Molecular cloning and characterization of an alkalophilic *Bacillus* sp. C125 gene homologous to *Bacillus subtilis* *secY*

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A 1·8 kb *Hind*III DNA fragment containing the *secY* gene of alkalophilic *Bacillus* sp. C125 has been cloned into plasmid pUC119 using the *B. subtilis* *secY* gene as a probe. The complete nucleotide sequence of the cloned DNA indicated that it contained one complete ORF and parts of two other ORFs. The similarity of these ORFs to the sequences of the *B. subtilis* proteins indicated that they were the genes for ribosomal protein L15–SecY–adenylate kinase, in that order. The gene product of the alkalophilic *Bacillus* sp. C125 *secY* homologue was composed of 431 amino acids and its M_r value has been calculated to be 47 100. The distribution of hydrophobic amino acids in the gene product suggested that the protein was a membrane integrated protein with ten transmembrane segments. The total amino acid sequence of alkalophilic *Bacillus* sp. C125 *secY* homologue showed 69·7% homology with that of *B. subtilis* *secY*. Regions of remarkably high homology (78% identity) were present in transmembrane regions, and cytoplasmic domains (73% identity) with less homologous regions present in extracellular domains (43% identity).

Introduction

Genetic studies suggest that a secretion machinery exists for the export of protein to the cell envelope (Michaelis & Beckwith, 1982). The *prl* series of genes, *prlA* (*secY*) (Ito *et al.*, 1983), *prlB*, *prlC* and *prlD* (Emr *et al.*, 1981; Bankatis & Bassford, 1985) identified by their ability to suppress mutations of an export-defective signal peptide, are believed to be involved in the secretion machinery of *Escherichia coli*. One of these genes, *secY* (*prlA*), encodes an integral membrane protein of 10 transmembrane segments (Akiyama & Ito, 1987) which is essential for protein export from the cytosol to the periplasm or outer membrane.

Bacillus sp. strains have a remarkable ability to secrete many proteins, such as amylases, cellulases or proteases, into the culture medium. Recently several groups isolated and analysed the *secY* gene from *B. subtilis* (Yoshikawa & Doi, 1990; Nakamura *et al.*, 1990). However, the protein export pathway or other protein components related to the protein secretion process in *Bacillus* remain unclear.

Our group has a specific interest in alkalophilic

bacteria (Horikoshi, 1991) and have recently selected alkalophilic *Bacillus* sp. C125 as a model alkalophile for molecular biological study (Kudo *et al.*, 1990). In alkalophiles a cell-membrane-maintained pH difference between the internal (pH 8–8·6) and the external (pH 10–11) environments exists. A membrane protein is likely to show special adaptations to alkaline conditions. In order to clarify the protein secretion system in alkalophilic *Bacillus* and to study the *secY* protein as a model membrane protein, the *secY* gene of alkalophilic *Bacillus* sp. C125 was cloned using the *B. subtilis* *secY* gene as a probe, and the cloned gene was characterized.

Methods

Bacterial strains and plasmid. Alkalophilic *Bacillus* sp. C125 (Trp[−] Ura[−] Cm^s) (Kudo *et al.*, 1990) was used as the DNA source for Southern hybridization analysis and cloning of the *secY* gene. *E. coli* JM109 [*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 λ[−] Δ(lac-proAB)* (F' *proAB lacF⁺ΔM15 traD36*)] was used as the host (Nakamura *et al.*, 1990). Plasmid pSY912 containing the *B. subtilis* *secY* gene was kindly provided by Dr H. Yoshikawa (Tokyo University, Tokyo, Japan) and used as a probe (Yoshikawa *et al.*, 1990).

Media and maintenance of strains. *E. coli* strain JM109 was grown and maintained on LB medium (Maniatis *et al.*, 1982). When necessary, ampicillin was added to the medium (100 µg ml^{−1}). Alkalophilic *Bacillus* sp. C125 was grown and maintained on Horikoshi II medium (Kudo *et al.*, 1990).

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The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number D10360.

Abbreviation: Adk, adenylate kinase.

General DNA techniques. DNA manipulations and transformation of *E. coli* were accomplished by the methods outlined by Maniatis *et al.* (1982).

