The essential two-component regulatory system encoded by *yycF* and *yycG* modulates expression of the *ftsAZ* operon in *Bacillus subtilis*

Keisuke Fukuchi, Yasuhiro Kasahara, Kei Asai, Kazuo Kobayashi, Shigeki Moriya and Naotake Ogasawara

Author for correspondence: Naotake Ogasawara. Tel: +81 743 72 5430. Fax: +81 743 72 5439.
e-mail: nogasawa@bs.aist-nara.ac.jp

Department of Cell Biology, Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5, Takayama, Ikoma, Nara 630-0101, Japan

Essential two-component systems are now being identified in bacteria. The *Bacillus subtilis* *yycF* gene encoding a response regulator, and its orthologue in *Staphylococcus aureus*, were reported recently to be essential for cell growth, although genes under their control have yet to be identified. The essential nature of the *yycF* regulator gene and its cognate kinase gene, *yycG*, in *B. subtilis* was also noted during the course of construction of a knockout mutant bank of newly identified genes in the genome sequence project. It was found that *yycG* could be deleted in the presence of an active form of the YycF protein, thereby suggesting direct interaction between YycG and YycF. Production of mini-cells and reduction in cell length occurred when the YycF regulator was overproduced in *B. subtilis*. These observations led to the finding that YycF overproduction up-regulated the expression from the P1 promoter of the cell division operon, *ftsAZ*. In addition, the YycF protein binds to the P1 promoter region *in vitro*. These results clearly indicate that the essential two-component regulatory system encoded by *yycF* and *yycG* genes has the potential to modulate expression of the *ftsAZ* operon in *B. subtilis*.

Keywords: YycFG essential two-component system, *Bacillus subtilis*, two-component regulatory system, *ftsAZ* gene

INTRODUCTION

The two-component signal-transduction system is a ubiquitous mechanism for sensing and responding to various environmental stimuli in bacteria (Parkinson & Kofoed, 1992). This system also plays an important role in lower eukaryotes and plants (Loomis *et al.*, 1998). A typical two-component system consists of two protein components: sensor kinase and response regulator. The sensor kinases monitor environmental signals, and modulate functions of response regulators through phosphotransfer reactions. In many cases, the response regulators modulate gene expression directly as a transcriptional repressor or activator. The two-component system can mediate diverse cellular processes, including adaptation to osmolarity, the redox state, turgor pressure, availability of nutrients, etc. The system enables genetic networks to adapt and survive in specific

environmental conditions and is dispensable for growth under usual conditions of culture. However, essential two-component systems are now being identified in bacteria. In *Caulobacter crescentus*, the CrtA response regulator acts directly at cell cycle-regulated promoters to control DNA replication, DNA methylation and flagellar biogenesis, and is essential for growth (Quon *et al.*, 1996). An essential sensor kinase, CckA, that is responsible for the CtrA activation has been identified (Jacobs *et al.*, 1999). Furthermore, the *Bacillus subtilis* *yycF* gene and its orthologue in *Staphylococcus aureus* encode a response regulator and seem to be essential for cell growth, although genes under their control have yet to be identified (Fabret & Hoch, 1998; Martin *et al.*, 1999).

We also found the essential nature of the *yycFG* genes in *B. subtilis* during construction of a knockout mutant bank of newly identified genes by genome sequencing. The entire genome sequence of *B. subtilis* revealed the existence of over 4000 ORFs, two-thirds of which had

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DIG, digoxigenin.

not been characterized in previous genetic and biochemical studies (Kunst *et al.*, 1997). Systematic analysis of functions of uncharacterized genes is being carried out in two research consortia, one in Japan and the other in Europe (Ogasawara, 2000). The main approach to assessing gene function is the construction of mutants in target genes and the analysis of the mutant phenotypes. Our group sequenced a 220 kb region containing the replication origin and identified 134 new genes (Ogasawara *et al.*, 1994; Kasahara *et al.*, 1997). When we carried out systematic knockout mutagenesis of the 134 genes, several genes were difficult to disrupt; these included two-component sensor kinase and regulator genes, *ycyF* and *ycyG*. Growth of the mutants in which an IPTG-inducible promoter, *Pspac*, regulates the expression of *ycyFG* became IPTG dependent. We now report that in *B. subtilis* expression of a cell division gene, *ftsZ*, is potentially under the direct control of the YcyFG system.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2. Bacterial strains were grown in LB medium (5 g yeast extract l⁻¹, 10 g tryptone l⁻¹, 5 g NaCl l⁻¹, pH 7.2) at 37 °C. A sporulation medium (DS medium; Schaeffer *et al.*, 1965) was used for *B. subtilis* sporulation. When required,

antibiotics were added at the following concentrations: ampicillin at 50 µg ml⁻¹, kanamycin at 10 µg ml⁻¹, erythromycin at 0.5 µg ml⁻¹, chloramphenicol at 5 µg ml⁻¹. IPTG was added at 1 mM.

Construction of plasmids and *B. subtilis* strains. Table 2 summarizes relevant characteristics of the plasmids we constructed. The primers for PCR amplification of *B. subtilis* chromosomal fragments used to construct the plasmids are listed in Table 3. Derivatives of pMutinT3 (Vagner *et al.*, 1998) were used to construct knockout or conditional-null mutants of *B. subtilis* genes. The *Bam*HI–*Sac*I fragment of pMutinT3 was cloned into the *Bam*HI–*Sac*I site of pDL (Yuan & Wong, 1995) to obtain pDL2, which was used to place the promoter–*lacZ* fusions in the *amyE* locus of the *B. subtilis* genome. Plasmids pRB373 (Bruckner, 1992) and pET15b (Novagen) were used to express the YcyF proteins in *B. subtilis* and *Escherichia coli*, respectively. Site-directed PCR mutagenesis was done to introduce a point mutation in the *ycyF* gene on pRBYcyF. Two primers, FKR021 and FKR022, having one base change which replaces the Asp-54 of the YcyF protein with His, were designed, and the 5′- and 3′-areas of *ycyF* were amplified, using primer sets FKR021-FKR021 and FKR022-FKR022, respectively. The two fragments were ligated by PCR with FKR021 and FKR022 primers, and cloned into pRB373 to obtain pRBYcyFD54H. Transformation of *B. subtilis* cells to obtain the strains described in Table 1 was done as described by Moriya *et al.* (1998). Further details of the construction of the plasmids and the *B. subtilis* strains used in this work are available on request.

