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Cytochrome c_{550} is Related to Initiation of Sporulation in *Bacillus subtilis*

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The effect of cytochrome c_{550} encoded by *cccA* in *Bacillus subtilis* during the event of sporulation was investigated. The sporulation of *cccA*-overexpressing mutant was significantly accelerated, while disruptant strain showed delayed sporulation in spite of the same growth rate. Activity of sporulation stage-0-specific enzyme, extracellular α-amylase of mutant strains was similar to that of the control strain, but *cccA*-overexpressing mutant exhibited higher activity of stage-II-specific alkaline phosphatase and stage-III-specific glucose dehydrogenase when compared to deletion mutant and control strain. Northern blot analysis also revealed that *cccA*-overexpressing mutant showed high level of *spo0A* transcripts, while the disruptant rarely expressed *spo0A*. These results suggested that although cytochrome c_{550} is dispensable for growth and sporulation, expression of *cccA* may play an important role for initiation of sporulation through regulation of *spo0A* expression.

Key words: *B. subtilis, cytochrome* c_{550}, *cccA, spo0A,* sporulation

Sporulation in *Bacillus subtilis* has been studied as a model for all other prokaryotic developmental system. In response to a variety of environmental stimuli including starvation of nutrients (Sonenshein, 1989) or high population density (Grossman and Losick, 1988), *B. subtilis* can produce spores which are resistant to heat, lysozyme cleavage and harsh chemicals (Setlow, 1993). Initiation of sporulation has been revealed to be controlled by the transcriptional regulatory program that is related to six different sigma subunits of RNA polymerase (Kroos *et al*., 1999). Also, the multi-component phosphorelay has been emphasized as a key that elucidates signaling of sporulation (Burbulys *et al*., 1991). Two component system of Spo0A as a response regulator and a transcription factor is especially the important regulatory element for the initiation of sporulation (Stephenson and Hoch, 2002). But it still remains a question as how environmental conditions can drive vegetative cells into a developmental pathway leading to spores formation (Burkholder and Grossman, 2000).

The *c*-type cytochromes are prevalent in biological systems and have been known to play important roles in electron transport systems. *B. subtilis* also contains four *c*-type cytochromes: 1) aa_3-type cytochrome *c* oxidase (van der Oost *et al*., 1991), 2) menaquinone:cytochrome *c* reductase (*bc* complex) (Yu and Le Brun, 1998), 3) cytochrome c_{550} (von Wachenfeldt and Hederstedt, 1990a) and 4) cytochrome c_{551} (Bengtsson *et al*., 1999). The heme of the *c*-type cytochromes is covalently bound to the polypeptide via cysteine residues in a consensus motif C-X-X-C-H, while that in other cytochromes it is non-covalently attached to the polypeptide (Meyer and Kamen, 1982; von Wachenfeldt and Hederstedt, 1993). The imidazole nitrogen of the histidine residue in this motif serves as the fifth axial ligand to the heme iron, and the reduced cysteines in this motif are necessary for the attachment of the heme iron (Schiött *et al*., 1997; Barker and Ferguson, 1999). Cytochrome c_{550}, 13 kDa protein, consists of two domains: one is the heme containing C-terminal domain of about 74 residues, located on the outer surface of the cytoplasmic membrane (von Wachenfeldt and Hederstedt, 1990b) and the other is the α-helical N-terminal transmembrane domain of about 30 residues, embedded in the cytoplasmic membrane as an anchor for the C-terminal domain (von Wachenfeldt and Hederstedt, 1990a).

The function of cytochrome c_{550}, encoded by *cccA*, has not yet been elucidated. The overexpressing mutant or disruptant of *cccA* gene have been reported to have no effect on the growth or respiration of *B. subtilis* (von Wachenfeldt and Hederstedt, 1990a). But, cytochrome c_{550} in the electron transport network of *Paracoccus denitrificans* serves as an alternative electron mediator protein between the cytochrome bc_1 complex and the cytochrome cd_1-type nitrite reductase (Davidson and Kumar, 1989; Otten *et al*., 2001; Pearson *et al*., 2003). In *Pseudomonas aeruginosa*, cytochrome c_{550} is an essential component of

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Table 1. Bacterial strains and plasmids used in the present work

