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Molecular Characterization and Functional Analysis of *ytsABCD, yvcPQRS,* and *yxdJKLM* Operons Encoding Two-component Regulatory Systems and ABC Transporters in *Bacillus subtilis*

（枯草菌における2成分制御系とABCトランスポーターをコードしている*ytsABCD*、*yvcPQRS*および*yxdJKLM*オペロンの機能解析）

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ABSTRACT

Two-component signal transduction system (TCS) is a major mechanism for sensing environmental signals in bacteria. In *Bacillus subtilis*, genes encoding TCS are often associated with the ABC transporter genes. Among them, three operons, *ytsABCD*, *yvcPQRS*, and *yxdJKLM*, are highly homologous to each other, and each gene on those operons encodes response regulator and sensor kinase of TCS and ATP binding and permease subunits of ABC transporter system, respectively. Transcription of the ABC transporter genes, but not of the TCS themselves, was induced in cells over-expressing the response regulator gene in the same operon. Furthermore DNAse I footprinting analysis using purified regulator proteins indicated that they bind directly to the promoter region of ABC transporter gene.

I found that the *ytsABCD* (renamed *bceRSAB*) operon has a role in bacitracin resistance, a peptide antibiotic produced by some strains of *Bacillus*. Disruption of *bceRS* TCS gene or *bceAB* ABC transporter genes resulted in hypersensitivity to bacitracin. Northern hybridization analysis indicated that expression of the *bceAB* operon was induced by bacitracin in the growth medium. Deletion analysis of the *bceAB* promoter sequence indicated that the BceR binding site revealed by DNase I footprinting experiments is essential for the *bceAB* induction by bacitracin. These results suggested that a sensor kinase, BceS, responds to extracellular bacitracin directly or indirectly and transmits a signal to a cognate response regulator, BceR, to induce the expression of BceAB that would work as subunits of a bacitracin efflux transporter. It had been reported that the *bcrC* transcription is also induced by bacitracin, and disruption of this gene also resulted in hypersensitivity of *B. subtilis* cells to baciracin. The expression of *bcrC* was dependent on the Extra Cytoplasmic Function (ECF) σ factors, σ^M and σ^X, but not on the BceRS two-component system. Furthermore the combined absence of BcrC and BceA or BceB had an additive effect on bacitracin resistance, indicating that BcrC and BceAB are involved in different mechanisms of bacitracin resistance in *B. subtilis.*

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To explore the function of the *yvcPQRS*, and *yxdJKLM* operons, I tested a number of antibiotics for the ability to induce the *yvcRS* and *yxdLM* expression, and found that the *yvcRS* ABC transporter transcription was induced by enduracidin, a cyclodepsipeptide isolated from *Streptomyces fungicidus*. Deletion analysis of the *yvcRS* promoter sequence and DNase I footprinting experiments revealed that the YvcP regulator binds directly on a sequence between –110 to –71 bp from the *yvcR* initiation codon *in vitro*, and the sequence is essential for *in vivo* induction of the *yvcRS* transcription by external enduracidin. Unexpectedly, deletion of the *yvcR* transporter gene abolished the activity of the *yvcPQ* two-component system, suggesting a physical link between the YvcPQ two-component system and the YvcRS transporter. However the induction of the *yvcRS* transcription by enduracin was transient and inactivation of either two-component system or ABC transporter gene resulted in no change in the enduracidin sensitivity of cells, suggesting there is another system contributed in the enduracidin resistance. Therefore I examined the enduracidin sensitivity of deletion mutants of ECF sigma genes, and found that ECF sigma factor, σ^I, is involved in enduracidin resistance. Transcription of the *sigI* gene was induced by enduracidin, essentially independent on the YvcPQ two-component system, and disruption of this gene increased the enduracidin sensitivity compared to wild type cells.

The bacterial cell envelope is the first and major line of defense against threats from the environment. It is also the target of numerous antimicrobial substances, many of which are produced by soil microbes, presumably to inhibit the growth of competitors. *B. subtilis* is a ubiquitously distributed soil microorganism. I found that the *bceRSAB* and the *yvcPQRS* operons response to bacitracin and enduracidin, respectively. Both antibiotics are inhibitor of cell wall synthesis; bacitracin inhibits dephosphorilation of C55-isoprenyl pyrophosphate

(IPP), while enduracidin inhibits the transglycosylation step in peptidoglycan synthesis. My results indicate that *B. subtilis* has evolved two-component signal transduction systems to sense and extrude various peptide antibiotics that inhibit cell wall synthesis. Similar combination of two-component and ABC transporter genes is wide spread among low GC gram-positive bacteria, and they would also involve in the defense system to inhibitors of cell wall synthesis. Furthermore my result suggested that ECF sigma factors also involve in antibiotics resistance through different mechanisms, indicating that *B. subtilis* has equipped diverse systems to cope with cell wall stresses. Further understanding of these systems will be important to reveal molecular bases for dynamism of bacterial population in nature.