Table 1. Bacterial strains

Strain	Genotype	Source or reference
<i>B. subtilis</i>		
168	<i>trpC2</i>	Pasteur stock
NIS8033	<i>trpC2 ycyF::pKF011(Pspac–ycyFG erm)</i>	pKF011 → 168
NIS8032	<i>trpC2 ycyG::pKF012(Pspac–ycyG erm)</i>	pKF012 → 168
MO566	<i>trpC2 pheA1 amyE::ftsAZ P1–lacZ cat</i>	P. Stragier*
MO685	<i>trpC2 pheA1 amyE::ftsAZ P2–lacZ cat</i>	P. Stragier
MO531	<i>trpC2 pheA1 amyE::ftsAZ P3–lacZ cat</i>	P. Stragier
MO1097	<i>trpC2 pheA1 amyE::ftsAZ P123–lacZ cat</i>	P. Stragier
NIS5001	<i>trpC2 amyE::ftsAZ P1–lacZ cat</i>	168 + MO566
NIS5002	<i>trpC2 amyE::ftsAZ P2–lacZ cat</i>	168 + MO685
NIS5003	<i>trpC2 amyE::ftsAZ P3–lacZ cat</i>	168 + MO531
NIS5004	<i>trpC2 amyE::ftsAZ P123–lacZ cat</i>	168 + MO1097
NIS5005	<i>trpC2 amyE::ftsAZ P1(–138 to +30)–lacZ cat</i>	pKF101 → p168
NIS5006	<i>trpC2 amyE::ftsAZ P1(–130 to +30)–lacZ cat</i>	pKF102 → p168
NIS5007	<i>trpC2 amyE::ftsAZ P1(–118 to +30)–lacZ cat</i>	pKF103 → p168
NIS5008	<i>trpC2 amyE::ftsAZ P1(–108 to +30)–lacZ cat</i>	pKF104 → p168
NIS5009	<i>trpC2 amyE::ftsAZ P1(–92 to +30)–lacZ cat</i>	pKF105 → p168
NIS5010	<i>trpC2 amyE::ftsAZ P1(–77 to +30)–lacZ cat</i>	pKF106 → p168
NIS5011	<i>trpC2 amyE::ftsAZ P1(–59 to +30)–lacZ cat</i>	pKF107 → p168
NIS5012	<i>trpC2 amyE::ftsAZ P1(–45 to +30)–lacZ cat</i>	pKF108 → p168
<i>E. coli</i>		
C600	<i>supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21</i>	Laboratory stock
DH5α	<i>supE44 Δlac U169(φ80lacZΔM15) hsdR17 recA1 endA1 glrA96 thi-1 relA1</i>	Takara
BL21(DE3)pLysS	F [–] <i>ompT hsdS_B (r_B[–] m_B[–]) gal dcm Δ(srl–recA)306::Tn10(DE3) pLysS</i>	Novagen

* P. Stragier, Institut de Biologie Physico-Chimique, Paris, France.

Table 2. Plasmids constructed in this study

Plasmid	Vector	Inserts*	Primers†
pKF001	pMutinT3	234 bp internal fragment of <i>yycF</i> (24 to 257)	FKF001, FKR001
pKF002	pMutinT3	487 bp internal fragment of <i>yycG</i> (24 to 510)	FKF002, FKR002
pKF003	pMutinT3	425 bp internal fragment of <i>yycH</i> (23 to 447)	FKF003, FKR003
pKF004	pMutinT3	349 bp internal fragment of <i>yycI</i> (23 to 371)	FKF004, FKR004
pKF005	pMutinT3	360 bp internal fragment of <i>yycJ</i> (17 to 377)	FKF005, FKR005
pKF006	pMutinT3	526 bp internal fragment of <i>yyxA</i> (10 to 555)	FKF006, FKR006
pKF011	pMutinT3	337 bp fragment containing the SD sequence and initiation codon of <i>yycF</i> (–80 to 257)	FKF011, FKR001
pKF012	pMutinT3	539 bp fragment containing the SD sequence and initiation codon of <i>yycG</i> (–29 to 510)	FKF012, FKR002
pRBYycF	pRB373	1046 bp fragment covering the promoter region and the entire coding region of <i>yycF</i> (–330 to 711)	FKF021, FKR022
pRBYycFD54H	pRB373	1046 bp fragment covering the promoter region and the entire coding region of <i>yycF</i> (–330 to 711) having one base change that replaces the Asp-54 by His	FKF021, FKR021 FKF022, FKR022
pKF101	pDL2	–138 to +30 region of <i>ftsZA</i> P1	FKF101, FKR101
pKF102	pDL2	–130 to +30 region of <i>ftsZA</i> P1	FKF102, FKR101
pKF103	pDL2	–118 to +30 region of <i>ftsZA</i> P1	FKF103, FKR101
pKF104	pDL2	–108 to +30 region of <i>ftsZA</i> P1	FKF104, FKR101
pKF105	pDL2	–92 to +30 region of <i>ftsZA</i> P1	FKF105, FKR101
pKF106	pDL2	–77 to +30 region of <i>ftsZA</i> P1	FKF106, FKR101
pKF107	pDL2	–59 to +30 region of <i>ftsZA</i> P1	FKF107, FKR101
pKF101	pDL2	–45 to +30 region of <i>ftsZA</i> P1	FKF108, FKR101
pETYycF	pET15b	Entire coding region of <i>yycF</i> (1 to 711)	FKF301, FKR301

* Numbers in parentheses indicate the nucleotide numbers of each gene.

