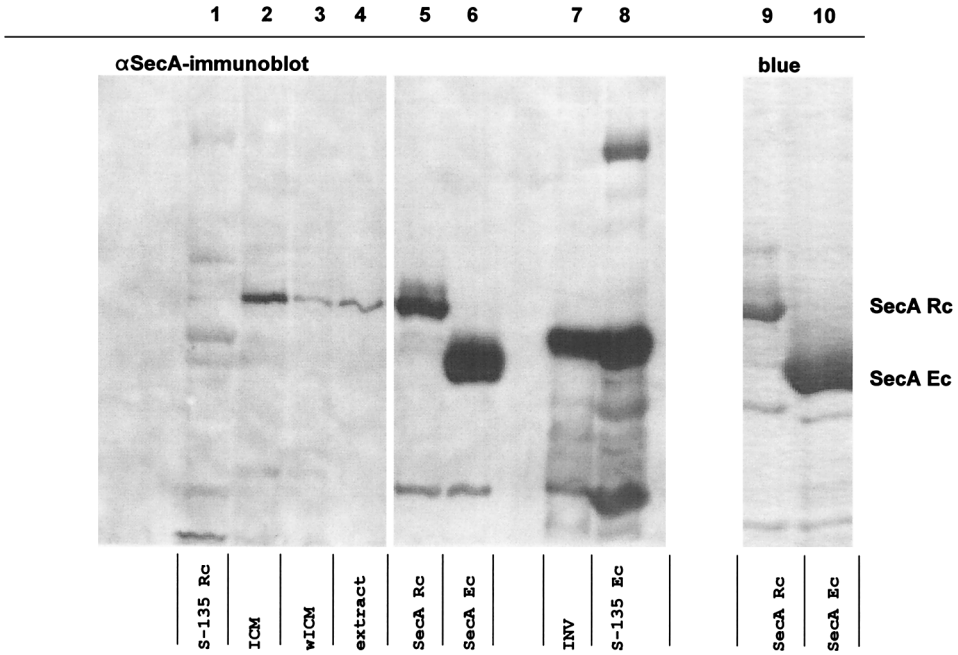

FIG. 6. *R. capsulatus* SecA is mostly membrane associated. Proteins contained in the indicated fractions were resolved by SDS-PAGE. Of each fraction, six times the amount used for a 25-μl synthesis-translocation reaction (Fig. 7) was applied. Lanes 1 to 8 represent an immunoblot developed with antibodies directed against *B. subtilis* SecA. Lanes 9 and 10 show proteins stained with Coomassie blue from the fractions blotted in lanes 5 and 6.

rial *sec* genes (22a). Therefore, the export of proteins from the matrix of mitochondria does not involve SecA and SecY. On the other hand, it has been well documented that chloroplasts descending from cyanobacteria use SecA for importing certain proteins into their thylakoids (8, 41, 69).

Our initial attempts to detect SecA in *R. capsulatus* by screening subcellular fractions with polyclonal antibodies raised against SecA of *E. coli* had failed (unpublished data). The reason for this is the lack of cross-reactivity of the *R. capsulatus* SecA with these anti-*E. coli* SecA antibodies. Con-

