GENERAL DISCUSSION

In bacteria, most proteins that are destined for export can only be translocated across the membrane in a more or less unfolded conformation that allows them to pass through the translocation channel of the Sec pathway. To facilitate this, cytosolic factors aid in maintaining these preproteins in a so-called translocation-competent state. Such factors, called chaperones, bind to preproteins and prevent their folding and aggregation. Some of these chaperones are secretion dedicated and assist in protein targeting to the translocase.

In *E. coli*, many secretory proteins are targeted to the inner membrane (IM) by molecular chaperons such as SecB, which keep them in a loosely folded and translocation-competent conformation (Kumamoto and Beckwith, 1985; Collier, *et al.*, 1988). The chaperone-based targeting pathways promote the translocation of fully synthesized proteins *in vitro* and probably also function in a post-translational fashion at least to some extent *in vivo*. In contrast, recent studies have suggested that a variety of inner membrane proteins (IMPs) are targeted to the membrane by an essential ribonucleoprotein complex that is closely related to eukaryotic signal recognition particle (SRP) (de Gier, *et al.*, 1996; Ulbrandt *et al.*, 1997; Seluanow and Bibi, 1997). In mammalian cells, SRP is a complex composed of six polypeptides and a signal RNA that targets proteins to the secretory pathway in a strictly co-translational fashion (Walter and Johnson, 1994). The 54 kDa subunit of SRP (SRP54) binds to signal sequences of nascent polypeptides and guides ribosome-nascent chain complexes to transport sites in the endoplasmic reticulum (ER) via an interaction with the membrane-bound SRP receptor. Although the SRP found in *E. coli* and many other bacerial species contains only a single protein (a homologue of SRP54 called "Ffh") and a small RNA ("4.5S RNA") (Poritz *et al.*, 1990), many aspects of its function appear to be conserved, including co-translational binding to substrates (Brown, 1987) and a specific interaction with a homologue of the SRP receptor ("FtsY") (Miller *et al.*, 1994).

In *B. subtilis*, the only secretion-specific chaperone thus far identified is the Ffh protein,

a GTPase that is homologous to the 54 kDa subunit of the eukaryotic SRP (srp54) (Honda, *et al.*, 1994). This protein forms a complex (denoted SRP) with the small cytoplasmic RNA (scRNA) that is functionally related to the eukaryotic 7S RNA (called scR1 RNA in *S. cerevisiae*) and the *E. coli* 4.5S RNA (Nakamura, *et al.*, 1992, 1994). Recent data have shown that HBsu, a histone-like protein of *B. subtilis*, is also associated with the scRNA. Notably, HBsu was shown to bind to a region of scRNA that is not conserved in the 4.5S RNA of *E. coli*, suggesting that the *E. coli* SRP lacks an HBsu-like component (Nakamura, *et al.*, 1999; Yamazaki, *et al.*, 1999). I isolated a *B. subtilis* gene encoding a homologue of SRα, FtsY. Furthermore, I detected that the depletion of FtsY caused the defect of translocation of extracellular proteins (Section I; Oguro *et al.*, 1996). These findings indicate that the SRP-like particle and its receptor exist and are involved in membrane targeting of the secretory proteins in *B. subtilis*.

The *ftsY* constitutes an operon structure with two additional gene, one (*rncS*) encodes a homologue of RNase III and the other (*smc*) encodes a SMC family protein. In recent studies, the RncS was a processing enzyme of scRNA precursor (Oguro *et al.*, 1999). It is interesting that SRP receptor and processing enzyme of SRP RNA is located in an operon together. The Smc is implicated in cell division (Moriya *et al.*, 1998).