† Primers used to PCR-amplify the inserts.

Northern blot analysis. *B. subtilis* 168 cells were collected by centrifugation at various time points during growth in the sporulation medium. Each cell pellet (cell density 10 OD₆₀₀ units) was resuspended in 1 ml SET buffer (50 mM Tris/HCl pH 7.6, 50 mM EDTA, 20% sucrose) containing 2 mg lysozyme, and kept on ice for 10 min. Cells were then collected and treated with ISOGEN (Nippon Gene) to prepare RNA fractions. Ten micrograms of total RNA was loaded into each lane of a 1% (w/v) agarose gel. After electrophoresis, RNA was blotted on a positively charged nylon membrane (Hybond-N⁺, Amersham Life Science). Digoxigenin (DIG)-labelled antisense RNA probes encompassing each of the *yycFGHIJ* and *yyxA* genes were prepared *in vitro* with T7 RNA polymerase using PCR products as templates. Inserts of the pKF001, pKF002, pKF003, pKF004, pKF005 and pKF006 plasmids were amplified with T3T7R, which contains the T7 promoter sequence, and T3F, and used as templates for *in vitro* RNA synthesis using the DIG RNA Labelling Kit (Boehringer Mannheim). Hybridization and detection of the signals were done following the manufacturer's instructions (Boehringer Mannheim).

Fluorescence microscopy. Cell morphology and nucleoid distribution were examined by fluorescence microscopy after DAPI staining, as described by Hassan *et al.* (1997).

β -Galactosidase assay. *B. subtilis* cells growing in LB medium (OD₆₀₀ 0.4) were collected by centrifugation and the activity of the β -galactosidase was assayed according to Youngman *et al.*

(1985). One unit was defined as 1 nmol 4-methylumbelliferyl β -D-galactoside hydrolysed in 1 min per mg protein. Protein concentration was determined using the Bio-Rad Protein Assay Kit. All results are the means of three or more assays.

Two-dimensional PAGE and micro-sequencing of proteins.

B. subtilis cells grown in 200 ml LB medium were harvested at OD₆₀₀ 0.4, and washed with 10 mM Tris/HCl (pH 8.0) followed by 10 mM Tris/HCl (pH 8.0) containing 10% sucrose. The cells were then resuspended in 10 mM Tris/HCl (pH 8.0) containing 10% sucrose, 1 mM PMSF and 1 mg lysozyme ml^{–1}, and incubated at 37 °C for 10 min, followed by addition of 9 vols lysis solution [8 M urea, 1 mM DTT, 1 mM PMSF and 1.5% IPG phor (Pharmacia Biotech)]. After removal of insoluble materials by centrifugation at 18000 g for 15 min at 25 °C, protein mixtures were isoelectrophoretically focused at room temperature on 180 mm IPG Drystrip pH 4–7, using the Multiphor II 2-D system and following the manufacturer's instructions (Pharmacia Biotech). The second dimension was run using a precast 12–14% gradient gel (Pharmacia Biotech). Proteins separated by two-dimensional PAGE were electroblotted onto a PVDF membrane (Bio-Rad) and the N-terminal sequences were determined using a 470A automated gas-phase sequencer (PE Biosystems).

Purification of the His₆-YycF protein. *E. coli* BL21(DE3)pLysS cells (Novagen) containing the pETYycF plasmid were incubated at 30 °C in 10 ml LB medium containing ampicillin

Table 3. Primers used in this study

Primer	Sequence*	Location in <i>B. subtilis</i> 168 chromosome†
FKF001	cgaggcagatctAGTAGATGATGAAAAACC	4153590 ... 4153573
FKR001	cgaggcagatctgaattcTCTGAATCCTTAGCCGTC	4153357 ... 4153374
FKF002	cgaggcagatctGTCGATCCAATTTAAGATTAC	4152872 ... 4152852
FKR002	cgaggcagatctgaattcCATACTCGCGACAACGTAG	4152386 ... 4152404
FKF003	cgaggcagatctCGATATTACTTACGGTAC	4151048 ... 4151031
FKR003	cgaggcagatctgaattcGTATTCAAACGACTGATTAG	4150624 ... 4150643
FKF004	cgaggcagatctCAATCTTCATCGTTGCC	4149694 ... 4149678
FKR004	cgaggcagatctgaattcACAAGTGCCTGAGCATCG	4149346 ... 4149363
FKF005	cgaggcagatctCGTACTTGCGAGCGGGAG	4148835 ... 4148818
FKR005	cgaggcagatctgaattcGCCGCATCGTGGGAAACG	4148476 ... 4148493
FKF006	cgaggcagatctACGAACGTGAGGAAGAAC	4147967 ... 4147950
FKR006	cgaggcagatctgaattcCCCAGACTTCACCTTATCTGA	4147423 ... 4147443
FKF011	cgaggcagatctCCATTCTTTACATGGGCC	4153693 ... 4153676
FKF012	cgaggcagatctGAGAAACCCAGAACAGGAC	4152924 ... 4152906
FKF021	tgtaaacgacggccagtaggatccGGCAAACATGAGGAGTG	4153943 ... 4153927
FKR021	cgaggcagatctGGGCATTAGTCCTGTTCTG	4152898 ... 4152916
FKR022	CCGTCTTTATTTGGGAGCATAATATGTAAGAATTAATCAGGC	4153429 ... 4153473
FKF022	GCCTGATTAAATTCTTTTACATATTATGCTCCCAAATAAAGACGG	4153473 ... 4153429
FKF101	ttttcggtagaattcAGCCACATTCATTGTATTGTTGTTCC	1595797 ... 1595822
FKF102	ttttcggtagaattcTTACACACTTGTAAGCCACATTCATTGTATTGTTGTTCC	1595783 ... 1595822
FKF103	cggtagaattcTCTGTTGTTATTTTTGTTACACAC	1595766 ... 1595790
FKF104	cggtagaattcAAGCACATAAAAAATATTCTGTTG	1595750 ... 1595772
FKF105	cggtagaattcAAACATAAAATGTGAAAAGCAC	1595734 ... 1595755
FKF106	cggtagaattcTTTTCAATAAACATAAAATGTGAAAAGC	1595726 ... 1595753
FKF107	cggtagaattcGGATATAACGAATATTTTCAATAAAC	1595712 ... 1595737
FKF108	cggtagaattcTATACATAGGATATAACGAATATTTTC	1595704 ... 1595730
FKR101	cgaggcagatctCATTCTATGGCACCTCCTC	1595872 ... 1595854
FKF201	AAAAAATGTGATATAAAAGAGG	1595680 ... 1595702
FKR201	AATGAATGTGGCTTTACAAG	1595809 ... 1595790
FKF203	GAAATGGTATTTGTTTCCGG	1595493 ... 1595512
FKR203	AAATACAAGTCTACACCAAG	1595565 ... 1595584
T3F	TGTA AACGACGGCCAGTTTGTGAGCGGATAACAATTA	(pMutinT3)
T3T7R	CAGGAAACAGCTATGACCTAATACGACTCACTATAGGGCGAGTGTATCAACAAGCT GGGGATC	(pMutinT3)
FKF301	tgtaaacgacggccagtcataatgATGGATAAAAAGATCCTTG TAG	4153610 ... 4153589
FKR301	caggaaacagctatgaccctcgagCATTAGTCCTGTTCTGGG	4152901 ... 4152918
pDLF	tgtaaacgacggccagtcCTTGATAATAAGGGTAAC	(pDL2)