Strain or plasmid	Description	Source or reference
Bacterial strains		
PS832	Trp^+ revertant of strain 169, wild type	P. Setlow
IS100	PS832 derivative, pHP13	This study
IS101	PS832 derivative, *cccA*::pHP13 [a]Cm^r [b]Em^r	This study
IS102	PS832 derivative, Δ*cccA* [c]Nm^r	This study
Plasmids		
pHP13	*lacI lacZ* Cm^r Em^r, Shuttle vector	P. J. Piggot
pBO101	*cccA* in pHP13 for overexpression	This study
pMLK117	*lacI gus neo bla*, Integration vector	P. J. Piggot
pBD101	*cccA* in pMLK117 for disruption	This study
pGEM-T Easy	[d]Amp^r T7 promoter, for cloning and sequencing	Promega

[a]Cm^r, chloramphenicol resistance
[b]Em^r, erythromycin resistance
[c]Nm^r, neomycin resistance
[d]Amp^r, ampicillin resistance

the ethanol oxidation system (Schobert and Görisch, 1999). In our present study, to understand the physiological role of cytochrome c_{550} in *B. subtilis*, we have prepared *cccA*-overexpressing strain and deletion mutant, and investigated the effect of cytochrome c_{550} on sporulation of *B. subtilis*.

Materials and Methods

Bacterial strains and culture conditions

The bacterial strains used in this work are listed in Table 1. The cultures of *B. subtilis* were grown at 37°C in 2 × SG media, consisting of 1.6% nutrient broth (w/v), 0.05% $MgSO_4$ (w/v), 0.2% KCl (w/v), 0.1% glucose (w/v), 1 μM $FeSO_4$, 1 mM $CaCl_2$, and 100 μM $MnCl_2$. *B. subtilis* mutants obtained by transformation were selected and grown on Luria-Bertani (LB) agar plates or 2 × SG media, containing 15 μg/ml chloramphenicol (all mutants) or 10 μg/ml neomycin (only IS102). For homogenizing cell state of *B. subtilis*, the cells used as seeds were pregrown at 37°C for 8 h on 2 × SG agar plates containing appropriate antibiotics.

Plasmid construction and genetic transformation

To analyze the possible role of *cccA* in sporulation, its overexpression vector and plasmid for disruption were constructed. The full length *cccA* was amplified by PCR by using primers, 5'-GGTATATGGATAAGCTTCCTTTATTTTACTGAAAAATGATGTC-3' and 3'-CCCACAGTTTTTAATTTATTTTCCTAGGAAAAAGAG-5' (the underlined sequences indicate *Hin*dIII and *Bam*HI site, respectively) and was ligated with pGEM-T Easy. The resultant plasmid, pGEM-*cccA*, was transformed into *E. coli* DH5α. *Hin*dIII/*Bam*HI-digested fragment from pGEM-*cccA* was inserted into pHP13 expression vector and transformed into DH5α. The pHP13 harboring *cccA* gene, pBO101 was transformed again into *B. subtilis* PS832 by using natural competency according to the modified method of Dubnau and Davidoff-Abelson (1971). The *cccA*-overexpressing strain, IS101 was selected on 2 × SG media containing 15 μg/ml of chloramphenicol. The disruption of *cccA* gene was carried out by using pMLK117 integration vector. The 235-bp length fragment of *cccA* gene including heme binding site was amplified by PCR by using two primers, 5'-TTTTTGCGGATCCCTGTTTT-3' and 3'-TCTTTCTACAGCTGCTTTAA-5' (the underlined sequences indicate *Bam*HI and *Sal*I site, respectively). PCR product was inserted into pGEM-T Easy to yield pGEM-*cccA*C. *Bam*HI/*Sal*I-digested fragment from pGEM-*cccA*C was cloned into pMLK117 and transformed into DH5α. The resultant plasmid, pBD101, was transformed into IS100, *B. subtilis* PS832 containing pHP13. The disruptant mutant, IS102, was selected on 2 × SG medium containing 10 μg/ml of neomycin and 15 μg /ml of chloramphenicol (Karow and Piggot, 1995).