CONTENT

I. INTRODUCTION

I. 1. Two-component regulatory system

Adaptability is a crucial characteristic of unicellular organisms that are routinely challenged by drastic changes in their extracellular environments. Recognition of a specific signal and conversion of this information into a specific response is the essence of signal transduction. A mechanism commonly found in bacteria for such signal transduction is a phosphotrannsfer pathway referred to as two-component regulatory systems, consisting of the histidine protein kinase (sensor kinase) and the response regulator protein as a core module (Parkinson and Kofoid, 1992; Hoch and Varughese, 2001).

The two-component system can mediate diverse cellular processes, including host detection and invasion leading to symbiosis or pathogenesis; metabolic adaptation to changes in carbon, nitrogen, electron acceptor, and phosphate sources; physiological responses to changes in medium osmolarity; chemotaxis; adaptation to stresses such as antibiotics and heavy metals; and differentiation such as sporulation and fruiting bodies formation (Parkinson and Kofoid, 1992; Stock *et al*., 2000). The accumulated experimental results indicate that two-component systems are involved in a variety of important biological functions. However, genome sequencing of diverse organisms is unveiling the existence of new classes of signaling proteins, whose function awaits further examination.

Signal propagations in two-component pathways require precise interaction between phosphoryl donors (sensor kinases) and acceptors (response regulators) to ensure the correct responses. Figure 1 shows a signal transduction scheme by a prototypical two-component system, consisting of a transmembrane sensor kinase and a cytoplasmic response regulator. The signal transduction cascade starts with the sensing of an environmental signal by the input domain of sensor kinase. Then input domain activates the histidine kinase activity in the

transmitter domain and induces an ATP-dependent autophosphorylation of a conserved histidine residue. The phosphorylated histidine kinase interacts with the cognate response regulator, and the regulator catalyzes transfer of the phosphoryl group to a conserve aspartate residue in own receiver domain. Finally phosphorylation of the receiver domain activates the adjoining output domain that elicits the specific output response. In most of bacterial systems, the output domain contains a HTH DNA binding motif and the response is regulation of gene expression, but some response regulators control more diverse responses such as the regulation of motility.