* Underlining indicates artificial recognition sites of restriction enzymes. Bold and lower-case characters indicate mutations and artificial sequences, respectively.

† Numbers were according to <http://bacillus.genome.ad.jp/BSORF-DB.html>.

(50 µg ml⁻¹). When the culture reached an OD₆₀₀ of 0.6, IPTG was added to a final concentration of 1 mM. The cells were incubated for another 3 h, then harvested by centrifugation, and the His₆-YycF protein was purified according to the manual of the PET system (Novagen). Collected cells were washed with a binding buffer (0.5 M NaCl, 5 mM imidazole, 20 mM Tris/HCl pH 7.9), and resuspended in 25 ml of the same buffer. The cells were broken by sonication on ice, and the lysate was centrifuged at 39000 g for 30 min at 4 °C. The supernatant fraction was loaded on the His-Bind Resin, and the resin was washed with 10 ml of the binding buffer followed by 10 ml of a washing buffer (0.5 M NaCl, 60 mM imidazole, 20 mM Tris/HCl pH 7.9). Protein bound to the resin was

eluted with 10 ml each of the binding buffers containing an increasing amount of imidazole (0.1 to 1 M). The fraction containing His₆-YycF protein was stored at -80 °C after the addition of glycerol to 10% (v/v).

Gel mobility-shift assay. DNA fragments covering the -168 to -35 region of the *ftsAZ* P1 promoter and the -92 to +1 region of the *ftsAZ* P3 promoter were PCR-amplified, using primer sets FKF201-FKR201 and FKF203-FKR203, respectively. DNA fragments covering the -59 to -35, -77 to -35 and -92 to -35 regions of the P1 promoter were PCR-amplified from plasmids pKF105, pKF106 and pKF107, respectively, using primer set pDLF-FKR201. PCR products were purified using TaKaRa RECOCHIP (Takara) after

separation in agarose gel, and end-labelled with DIG using the DIG Labelling Kit (Boehringer Mannheim). The binding reactions (10 µl final volume) were performed by incubating 20 fmol each of the DIG-labelled DNA fragments, 0.5 µg poly(dI-dC) and 0–24 pmol purified His₆-YycF protein in 50 mM PIPES (pH 6.1), 200 mM NaCl, 1 mM EDTA, 4 mM MgCl₂, 4 mM DTT, 0.5% Tween 20 and 10% (v/v) glycerol. For binding competition assays, 20 pmol unlabelled fragment was added to the reaction mixture. After incubation at 25 °C for 30 min, 2 µl loading dye (0.1% xylene cyanol in 40%, v/v, glycerol, 22.25 mM Tris, 20 mM boric acid and 0.5 mM EDTA, pH 8.0) was added to stop the reactions and the samples were loaded on 5% polyacrylamide nondenaturing gels (29:1 acrylamide to bisacrylamide in 0.5 × TBE buffer). After electrophoresis, DIG-labelled DNA fragments were transferred onto a Hybond-N+ membrane (Amersham Life Science) and detected using the DIG Gel Shift Assay Kit (Boehringer Mannheim).

RESULTS

Characterization of an operon containing *yycF* and *yycG*

The nucleotide sequence around the *yycF* and *yycG* genes suggested that they constitute an operon with additional four genes, *yycH*, *yycI*, *yycJ* and *yyxA* (Ogasawara *et al.*, 1994; Fig. 1a). Northern blot analysis using the *yycF*- and *yyxA*-specific RNA probes revealed a common 7.4 kb transcript covering six genes in the early vegetative growth phase (Fig. 1b, c). The same transcript was detected with the *yycG*-, *yycH*-, *yycI*- and *yycJ*-specific probes (data not shown). In addition, the

yycF probe detected a 2.4 kb transcript in the early vegetative phase and the *yyxA* probe a 1.4 kb transcript in the late sporulation phase.