Southern hybridization. Alkalophilic *Bacillus* sp. C125 chromosomal DNA was digested with restriction endonucleases, electrophoresed on a 1% (w/v) agarose gel and then blotted on to a nylon membrane (Amersham) by the method of Southern (Southern, 1975). The membrane was prehybridized and hybridized in a 20% (v/v) formamide hybridization solution at 42 °C. Subsequent washing and DNA detection were carried out according to the protocol provided with the digoxigenin DNA labelling and detection kit (Boehringer Mannheim).

Sequence analysis. Nucleotide sequencing was carried out by the method of Sanger *et al.* (1977) on an Applied Biosystems DNA sequencer (373A) using single-stranded DNA prepared by the method of Vieira & Messing (1987) and an Applied Biosystems sequencing kit. Exonuclease III deletions were carried out by the method of Henikoff (1984). The DNA sequences were analysed using Macmol software.

Results and Discussion

Cloning of *secY* DNA

To verify the existence of the *secY* gene in alkalophilic *Bacillus* sp. C125 chromosomal DNA, the DNA was digested with *Hind*III or *Eco*RI and then analysed by Southern hybridization using the *B. subtilis* *secY* gene as a probe (Fig. 1). A 1.8 kb *Hind*III fragment and two *Eco*RI fragments (5 and 0.5 kb) were detected. The 1.8 kb *Hind*III fragment was chosen for cloning since it gave a strong signal and the size was such that it was likely to contain the complete *secY* gene. Fragments of *Hind*III-digested *Bacillus* sp. C125 DNA of 1.8 kb were purified from a 1% (w/v) agarose gel by electroelution, cloned into the *Hind*III site of pUC119 and transformed into *E. coli*. Two-thousand Amp^r transformants were subjected to colony hybridization analysis and two positive colonies identified. Southern analysis of purified plasmid DNA from these two positive colonies showed positive bands. Restriction mapping of the two plasmids revealed that they contained the same 1.8 kb fragment in the same orientation. The plasmid was designated as pASY3. The physical map of the 1.8 kb *Hind*III fragment contained in pASY3 is presented in Fig. 2.

DNA sequence analysis of the *secY* gene of alkalophilic *Bacillus* sp. C125

The nucleotide sequence of the entire 1.8 kb fragment contained in pASY3 was determined. The nucleotide and deduced amino acid sequences are presented in Fig. 3. Three ORFs, all in the same orientation, were identified. The first ORF lacking the N-terminal region consisted of 66 amino acids. This ORF shared 79.1% amino acid identity (51/66) with the ribosomal protein

Fig. 1. Southern hybridization analysis of alkalophilic *Bacillus* sp. C125 chromosomal DNA. Lanes: 1, Size marker (*Hind*III-digested λ DNA and *Hae*III-digested ϕ X174 DNA mixture); 2, *Eco*RI-digested C125 DNA; 3, *Hind*III-digested C125 DNA. The plasmid pSY912 containing the *B. subtilis* *secY* gene was used as a probe.

Fig. 2. Physical map and alignment of the three ORFs in the cloned 1.8 kb *Hind*III fragment from alkalophilic *Bacillus* sp. C125 chromosomal DNA.

L15 of *B. subtilis* (Yoshikawa & Doi, 1990; Nakamura *et al.*, 1990). The second ORF, beginning with an ATG codon at position 202, consisted of 431 amino acids and showed 69.7% amino acid identity with the *B. subtilis* *secY* gene product (Yoshikawa & Doi, 1990; Nakamura *et al.*, 1990). The third ORF, beginning with a TTG codon at position 1570, consisted of 107 amino acids and was terminated by the *Hind*III site. This ORF had 78.5% amino acid identity with the *B. subtilis* *adk* gene product (Yoshikawa & Doi, 1990; Nakamura *et al.*, 1990). Typical sequences for ribosomal binding, upstream of the 2nd and 3rd ORFs were observed. These results indicated that the gene order around *secY*, given by the deduced gene product sequences was L15–SecY–adenylate kinase (Adk) and identical to that observed in *B. subtilis*.

The predicted amino acid sequence of the *secY* gene product of alkalophilic *Bacillus* sp. C125 was compared with that of the *B. subtilis* *secY* gene product (Fig. 4) (Yoshikawa & Doi, 1990). The number of amino acid residues of both gene products was the same (431 amino acids) and alkalophilic *Bacillus* sp. C125 SecY shared 69.7% amino acid identity with *B. subtilis* SecY. The hydrophobic profiles in the amino acid sequences were analysed by the procedure of Kyte & Doolittle (1982).

