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Proteome analysis of *Bacillus subtilis* extracellular proteins: a two-dimensional protein electrophoretic study

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To analyse the proteome of *Bacillus subtilis* extracellular proteins, extracellular protein samples were prepared from culture media (minimal medium containing 0·4% glucose) of parental *B. subtilis* 168, a *secA*-temperature sensitive mutant and an *ffh* conditional mutant, and examined by two-dimensional gel electrophoresis. Approximately 100 to 110 spots were visualized in a gel of *B. subtilis* 168 extracellular proteins. Over 90% and 80% of these disappeared in the absence of SecA and Ffh, respectively. Thirty-eight obvious spots on the gel of the *B. subtilis* 168 preparation were selected and compared with spots obtained under SecA- or Ffh-deficient conditions. The appearance of 36 of these 38 spots depended on SecA and Ffh. Nineteen additional extracellular proteins were detected in cultures maintained in cellobiose, maltose and soluble starch. Among 23 proteins of which the N-terminal amino acid sequences were determined, 17 were extracellular proteins having signal peptides in their precursor form. Two membrane proteins, YfnI and YflE, were cleaved behind ^{226}Ala-Tyr-Ala^{228} and ^{213}Ala-Leu-Ala^{215}, respectively, and of which products seemed to be liberated into the culture medium. The production of YfnI and YflE were also dependent on SecA and Ffh. These results indicate that most extracellular proteins target to and translocate across the cytoplasmic membrane by co-operation between the signal-recognition particle and Sec protein-secretion pathways. In contrast, a spot for Hag appeared independent from SecA and Ffh. Intracellular proteins Gap, SodA and KatA were identified in the extracellular protein samples. On the basis of these results and computer searches, it was predicted that *B. subtilis* produces 150 to 180 proteins extracellularly.

Keywords: *Bacillus subtilis*, extracellular proteins, proteome analysis, *secA* and *ffh* mutants, two-dimensional gel electrophoresis

INTRODUCTION

Bacillus subtilis secretes high levels of extracellular proteins and generates a heat-resistant endospore under poor nutrient conditions. However, the numbers and quantities of secreted proteins remain unknown. The European–Japanese *B. subtilis* genome project has determined that the chromosomal DNA of this organism is 4215 kb in length and 4100 genes encoding proteins and peptides have been predicted (Kunst *et al.*, 1997). Among the 4100 gene products, the number of extracellular proteins and the secretion machinery proteins that facilitate their extracellular production, remain unknown. To determine the numbers of extracellular proteins, we resolved the proteome of *B. subtilis* 168 extracellular proteins by two-dimensional (2D) gel protein electrophoresis. The dependence of each protein on SecA and the signal-recognition particle (SRP) was

Abbreviations: 2D, two dimensional; SRP, signal-recognition particle.

The SWISS-PROT accession numbers for the N-terminal amino acid sequences reported in this paper are: P00691 for AmyE; P54507 for CotN; O07921 for Csn; P09124 for Gap; P26901 for KatA; P39116 for Pel; P39824 for PenP; P54375 for SodA; P29141 for Vpr; Q07833 for WapA; P54423 for WprA; P54327 for XkdG; Q45071 for XynD; P94421 for YclQ; O31803 for YcnM; O05512 for YdhT; O34952 for YflE; O06487 for YfnI; O31737 for YlqB; P96740 for YwtD; P42110 for YxaK; P94356 for YxkC.

determined by comparing the 2D profiles of proteins resolved from *B. subtilis* 168 preparations with those from SecA and Ffh conditional mutants.

Protein-secretion pathways have been extensively studied in *Escherichia coli*, yeast and mammals. A presecretory protein precursor in *E. coli* is thought to be recognized by a molecular chaperone such as SecB and is targeted to membrane-bound SecA, which is the peripheral ATPase subunit of translocase (Collier *et al*., 1988, Hartle *et al*., 1990, Valent *et al*., 1998). The precursor is then translocated across the cytoplasmic membrane through a SecA ATPase-dependent translocase consisting of SecA, SecE, SecG, SecY and other membrane proteins (Douville *et al*., 1995). SecA promotes protein translocation during cycles of SecA insertion into and removal from SecEYG on the membrane (Nishiyama *et al*., 1996). Therefore, SecA plays a central role in the protein-secretion pathway in *E. coli*. A *secA* mutant of *B. subtilis* has been isolated as the cell-division mutation *div341* (Miyakawa & Komano, 1980). After cloning and characterizing the wild-type gene that can complement the *div341* mutation, the wild-type gene was designated *secA*. The predicted amino acid sequence of the gene show 50% identity with that of *E. coli* SecA. The *div341* mutation is a nucleotide replacement of C to T at position 1292 of *secA* (Sadaie *et al*., 1991, Takamatsu *et al*., 1992). SecA homologues have been found in all eubacteria and chroloplasts (Nohara *et al*., 1995). Homologues of *E. coli* SecE, SecG, SecD, SecF and SecY have been also identified in *B. subtilis*, but not SecB (Bolhuis *et al*., 1998; van Wely *et al*., 1999).

In contrast, a SecA homologue has not been identified in yeast and mammalian cells (Rapoport *et al*., 1996). The SRP plays a central role in recognizing and targeting presecretory proteins to the endoplasmic reticulum membrane in mammalian cells (Walter & Blobel, 1982). SRP is a ribonucleoprotein complex composed of one RNA (SRP 7S RNA) and six proteins (SRP9, SRP14, SRP19, SRP54, SRP68 and SRP72; Walter & Johnson, 1994). SRP54 binds to the signal peptide of a nascent presecretory protein, probably through direct interaction of the hydrophobic region of its M-domain with the hydrophobic region of the signal peptide (Lutcke *et al*., 1992; Lutcke, 1995). Furthermore, homologues of SRP54, Ffh (fifty-four homologues) in bacteria, have also been identified in animals, plants, yeast, eubacteria, archaea and chloroplasts (Keenan *et al*., 1998). *B. subtilis* Ffh forms an SRP-like particle complexed with small cytoplasmic RNA (scRNA, a homologue of SRP RNA) and HBsu, a histone-like protein (Nakamura *et al*., 1999).

In *B. subtilis*, SecA and Ffh are essential for normal cell growth and protein translocation. SecA or Ffh depletion inhibits the secretion of extracellular α-amylase, β-lactamase and β-lactamase fusion proteins carrying the signal peptide of *B. subtilis* alkaline protease, penicillin-binding protein 5* or cyclodextrin glucanotransferase (Honda *et al*., 1993, Bunai *et al*., 1996, Takamatsu *et al*., 1997). Furthermore, SecA and Ffh can intrinsically bind to precursors of β-lactamase and its fusion proteins *in vitro* but not to their mature forms. Since Ffh interacts with SecA and Ffh enhances the binding of SecA to precursors, we have proposed that the co-operation of *B. subtilis* SecA and Ffh constitutes an efficient protein-secretion pathway (Bunai *et al*., 1999).