To characterize functions of *yycFGHIJ* and *yyxA* genes, we attempted to knock out each of them by integrating the pMutinT3 plasmid harbouring an internal fragment of each gene into the genome by a Campbell-type recombination (Vagner *et al.*, 1998). Knockout mutants of *yycH*, *yycI*, *yycJ* and *yyxA* were easily obtained, and growth defects, including the formation of heat-resistant spores, were not observed in the mutants (data not shown). By contrast, *yycF* and *yycG* disruptants could not be obtained, suggesting that *yycF* and *yycG* are essential for cell growth. Therefore, we constructed conditional-null mutants by integrating pMutinT3 harbouring the SD sequence and the N-terminal portion of *yycF* or *yycG* (Fig. 2a). If these genes are indeed essential, integration of the plasmids was expected to occur only in the presence of IPTG, where the expression of *yycF* and *yycG* would be maintained by an IPTG-inducible promoter, *Pspac*. As expected, transformants in which the pMutinT3 derivatives were properly integrated into the genome (NIS8033 and NIS8032) were obtained in the presence, but not in the absence, of IPTG.

The growth of the conditional-null mutant of *yycFG* (NIS8033) was monitored in liquid LB medium, with and without IPTG (Fig. 2b). When IPTG was removed from the medium, the increase in optical density halted within 2 h and cells began to lose viability. Similar

Fig. 1. Gene organization and transcriptional units of the *yycFGHIJ* and *yyxA* region. (a) Arrangement of the *yycFGHIJ* and *yyxA* genes is shown schematically. T indicates the location of a possible rho-independent termination signal of transcription. Below the map, transcripts detected by Northern blot analysis are indicated by arrows. (b, c) Northern blot analysis with *yycF*- (b) and *yyxA*-specific (c) RNA probes. Each lane contained 10 µg total RNA from the *B. subtilis* wild-type strain 168 at $t_{-2.5}$ (lane 1), t_{-2} (2), $t_{-1.5}$ (3), t_{-1} (4), t_0 (5), t_1 (6), t_2 (7), t_3 (8), t_4 (9) and t_5 (10) during growth in sporulation medium. An RNA marker (Boehringer Mannheim) was loaded in lane M and sizes are indicated on the left of the blots. Positions of rRNA are indicated (23S, 2.93 kb 23S rRNA; 16S, 1.55 kb 16S rRNA).

Fig. 2. IPTG-dependent growth of the conditional-null mutant of *yycFG*. (a) Structures of conditional-null mutants of *yycFG* (NIS8033) and *yycG* (NIS8032). Filled and hatched arrows indicate genes on the pMutinT3 plasmid and on the *B. subtilis* genome, respectively. The filled box indicates the region cloned in pKF011 or pKF012. (b) OD_{600} and viable cell number (colony formation on LB plates with IPTG per ml culture) of NIS8033 in liquid LB medium with ($\blacktriangle$) and without IPTG ($\triangle$) were monitored after inoculating the cells at time 0 from an overnight culture with IPTG. As a control, both parameters of the wild-type 168 in the absence of IPTG are indicated ($\blacklozenge$). (c) DAPI-stained images of wild-type 168 (upper) and NIS8033 cells (lower) after incubation for 2 h in the absence of IPTG. Cells in which the cytoplasmic contents appear to have been lost are indicated by arrows.

growth defects were also observed in both the sporulation and a defined salt medium (data not shown). Microscopic examination of the mutant cells cultivated for 2 h in the absence of IPTG revealed the presence of ghost cells in which the cytoplasmic contents seemed to be lost (Fig. 2c). Fabret & Hoch (1998) reported the same phenomenon for the *yycF*-ts mutant at the non-permissive temperature. The defect of growth was not clearly observed for the *Pspac*-*yycG* mutant (NIS8032) in liquid medium without IPTG, suggesting that a weak residual expression of *yycG* from *Pspac* in the absence of IPTG would be sufficient to maintain apparently normal cell growth for several generations (data not shown).

Inactivation of the *yycG* sensor gene in the presence of a mutated YycF regulator

We then asked if the growth defect in the YycG sensor mutant was due to a deficiency in activation of the YycF regulator. A 1046 bp fragment covering the promoter and the complete coding region of the *yycF* gene was cloned into a shuttle plasmid, pRB373 (Bruckner, 1992), and a mutation which replaced the conserved Asp

residue (Asp-54, probable phosphorylation site) with His was introduced by site-directed mutagenesis (pRBYycFD54H, Fig. 3a). We then transformed cells carrying pRBYycFD54H by the pMutinT3-*yycG* plasmid (pFK002), and inactivated the *yycG* sensor gene (Fig. 3b). This finding indicated that the YycFD54H protein was locked in an active form independently of the YycG kinase. Furthermore, this evidence strongly suggested a direct interaction between the YycG kinase and the YycF regulator. The growth defect in the absence of YycG is attributed to a deficiency in activation (phosphorylation) of the YycF regulator.

Overproduction of the YycF regulator induces excess cell division

The copy number of pRB373 in *B. subtilis* was reported to be about 50 (Bruckner, 1992). Thus, the YycF or YycFD54H protein was expected to be overproduced within cells carrying pRBYycF or pRBYycFD54H, and this was confirmed by two-dimensional PAGE analysis of whole-cell extracts (Fig. 4a). The molecular mass and pI value of the YycF protein were calculated to be

Fig. 3. Inactivation of the *yycG* sensor gene in the presence of a mutated YycF regulator, YycFD54H. (a) Design of primers, FKR022 and FKF022, used to introduce a base change that replaces Asp-54 of the YycF protein with His. The partial nucleotide sequence and the deduced amino acid sequence of *yycF*, containing the XXXXD motif (X indicates non-polar residues, underlined) conserved in response regulators (Parkinson & Kofoed, 1992), are shown. Nucleotide sequences of FKR022 and FKF022 primers are shown below. (b) Wild-type 168 and its derivatives harbouring pRB373, pRBYycF or pRBYycFD54H were transformed with pKF002 containing an internal fragment of *yycG*. The number of erythromycin-resistant transformants (disruptants of *yycG*) per ml of competent cells is shown.

27.4 kDa and 5.06, respectively. In cells harbouring pRBYycF, the intensity of a spot with an apparent molecular mass of 31 kDa and pI value of 5.15 markedly increased compared with that of the cells harbouring the pRB373 vector. The N-terminal 12 amino acid sequence of the protein in the spot perfectly matched that deduced from the nucleotide sequence of the *yycF* gene (data not shown). In cells harbouring pRBYycFD54H, there was an additional intense spot with a slightly higher pI value, and the N-terminal amino acid sequence was again identical to that of YycF (data not shown).