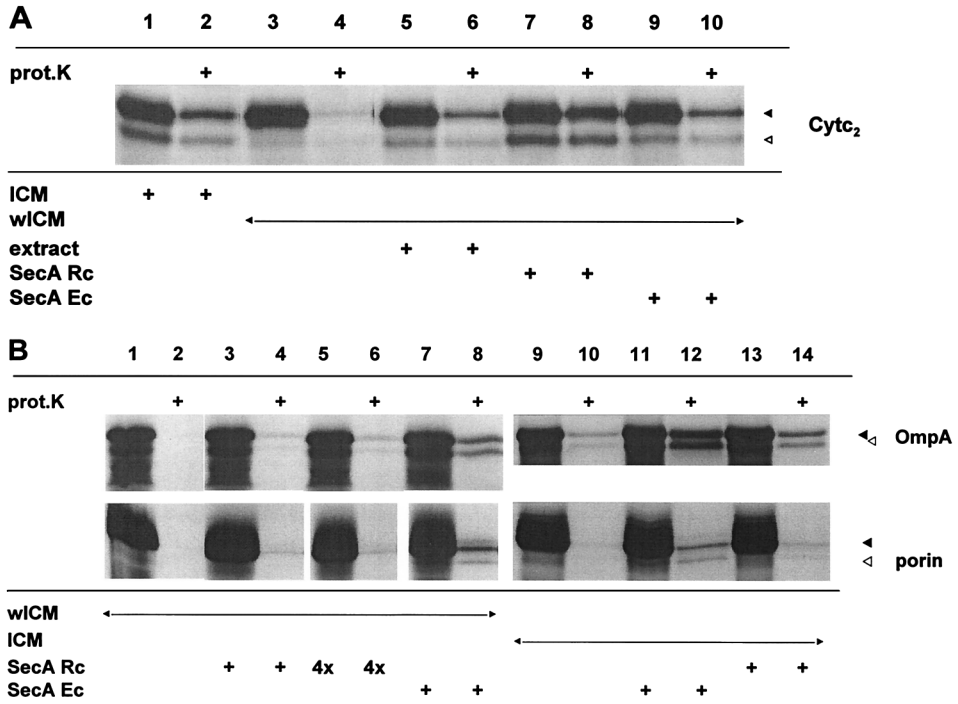

FIG. 7. The SecA proteins of *R. capsulatus* and *E. coli* have different precursor specificities. The precursors of cytochrome c_2 (*R. sphaeroides*), OmpA (*E. coli*), and porin (*Rhodopseudomonas blastica*) were synthesized in vitro with an S-135 prepared from *R. capsulatus*. The additions indicated at the bottom of each panel were made either cotranslationally (A) or posttranslationally (B). Reaction mixtures analyzed in lanes 1 to 8 of panel B contained 2 μg of purified *E. coli* SecB. For further details, see the legend to Fig. 1.

trary to a previous report (16), heterologous anti-SecA antibodies are therefore not appropriate to establish the occurrence or absence of SecA from a particular bacterial strain.

Purification of SecA from *R. capsulatus*. Crucial to the purification of the *R. capsulatus* SecA was its separation from the *E. coli* counterpart by gel filtration, because only then could the translocation activity recorded be ascribed solely to *R. capsulatus* SecA. Similar conditions of gel filtration, including the same gel matrix (Superdex 200), were used by others to demonstrate that *E. coli* SecA occurs as homodimers (18). When the column used here was calibrated with marker proteins, the elution volume of the *R. capsulatus* SecA suggested (at least) dimer formation whereas the *E. coli* protein appeared to be eluted as oligomeric complexes. The reason for the difference from previous reports (4, 18) is not clear. The occurrence of oligomeric forms of *E. coli* SecA, however, has also been described by others (64).

SecA-responsive in vitro translocation systems. Except for SecA from *E. coli*, the only isolated SecA species shown to exhibit translocation activity are those of *Staphylococcus carnosus* (49), pea chloroplasts (69), and *R. capsulatus*. This is because (i) these are the only cases in which a SecA-responsive in vitro translocation system was available and (ii) in general, in vivo assays employing complementation of *E. coli secA* mutants rarely give satisfactory results (see below). The SecA responsiveness of the cell-free translocation system prepared from *R. capsulatus* results on the one hand from a priori subsaturating amounts of SecA rendering this system stimulable by externally added purified SecA. The major part of SecA present in the *R. capsulatus* in vitro system is, however, removed by extracting membranes at low concentrations of salt.