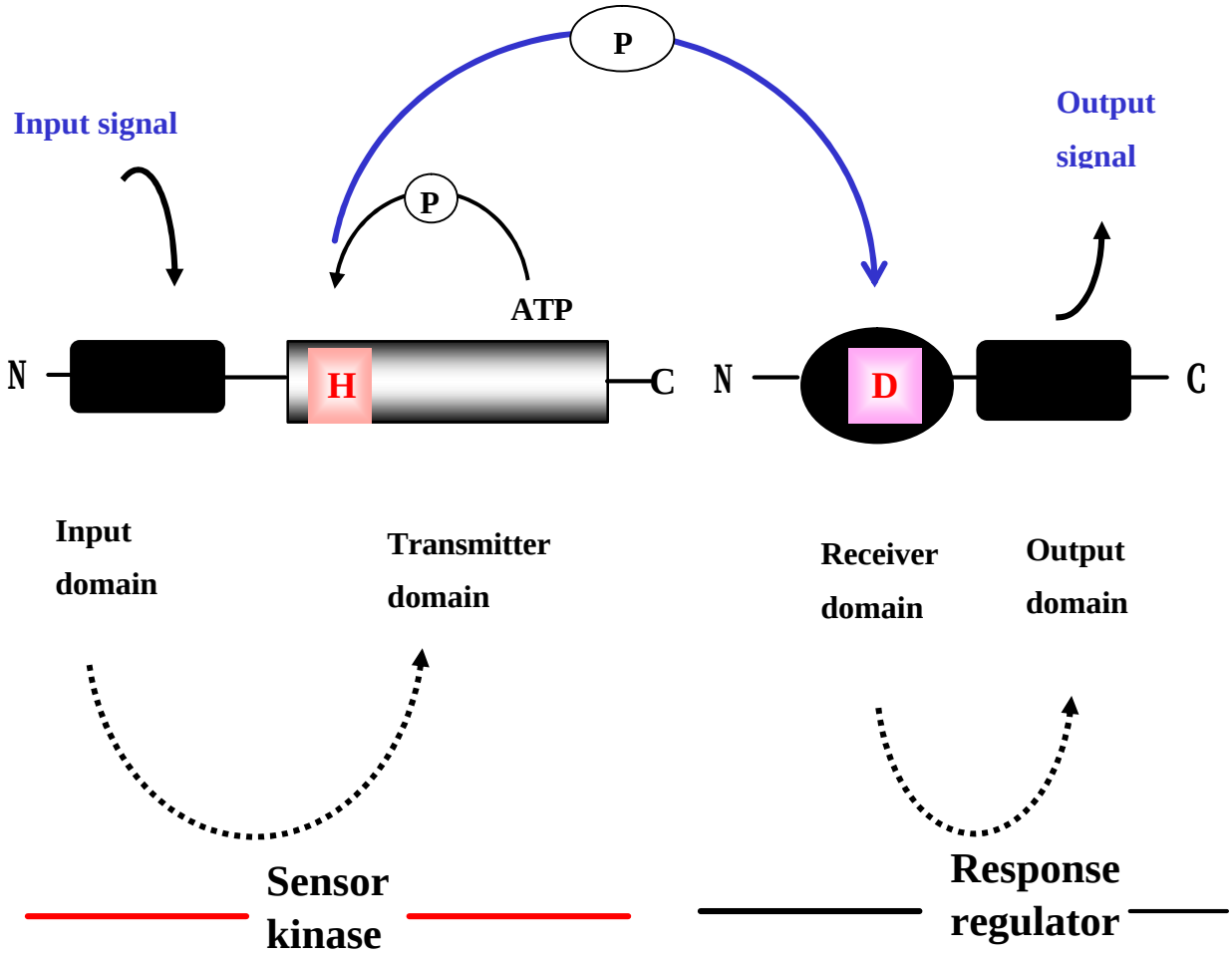

Figure 1. The "two-component" paradigm for sensory signaling via communication module. Information flows through non covalent control exerted by one domain upon another (dashed arrows) and by phosphorylation reactions (arrows labeled P) involving histidine (H) and aspatrate (D) residues are indicated (Parkinson and Kofoid, 1992).

While the naming of “two-component” system has been well accepted, it is often misleading. Signal transduction system through His-Asp phosphotransfer may have originated as a simple one consisting of sensor kinase and response regulator pair, but the system has evolved into multi-component pathways in many organisms. For example, in cyanobacteria, kinase and receiver domains have been found to be connected to various kinds of functional domains within a single protein (Ohmori *et al*., 2001). In addition, it is well known that the His-Asp phosphorelay often resides within a single protein (hybrid kinase) (Hoch and Silhavy, 1995).

The availability of complete genome sequences of many organisms has allowed definitive assessment of the prevalence of two-component system protein. In *E. coli* there are 30 histidine kinases (5 of which are hybrid kinase) and 32 response regulators (Mizuno, 1997). However, the number of two-component genes differs greatly in different bacteria, ranging from 0 in *Mycoplasma genetalium* to 211 in *Anabaena* PCC7120. Analyses of other representative completed bacterial genomes have estimated the number of genes encoding two-component system to be as follow: *Haemophilus influenzae*, 9; *Helicobacter pylori*, 11; *Thermatoga maritima*, 19; *Streptococcus pneumoniae*, 27; *Bacillus subtilis*, 70; *Synecocystis* sp., 80 (Stock *et al*., 2000). Surprisingly, cyanobacteria *Anabaena* PCC 7120 genome contains 211 genes having two-component signaling element. These genes together represent 4.2% of the coding capacity of the whole genome, making this bacterium a leading member among prokaryotes in terms of its signaling potential (Wang *et al*., 2002). Two-component proteins are abundant in most bacteria, but they are also found in archaea and eukaryotes, although the number is limited. In fungi, two-component systems mediate environmental stress responses (Rui and Schuller, 1995), and they are involved in important processes such as osomoregulation, cell growth and differentiation in plants (Loomis et al., 1998). However, no two-component genes have been identified in animals.