In this study, we predict that 150 to 180 *B. subtilis* proteins are extracellular. By comparing 2D extracellular protein profiles from *B. subtilis* 168, and SecA and Ffh conditional mutants, 17 out of 23 proteins where the N-terminal amino acid sequence was determined were extracellular, and two were membrane proteins that appeared to be librated into the medium after processing. The appearance of these 19 proteins depended on both SecA and Ffh. In contrast, Hag and Gap appeared even under SecA- and Ffh-deficient conditions and SodA appeared as a large spot under Ffh deficient conditions.

METHODS

Strains and culture conditions. *B. subtilis* 168 (*trpC2*) from laboratory stocks was the standard strain. The *secA* temperature-sensitive mutant *B. subtilis* TB301 (*trpC2*, *secA341*) is a transformant of *B. subtilis* 168 (Takamatsu *et al*., 1994). The *ffh* conditional mutant *B. subtilis* DF46 (*trpC2*, *ffh*::*pTUE905*) is also a transformant of *B. subtilis* 168, in which *ffh* expression is regulated by the IPTG-inducible promoter *spac-1*, derived from pDH88 (Henner, 1990, Honda *et al*., 1993). These strains were cultured in the minimal medium used by the *B. subtilis* genome project under the conditions described below.

Extracellular sample preparation for the proteome analysis. A final concentration of 3 mM PMSF was added to culture medium (100 ml each) at the late-exponential phase of growth to prevent proteolytic digestion, then cells were removed by centrifugation followed by filtration through a 0·2 nm nitrocellulose filter (Millipore). Then 4 ml 50% TCA was added to the medium, mixed well and placed on ice for 30 min. The aggregated proteins were precipitated by centrifugation, washed three times in cold 70% ethanol (−20 °C), dried and dissolved in sample solution consisting of 8 M urea, 1% Triton X-100, 15 mM DTT and 5 mM PMSF.

Analytical 2D gels. The first dimension in the 2D gel electrophoresis was isoelectrically foucused on an Electro Immobiline Dry Strip pH 3–10L (11cm) (Amersham Pharmacia). Extracellular preparations containing 20 μg protein mixture dissolved in the sample solution containing 1% (v/v) Formalyta 3–10·5 was applied to the first dimension. Gels were focused for 15 h at 400V followed by 1 h at 600V using a Multiphor II (Amersham Pharmacia). After placing in equilibration buffer A [50 mM Tris/HCl, pH 6·8, containing 6 M urea, 30 % (v/v) glycerol, 2·5% SDS and 0·25% DTT] for 15 min and buffer B [50 mM Tris/HCl, pH 6·8, 6 M urea, 30% (v/v) glycerol, 2·5% SDS, 0·25% DTT and 4·5% iodoacetic acid] for 5 min, the isoelectric focusing gels were embedded in 0·25 M Tris/HCl, pH 6·8, 0·25% SDS, 1% agarose onto 12% SDS polyacrylamide gels (14 × 14 × 0·1 cm), and the proteins were resolved in the second dimension by a constant current of 20 mA until the bromphenol blue marker entered the stacking gel, followed by 40 mA for 3 h (Rabilloud

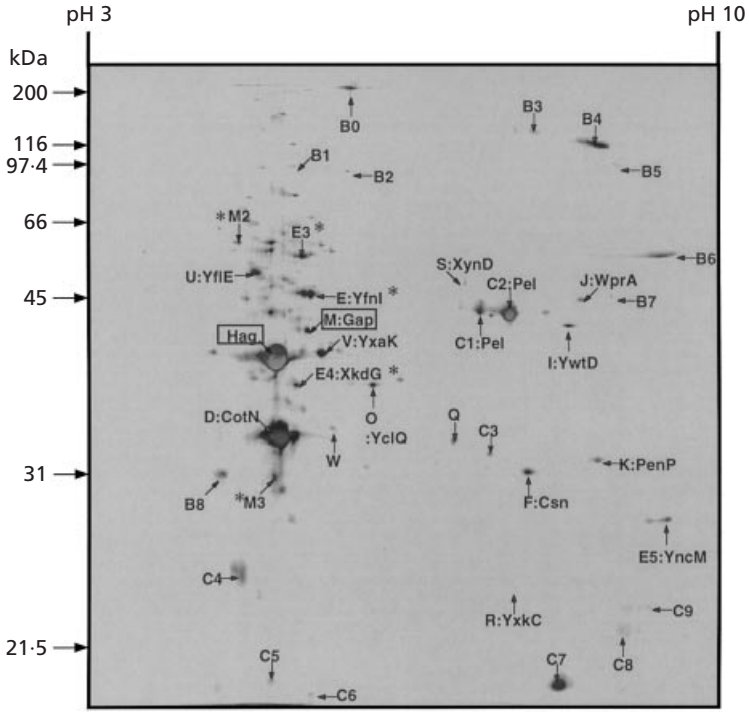

Fig. 1. Extracellular proteins of *B. subtilis* 168 profiled on a 2D gel. Cells were grown in minimal medium containing 0·4% glucose and 50 μg tryptophan ml^{-1} at 37 °C and sampled at the late-exponential phase of growth (see arrows, Fig. 2b). Thirty-eight spots were selected, of which 31 disappeared under SecA- and Ffh-deficient conditions, 5 spots (*) disappeared in the absence of SecA and were faint in the absence of Ffh and 2 (boxed) were still under both SecA- and Ffh-deficient conditions.

et al., 1997). The gels were fixed in an 80% ethanol/20% acetic acid mixture and silver stained as described by Morrissey (1981).

Preparative 2D gel electrophoresis and N-terminal amino acid sequencing. For preparative 2D gel electrophoresis, 200–250 μg proteins from the extracellular preparations were separated by 2D gel electrophoresis as described above. The protein spots in the gels were transferred onto PVDF membranes (Millipore) by the method of Matsudaira (1987), then stained with 0·1% Coomassie Brilliant Blue R250. Spots were excised from the membrane and sequenced using an Applied Biosystems 492 protein sequencer.