The reason for the considerably lower SecA content of the cell-free system prepared from *R. capsulatus* compared to that of *E. coli* is not clear. It is unlikely that major parts of *R. capsulatus* SecA were lost during the preparation because the only major subcellular fraction missing from our in vitro system is the cytoplasmic membranes (from which the ICM develop). However, when probed with the *B. subtilis* anti-SecA antibodies these membranes did not contain substantial amounts of SecA (not shown). Suboptimal levels of SecA might be the reason why so few in vitro translocation systems have been developed from other bacteria. Consistently, a cell-free translocation system prepared from *S. carnosus* shows low endogenous translocation efficiency for preprolipase unless stimulated with the homologous SecA (49).

Membrane specificity of SecA. The inability of the purified *R. capsulatus* SecA to efficiently support translocation of a precursor protein into *E. coli* membrane vesicles is consistent with its failure to complement the growth defect of two *E. coli secA* mutants, *secA51* (MM52) and *secA109*(Am) [MM66 carrying *su3*(Ts)] (not shown). In addition, the fact that the *R. capsulatus* SecA could be successfully expressed even after prolonged induction by IPTG in a *secA* wild-type *E. coli* strain suggests that the recombinant SecA did not interfere with the SecA-mediated export of the host. Contradictory reports have been presented with respect to the complementation of these and similar *E. coli secA* mutants by the *B. subtilis secA* gene *div*: whereas Klose et al. (30) reported recovery of growth provided that the *B. subtilis* SecA was expressed at moderate levels, McNicholas et al. (37) did not find functional substitution of the wild-type *div* gene product for *E. coli* SecA. Furthermore, purified *B. subtilis* SecA did not efficiently support translocation of precursor proteins into *E. coli* membrane vesicles (54, 62), suggesting that *B. subtilis* SecA does not functionally interact with *E. coli* membranes. On the other hand, the 242 NH_2-terminal amino acids of *B. subtilis* SecA were found to functionally replace the corresponding part of the *E. coli* protein (37), and this is the domain which mediates insertion-deinsertion into the lipid bilayer of the plasma membrane by the help of its high-affinity ATP binding site (20, 21, 44, 62). However, there are clearly more determinants for the membrane association of SecA than only a functional NH_2-terminal domain. For example, SecD and SecF stabilize the membrane-integrated form of SecA (21, 28). The SecA-SecD/F interaction could thus represent the basis of a species-specific association of SecA with membranes. Pending further analyses involving a greater number of purified SecA species from different sources, our results are consistent with several observations pointing towards a rather high specificity of individual SecA proteins with respect to their homologous membranes.

In the context of the association of SecA with membranes, the marked preponderance of the membrane-bound *R. capsulatus* protein over the soluble form is a noteworthy finding. Whereas the *B. subtilis* SecA, like that of *E. coli* (37), partitions nearly equally between cytosol and membranes, the SecAs of spinach (8) and pea (41) chloroplasts are predominantly soluble. This suggests that the intracellular distribution of SecA in a given organism does not necessarily reflect a certain functional property or state of SecA. Consistent with this notion is our finding that soluble SecA of *E. coli* can functionally replace ICM-bound SecA of *R. capsulatus* (Fig. 7B). In view of the well-documented binding of SecA to the membrane lipids, it is possible, for example, that the extent of membrane association of SecA merely varies with the lipid composition of the target membrane. The major difference between the membrane lipids of *E. coli* and those of *Rhodobacter* spp. is the relative abundance of phosphatidylcholine in the latter species (19a).

Precursor specificity of SecA. A comparison of the specific activities of the two SecA species tested here towards different precursors revealed that cytochrome c_2 was more efficiently translocated into wICM by *R. capsulatus* SecA than by the *E. coli* protein, whereas the opposite was observed for OmpA (*E. coli*) and porin (*Rhodopseudomonas blastica*). The results obtained when testing the two SecA species with Skp (Fig. 5, lanes 11 to 14) are not considered because, in this instance, a different ratio of SecA proteins had been used compared with that for Fig. 7. The difference in the responsiveness of single precursors towards individual SecA species cannot be explained simply by organism specificity, because cytochrome c_2 and porin are both α-subclass purple bacterial proteins and yet show different preferences for *E. coli* and *R. capsulatus* SecA. The finding that among the four precursors tested only precytochrome c_2 was recognized less efficiently by *E. coli* SecA than by its counterpart from *R. capsulatus* is most plausibly explained by some structural constraints. Consistent with this view, the precursor binding sites are among the least conserved sections of the various SecA molecules sequenced thus far.