In *E. coli*, several factors have been identified, by both genetic and biochemical means, that are required at different stages of the general secretory pathway. Presecretory proteins interact with molecular chaperones like SecB, DnaK/ DnaJ and GroEL/ GroES to maintain their translocation-component conformation in the cytosol (Kumamoto, 1991). Recent evidence indicates that SecB binds cotranslationally to only a limited subset of presecretory proteins (Kumamoto and Francetic, 1993). Determination of SecB-binding sites in precursor molecules has met with conflicting the results, but most of the available data indicate that SecB binds to multiple sites in the mature region of the presecretory protein (Pugsley, 1993; Kumamoto, 1991). GroEL was shown to interact with completed pre-β-lactamase by photocross-linking (Bochkareva *et al.*, 1988). Different presecretory proteins seem to prefer different chaperones but they can be quite promiscuous when circumstances change. For example, increased levels of

GroEL and DnaJ/ DnaK can compensate for the loss of SecB (Altman *et al.*, 1991: Wild *et al.*, 1992). Among these chaperones, SecB seems to be the most specific for exported proteins. This conclusion is supported by the fact that SecB also fulfills a "pilot" function by binding to the membrane-associated SecA protein (reviewed by Wolin, 1994). In contrast, genetic and biochemical evidence indicates that SRP-mediated targeting may also occur in E. coli (Hartl and Wiedmann, 1993; Luirink and Dobberstein, 1994). Thses observations give rise to a question what is the relationship between SRP dependent pathway and Sec proteins dependent pathway.

It was reported that depletion of either SecB or Ffh affects the translocation of certain secretory proteins more seriously than others. The differences are somewhat complementary: translocation of ribose-binding protein (RBP) and alkaline phosphatase (PhoA) are unaffected in a SecB null allele, yet are among the more strongly affected proteins in cells depleted of Ffh (Kumamoto and Beckwith, 1985; Phillips and Silhavy, 1992). Thus, it seems likely that some proteins are targeted to the membrane via an interaction with SecB or other chaperones, while others use the SRP pathway. There is probably considerable functional redundancy between the different pathways to some degree. In FtsY-depleted cells, the accumulation of Ffh-dependent proteins (such as RBP, PhoA, or OmpF) was observed. This suggests that FtsY is required for the SRP-dependent pathway. *In B. subtilis*, DnaK, GroEL and GroES were isolated as molecular chaperones. However, there is no experimental evidence that these chaperones are involved protein secretion in *B. subtilis*. Moreover, SecB has not yet been isolated in *B. subtilis*. Recently, the genome sequence of *B. subtilis* was determined complete by the *Bacillus subtilis* genome project, but no SecB homologue was found (Kunst, F., *et al.*, 1997). So, it is difficult to mention about the relationship between SRP-like particle and chaperones in *B. subtilis*. How does SecA function in SRP-SRP receptor dependent pathway? The SRP components and SRP receptor are required for pre-β-lactamase translocation in *B. subtilis* (Nakamura *et al.*, 1992; Honda *et al.*, 1993; Section I; Oguro *et al.*, 1996). If the SRP-SRP receptor dependent pathway is separated from the SecA function, some of pre-β-lactamase was completely inhibited in the *secA341* mutant under the non-permissive temperature conditions at which almost all the mutant SecA

protein was degraded by proteolysis (Takamatsu *et al.*, 1994). Therefore, I assume that SRP recognizes some presecretory proteins and transfers them to SecA. In *B. subtilis*, several lines of experimental evidence suggest that the presecretory protein binds to Ffh first and then is delivered to SecA by *in vitro* binding assay (Bunai *et al.*, 1999). Release of Ffh from polypeptide may be mediated by direct interaction with FtsY (Fig. 1). A further analysis is nessessary to conform to this model.

In response to nutrient starvation, *B. subtilis* develops endospores to resist a variety of harsh conditions, such as extreme cold or heat (Stragier and Losick, 1996). At the beginning of the sporulation process, an asymmetrically positioned septum is formed that divides the cell into two unequally sized compartments. Subsequently, the larger compartment (the mother cell) engulfs the smaller compartment (the forespore), which ultimately becomes the spore. Thus, the forespore is surrounded by two membranes. The inter membrane space (IMS) between these two membranes is the assembly site of two layers of specialized peptidoglycan, called the germ cell wall and the cortex. Consequently, proteins residing in the germ cell wall or cortex must be sorted to the IMS between the forespore and the mother cell. This subcellular compartmentalization imposes a requirement for intracellular protein sorting on the cells, because protein synthesis is limited to the cytoplasm of the mother cell and the forespore.