Detection of SecA and Ffh. Cells were harvested by centrifugation and cell lysates were prepared as described by Takamatsu *et al*. (1994, 1997). Total lysate proteins (10 mg each) were resolved by SDS-PAGE and blotted onto PVDF membranes. Bands for SecA and Ffh were detected using anti-*B. subtilis* SecA antibody and anti-*B. subtilis* Ffh antibody, respectively, by immunoblotting as described by Takamatsu *et al*. (1994, 1997)

RESULTS

A computer search for extracellular proteins

We used the PSORT software to predict that about 250 candidates were secretory proteins among the whole 4100 proteins of *B. subtilis*, which were deposited in a *B. subtilis* database BSORF. Among these 250 candidate proteins, 138 had N-terminal amino acid sequences similar to that of a typical signal pepetide of *B. subtilis*. A few basic amino acids at the N terminus were followed by a hydrophobic amino acid core and an Ala-X-Ala motif for signal peptide cleavage. Of these 138 proteins, 63 were known or their functions were predicted by a database search. These included 13 enzymes related to carbohydrates and their metabolism, 11 proteases or peptidases, 6 enzymes related to phosphate metabolism, 6 peptide hormones, 6 penicillin-binding proteins, 6 proteins related to the cell wall and its metabolism, 5 sporulation-specific proteins, 4 proteins concerned with competence for DNA incorporation, 2 lipases, 2 penicillinases, and YacD and YliL. The functions of the remaining 75 proteins remain unknown.

2D analysis of the extracellular proteome

Bacillus subtilis 168 produces high levels of extracellular proteins at the early stationary phase of growth. We made extracellular preparations at the late-exponential phase of growth in minimal medium containing 0·4% glucose to avoid possible contamination with cytoplasmic proteins due to partial cell lysis. The protein composition of the samples was then examined by 2D gel electrophoresis (Fig. 1). *B. subtilis* 168 generated 100–110 detectable spots on the gel. Among these, thirty eight obvious spots were selected and assigned names as shown in Fig. 1. Their N-terminal amino acid sequences were analysed; the results of 20 of these are summarized in Table 1. After searching the database, spots C1 and C2 were defined as the gene products of *pel* and spots J and C4 were those of *wprA*. Therefore, a total of eighteen gene products were identified. The N-terminal amino acids of spots J and C4 corresponded to Ala-414 and Ala-32 of the predicted amino acid sequence of *wprA*, respectively. Therefore, spot J was CWBP52 serine protease and spot C4 was CWBP23 (Margot & Karamata, 1996; Stephenson & Harwood, 1998). Spot B8 was the predicted gene product of *wapA*. The predicted amino acid sequence of *wapA* indicates that a signal peptide (Met-1 to Ala-28) is located in its precursor. However, the N-terminal amino acid of spot

Table 1. N-terminal amino acid sequences of spots on 2D gel, predicted genes and subcellular location of gene products.

+, Obvious spots on 2D gels stained by silver by the method of Morrissey (1981). +/− or − indicate faint or no spots under SecA- or Ffh-deficient conditions.

Spot	TB301		DF46		Gene	Function	Predicted subcellular location	Putative signal peptide and/or prosequence	Analysed sequence	Reference
	30 °C	42 °C	+ IPTG	− IPTG						
Cl	+	−	+	−	*pel*	Pectate lyase	Extracellular	MKKVMLATALFLGLTPAGANS	ADLGHQTLGSN	Nasser *et al.* (1993)
C2	+	−	+	−	*pel*	Pectate lyase	Extracellular	MKKVMLATALFLGLTPAGANS	ADLGHQTLGSN	Nasser *et al.* (1993)
D	+	−	+	−	*cotN* (*tasA*)	Spore-coat-associated protein	Extracellular	MGMKKKLSLGVASAALGLALVGGGTWA	AFNDIKSKDATF	Stover & Driks (1999)
F	+	−	+	−	*csn*	Chitosanase	Extracellular	MKISMQKADFWKKAAISLLVFTMFFTLMMSETVFA	AGLNKDQKRR	Tominaga & Tsujisaka (1975)
I	+	−	+	−	*ywtD*	Similar to murein hydrolase	Extracellular	VNTLANWKKFLLVAVIICFLVPIMTKAEIAEA	DTSSELIVSEA	Kunst *et al.* (1997)
J	+	−	+	−	*wprA*	Cell-wall-associated protein	Extracellular	MKRRKFSSVVAAVLIFALIFSLFSPGTKAAA, 32–413 aa	ANDIQYPYQWP	Margot & Karamata (1996)
K	+	−	+	−	*penP*	Penicillinase	Extracellular	MKLKTKASIKFGICVGLLCLSITGFTPFFNSTFLAEA	KSIEDTNMASC	Izui *et al.* (1980)
O	+	−	+	−	*yclQ*	Unknown	Extracellular	MKKFALLFIALVTAVVISA, CGNQSTSSKG	SDTKKEQITVK	Kunst *et al.* (1997)
R	+	−	+	−	*yxkC*	Unknown	Extracellular	MRHKIITFILAVVVIIIIGNMIGGGGGSEA	TSKTSSSSKAE	Kunst *et al.* (1997)
S	+	−	+	−	*xynD*	Endo-1,4-*β*-xylanase	Extracellular	MRKKCSVCLWILVLLLSCLSGKSAYA	ATSTTIAKHIG	Gosalbes *et al.* (1991)
U	+	−	+	−	*yflE*	Similar to anion-binding protein	Membrane	1–215 aa	DSSDVTEVENY	Kunst *et al.* (1997)
V	+	−	+	−	*yxaK*	Unknown	Extracellular	MVKSFRMKALIAGAAVAAAVSAGAVSDVPAAKVLQ-PTAAYA	AETVFSQNNGA	Kunst *et al.* (1997)
E5	+	−	+	−	*yncM*	Unknown	Extracellular	MAKPLSKGGILVKKVLIAGAVGTAVLF, GTLSSGIPGLPAADAQVAKA	ASELPNCDGGR	Kunst *et al.* (1997)
B8	+	−	+	−	*wapA*	Cell-wall-associated protein	Extracellular	MKKRKRRNFKRFIAAFLVLALMISLVPA, 29–96 aa	YLDPIHTKETP	Foster (1993)
C4	+	−	+	−	*wprA*	Cell-wall-associated protein	Extracellular	MKRRKFSSVVAAVLIFALIFSLFSPGTKAAA	AGAIDQAAL	Margot & Karamata (1996)
C7	+	−	+	−	*YlqB*	Unknown	Extracellular	MKKIGLLFMLCLAALFTIGFPAQQADA	AEAPYKASITN	Kunst *et al.* (1997)
E	+	−	+	+/−	*yfnI*	Unknown	Membrane	1–228 aa	SSDDLTSVEM	Kunst *et al.* (1997)
E4	+	−	+	+/−	*xkdG*	Unknown	Extracellular	MRNQEIIRK	AEMSLSALKS	†
Hag	+	+	+	+	*hag*	Flagellin	Extracellular		MRINHNIAALN	De Lange *et al.* (1976)
M	+	+	+	+	*gap*	Glyceraldehyde-3-phosphate dehydrogenase	Cytosol	M	AVKVIGINGFGR	Graumann *et al.* (1996)
P	+	+	+	+	*sodA*	Superoxide dismutase	Cytosol	M	AYELPELPYAY	Antelmann et al. (1997)

*SWISS-PROT accession no. BLAC_BACSU.