The precursor-selective translocation activity of *R. capsulatus* SecA appears also to be linked to the differing dependence on SecB. While OmpA is a precursor whose translocation is stimulated by SecB (5), porin of *Rhodopseudomonas blastica* behaves like a SecB-dependent protein when tested in a SecB-sensitive *E. coli* cell-free translocation system (38a). Cytochrome c_2 translocates independently of SecB (27a), as does Skp (22, 55). We show here that *R. capsulatus* SecA exhibits appreciable translocation activity only towards those precursors which are independent of SecB. This suggests that a putative SecB analog cannot occur in saturating amounts in the S-135 prepared from *R. capsulatus* and used for the synthesis of OmpA and porin. By the same token, since *E. coli* SecB did not greatly improve the very low activity of *R. capsulatus* SecA towards OmpA and porin (the reactions shown in Fig. 7B,

lanes 1 to 8, had been supplemented with SecB purified from *E. coli*), we conclude that the *R. capsulatus* SecA does not productively interact with *E. coli* SecB. Thus, SecB would be required for an efficient recognition by SecA of a SecB-dependent precursor, a scenario for which the previous finding of a soluble, ternary complex among precursor, SecA, and SecB (26) provides circumstantial evidence. Although we have thus far not been able to detect a SecB-like activity in *R. capsulatus*, the SecB dependence of porin described above would actually point towards the existence of a similar protein in this organism.

SecA-dependent translocation of precytochrome c_2. In a previous report from this laboratory (66), we demonstrated the requirement of precytochrome c_2 translocation for a fraction of peripheral membrane proteins, which could be obtained by low-salt extraction of ICM. By then we had not been able to find a SecA-like activity in the low-salt extract, which was intriguing in view of the possibility that *c*-type apocytochromes might use a Sec-independent, ATP-binding cassette transporter-based export into the bacterial periplasm (see reference 56). In contrast, we show here that precytochrome c_2 strictly depends on SecA and that SecA is probably the major translocation-promoting activity of the low-salt extract of ICM. The discrepancy with our previous findings stems from the fact that formerly the low-salt extract was tested against SecA-depleted *E. coli* membranes, which, as we show here, can only poorly interact with *R. capsulatus* SecA.

ACKNOWLEDGMENTS

We thank Gaby Decker, Institut für Biotechnologie, Forschungszentrum Jülich, Jülich, Germany, for her expert help with the preparation of antibodies against *B. subtilis* SecA; Vincent Geli, Institut de Biologie Structurale et Microbiologie, CNRS, Marseille, France, for a gift of antiserum against *E. coli* elongation factor EF-Tu; Georg E. Schulz and Benedikt Schmid, Institut für Organische Chemie und Biochemie, Universität Freiburg, for providing plasmid pBSRB-1; Michael Klose and Roland Freudl, Institut für Biotechnologie, Forschungszentrum Jülich, for plasmid pMKL180; Juan MacFarlane, for a gift of plasmid pJMETB7 containing *secB* and his critical reading of the manuscript; and Brigitte Fleischner, for secretarial assistance.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft, Dr 29/31-8 C; the Sonderforschungsbereich 184; and the Fonds der Chemischen Industrie.