RNA preparation and northern blot analysis

Total RNA isolation from *B. subtilis* mutants was performed by using 'Modified Kirby Mix', designed by Van Dessel *et al.* (2004). Cells were homogenized and disrupted by Modified Kirby Mix, which contained 1% *N*-lauroylsarcosine, 6% *p*-aminosalicylic acid sodium salt, and 6% phenol in 0.1 M Tris-HCl (pH 8.0) and 0.1% diethyl pyrocarbonic acid (DEPC). Cell debris and proteins were removed by extraction with phenol:chloroform:isoamylalcohol (25:24:1). Total nucleic acids were precipitated from the aqueous phase by adding 1/10 volume of 3 M sodium acetate (pH 6.0) and one volume of isopropanol, and treated by RNase-free DNase I (Roche, Germany) to remove DNA contaminants. Northern blot

Table 2. Primers used in constructing northern probes

Primers	Sequences
cccA-NF	5'-AGATATATGGATAATATGCCT-3'
cccA-NR	5'-GGGTGTCAAAAATTAAATAAA-3'
spo0A-NF	5'-ATAAGCTCATGTTTAAGAAGC-3'
spo0A-NR	5'-GGAGGAAGAAACGTGGAGAAA-3'

analysis was adopted from general method created by Kenney and Moran (1987). RNA samples were separated on 1% agarose gel containing 0.22 M formaldehyde and were blotted onto Magna nylon membrane (GE Osmonics, USA). The coding regions of various genes were amplified by PCR and labeled with [α-^{32}P]-dATP. The primers used in constructing the probes are listed in Table 2. All the blots were pre-hybridized for 1 h, hybridized for 2 h, and washed with Buffer 1 (2% SSC, 0.1% SDS) and Buffer 2 (0.2% SSC, 0.1% SDS) for 20 min at 65°C.

Assay of sporulation stage-specific enzyme

Measurement of all enzyme activities was made on a UV-1601 spectrophotometer (Shimadzu, Japan). Units of each stage-specific enzyme activity per 10^3 or 10^6 CFU (colony forming units), were used to describe the number of cells entering into each sporulation stage. The assay of extracellular α-amylase activity was performed to check the end of zero stage of sporulation (Nicholson and Chambliss, 1985). Samples for extracellular α-amylase activity were prepared from culture supernatants, which were treated with 0.05% soluble potato starch in 50 mM Tris-HCl (pH 6.8) and 25 mM $CaCl_2$. After incubation at 37°C for 30 min, 0.01% of I_2 and 0.1% of KI in 1 M HCl were added. One unit of α-amylase was defined as a decrease in A_{620} of 0.1 under the above mentioned conditions. Alkaline phosphatase was used as stage-II-specific marker (Akrigg, 1978; Akrigg and Mandelstam, 1978). In order to measure the activity of alkaline phosphatase, one drop of toluene and 1 mg/ml of *p*-nitrophenyl phosphate were added to the sporulating cells. When samples exhibited yellow color, the reaction was stopped by the addition of 2 M NaOH. One unit of alkaline phosphatase was defined as the amount of enzyme that hydrolyzes 1 nmol of *p*-nitrophenyl phosphate in 1 min at 30°C. Activity of glucose dehydrogenase was measured as stage-III-specific enzyme activity (Sadoff, 1966; Fujita *et al.*, 1997). Cells employed for measuring the activity of glucose dehydrogenase were suspended in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol, pH 7.0). After incubation with 0.4 mg/ml lysozyme and 0.08% Triton X-100, soluble extracts were obtained by centrifugation. Since this assay monitored the reduction of NAD^+ to NADH, reaction mixture contained 0.3 ml of cell extract, 0.1 ml of NAD, 0.1 ml of 2-deoxyglucose, and 0.5 ml reaction buffer (0.5 M Tris-HCl, pH 8.0; 5 mM EDTA; 200 mM KCl). The increase in A_{340} was monitored for 2 min at room temperature in a spectrophotometer and was used for calculating units of glucose dehydrogenase activity as references.

Determination of sporulation rate

The ratio of spores to total viable cells, indicating the sporulation rate, was determined on the basis of resistance of spores to heat treatment (Milhaud and Balassa, 1973). Samples at each indicated time were taken from cultures grown in 2 × SG medium at 37°C and were heated at 80°C for 10 min. After samples were serially diluted in steps of 100-fold in distilled water, appropriate volumes of the dilutions were spread on LB agar plates, which were incubated overnight at 37°C (Yoon *et al.*, 2004). The proportion of survivors was determined as ratio of spores in each sample by colony counting.