L15	
Lys Leu Asn Arg Phe Glu Asp Gly Thr Glu Val Ser Pro Glu Leu Leu Leu Glu	
<u>AAG CTT</u> AAT CGT TTT GAA GAT GGA ACA GAG GTT TCA CCA GAA CTT TTG CTT GAA	54
<i>HindIII</i>	
Thr Gly Val Val Ser Asn Ala Lys Asp Gly Ile Lys Ile Leu Gly Asn Gly Lys Leu Glu	
ACA GGT GTT GTT AGC AAC GCA AAG GAC GGC ATT AAG ATC CTC GGT AAT GGA AAG CTA GAG	114
Lys Lys Leu Thr Val Lys Ala Asn Lys Phe Ser Ala Ser Ala Val Glu Ala Ile Glu Ala	
AAG AAG <u>TTA ACA</u> GTG AAA GCC AAC AAA TTC TCT GCT <u>TCT GCA</u> GTG GAA GCT ATT GAA GCT	174
<i>HpaI</i> SecY <i>PstI</i>	
Ala Gly Gly Lys Thr Glu Val Ile TER Met Phe Arg Thr Ile Ser Asn Ile Phe Arg Val	
GCC GGC GGG AAA ACT <u>GAG GTG</u> ATC TAA ATG TTC CGA ACG ATC TCC AAC ATT TTT CGA GTG	234
SD	
Gly Asp Leu Arg Arg Lys Val Ile Phe Thr Leu Leu Met Leu Ile Val Phe Arg Ile Gly	
GGT GAT TTG CGC CGT AAG GTC ATT TTC ACC CTG CTC ATG CTC ATC GTT TTT CGG ATC GGA	294
Ser Phe Ile Pro Val Pro Gly Thr Asn Arg Glu Val Leu Asp Phe Val Asp Gln Ala Asn	
AGT TTT ATC CCC GTT CCA GGT ACA AAC CGA GAA GTG CTA GAT TTT GTC GAT CAG GCA AAT	354
Ala Phe Gly Phe Leu Asn Thr Phe Gly Gly Gly Ala Leu Gly Asn Phe Ser Ile Phe Ala	
GCA TTC GGT TTT TTA AAT ACG TTT GGA GGA GGG GCA CTT GGG AAT TTC TCC ATC TTT GCG	414
Met Gly Ile Met Pro Tyr Ile Thr Ala Ser Ile Val Met Gln Leu Leu Gln Met Asp Val	
ATG GGG ATC ATG CCA TAC ATT ACA GCA TCC ATT GTC ATG CAA TTA TTG CAG ATG GAT GTC	474
Val Pro Lys Phe Ala Glu Trp Ala Lys Glu Gly Glu Ala Gly Arg Arg Lys Leu Ala Gln	
GTT CCG AAA TTT GCT GAG TGG GCG AAA GAG GGC GAA GCA GGG CGT CGT <u>AAG CTA</u> GCT CAA	534
<i>NheI</i>	
Phe Thr Arg Tyr Gly Thr Ile Val Val Leu Gly Phe Ile Gln Ala Leu Gly Met Ser Val	
TTT ACC CGC TAT GGA ACG ATT GTT GTT TTA GGG TTT ATT CAG GCA CTC GGG ATG TCG GTT	594
Gly Phe Asn Asn Phe Phe Pro Gly Leu Ile Pro Asn Pro Ser Val Ser Val Tyr Leu Phe	
GGT TTT AAC AAC TTC TTC CCA GGA TTA ATT CCT AAT CCA AGC GTG TCG GTG TAT CTC TTT	654
Ile Ala Leu Val Leu Thr Ala Gly Thr Ala Phe Leu Met Trp Leu Gly Glu Gln Ile Thr	
ATC GCA CTT GTC CTT <u>ACT GCA</u> GGA ACG GCT TTC TTA ATG TGG CTC GGG GAG CAG ATT ACA	714
<i>PstI</i>	
Ala Lys Gly Val Gly Asn Gly Ile Ser Ile Ile Ile Phe Ala Gly Ile Ala Ala Gly Ile	
GCT AAG GGA GTC GGA AAC GGA ATC TCC ATT ATC ATC TTT GCA GGG ATT GCT GCT <u>GGA ATT</u>	774
<i>EcoRI</i>	
Pro Asn Gly Leu Asn Leu Ile Tyr Ser Thr Arg Ile Gln Asp Ala Gly Glu Gln Leu Phe	
CCA AAT GGG TTA AAT CTC ATT TAC TCA ACA <u>CGG ATC</u> CAA GAC GCA GGG GAG CAA TTG TTC	834
<i>BamHI</i>	
Leu Asn Ile Val Val Ile Leu Leu Leu Ala Leu Ala Ile Leu Ala Ile Ile Val Gly Val	
TTG AAC ATT GTG GTG ATC TTG TTG CTT GCT CTT GCG ATT CTT GCC ATC ATT GTG GGC GTT	894
Ile Phe Val Gln Gln Ala Leu Arg Lys Ile Pro Val Gln Tyr Ala Lys Arg Leu Val Gly	
ATT TTC GTC CAA CAA GCG TTG CGT AAA ATC CCT GTT CAG TAT GCC AAG CGT CTA GTA GGG	954
Arg Asn Pro Val Gly Gly Gln Ser Thr His Leu Pro Leu Lys Val Asn Ala Ala Gly Val	
AGA AAC CCT GTT GGA GGT <u>CAG TCG ACC</u> CAT CTT CCG TTA AAA GTG AAT GCC GCA GGC GTT	1014
<i>SalI</i>	
Ile Pro Val Ile Phe Ala Leu Ser Leu Leu Ile Phe Pro Pro Thr Val Ala Gly Leu Phe	
ATT CCG GTC ATT TTT GCG TTA TCG TTG CTC ATT TTT CCA CCA ACG GTT GCT GGG CTG TTT	1074
Gly Ser Asp His Pro Val Ala Ala Trp Val Ile Glu Thr Phe Asp Tyr Thr His Leu Ile	
GGC AGT GAT CAT CCG GTC GCT GCA TGG GTG ATT GAG ACA TTT GAC TAT ACC CAC TTG ATT	1134
Gly Met Ala Val Tyr Ala Leu Arg Ile Ile Gly Phe Thr Tyr Phe Tyr Ala Phe Ile Gln	
GGG ATG GCG GTA TAT GCC TTG CGC ATC ATC GGA TTT ACG TAT TTC TAT GCG TTT ATC CAA	1194
Val Asn Pro Glu Arg Met Ala Glu Asn Leu Lys Lys Gln Gly Gly Tyr Ile Pro Gly Ile	
GTC AAC CCA GAG CGA ATG GCG GAG AAC TTG AAA AAA CAG GGT GGC TAT ATT CCT <u>GGA ATT</u>	1254
<i>EcoRI</i>	
Arg Pro Gly Lys Ala Thr Gln Thr Tyr Ile Thr Pro Ile Leu Tyr Arg Leu Thr Phe Val	
CGT CCA GGG AAA GCA ACG CAG ACG TAC ATC ACG CCT ATC TTG <u>TAT CGA</u> TTA ACG TTT GTC	1314