REFERENCES

1. **Ahn, T., and H. Kim.** 1994. SecA of *Escherichia coli* traverses lipid bilayer of phospholipid vesicles. Biochem. Biophys. Res. Commun. **203:**326–330.
2. **Ahrem, B., H. K. Hoffschulte, and M. Müller.** 1989. In vitro membrane assembly of a polytopic, transmembrane protein results in an enzymatically active conformation. J. Cell Biol. **108:**1637–1646.
3. **Akita, M., S. Sasaki, S. Matsuyama, and S. Mizushima.** 1990. SecA interacts with secretory proteins by recognizing the positive charge at the amino terminus of the signal peptide in *Escherichia coli*. J. Biol. Chem. **265:**8164–8169.
4. **Akita, M., A. Shinkai, S. Matsuyama, and S. Mizushima.** 1991. SecA, an essential component of the secretory machinery of *Escherichia coli*, exists as homodimer. Biochem. Biophys. Res. Commun. **174:**211–216.
5. **Altman, E., S. D. Emr, and C. A. Kumamoto.** 1990. The presence of both the signal sequence and a region of mature LamB protein is required for the interaction of LamB with the export factor SecB. J. Biol. Chem. **265:**18154–18160.
6. **Amann, E., J. Brosius, and M. Ptashne.** 1983. Vectors bearing a hybrid trp-lac promoter useful for regulated expression of cloned genes in *Escherichia coli*. Gene **25:**167–178.
7. **Amann, E., B. Ochs, and K. J. Abel.** 1988. Tightly regulated tac promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. Gene **69:**301–315.

7a. **Behrmann, M., and M. Müller.** Unpublished data.

8. **Berghöfer, J., I. Karnauchov, R. G. Herrmann, and R. B. Klösgen.** 1995. Isolation and characterization of a cDNA encoding the SecA protein from spinach chloroplasts. J. Biol. Chem. **270:**18341–18346.
9. **Breukink, E., R. A. Demel, G. de Korte-Kool, and B. de Kruijff.** 1992. SecA insertion into phospholipids is stimulated by negatively charged lipids and inhibited by ATP: a monolayer study. Biochemistry **31:**1119–1124.
10. **Breukink, E., N. Nouwen, A. van Raalte, S. Mizushima, J. Tommassen, and B. de Kruijff.** 1995. The C terminus of SecA is involved in both lipid binding and SecB binding. J. Biol. Chem. **270:**7902–7907.
11. **Cabelli, R. J., K. M. Dolan, L. Qian, and D. B. Oliver.** 1991. Characterization of membrane-associated and soluble states of SecA protein from wild-type and secA51(ts) mutant strains of *Escherichia coli*. J. Biol. Chem. **266:**24420–24427.
12. **Chun, S.-Y., and L. L. Randall.** 1994. In vivo studies of the role of SecA during protein export in *Escherichia coli*. J. Bacteriol. **176:**4197–4203.
13. **Corpet, F.** 1989. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. **16:**10881–10890.
14. **Cunningham, K., and W. Wickner.** 1989. Specific recognition of the leader region of precursor proteins is required for the activation of translocation ATPase of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **86:**8630–8634.
15. **Cunningham, K., R. Lill, E. Crooke, M. Rice, K. Moore, W. Wickner, and D. Oliver.** 1989. SecA protein, a peripheral protein of the *Escherichia coli* plasma membrane, is essential for the functional binding and translocation of proOmpA. EMBO J. **8:**955–959.
16. **de Cock, H., and J. Tommassen.** 1991. Conservation of components of the *Escherichia coli* export machinery in prokaryotes. FEMS Microbiol. Lett. **80:**195–200.
17. **de Cock, H., and J. Tommassen.** 1992. SecB-binding does not maintain the translocation-competent state of prePhoE. Mol. Microbiol. **6:**599–604.
18. **Driessen, A. J. M.** 1993. SecA, the peripheral subunit of the *Escherichia coli* precursor protein translocase, is functional as a dimer. Biochemistry **32:**13190–13197.
19. **Driessen, A. J. M.** 1994. How proteins cross the bacterial cytoplasmic membrane. J. Membr. Biol. **142:**145–159.