†SWISS-PROT accession no. XKDG_BACSU.

B8 corresponded to Tyr-98 of the sequence. Therefore, WapA is processed after secretion (Foster, 1993). The N-terminal amino acid of spot O corresponded to Gly-31 of the predicted sequence of *yclQ*. Met-1 to Ala-20 of the precursor of YclQ was the predicted signal peptide. Therefore Cys-21 to Gly-30 constitutes the propeptide. These results indicate that 13 of the 18 gene products are secretion proteins. However the 18 gene products contained two membrane proteins (YflE and YfnI), flagellin monomer (Hag), the intracellular protein Gap (glyceraldehyde-3-phosphate dehydrogenase) and XkdG, an extracellular protein encoded in the PBSX genome.

To determine which proteins are dependent on SecA and/or Ffh for secretion, extracellular proteins were prepared from strains TB301 and DF46. Fig. 2(a) shows that the growth of *B. subtilis* TB301 at 30 °C was similar to that of 168 after replacing the medium at the late-exponential phase of growth followed by a further 1 h incubation at 30 °C or 42 °C, while the growth of strain TB301 at 42 °C was delayed. Under these conditions, we found that SecA in the cell lysate of TB301 at 42 °C completely disappeared within 15 min, while a similar amount of SecA was detected in the TB301 cell lysate cultured at 30 °C and in 168 cell lysates at 30 °C and 42 °C (Fig. 3a). The incorporation of ^{35}S-Met into the TCA-precipitable fraction of strain TB301 was not affected after 1 h cultivation under non-permissive conditions (data not shown).

We prepared extracellular proteins from TB301 after a 1 h incubation at either 30 °C or 42 °C, then compared the 2D profiles of the resolved proteins with those of the 168 preparation. The spots in the 2D gel of TB301 at 30 °C were faint but the 38 selected spots were clear (Fig. 4a). In contrast, the 2D profiles of TB301 at 42 °C (Fig. 4b) completely differed from those at 30 °C and 168 (Fig. 1). Over 90% of the spots resolved from *B. subtilis* 168, except for Hag, Gap and a few others, disappeared. The presence and absence of each spot from the 42 °C and 30 °C preparations are summarized in Table 1.

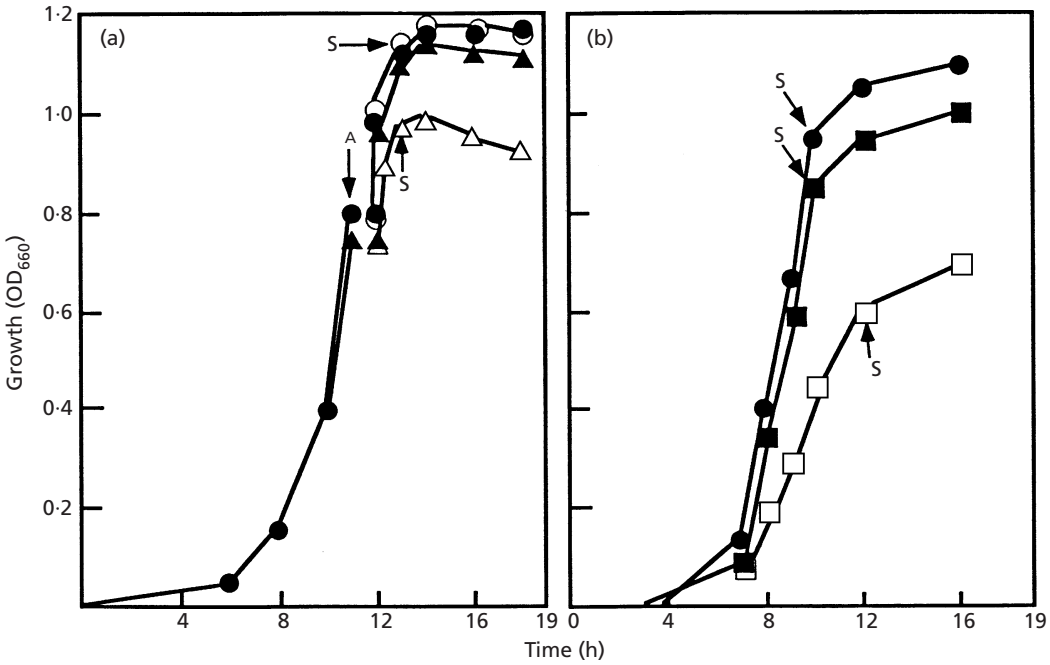

Fig. 2. Growth courves for *B. subtilis* 168, TB301 and DF46. (a) To examine the effect of SecA and on the production of secretory proteins, *B. subtilis* 168(●, ○) and TB301 (▲, △) were incubated at 30 °C for 10 h (arrow A) in minimal medium containing 0·4% glucose and 50 μg tryptophan ml^{-1}. Cells were precipitated by centrifugation at 20 °C, 3000 ***g*** for 15 min and suspended in the original volume of medium warmed at 30 °C and 42 °C. Cell suspensions were then incubated at 30 °C (●, ▲) and 42 °C (○, △). Samples for 2D resolution were withdrawn after 1 h at each temperature (arrow S). To analyse the presence and absence of SecA by Western blotting (Fig. 3), the culture media at 30 °C and 42 °C were sampled after 0, 15 and 60 min. (b) To analyse the effect of Ffh on the protein composition of secretory proteins, strains 168 (●) and DF46 (■, □) were cultured at 37 °C in the presence (■) or absence (●, □) of 1 mM IPTG. Extracellular proteins and cells with which to analyse the effect of Ffh were sampled at the periods indicated (arrow S).