Results

Overexpression and disruption of cccA in B. subtilis

The mutant strains in *B. subtilis* were prepared as described in Materials and Methods. As the mutant strains were grown in media containing chloramphenicol for maintenance of vectors, IS100 instead of PS832 was considered as control strain and used as host cell when deletion mutant IS102 was constructed. To confirm whether mutants were prepared successfully, northern blot analysis was performed. The *cccA* mRNA level of IS101 was increased significantly when compared to IS100, while that of IS102 was not detected (Fig. 1). Growth and development of mutants were monitored in order to investigate whether cytochrome c_{550} plays any role during growth and sporulation. Growth in 2 × SG medium did not exhibit any difference between control strain and cytochrome c_{550} mutants (Fig. 2A), which was consistent with the previous report by von Wachenfeldt and Hederstedt (1990a). However, microscopic observation represented the difference in sporulation between the two strains. By considering the end of exponential phase as T_0, there were no spores in IS100 and IS102 samples at T_6 (Fig. 2B). But at the same time, IS101 had already made lots of spore bodies at 6 h after the end of exponential growth (T_6). This suggested

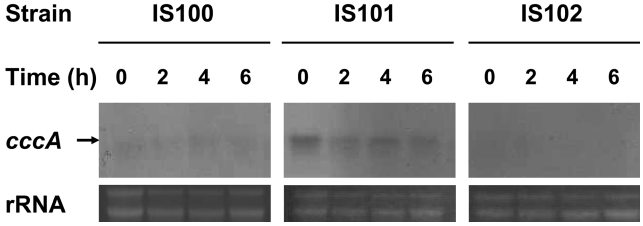

Fig. 1. Northern blot analysis of *cccA*. Total RNA (20 μg) was prepared from each mutant strain at indicated time, and detected by northern hybridization by using the probe specific for *cccA*. The arrowhead indicates the position of *cccA* mRNA hybridizing with the [α-^{32}P]-dATP labeled probe.

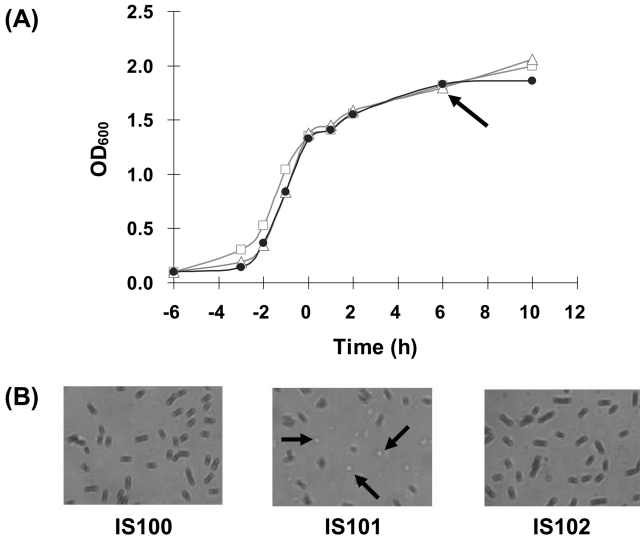

Fig. 2. Growth curves and microscopic images of cytochrome c_{550} mutant; IS100, IS101, and IS102 in 2 × SG media. The end point of exponential phase was considered as T_0 time in graph of growth curve. Microscopic analysis was made at T_6 time. The arrow in (A) means T_6 time when the cells enter into the initiation step of sporulation. The arrows in (B) show phase-bright spore bodies. Circles, squares, and triangles denote IS100, IS101, and IS102 strains, respectively.

that the overexpression of cytochrome c_{550} may influence sporulation rate.

The effect of cccA expression on sporulation rate

To determine the sporulation rate, the number of spores in each mutant strain samples at indicated time was counted by *B. subtilis* surviving heat treatment (Milhaud and Balassa, 1973). Cells were harvested at the end of exponential phase (T_0) and at the indicated times (T_N). The sporulation efficiency of IS101 at T_{17} was similar to that of IS100 (Fig. 3A). But about 90% of IS101 had already sporulated before T_7, showing higher sporulation rate than that of IS100 sample. On the other hand, the sporulation efficiency of IS102 at T_{17} was lower than that of IS100. These data clearly revealed that cytochrome c_{550} was dispensable for sporulation, but expression of cytochrome c_{550} accelerated initiation of sporulation. The expression effect of *cccA* gene on sporulation was confirmed by assays of sporulation stage-specific enzymes. Among many assays for the enzymes, extracellular α-amylase (stage-0-specific), alkaline phosphatase (stage-II-specific), and glucose dehydrogenase (stage-III-specific) are very useful tools for determining sporulation rate of cell at each stage of sporulation. From the results of enzyme