19a. **Dryden, S. C., and W. Dowhan.** 1996. Isolation and expression of the *Rhodobacter sphaeroides* gene (*pgsA*) encoding phosphatidylglycerophosphate synthase. J. Bacteriol. **178:**1030–1038.

20. **Economou, A., and W. Wickner.** 1994. SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion. Cell **78:**835–843.
21. **Economou, A., J. A. Pogliano, J. Beckwith, D. B. Oliver, and W. Wickner.** 1995. SecA membrane cycling at SecYEG is driven by distinct ATP binding and hydrolysis events and is regulated by SecD and SecF. Cell **83:**1171–1181.
22. **Ernst, F., H. K. Hoffschulte, B. Thome-Kromer, U. E. Swidersky, P. K. Werner, and M. Müller.** 1994. Precursor-specific requirements for SecA, SecB, and $\Delta\mu_H+$ during protein export in *Escherichia coli*. J. Biol. Chem. **269:**12840–12845.

22a. **Glick, B. S., and G. von Heijne.** 1996. *Saccharomyces cerevisiae* mitochondria lack a bacterial-type Sec machinery. Protein Sci. **5:**2651–2652.

23. **Grimberg, J., S. Maguire, and L. Belluscio.** 1989. A simple method for the preparation of plasmid and chromosomal *E. coli* DNA. Nucleic Acids Res. **17:**8893.
24. **Hartl, F.-U., S. Lecker, E. Schiebel, J. P. Hendrick, and W. Wickner.** 1990. The binding cascade of SecB to SecA to SecY/E mediates preprotein targeting to the *E. coli* plasma membrane. Cell **63:**269–279.
25. **Hendrick, J. P., and W. Wickner.** 1991. SecA protein needs both acidic phospholipids and SecY/E protein for functional high-affinity binding to the *Escherichia coli* plasma membrane. J. Biol. Chem. **266:**24596–24600.
26. **Hoffschulte, H. K., B. Drees, and M. Müller.** 1994. Identification of a soluble SecA/SecB-complex by means of a subfractionated cell-free export system. J. Biol. Chem. **269:**12833–12839.
27. **Kang, P. J., and L. Shapiro.** 1994. Cell cycle arrest of a *Caulobacter crescentus secA* mutant. J. Bacteriol. **176:**4958–4965.

27a. **Kiefer, D., B. Wieseler, and M. Müller.** Unpublished results.

28. **Kim, Y. J., T. Rajapandi, and D. Oliver.** 1994. SecA protein is exposed to the periplasmic surface of the E. coli inner membrane in its active state. Cell **78:**845–853.
29. **Kimura, E., M. Akita, S. Matsuyama, and S. Mizushima.** 1991. Determination of a region in SecA that interacts with presecretory proteins in *Escherichia coli*. J. Biol. Chem. **266:**6600–6606.
30. **Klose, M., K. L. Schimz, J. van der Wolk, A. J. M. Driessen, and R. Freudl.** 1993. Lysine 106 of the putative catalytic ATP-binding site of the Bacillus subtilis SecA protein is required for functional complementation of *Escherichia coli* secA mutants in vivo. J. Biol. Chem. **268:**4504–4510.
31. **Kreusch, A., A. Neubüser, E. Schiltz, J. Weckesser, and G. E. Schulz.** 1994. The structure of the membrane channel porin from Rhodopseudomonas blastica at 2.0 Å resolution. Protein Sci. **3:**58–63.
32. **Kumamoto, C. A., and O. Francetić.** 1993. Highly selective binding of nascent polypeptides by an *Escherichia coli* chaperone protein in vivo. J. Bacteriol. **175:**2184–2188.
33. **Kusters, R., R. Huijbregts, and B. de Kruijff.** 1992. Elevated cytosolic concentrations of SecA compensate for a protein translocation defect in *Escherichia coli* cells with reduced levels of negatively charged phospholipids. FEBS Lett. **308:**97–100.
34. **Liebke, H. H.** 1987. Multiple SecA protein isoforms in *Escherichia coli*. J. Bacteriol. **169:**1174–1181.