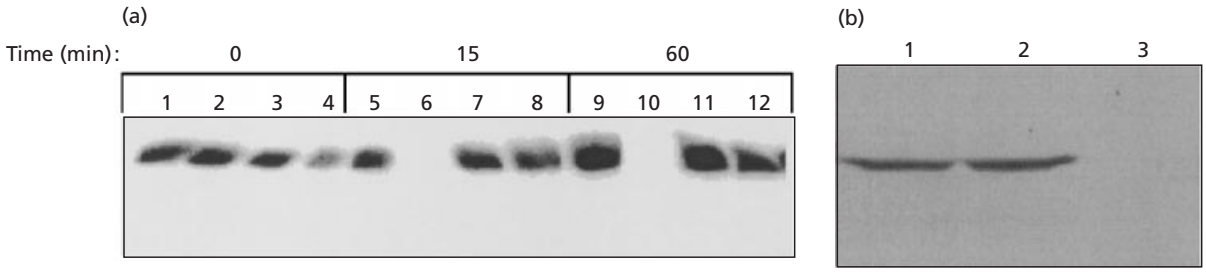

Fig. 3. (a) Absence of SecA in TB301 cell lysate cultured at 42 °C. After the replacement of the medium as described in Fig. 2a, cells grown at 30 °C and 42 °C were harvested at 0 (lanes 1–4), 15 (lanes 5–8) and 60 min (lanes 9–12). Cell lysates were prepared and SecA in each preparation was resolved by PAGE, then detected by immunoblotting using rabbit antiserum against *B. subtilis* SecA. Lanes 1, 5 and 9, TB301 cells grown at 30 °C; lanes 2, 6 and 10, TB301 cells grown at 42 °C; lanes 3, 7 and 11, strain 168 cells grown at 30 °C and lanes 4, 8 and 12, strain 168 cells grown at 42 °C. (b) Presence and absence of Ffh in DF46 cells grown with and without IPTG. Ffh was resolved by PAGE, then detected by immunoblotting using rabbit antiserum against *B. subtilis* Ffh. Lane 1, 168 cells grown in the absence of IPTG; Lane 2, DF46 cells grown in the presence of IPTG; Lane 3, DF46 cells grown in the absence of IPTG.

Similarly, 2D profiles of DF46 preparations cultured in the presence or absence of IPTG were compared to that of *B. subtilis* 168 (Fig. 4c, d). At the sampling times shown in Fig. 2b, we detected a clear band for Ffh by Western blotting the DF46 cell lysate in the presence, but not in the absence of IPTG (Fig. 3b). The 2D profile of the DF46 preparation in the presence of IPTG was almost identical to that of strain 168. In contrast, the profile in the absence of IPTG differed from those of DF46 in the presence of IPTG and of strain 168. Hag, Gap and the additional 12 spots indicated, were identical to those found in the preparation from stain 168. The remaining 30 or so spots seemed to be artifacts caused by partial cell lysis during culture. Spot P is superoxide dismutase (SodA), which is a cytosolic protein (Antelmann *et al*., 1997).

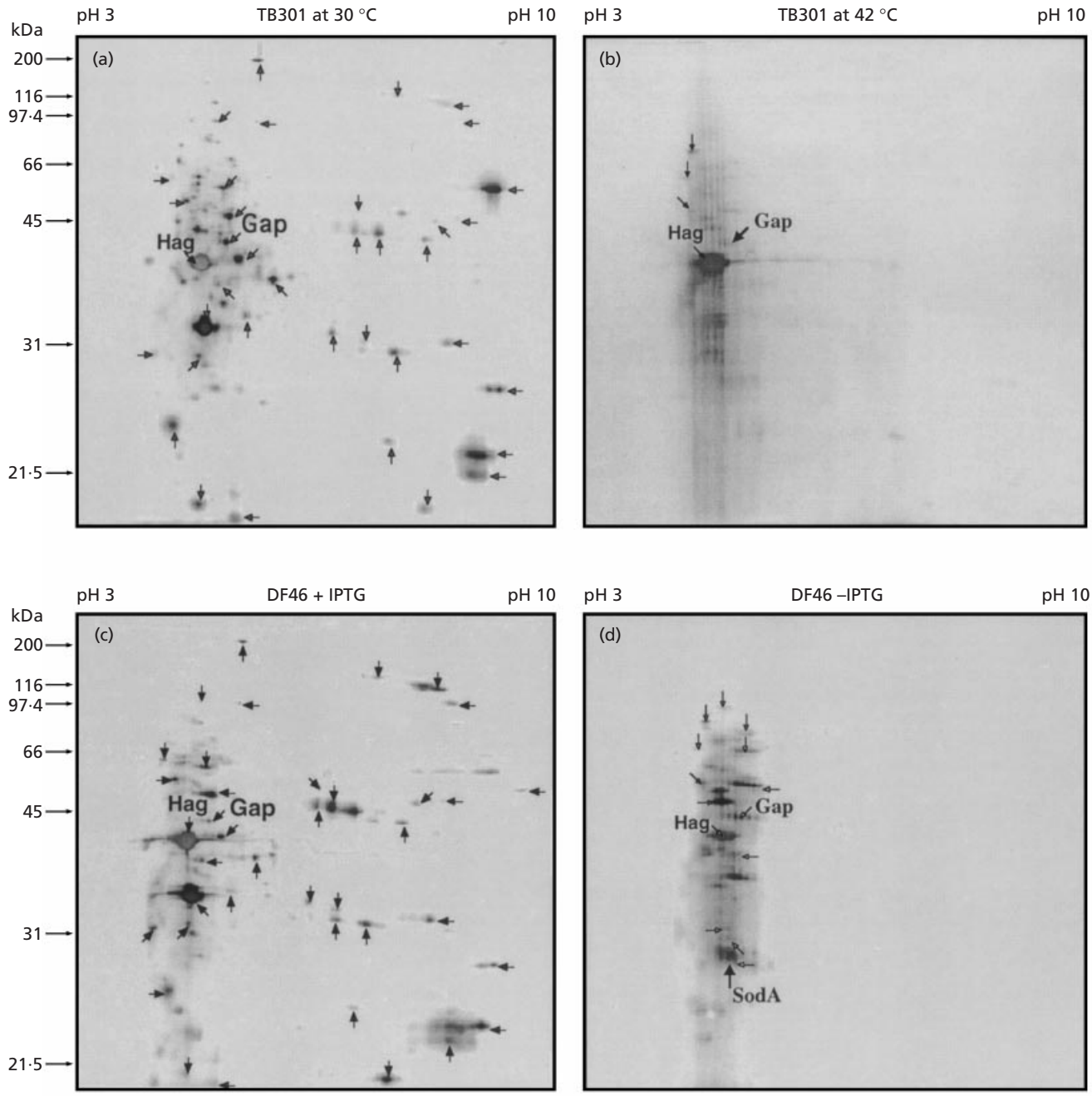

Fig. 4. 2D profiles of extracellular proteins obtained from TB301 incubated at 30 °C (a) and 42 °C (b) and DF46 incubated in the presence (c) and absence (d) of IPTG. The preparations were sampled at the periods indicated in Fig. 2. The 38 spots shown in Fig. 1 are indicated by arrows in (a) and (c). The same spots as those of the preparation from strain 168 except SodA are indicated by arrows in (b) and (d).