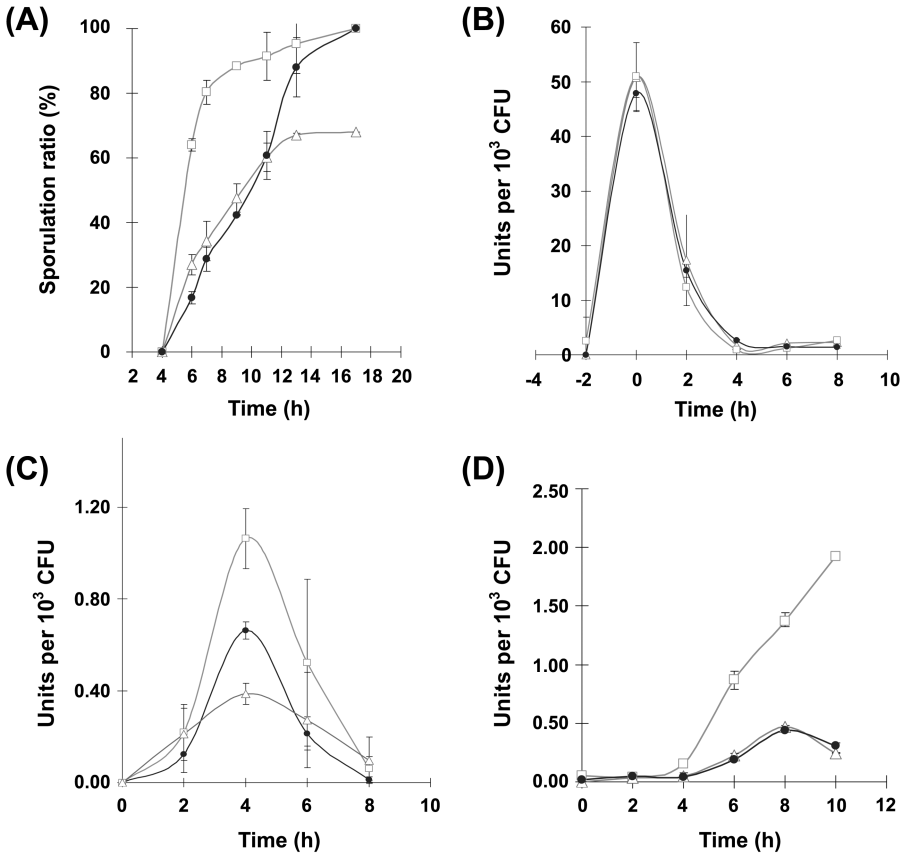

Fig. 3. Effects of cytochrome c_{550} mutation on sporulation rate (A) and stage specific enzyme activity (B, α-amylase; C, alkaline phosphatase; D, glucose dehydrogenas). All experiments were performed as described in Materials and Methods. Circles, squares, and triangles denote IS100, IS101, and IS102 strains, respectively.

assay (Fig. 3B-D), we found that each strain exhibited different expression patterns of marker enzymes. The level of extracellular α-amylase was maximal at T_0 time in all the mutants. But assay data for alkaline phosphatase showed different enzyme activities in sporulation stage-II of each mutant. IS101 contained higher activities of alkaline phosphatase than IS100, while IS102 showed lower activities and stage-delayed aspect. These data revealed that cytochrome c_{550} may have important roles on sporulation between satge-0 and stage-II. Moreover, activities of glucose dehydrogenase in IS101 increased dramatically and lasted longer than other strain, supporting that the expression of *cccA* affected sporulation process of *B. subtilis*. Therefore, it is suggested that cytochrome c_{550} is dispensable for growth and sporulation, but expression level of cytochrome c_{550} may regulate sporulation rate.

Relations of cytochrome c_{550} to spo0A expression in the sporulation initiation

To investigate further as how cytochrome c_{550} can activate sporulation, transcripts level of spore specific gene, *spo0A* was analyzed. Because Spo0A is expressed at the initiation of sporulation and accumulation of Spo0A to a critical concentration is required for the commitment of cells to the sporulating pathway (Hoch, 1993; Spiegelman *et al.*, 1995), northern blot analysis of *spo0A* could give more precise sporulation rate of each cytochrome c_{550} mutant strains. The *spo0A* mRNA in control strain IS100 increased after the end of exponential phase and accumulated upto the next step (Fig 4). But IS101 showed that very large amount of *spo0A* was transcribed earlier than other strains, while IS102 had extremely low level of *spo0A* transcripts. Thus, these results indicated that the overexpression of cytochrome c_{550} in *B. subtilis* promotes sporulation, but the disruption makes some defects in sporulation process. It is likely that cytochrome c_{550} activates the expression of *spo0A* to initiate signaling of sporulation. This suggested that cytochrome c_{550} may be a type of activator for initiation of sporulation through an unknown mechanism.