35. **Lill, R., W. Dowhan, and W. Wickner.** 1990. The ATPase activity of SecA is regulated by acidic phospholipids, SecY, and the leader and mature domains of precursor proteins. Cell **60:**271–280.
36. **Mayer, A., W. Neupert, and R. Lill.** 1995. Translocation of apocytochrome c across the outer membrane of mitochondria. J. Biol. Chem. **270:**12390–12397.
37. **McNicholas, P., T. Rajapandi, and D. Oliver.** 1995. SecA proteins of *Bacillus subtilis* and *Escherichia coli* possess homologous amino-terminal ATP-binding domains regulating integration into the plasma membrane. J. Bacteriol. **177:**7231–7237.
38. **Mitchell, C., and D. Oliver.** 1993. Two distinct ATP-binding domains are needed to promote protein export by *Escherichia coli* SecA ATPase. Mol. Microbiol. **10:**483–497.

38a. **Müller, M.** Unpublished results.

39. **Müller, M., and G. Blobel.** 1984. In vitro translocation of bacterial proteins across the plasma membrane of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **81:**7421–7425.
40. **Müller, M., R. P. Fisher, A. Rienhöfer-Schweer, and H. K. Hoffschulte.** 1987. DCCD inhibits protein translocation into plasma membrane vesicles from *Escherichia coli* at two different steps. EMBO J. **6:**3855–3861.
41. **Nakai, M., A. Goto, T. Nohara, D. Sugita, and T. Endo.** 1994. Identification of the SecA protein homolog in pea chloroplasts and its possible involvement in thylakoidal protein transport. J. Biol. Chem. **269:**31338–31341.
42. **Oliver, D. B.** 1993. SecA protein: autoregulated ATPase catalysing preprotein insertion and translocation across the *Escherichia coli* inner membrane. Mol. Microbiol. **7:**159–165.
43. **Oliver, D. B., and J. Beckwith.** 1982. Regulation of a membrane component required for protein secretion in *Escherichia coli*. Cell **30:**311–319.
44. **Rajapandi, T., and D. Oliver.** 1996. Integration of SecA protein into the *Escherichia coli* inner membrane is regulated by its amino-terminal ATP-binding domain. Mol. Microbiol. **20:**43–51.
45. **Sadaie, Y., H. Takamatsu, K. Nakamura, and K. Yamane.** 1991. Sequencing reveals similarity of the wild-type div^+ gene of Bacillus subtilis to the *Escherichia coli* secA gene. Gene **98:**101–105.
46. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
47. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
48. **Schiebel, E., A. J. M. Driessen, F. U. Hartl, and W. Wickner.** 1991. $\Delta\mu_H+$ and ATP function at different steps of the catalytic cycle of preprotein translocase. Cell **64:**927–939.
49. **Schimz, K.-L., G. Decker, E. Frings, J. Meens, M. Klein, and M. Müller.** 1995. A cell-free protein translocation system prepared entirely from a Gram-positive organism. FEBS Lett. **362:**29–33.
50. **Schmidt, M. G., E. E. Rollo, J. Grodberg, and D. B. Oliver.** 1988. Nucleotide sequence of the *secA* gene and *secA*(Ts) mutations preventing protein export in *Escherichia coli*. J. Bacteriol. **170:**3404–3414.
51. **Shinkai, A., L. H. Mei, H. Tokuda, and S. Mizushima.** 1991. The conformation of SecA, as revealed by its protease sensitivity, is altered upon interaction with ATP, presecretory proteins, everted membrane vesicles, and phospholipids. J. Biol. Chem. **266:**5827–5833.
52. **Sugimura, K., and T. Nishihara.** 1988. Purification, characterization, and primary structure of *Escherichia coli* protease VII with specificity for paired basic residues: identity of protease VII and OmpT. J. Bacteriol. **170:**5625–5632.