One of the processes that requires protein transport during sporulation is the communication between the mother cell and the forespore. Two proteins were shown to be exported by the forespore and to interact with membrane proteins of the mother cell. First, the SpoIIR protein, synthesized in the forespore prior to engulfment, contains a functional signal peptide that can drive the export of the mature part of the protein. The mature SpoIIR protein is thought to activate, directly or indirectly, the receptor/protease SpoIIGA, which is required for pro-σ^E processing (Hofmeister, 1988; Hofmeister *et al.*,1995). Second, the SpoIVB protein is synthesized in the forespore and transported across the forespore inner membrane after engulfment has taken place. SpoIVB probably remains anchored to the latter membrane, but a smaller form seems to be released into the IMS of the spore, allowing the activation of receptors

and proteases on the outer forespore membrane that are responsible for pro-σ^K processing (Cutting *et al.*, 1991). As the amino-terminus of SpoIVB contains a putative signal peptide but lacks a putative SPaseI cleavege site, it is presently unclear which protease is reponsible for the processing and subsequent release of SpoIVB in the IMS of the forespore. Other processes in sporulation which require transport of proteins are the biogenesis of the germ wall and spore-cortex in the IMS of the forespore and the degradation of the spore peptidoglycan during germination. CwlD and DacB (Popham *et al.*, 1995; Popham *et al.*, 1996) are the only proteins with a putative signal peptide that were reported to be involved in cortex synthesis. However, the precise subcelluar localization of DacB and CwlD has not yet been documented. The germination-specific amidase SleB was found to be localized on the exterior side of the cortex in spores, while its synthesis is forespore specific (Moriyama *et al.*, 1999). The fact that pre-SleB has to be transported across the forespore inner membrane and processed into its mature form to reach the IMS implies that a functional protein translocation machinery and at least one of the type I SPases are present in the forespore inner membrane. Other proteins involved in spore-cortex synthesis, such as SpoVB and SpoVE (Stragier and Losick, 1996), are predicted to be transmembrane proteins with loops exposed in the IMS of the forespore. Finally, the recent finding that TasA (for translocated anti bacterial spore-associated protein), a protein with a broad spectrum of antibacterial activity, is transported to *B. subtilis* endospores provides another example of spore-specific protein sorting. TasA is thought to confer a competitive advantage to the spore during the oneset of sporulation and later, during germination, by inhibiting the growth of other organisms (Stöver and Driks, 1999). In addition, TasA has been suggested to be required for proper spore coat assembly (Serrano *et al.*, 1999). Although not much is known about the factors involved in protein transport from one sporulation-specific compartment to another.

The cellular distribution of FtsY in sporulating cell was examined by immunoelectron microscopy using affinity purified anti-FtsY antiserum and colloidal gold-labeled second antibody on ultrathin sections. The gold particles were located on the forespore membrane as well as the cytoplasmic membrane (Section II; Kakeshita *et al.*, 2000a). These results suggest

that the SRP-SRP receptor system is involved in the localization of spore-forming proteins (Fig. 2). The sporulation of *B. subtilis* is the simplest model of cell differentiation. In mammals, the SRP-SRP receptor system is involved in only targeting of the pre-secretory proteins to ER membrane. The eukaryotic cells have the other organelles; nucleus, mitochondrium, chloroplast and so on. Each organelle has individual protein localization system which is independent of the SRP-SRP receptor system. However, in the developing process, the SRP-SRP receptor system may be involved in the localization of proteins to each organelle which is required for progressive differentiation of the cell structure.

In Section II, I found that, in addition to the expression of *ftsY* in vegetative cells by the σ^A promoter, *ftsY* is expressed solely at T_8 during sporulation, from a promoter that is controlled by σ^K and GerE and located immediately upstream of *ftsY* and inside the *smc* gene (Kakeshita *et al.*, 2000a). In *B. subtilis*, to produce mature to spores, many proteins relating to spore formation should be transported from mother cell to forespore (or prespore) and also *vise vasa*. Therefore FtsY is required for spore formation. Electron microscopic study showed that the outer coat of the *ftsY* mutant spores is composed of thin layers and immunoelectron microscopy localized FtsY in the coat. Moreover, the amount of several coat proteins, such as CotA and CotE, is reduced on the outer coat of FtsY mutant spores, and molecular weight of CotB is significantly reduced (Section II; Kakeshita *et al.*, 2000b). These results suggest that FtsY is essential for the systematic assembly of spore coat proteins into outer coat. However, CotA, CotB and CotE do not have signal peptide. I think that FtsY function is required for the assembly of other proteins that have signal peptide and directly regulates the assembly of spore coat proteins. A further study is necessary to determine the physiological roles of FtsY in spore coat protein assembly.