When cells carrying pRBYycF or pRBYycFD54H were examined by phase-contrast microscopy, we noticed production of mini-cells (Fig. 4b). The proportion of mini-cells was calculated to be 4.8% (49 mini-cells/1020 cells observed) and 2.9% (31/1080) for cells harbouring pRBYycF and pRBYycFD54H, respectively. By contrast, only two mini-cells (0.2%) were found among 1074 cells harbouring pRB373. Furthermore, statistical analysis of cell length revealed that the overproduction of the YycF or YycFD54H protein resulted in shorter cells (Fig. 4c). Therefore, excess cell division was suggested to occur in cells carrying pRBYycF or pRBYycFD54H.

Overproduction of the YycF regulator stimulates expression from the P1 promoter of the *ftsAZ* operon

The observation described above suggested that the overproduced YycF regulator promoted the expression

Fig. 4. Effects of YycF and YycFD54H overproduction on cell size. (a) *B. subtilis* 168 cells harbouring pRB373, pRBYycF or pRBYycFD54H in LB medium were harvested in the vegetative growth phase (OD_{600} 0.4) and crude extracts were analysed by two-dimensional PAGE. Gels were stained using the Pharmacia Biotech Silver Staining Kit. Parts of two-dimensional gels including YycF and YycFD54H spots are shown. (b) Phase-contrast images of 168 cells harbouring pRB373, pRBYycF or pRBYycFD54H. Mini-cells are indicated by arrows. (c) Histograms showing the frequency distribution of cell length of each strain. About 100 cells from the each strain were measured. The mean cell length was 5.6 μm, 4.1 μm and 4.1 μm for cells carrying pRB373, pRBYycF and pRBYycFD54H, respectively (indicated by the vertical arrows in each panel).

Fig. 5. Structure of the *B. subtilis* *ftsAZ* operon. (a) The organization of the *ftsA* and *ftsZ* genes and their promoters are schematically shown (Gonzy-Treboul *et al.*, 1992). Below the map, DNA fragments transcriptionally fused to the *lacZ* reporter are shown by bars. DNA fragments used in the gel mobility-shift assay are also shown. Nucleotide numbers of the boundaries of the fragments are indicated. Nucleotide number +1 corresponds to the transcriptional start site from the P1 promoter. (b) Nucleotide sequence of the *ftsAZ* promoter region. Nucleotide numbering is the same as in (a). In addition to -10 and -35 sequences of the *ftsAZ* promoters, the end-points of the DNA fragments used for the *lacZ* assays shown in Fig. 7 are indicated.

of cell-division gene(s). Therefore, we analysed the effect of YycF overproduction on expression of an essential cell division operon, *ftsAZ*. Three promoter sequences, P1, P2 and P3, have been identified in the *B. subtilis* *ftsAZ* operon (Fig. 5; Gonzy-Treboul *et al.*, 1992). The P1 and P3 promoters were sigma-A dependent and active in the vegetative growth phase, whereas the P2 promoter was sigma-H dependent and important for asymmetric cell division at the onset of sporulation. We constructed cells in which P1, P2, P3 or the whole promoter region was placed in front of the *lacZ* reporter gene at the *amyE* locus of the genome (Fig. 5), then pRB373, pRB_{YycF} and pRB_{YycFD54H} plasmids were introduced into them. The LacZ activities in the vegetative growth phase of the cells thus constructed indicated clearly that overproduced YycF or YycFD54H activates specifically the expression from the P1 promoter about threefold (Fig. 6). As a consequence, an approximately 1.5-fold increase in the total activity of the three promoters was observed in YycF- or

YycFD54H-overproducing cells; this would explain the excess division observed in these cells.

Deletion analysis of a regulatory region of the *ftsAZ* P1 promoter

To further characterize regulation of expression from the P1 promoter by the YycF regulator, we gradually shortened the upstream sequence of the P1 promoter in the P1-*lacZ* assay (Fig. 7; see also Fig. 5). The P1 fragment used in the previous assay contained a 118 bp sequence upstream of the start site of the transcription, and the same level of activation by YycF was observed in cells containing a 16 bp shorter sequence (-92). By contrast, deletion of a further 15 bp abolished the activation (-77), although a basal level of expression from the P1 promoter was maintained. These results indicated that a region between -92 and -77 was essential for activation of the P1 promoter by the YycF protein.

Fig. 6. Effect of YycF and YycFD54H overproduction on *ftsAZ* promoter activity. *B. subtilis* strains containing a P1– (NIS5001), P2– (NIS5002), P3– (NIS5003) or P123–*lacZ* fusion (NIS5004) were transformed with pRB373, pRBYycF or pRBYycFD54H, cultured in LB medium at 37 °C to OD₆₀₀ 0.4 and β-galactosidase activities assayed. All results are the means of three assays, and the error ranges are indicated by vertical lines.

Fig. 7. Deletion analysis of the regulatory sequence by the YycF regulator. *B. subtilis* strains containing P1(–138 to +30)– (NIS5005), P1(–130 to +30)– (NIS5006), P1(–118 to +30)– (NIS5007), P1(–108 to +30)– (NIS5008), P1(–92 to +30)– (NIS5009), P1(–77 to +30)– (NIS5010), P1(–59 to +30)– (NIS5011) or P1(–45 to +30)–*lacZ* (NIS5012) were transformed with pRB373 or pRBYycF. They were cultured in LB medium at 37 °C to OD₆₀₀ 0.4, and the β-galactosidase activities were assayed. All results are the means of three assays, and error ranges are indicated by vertical lines.