Fig. 3.

Clal

Gly Ser Leu Phe Leu Ala Val Val Ala Ile Leu Pro Val Phe Phe Ile Lys Phe Ala Asp
GGA TCA CTT TTC CTC GCA GTG GTG GCG ATC TTG CCG GTA TTC TTT ATT AAG TTT GCC GAC 1374

Leu Pro Gln Ala Ile Gln Ile Gly Gly Thr Gly Leu Leu Ile Val Val Gly Val Ala Leu
TTG CCA CAG GCG ATT CAA ATT GGT GGT ACG GGC TTG CTC ATC GTC GTC GGG GTT GCC CTT 1434

Asp Thr Met Lys Gln Ile Glu Ala Gln Leu Ile Lys Arg Ser Tyr Lys Gly Phe Ile Lys
GAT ACG ATG AAG CAA ATT GAA GCA CAG TTG ATC AAA CGT TCT TAT AAA GGG TTT ATT AAG 1494

TER

TAG GGG GAG CAG GGA AGC GTG GTG TCT TCC CGT TCG CCT TCT GTT CTT AAA TTA TTA GAC 1554

Adk

Met Asn Leu Asn Leu Met Gly Leu Pro Gly Ala Gly Lys Gly Thr
GTG GAG GGG ATA GAC TTG AAT CTG AAC CTT ATG GGA CTT CCT GGT GCT GGT AAA GGT ACA 1614

SD

Gln Ala Glu Lys Ile Ile Glu Lys Tyr Gly Ile Pro His Ile Ser Thr Gly Asp Met Phe
CAG GCA GAA AAG ATC ATT GAG AAG TAC GGC ATC CCA CAC ATT TCA ACA GGT GAC ATG TTT 1674