Fig. 1. Schematic model for a putative targeting pathway exported presecretory proteins to the *B. subtilis* cytoplasmic membrane.

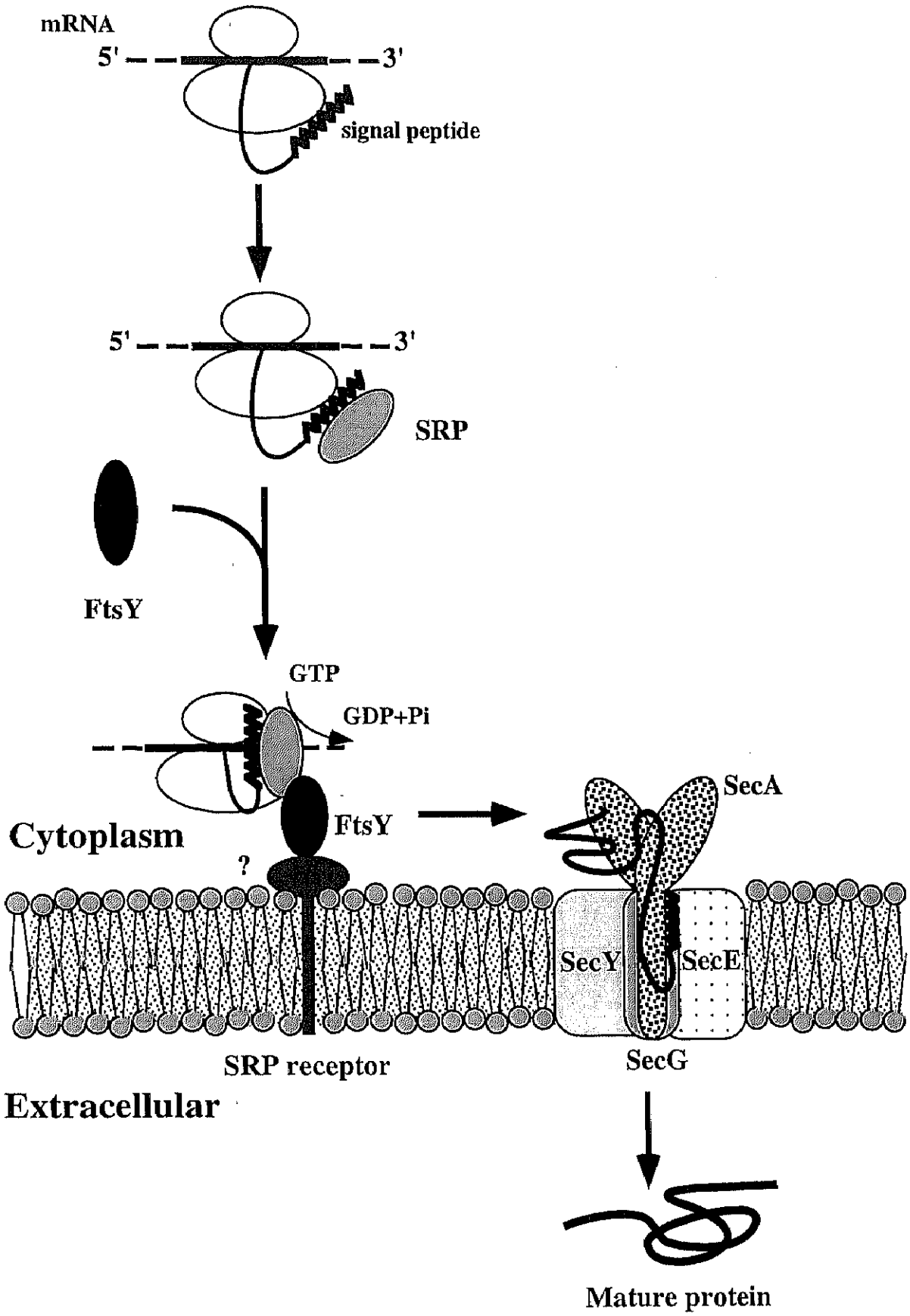
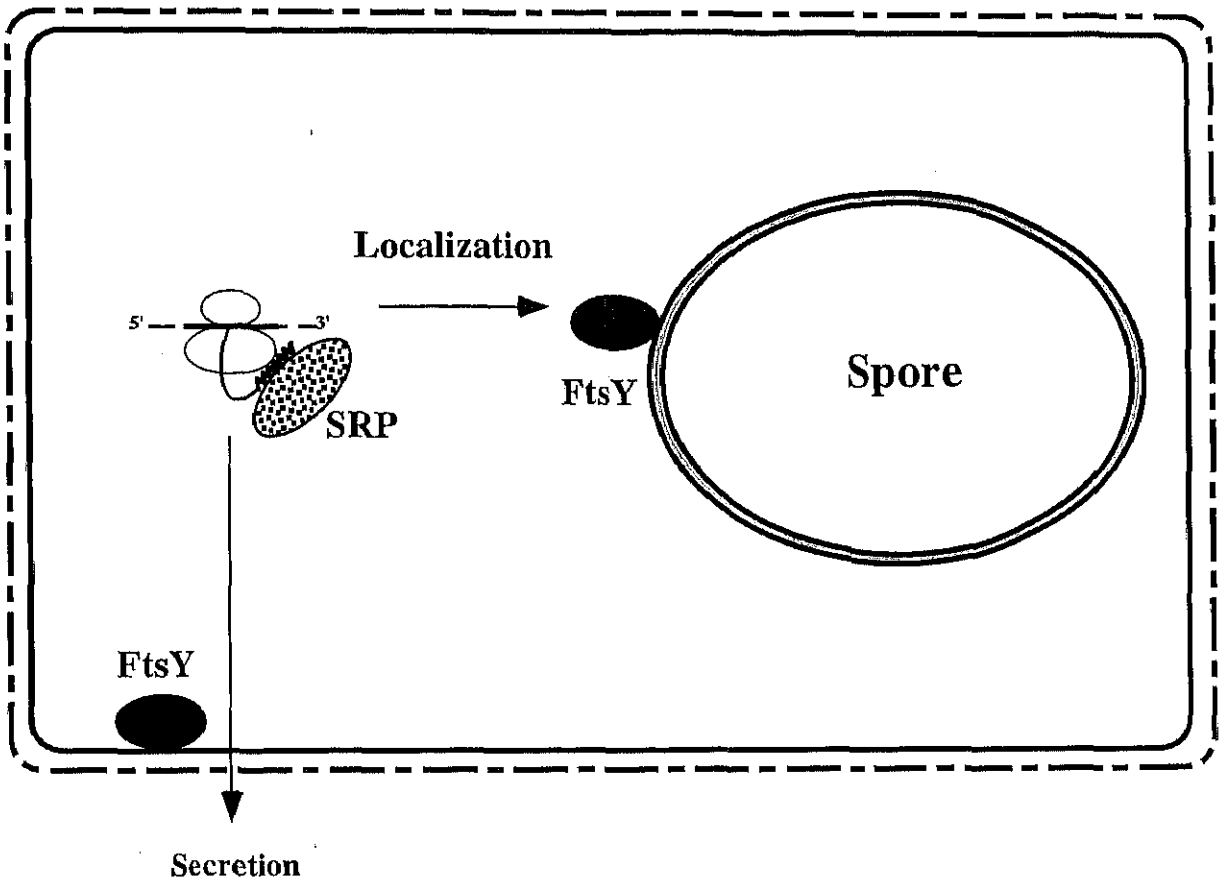

mRNA
5'
3'
signal peptide
5'
3'
SRP
FtsY
GTP
GDP+Pi
FtsY
Cytoplasm
?
SecA
SecY
SecE
SecG
SRP receptor
Extracellular
Mature protein

Fig. 2. Schematic model for SRP-SRP receptor-mediated protein localization system in *B. subtilis*.


Localization
5'
3'
SRP
FtsY
Spore
FtsY
Secretion