The YycF regulator binds to the P1 promoter sequence *in vitro*

Finally, we examined the direct interaction between the YycF protein and the P1 promoter sequence in gel mobility-shift assays. The YycF protein was expressed in *E. coli* as a fused protein with a histidine tag (His₆) and purified to near homogeneity as described in Methods (Fig. 8a). A 134 bp fragment covering the P1 promoter and a 92 bp fragment covering the P3 promoter were PCR-amplified (see Fig. 5), end-labelled with DIG, and used as probes for gel mobility-shift assays (Fig. 8b). When incubated with 20 fmol each of the DIG-labelled DNA fragments, 18 pmol His₆-YycF caused a complete shift of the P1 but not the P3 fragment (lane 5). Specificity of the interaction of His₆-YycF with the P1 sequence was further tested in competition experiments. When 20 pmol unlabelled P1 fragment was added to the reaction mixture, a clear competition was observed (lane 7). These results demonstrated that the YycF protein bound specifically to the *ftsAZ* P1 promoter sequence. The *in vivo* deletion analysis of the regulatory sequence for the P1 activation by YycF suggested that the –92 to –77 region would be the binding site of YycF. When a fragment covering –92 to –35 of the P1 sequence was used as probe, the same mobility shift was observed as with the 134 bp P1 fragment (Fig. 8c, lanes 9–12). As expected, deletion of the –92 to –78 region from the probe markedly reduced the binding affinity to YycF (lane 5–8), and further deletion to –59 completely abolished the YycF binding (lanes 1–4). Thus the DNA-binding property of the YycF protein *in vitro* was in agreement with the result of the deletion analysis of the *in vivo* regulatory sequence.

The *in vitro* binding affinity seemed to be relatively low, as about 1000-fold excess molecules of the His₆-YycF protein were needed for a complete shift, suggesting that our His₆-YycF preparation was in a non-phosphorylated form. We purified YycG kinase, using the His-tag system in *E. coli*, and attempted to phosphorylate the YycF regulator *in vitro*, so far without success. Acetyl phosphate also failed to activate the purified YycF protein.

DISCUSSION

The *B. subtilis* *yycF* gene encoding a response regulator and its orthologue in *S. aureus* was found by other researchers to be essential for cell growth (Fabret & Hoch, 1998; Martin *et al.*, 1999). We obtained supportive evidence for these findings during construction of a knockout mutant bank of genes newly identified in the genome sequence project. Furthermore we found that the *yycG* gene could be deleted in the presence of an active form of the YycF protein, YycFD54H, thereby suggesting direct interactions between YycG and YycF. The molecular mechanism involved in locking YycF in the activated form by replacement of Asp-54 with His has yet to be clarified.

Fig. 8. Direct binding of the YycF regulator to the *ftsZA* P1 promoter region. (a) SDS-PAGE of protein samples from each purification step of His₆-YycF. The proteins were separated in a 15% acrylamide gel and stained with Coomassie brilliant blue. Lanes: 1, standard proteins (Bio-Rad), with the molecular masses given on the left; 2, crude extract; 3, flowthrough from the nickel resin; 4, wash by 1 mM imidazole; 5, wash by 30 mM imidazole; 6, elution by 100 mM imidazole (lane 6) was used for the gel mobility-shift assay. (b) Gel mobility-shift assay for the binding of His₆-YycF to the P1 and P3 promoter regions. A 134 bp fragment covering the P3 promoter and a 92 bp fragment covering the P1 promoter (see Fig. 5) were end-labelled with DIG. Twenty femtomoles each of the DIG-labelled DNA fragments were incubated with 0 pmol (lane 1), 3 pmol (lane 2), 6 pmol (lane 3), 12 pmol (lane 4), 18 pmol (lane 5) or 24 pmol (lanes 6 and 7) of purified His₆-YycF. Non-labelled P1

The YycFG two-component regulatory system is expected to regulate an essential gene function in *B. subtilis* and *S. aureus*. Based on the observation that overproduction of the YycF regulator caused excess cell division, we demonstrated that the overproduced YycF protein stimulated the P1 promoter activity of the cell-division operon *ftsAZ*. Furthermore, we showed that the region between -92 and -77 was essential for the activation of the P1 promoter *in vivo*, and indeed the YycF protein was shown to bind specifically to the -92 to -59 region of the P1 sequence *in vitro*. These results clearly indicate that the essential two-component regulatory system encoded by *yycF* and *yycG* has the potential to modulate expression of the *ftsAZ* operon in *B. subtilis*. However, characteristic features in the sequence between -92 and -59 of the P1 promoter remain to be identified.

In *E. coli*, the expression of *ftsZ* is controlled by at least six promoters. In addition, at least two transcriptional activators – SdiA, which is a part of a quorum-sensing pathway, and RcsB, which is involved in the regulatory pathway of capsular polysaccharide biosynthesis – have been found to modulate *ftsZ* expression (reviewed by Vicente *et al.*, 1999). In *B. subtilis*, three promoter sequences for *ftsZ* expression were identified, and our results show that YycF is the transcriptional factor with the potential to regulate *ftsZ* expression. Gonzy-Treboul *et al.* (1992) reported that the P1 promoter could be deleted without interference with cell growth. We confirmed that the P1 promoter could be deleted, but the resultant cells became elongated, indicating that the P2 and P3 promoters alone are not sufficient to maintain normal cell division (data not shown). However, deletion of a region necessary for YycF regulation did not clearly affect P1 promoter activity in wild-type cells (Fig. 7). Therefore, the precise role of the YycFG system under normal expression in the control of cell division awaits further characterization.