NspI

Arg Ala Ala Met Lys Asn Glu Thr Glu Leu Gly Leu Lys Ala Lys Ser Tyr Met Asp Ala
CGT GCT GCG ATG AAG AAC GAG ACC GAG CTT GGA TTA AAA GCA AAA TCG TAC ATG GAT GCA 1734

Gly Glu Leu Val Pro Asp Glu Val Thr Ile Gly Ile Val Arg Asp Arg Leu Ser Gln Asp
GGG GAA CTG GTT CCT GAT GAA GTA ACG ATT GGT ATC GTT CGG GAT CGT CTC AGT CAA GAC 1794

Asp Cys Gln Asn Gly Phe Leu Leu Asp Gly Phe Pro Arg Thr Val Ala Gln Ala Glu Ala
GAT TGC CAA AAT GGC TTT TTG CTT GAC GGG TTT CCA CGG ACT GTC GCT CAA GCA GAA GCG 1854

Leu Glu Asp Ile Leu Ala Ser Leu Asp Lys Lys Leu
TTA GAA GAT ATT TTG GCG TCA CTT GAT AAA AAG CTT 1890

HindIII

Fig. 3. Nucleotide and deduced amino acid sequences of the 1.8 kb *Hind*III fragment. Nucleotides are numbered from the 5'-end. The possible ribosome binding sites (SD) preceding each of the ORFs are underlined.

Fig. 4. Hydrophobic profiles for the SecY protein of alkalophilic *Bacillus* sp. C125 and *B. subtilis*. Hydrophobic profiles were calculated by the procedure of Kyte & Doolittle (1982).

The hydrophobic profile of alkalophilic *Bacillus* sp. C125 SecY was similar to that of *B. subtilis* SecY (Fig. 4). It appeared that alkalophilic *Bacillus* sp. C125 SecY contains the same 10 hydrophobic regions as those of *B. subtilis* SecY. Highly homologous regions between *B. subtilis* and alkalophilic *Bacillus* sp. C125 SecY were found in the membrane domains (about 78% identity) and the cytoplasmic domains (73% identity). Less homologous regions (43% identity) were present in extracellular domains (Fig. 5). Two possibilities are considered. First, more variation is tolerated in the extracellular region and the greater divergence is due to genetic drift because *E. coli* and *B. subtilis* SecY extracellular regions were not so conserved compared with those of membrane or intracellular domains. Second, higher sequence variation seen with the extracellular domains of the protein may be a response to the alkaline environment. Due to the high level of variation in the extracellular regions it is difficult to pinpoint and investigate possible changes in the alkalophilic *Bacillus* sp. C125 SecY protein that may contribute to its adaptation to alkaline conditions. A comparison of the amino acid composition of the extracellular domains,

Fig. 5. Alignment of identical residues (*) between the predicted amino acid sequences for alkalophilic *Bacillus* sp. C125 *secY* and *B. subtilis* *secY* gene products. The membrane-spanning regions (I-X) were deduced according to the results of hydrophobic profiles calculated by the procedure of Kyte & Doolittle (1982). Extracellular regions (1*-6*) and intracellular regions (1-6) are also indicated. A dot indicates similar amino acids.

Table 1. Comparison of amino acid composition of the extracellular region of alkalophilic *Bacillus* C-125 and *Bacillus subtilis* SecY proteins

Extracellular region			Extracellular region		
Amino acid	C125	<i>B. subtilis</i>	Amino acid	C125	<i>B. subtilis</i>
Ile	4	6	Thr	6	7
Leu	10	8	Gln	4	10
Gly	11	10	Asn	4	6
Ala	8	7	Ser	4	5
Val	5	4	Arg	2	1
Phe	11	9	Lys	1	3
Pro	7	2	Thr	2	3
Met	0	1	Glu	3	3
Trp	1	1	Asp	6	4
His	2	2			

however, indicated that in the alkalophilic *Bacillus* sp. C125 protein, the number of proline residues increased mainly in extracellular domains 2* and 4* and the number of glutamine residues decreased mainly in extracellular domain 3*, compared with those of the *B.*

subtilis protein (Table 1). The role of the extracellular region of the SecY protein is not clear but it appears that these amino acid residues may play some role in alkaline conditioning of the protein from alkalophilic *Bacillus* sp. C125.

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