53. **Swidersky, U. E., H. K. Hoffschulte, and M. Müller.** 1990. Determinants of membrane-targeting and transmembrane translocation during bacterial protein export. EMBO J. **9:**1777–1785.
54. **Takamatsu, H., S. I. Fuma, K. Nakamura, Y. Sadaie, A. Shinkai, S. I. Matsuyama, S. Mizushima, and K. Yamane.** 1992. In vivo and in vitro characterization of the *secA* gene product of *Bacillus subtilis*. J. Bacteriol. **174:**4308–4316.
55. **Thome, B. M., and M. Müller.** 1991. Skp is a periplasmic *Escherichia coli* protein requiring SecA and SecY for export. Mol. Microbiol. **5:**2815–2821. (Erratum, **6:**1077.)
56. **Thöny-Meyer, L., D. Ritz, and H. Hennecke.** 1994. Cytochrome c biogenesis in bacteria: a possible pathway begins to emerge. Mol. Microbiol. **12:**1–9.
57. **Troschel, D., S. Eckhardt, H. K. Hoffschulte, and M. Müller.** 1992. Cell-free synthesis and membrane-integration of the reaction center subunit H from *Rhodobacter capsulatus*. FEMS Microbiol. Lett. **91:**129–134.
58. **Troschel, D., and M. Müller.** 1990. Development of a cell-free system to study the membrane assembly of photosynthetic proteins of *Rhodobacter capsulatus*. J. Cell Biol. **111:**87–94.
59. **Uchida, K., H. Mori, and S. Mizushima.** 1995. Stepwise movement of preproteins in the process of translocation across the cytoplasmic membrane of *Escherichia coli*. J. Biol. Chem. **270:**30862–30868.
60. **Ulbrandt, N. D., E. London, and D. B. Oliver.** 1992. Deep penetration of a portion of *Escherichia coli* SecA protein into model membranes is promoted by anionic phospholipids and by partial unfolding. J. Biol. Chem. **267:**15184–15192.
61. **Valentin, K.** 1993. SecA is plastid-encoded in a red alga: implications for the evolution of plastid genomes and the thylakoid protein import apparatus. Mol. Gen. Genet. **236:**245–250.
62. **van der Wolk, J., M. Klose, E. Breukink, R. A. Demel, B. de Kruijff, R. Freudl, and A. J. M. Driessen.** 1993. Characterization of a *Bacillus subtilis* SecA mutant protein deficient in translocation ATPase and release from the membrane. Mol. Microbiol. **8:**31–42.
63. **Watanabe, M., and G. Blobel.** 1993. SecA protein is required for translocation of a model precursor protein into inverted vesicles of *Escherichia coli* plasma membrane. Proc. Natl. Acad. Sci. USA **90:**9011–9015.
64. **Weaver, A. J., A. W. McDowall, D. B. Oliver, and J. Deisenhofer.** 1992. Electron microscopy of thin-sectioned three-dimensional crystals of SecA protein from *Escherichia coli*: structure in projection at 40 Å resolution. J. Struct. Biol. **109:**87–96.
65. **Wickner, W., A. J. M. Driessen, and F. U. Hartl.** 1991. The enzymology of protein translocation across the *Escherichia coli* plasma membrane. Annu. Rev. Biochem. **60:**101–124.
66. **Wieseler, B., and M. Müller.** 1993. Translocation of precytochrome c_2 into intracytoplasmic membrane vesicles of *R. capsulatus* requires a peripheral membrane protein. Mol. Microbiol. **7:**167–176.
67. **Wieseler, B., E. Schiltz, and M. Müller.** 1992. Identification and solubilization of a signal peptidase from the phototrophic bacterium *Rhodobacter capsulatus*. FEBS Lett. **298:**273–276.
68. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.
69. **Yuan, J., R. Henry, M. McCaffery, and K. Cline.** 1994. SecA homolog in protein transport within chloroplasts: evidence for endosymbiont-derived sorting. Science **266:**796–798.