The essential nature of the YycFG two-component system cannot be explained solely by its potential to modulate *ftsAZ* expression; it must regulate hitherto unidentified essential gene(s). Alternatively, it may regulate several genes important for cell growth, and simultaneous inhibition of their expression may be lethal. We and others (Fabret & Hoch, 1998) observed ghost cells that had apparently lost cellular contents by depletion of the *B. subtilis* YycF protein or by thermal inactivation of the ts-mutant protein. The temperature-

fragment (20 pmol) was added to the reaction mixture in lane 7. The samples were run on 5% polyacrylamide nondenaturing gel and the DIG-labelled fragments were detected using the DIG Gel Shift Assay Kit (Boehringer Mannheim). (c) Deletion analysis of the YycF-binding sequence. DNA fragments covering the -59 to -35 (lanes 1–4), -77 to -35 (lanes 5–9) or -92 to -35 (lanes 9–12) region of the P1 sequence were labelled with DIG and incubated with 0 pmol (lanes 1, 5, 9), 12 pmol (lane 2, 6, 10), 18 pmol (lanes 3, 7, 11) or 24 pmol (lanes 4, 8, 12) of purified His₆-YycF. Then the DIG-labelled fragments were separated and detected as described in (b).

sensitive *yycF* mutant of *S. aureus* became hypersensitive to macrolide antibiotics at the permissive temperature, probably due to defects in the permeability barrier (Martin *et al.*, 1999). Based on these observations, we speculate that the YycFG system may perhaps be involved in global regulation of cell envelope synthesis. Further characterization of genes under the control of the YycFG system is essential to determine precise roles in the control of cell growth and to identify the effector molecule that activates the YycFG system. We are currently characterizing the YycF-binding motif in the *ftsZA* P1 promoter sequence; once the motif is known, we will survey candidate genes that may belong to the *yycFG* regulon on the complete genome sequence of *B. subtilis*.

ACKNOWLEDGEMENTS

We are grateful to Dr Tatsuo Nakayama for determining the N-terminal sequences of YycF and YycFD54H proteins. We also thank colleagues in our laboratory for valuable discussions and M. Ohara for language assistance. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas and a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Science, Sports and Culture of Japan, and grant JSPS-RFTF96L00105 from the Japan Society for the Promotion of Science.

REFERENCES

- Bruckner, R. (1992). A series of shuttle vectors for *Bacillus subtilis* and *Escherichia coli*. *Gene* **122**, 187–192.
- Fabret, C. & Hoch, J. A. (1998). A two-component signal transduction system essential for growth of *Bacillus subtilis*: implications for anti-infective therapy. *J Bacteriol* **180**, 6375–6383.
- Gonzy-Treboul, G., Karmazyn-Campelli, C. & Stragier, P. (1992). Developmental regulation of transcription of the *Bacillus subtilis* *ftsAZ* operon. *J Mol Biol* **224**, 967–979.
- Hassan, A. K., Moriya, S., Ogura, M., Tanaka, T., Kawamura, F. & Ogasawara, N. (1997). Suppression of initiation defects of chromosome replication in *Bacillus subtilis* *dnaA* and *oriC*-deleted mutants by integration of a plasmid replicon into the chromosomes. *J Bacteriol* **179**, 2494–2502.
- Jacobs, C., Domian, I. J., Maddock, J. R. & Shapiro, L. (1999). Cell cycle-dependent polar localization of an essential bacterial histidine kinase that controls DNA replication and cell division. *Cell* **97**, 111–120.
- Kasahara, Y., Nakai, S. & Ogasawara, N. (1997). Sequence analysis of the 36-kb region between *gntZ* and *trnY* genes of *Bacillus subtilis* genome. *DNA Res* **4**, 155–159.
- Kunst, F., Ogasawara, N., Moszer, I. & 148 other authors (1997). The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature* **390**, 249–256.
- Loomis, W. F., Kuspa, A. & Shaulsky, G. (1998). Two-component signal transduction systems in eukaryotic microorganisms. *Curr Opin Microbiol* **1**, 643–648.
- Martin, P. K., Li, T., Sun, D., Biek, D. P. & Schmid, M. B. (1999). Role in cell permeability of an essential two-component system in *Staphylococcus aureus*. *J Bacteriol* **181**, 3666–3673.
- Moriya, S., Tsujikawa, E., Hassan, A. K., Asai, K., Kodama, T. & Ogasawara, N. (1998). A *Bacillus subtilis* gene-encoding protein homologous to eukaryotic SMC motor protein is necessary for chromosome partition. *Mol Microbiol* **29**, 179–187.
- Ogasawara, N. (2000). Systematic function analysis of *Bacillus subtilis* genes. *Res Microbiol* **151**, 129–134.
- Ogasawara, N., Nakai, S. & Yoshikawa, H. (1994). Systematic sequencing of the 180 kilobase region of the *Bacillus subtilis* chromosome containing the replication origin. *DNA Res* **1**, 1–14.
- Parkinson, J. S. & Kofoid, E. C. (1992). Communication modules in bacterial signaling proteins. *Annu Rev Genet* **26**, 71–112.
- Quon, K. C., Marczyński, G. T. & Shapiro, L. (1996). Cell cycle control by an essential bacterial two-component signal transduction protein. *Cell* **84**, 83–93.
- Schaeffer, P., Millet, J. & Aubert, J.-P. (1965). Catabolite repression of bacterial sporulation. *Proc Natl Acad Sci USA* **54**, 704–711.
- Vagner, V., Dervyn, E. & Ehrlich, S. D. (1998). A vector for systematic gene inactivation in *Bacillus subtilis*. *Microbiology* **144**, 3097–3104.
- Vicente, M., Chater, K. F. & De Lorenzo, V. (1999). Bacterial transcription factors involved in global regulation. *Mol Microbiol* **33**, 8–17.
- Youngman, P., Perkins, J. & Sandman, K. (1985). Use of Tn917-mediated transcriptional gene fusions to *lacZ* and *cat-86* for the identification and study of regulated genes in the *Bacillus subtilis* chromosome. In *Molecular Biology of Microbial Differentiation*, pp. 47–54. Edited by J. A. Hoch & P. Setlow. Washington, DC: American Society for Microbiology.
- Yuan, G. & Wong, S. L. (1995). Regulation of *groE* expression in *Bacillus subtilis*: the involvement of the σ^A -like promoter and the roles of the inverted repeat sequence (CIRCE). *J Bacteriol* **177**, 5427–5433.

Received 28 March 2000; accepted 17 April 2000.