Discussion

Four different *c*-type cytochromes have been identified in *B. subtilis* and their physico-chemical characterizations were carried out. However, it has been far difficult to figure out their physiological roles since deletion mutant did not show clear phenotype. The present work sought to elucidate the roles of cytochrome c_{550} in sporulation of *B. subtilis*. The overexpression of *cccA* accelerated sporulation while the lack of *cccA* resulted in low efficiency of sporulation when compared to the control strain.

Several deleted mutants for each genes encoding cytochrome *c* have been characterized and they exhibited no growth, sporulation, and germination defect. In this work, IS102, *cccA*-disruptant in 2 × SG media also exhibited similar growth pattern (Fig. 2A) because function of *c*-type cytochromes would be redundant or subtly different during the growth process. However, expression of *cccA* influenced initial rate and efficiency of sporulation (Figs. 2B and 3A). In *Bacillus cereus*, there was marked increase in the level of cytochromes prior to sporulation (Felix and Lundgren, 1973; Lang *et al.*, 1972), implicating that expression of cytochromes is somehow involved in sporulation. Since cytochromes function as mediator in electron transport network and the role of cytochrome c_{550} in respiration and growth is unclear, we cannot rule out the possibility that level of cytochrome c_{550} affects sporulation through changes in metabolism.

Changes in activities of several enzymes are useful indicator events in sporulation stage. To validate earlier formation of spore in IS101 (Fig. 2B), activities of three stage-specific enzymes were monitored (Fig. 3). While the results of activities of alkaline phosphatase and glucose dehydrogenase coincide with sporulation formation of each strain, activities of extracellular α-amylase in three strains were almost the same, indicating the discrepancy in sporulation pattern. Even though α-amylase is known to be stage-0-specific enzyme, the appearance of its activity does not correlate with sporulation directly because mutant defective in α-amylase can sporulate normally (Schaeffer, 1969) and sporulation-defective *ftsH* null mutant showed an increase in α-amylase after the entry into stationary phase (Deuerling *et al.*, 1997). We infer from these results that acceleration of sporulation caused by overexpression of *cccA* is not related to pathway leading to secretion of α-amylase.

Differentiation of *B. subtilis* into spore has been known to be activated by the master transcription regulator Spo0A through the phosphorelay. Spo0A directly or indirectly regulates the expression of more than 500 genes during early stages of development (Molle *et al.*, 2003; Kim *et al.*, 2004). A threshold level of Spo0A is also necessary for sporulation and genes regulated by Spo0A require different level of this master regulator (Fujita *et al.*, 2005). Overexpression of *cccA* in *B. subtilis* dramatically increased the level of *spo0A* transcripts and its dis-

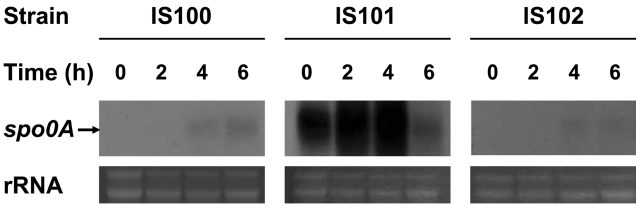

Fig. 4. Northern blot analysis of *spo0A*. Total RNA (20 μg) was prepared from each mutant strain at indicated time, and detected by northern hybridization by using the probe specific for *spo0A*. The arrowhead indicates the position of *spo0A* mRNA hybridizing with the [α-^{32}P]-dATP labeled probe.

ruption led to lower levels of transcripts (Fig. 4). The change in the amount of *spo0A* transcript caused by *cccA* expression should result in alteration in initial stage of sporulation. It is suggested that cytochrome c_{550} may be an activator of Spo0A. And it is possible that unknown function of cytochrome c_{550} may mimic the regulator of *spo0A* expression, since cytochrome c_{550} is dispensable for sporulation and its exact function is not uncovered yet. Therefore, we are further investigating novel function of cytochrome c_{550} and its possible relation with sporulation.

In conclusion, we have demonstrated that expression of cytochrome c_{550} may influence initial stage of sporulation of *B. subtilis* through regulation of Spo0A. These findings may provide new insight into novel function or activity of cytochrome c_{550} in *B. subtilis*.

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