A comparison of the 2D profiles of strains 168, TB301 at 30 °C and 42 °C and DF46 in the presence or absence of IPTG, classified the 38 spots (Fig. 1) into three groups as follows. Thirty-one spots completely disappeared under both SecA- and Ffh-deficient conditions and the 5 spots marked with asterisks (spots M2, E3, E, E4 and M3) disappeared under SecA-deficient conditions and were faint in the absence of Ffh. These 36 proteins seem to be extracellular, although two (YflE, spot U, and YfnI, spot E) were predicted membrane proteins. These results indicate that both SecA and Ffh function in the secretion of most extracellular proteins.

On the other hand, Hag and Gap were not inhibited by the absence of SecA or Ffh. In *E. coli*, the production of Hag in the culture medium depends on a specific pathway (Hueck, 1998, Namba *et al*., 1989). Therefore, the extracellular production of Hag in *B. subtilis* may

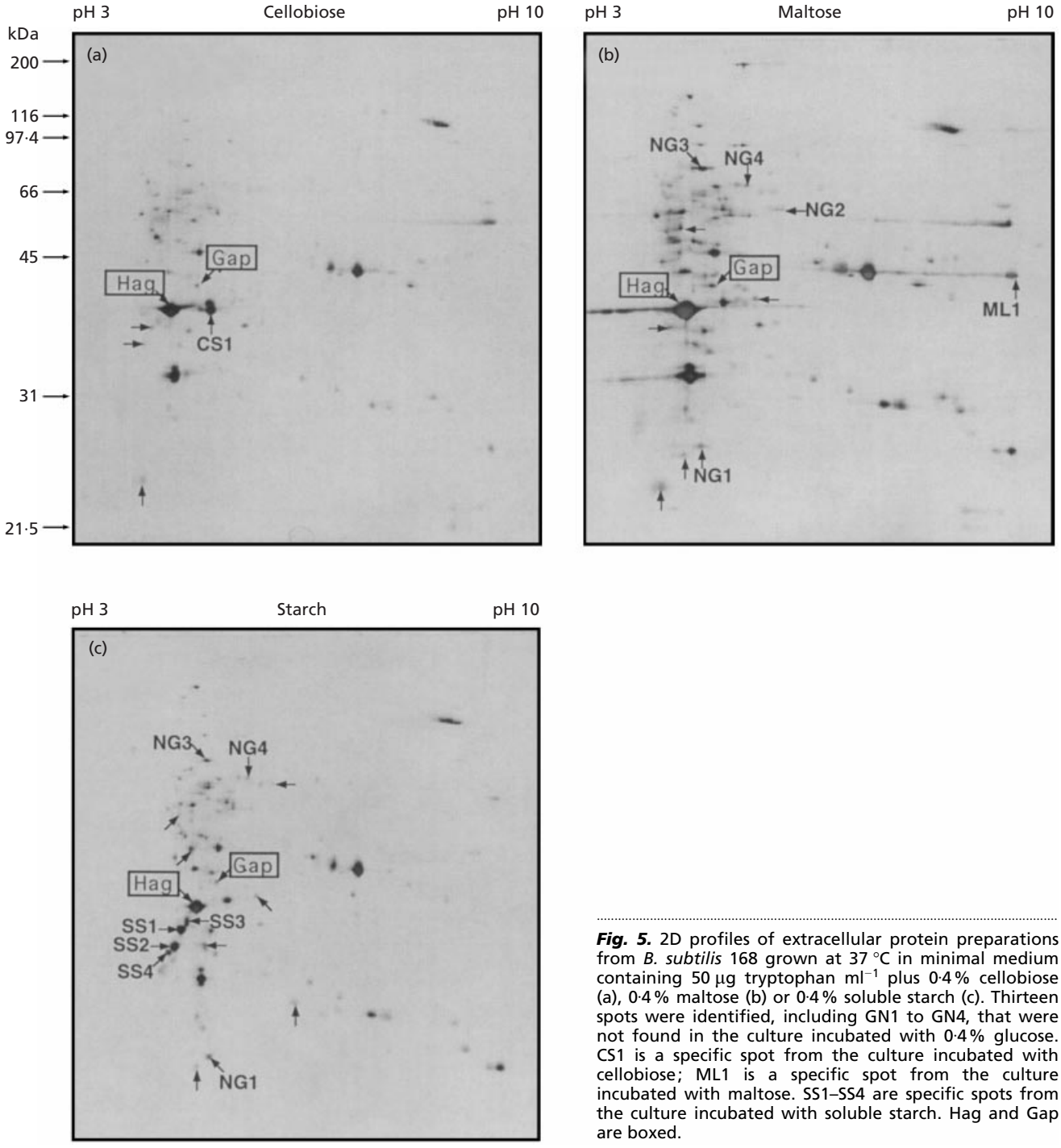

Fig. 5. 2D profiles of extracellular protein preparations from *B. subtilis* 168 grown at 37 °C in minimal medium containing 50 μg tryptophan ml^{-1} plus 0·4% cellobiose (a), 0·4% maltose (b) or 0·4% soluble starch (c). Thirteen spots were identified, including GN1 to GN4, that were not found in the culture incubated with 0·4% glucose. CS1 is a specific spot from the culture incubated with cellobiose; ML1 is a specific spot from the culture incubated with maltose. SS1–SS4 are specific spots from the culture incubated with soluble starch. Hag and Gap are boxed.

also be independent of the SRP/Sec protein-secretion system. Gap is a predicted cytosolic protein (Graumann *et al.*, 1996).

Effect of carbon source on the protein composition of the extracellular preparations

We did not identify any spots for typical extracellular enzymes such as α-amylase and proteases. We surmised that this was caused by using 0·4% glucose as the carbon source in the medium, which would cause catabolite repression.

Fig. 5 (a, b and c) shows the 2D profiles of the extracellular proteins prepared from strain 168 cultured in minimal medium containing 0·4% cellobiose, 0·4% maltose or 0·4% soluble starch, respectively. Growth rates in cellobiose were similar to those in glucose, but were slightly delayed in maltose and soluble starch. Spots for Hag and Gap were detected in all three 2D profiles, and the other spots were essentially identical to

Table 2. N-terminal amino acid sequences of spots found on 2D gels of *B. subtilis* 168 preparations grown on cellobiose, maltose and soluble starch.

Spot	Gene	Function	Predicted subcellular location	Putative signal sequence and/or prosequence	Analysed sequence	Reference
CS1	*ydhT*	Mannan endo-l,4-*β*-mannosidase	Extracellular	MFKKHTISLLIIFLLASAVLAKPIEA	HTVSPVNPNAQ	Kunst *et al.* (1997)
NG1	*sodA*	Superoxide dismutase	lntracellular	M	AYELPELPYAY	Antelmann *et al.* (1997)
NG2	*katA*	Vegetative catalase (catalase I)	lntracellular	M	SSNKLTTSWG	Bol & Yasbin (1991)
NG3	*vpr*	Minor extracellular serine protease	Extracellular	MKKGIIRFLLVSFVLFFALSTGITGVQAAPA, 32–160 aa	MDDSAPYIGA	Sloma *et al.* (1991)
NG4	*amyE*	α-Amylase	Extracellular	MFAKRFKTSLLPLFAGFLLLFHLVLAGPAAASA	ETANKSNELT	Yang *et al.* (1983); Yamazaki *et al.* (1983)
SS1	*hag*	Flagellin monomer	Extracellular	MRINHNIAALNTLNRLS	SNNSASQKN	DeLange *et al.* (1976)
SS2	*hag*	Flagellin monomer	Extracellular	MRINHNIAALNTLNRLSSNNSASQKNMEKLS	SGLRINRAGD	DeLange *et al.* (1976)
SS3	*hag*	Flagellin monomer	Extracellular	MRINHNIAALN	TLNRLSSNNS	DeLange *et al.* (1976)
SS4	*hag*	Flagellin monomer	Extracellular	MRINHNIAALNTLNRLSSNNSASQKNMEKLSSGLRIN	RAGDDAAGLA	DeLange *et al.* (1976)

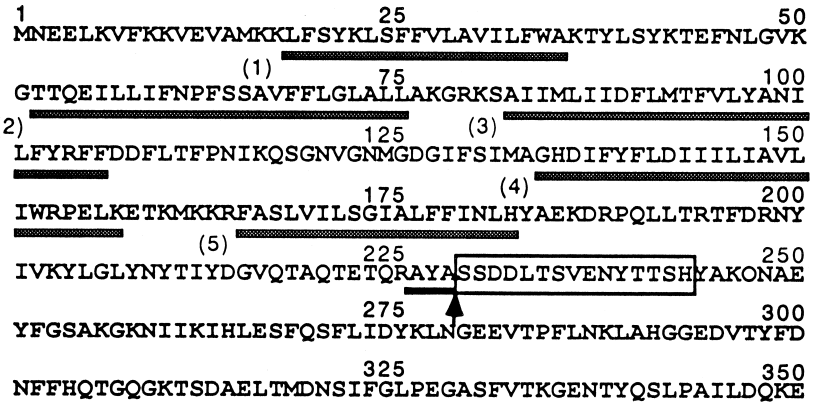

Fig. 6. Comparison of the predicted amino acid sequence from the nucleotide sequence of *ynfI* and the determined N-terminal amino acid sequence of spot YfnI. The determined amino acid sequence is shown in the box. Shaded boxes labelled (1) to (5) indicate transmembrane regions. An Ala-X-Ala sequence motif is underlined. ↑ indicates the putative cleavage site. The figure shows the sequence of aa 1–350 of YfnI, which consists of 653 aa.

the profile of strain 168 (Fig. 1). Nineteen spots (arrowed) that were absent from strain 168 cultured in glucose, were novel. Spots for NG1 to NG4, CS1, ML1 and SS1 to SS4 were extracted and their N-terminal amino acids were sequenced (Table 2). This analysis revealed AmyE, Vpr, KatA and YhdT (mannan endo-1,4-*β*-mannosidase). AmyE and Vpr are extracellular proteins and YdhT was also predicted to be extracellular. KatA is a cytoplasmic protein that locates in the culture medium during the stationary phase of growth (Bol & Yasbin, 1991). Spots SS1 to SS4 were degradation products of Hag. Spot ML1 could not be transferred onto PVDF membranes. These spots were not found in the 2D profiles preparations obtained from TB301 cultured in 0·4% maltose and soluble starch at 42 °C and from DF46 cultured in 0·4% maltose in the absence of IPTG.

Secretion of YfnI and YflE

We predicted that YflE and YfnI were membrane proteins because of the absence of signal peptides at their N-terminal regions and the presence of transmembrane domains. However the two proteins migrated as obvious spots in the extracellular preparation of strain 168. Fig. 6 shows the predicted amino acid sequence of *yfnI* and the determined N-terminal amino acid sequence of the YfnI spot. The determined sequence was located at position 229 to 243 in the predicted amino acid sequence. An Ala-X-Ala motif was found in front of the analysed cleavage site and five transmembrane domains were located on the N-terminal side of the cleavage site. The amino acid sequence of YfnI shares 55% identity to that of YflE. The detected amino acid sequence of the YflE spot was located at position 216 to 227 from the putative translation-initiation site, the Ala-X-Ala motif was located in front of the putative cleavage site and five transmembrane domains were also on the N-terminal side of the predicted cleavage site. A search of the databases revealed three more homologous proteins, YqgS, YvgJ of *B. subtilis*, as well as a rat protein (Fig. 7a). The four proteins of *B. subtilis* were predicted to contain five transmembrane domains in their N-terminal regions and an Ala-X-Ala motif was located at around 210 amino acids from the putative translation-initiation sites. These proteins are probably synthesized initially as high-molecular-mass membrane proteins, then liberated into the culture medium by processing at the cleavage site behind the Ala-X-Ala motif (Fig. 7b). The function of these proteins remains unknown, but they are structurally similar to an anion-binding protein.

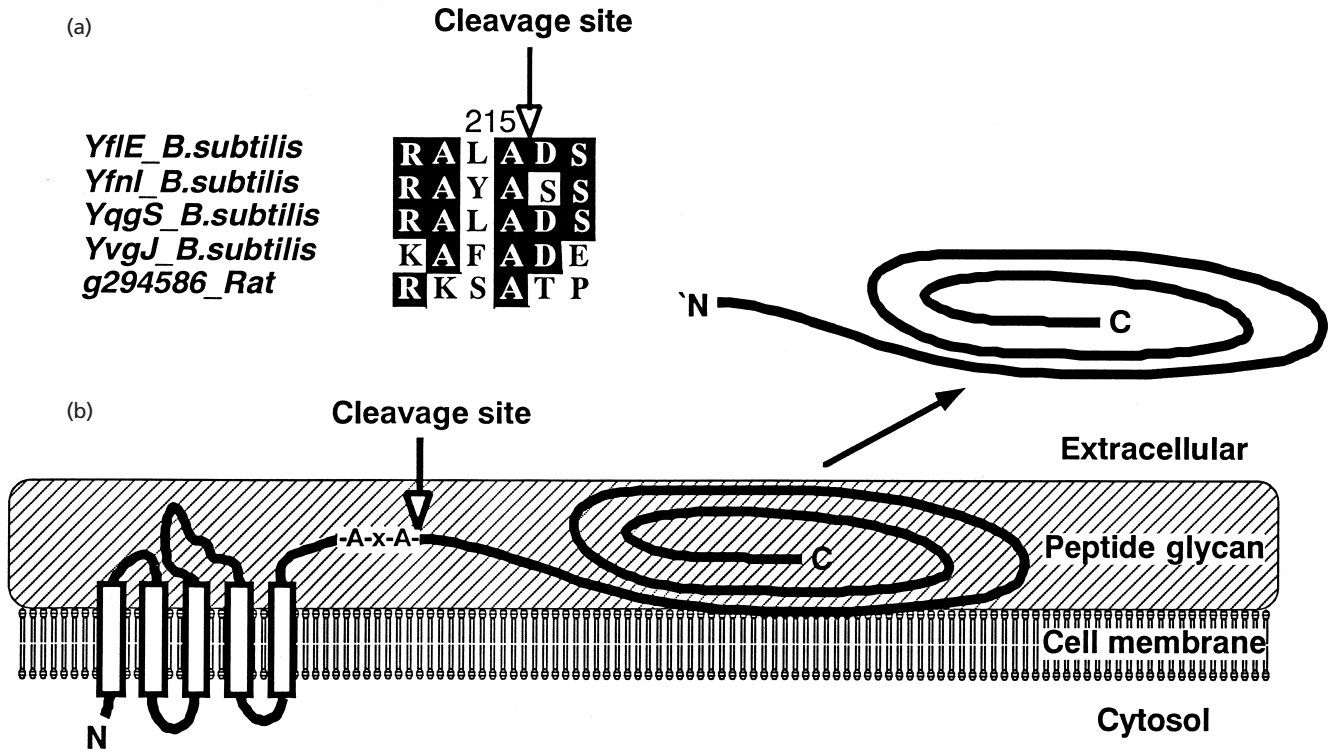

Fig. 7. (a) Membrane proteins (*B. subtilis* YflE, YqgS and YvgJ, and a rat protein homologous to YfnI) and possible or predicted cleavage sites (↓) in their precursors. 215 indicates the amino acid position for Ala from the putative translation-initiation site of *yflE*. (b) Possible model for YfnI illustrating liberation into culture medium from the cytoplasmic membrane. White boxes indicate transmembrane domains.

DISCUSSION

We defined 23 gene products from *B. subtilis* 168 by 2D gel electrophoresis and N-terminal amino acid sequencing of extracellular protein preparations from cultures in glucose, cellobiose, maltose and soluble starch. Except for Gap, SodA and KatA, 20 proteins were predicted to be extracellular, and secreted by the three following pathways. (1) Seventeen proteins including XkdG that had predicted signal peptides in their precursor forms, disappeared from 2D gels under SecA-deficient conditions and disappeared or became faint in the absence of Ffh. (2) YflE and YfnI were predicted membrane proteins but they were apparently liberated into the culture medium after processing. This secretion into the medium was also dependent on SecA and Ffh. Although the Ala-X-Ala sequence in YfnI and YflE is predicted to locate after five transmembrane domains, the predicted cleavage behind the Ala-X-Ala sequence will be done by type I signal peptidase(s) as reported by Beltzer *et al.* (1989). *B. subtilis* has at least five different type I signal peptidases (Tjalsma *et al.*, 1998) and some of them will be responsible for the cleavage. (3) Flagellin was produced into the medium through a specific pathway that is independent of SecA and Ffh. Therefore, 19 of 20 proteins were secreted by the SRP/Sec protein-secretion system.

Weiner *et al.* (1998) and Sargent *et al.* (1998) reported a fourth secretion pathway in *E. coli*, called the Tat system, for twin-arginine leader peptides of a group of secretory proteins. *B. subtilis* counterparts of *E. coli tatA*, *C*, *D* and *E* were identified as *ydiI*/*ycbB*, *ydiJ*/*ycbI*, *yabD* and *ynzA*, respectively, by a computer search of the databases. Therefore a similar protein pathway to the *E. coli* Tat system may also be present in *B. subtilis*. However, we found two signal peptide amino acid sequences of WprA and WapA precursors, which were similar to the twin-arginine leader motif, (S/T)RRXFLK (Berks, 1996). Their production into the culture medium was dependent on SecA and Ffh. Therefore, a *B. subtilis* Tat system may be involved in the secretion and membrane location of some specific protein groups.

We ascertained that the AmyE precursor accumulates in the absence of SecA or Ffh (Takamatsu *et al.*, 1992). Since a homologue of *E. coli* SecB has not been identified in *B. subtilis* either by individual investigation and/or the genome project, SRP in *B. subtilis* must recognize presecretory proteins as a chaperone and target them to Sec protein translocase for transport across the membrane. Therefore the co-operation of SRP and Sec translocase should be the major protein-secretion pathway in *B. subtilis*, although we have not yet confirmed the accumulation of precursors of extracellular proteins other than AmyE. Furthermore, we have to ascertain whether or not the disappearance of these extracellular

proteins is due to a primary effect of the depletion of SecA or Ffh.

Ulbrandt *et al*. (1997) and Valent *et al*. (1998) reported that *E. coli* SRP is mainly required for the localization of inner-membrane proteins. The requirement of SRP for the localization of membrane proteins has not been analysed in *B. subtilis*. However it is quite possible that the location of membrane protein is due to the function of SRP and Sec protein translocase, as well as the secretion of extracellular proteins as shown in Table 1, where the production and liberation of membrane proteins YfnI and YflE also depend on SecA and Ffh.

On the other hand, Hag migrated as the largest spot in 2D gels of extracellular protein preparations. Hag is another protein, the secretion of which is not due to SRP and Sec translocase in *E. coli* (Hueck, 1998; Namba *et al*., 1989). Therefore the appearance of Hag in the absence of both SecA and Ffh does not conflict with the disappearance of many extracellular proteins under these conditions. Hag in *B. subtilis* must be secreted via a specific pathway like that in *E. coli*. The appearance of Gap, KatA and SodA in the extracellular preparations was probably due to leakage of cytoplasmic proteins.

A computer search of the databases and the similarity of the N-terminal amino acid sequences to typical signal peptides of extracellular proteins led to the selection of 138 secretory-protein candidates. We identified only 17 proteins that migrated as obvious spots on 2D gel electrophoresis as secretory proteins. However, YwtD and XynD in Table 1 were not included in the candidates. Approximately 120 to 140 spots were detected in our 2D gel system overall and many more proteins will be identifiable by studies using mutants with other features such as high protein-secretion ability and by using various culture conditions. These results indicate that *B. subtilis* 168 will produce 150 to 180 proteins into culture medium. This number is fully consistent with the earlier estimate of 180 secreted proteins by Tjalsma *et al*. (1999).

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