The genome sequencing of a gram-positive bacterium, *B. subtilis*, has revealed the presence 36 sensor kinase and 35 response regulator genes, among which each of 30 kinase-regulator pairs reside in an operon (Fabret *et al*., 1999; Kunst, F. *et al*., 1997). None of the *B. subtilis* proteins were composite kinase in which phosphorylatable response regulator domain was contiguous with the kinase domain. Among 30 kinase-regulator pairs, specific roles has been characterized for 8 pairs (reviewed in Ogura and Tanaka, 2002); DegS-DegU, regulation of degradation enzyme production and competence; ComP-ComA, regulation of early competence development; PhoP-PhoR, phosphate starvation responses; ResE-ResD, switching of aerobic and anaerobic respiration; DesK-DesR, cold temperature adaptation; DctS-DctR, C-4 dicarboxylate uptake; CssS-CssR, responses to protein secretion stress; CitS-CitT, citrate uptake. In addition, CheA-CheY pair and an orphan regulator, CheB, are known to regulate chemotaxis (Garrity and Ordal, 1997; Kirby *et al*., 2000). Very recently, YufL-YufM was reported to be involved in malate uptake and metabolism (Tanaka *et al*., 2003; Doan *et al*., 2003). In a directed knockout study of the two-component system genes, only the YycG-YycF system was found to be essential for cell viability (Fabret *et al*., 1998; Fukuchi *et al*., 2000); however signal it senses and genes it regulates is still unclear. Furthermore, three orphan kinases (KinA, KinB and KinC), two orphan response regulator (Spo0F and Spo0A) and a phosphotransferase domain protein (Spo0B) constitute a well characterized phosphorelay cascade leading to initiation of sporulation (Tzeng *et al*., 1998; Fujita and Losick 2003). The other response regulator null mutations did not noticeably affect colony morphology, growth, or sporulation on laboratory media (Fabret *et al*., 1998). Thus, although *B. subtilis* is a well characterized model bacterium for biological researches, function of about half of its two-component systems are still unknown.

I. 2. ABC transport system

All cells and sub cellular compartments are separated from the external milieu by lipid membranes. Cell survival requires the regulated and selective passage of specific molecules across these membranes; the uptake of essential nutrients, ions, and metabolites, as well as the expulsion of toxic compounds, cell envelope macromolecules, secondary metabolites, and the end products of metabolism. Transmembrane transport is mediated by specific proteins which associate with the membrane. The characterization of these proteins and their role in cellular physiology has been a focus of intensive research for many years (Schmitt and Tampe, 2002). The membrane transport system can be grouped into channel proteins that catalyze movement of solutes by an energy-independent process, secondary active transporters that use chemiosmotic energy, primary active transporters that use chemical, electrical or solar energy, and group translocating systems, such as bacterial phosphotransferase systems (Lage, 2003). ABC transport system, which utilizes the energy of ATP hydrolysis to pump solute across the membrane, is considered to be the largest and most diverse subfamily of primary active transport systems (Higgin, 1992).

A typical of ABC transport system consists of four membrane-embedded or associated domains that may be expressed as separate polypeptides or fused multidomain protein(s) (Figure 2). Two transmembrane domains span the membrane multiple times to form a pathway through which solute crosses the membrane and determine the substrate specificity/selectivity of the transporter. Two ATP-binding domains, located at the cytosolic face of the membrane, couple ATP hydrolysis to solute movement. The later domain shares 30-40% sequence identity with the equivalent domains from other ABC transporters, irrespective of their substrate specificity or species of origin, and is characterized by the walker A and B motif of nucleotide binding proteins and the ABC signature that distinguishes

these ATP binding cassettes from other nucleotide binding proteins (Higgins, 1995; Linton and Higgins, 1998). Membrane spanning domain of some ABC transporters have been shown to contain a conserved motif (referred as EAA motif) located on the cytoplasmic loop (Dassa and Hofnung, 1985; Saurin *et al*., 1994). This region is assumed to interact with ATP-binding domains (Pearce *et al*., 1992). ABC transporters can serve a wide range of important physiological role from bacteria to human. Nutrient uptake, protein export, drug and antibiotic resistance, and channel function are the general function of ABC transporters (Higgin, 1992). However, despite many elegant genetic and biochemical studies, molecular mechanisms by which ABC transporters mediate transmembrane translocation of solute are still largely unknown.

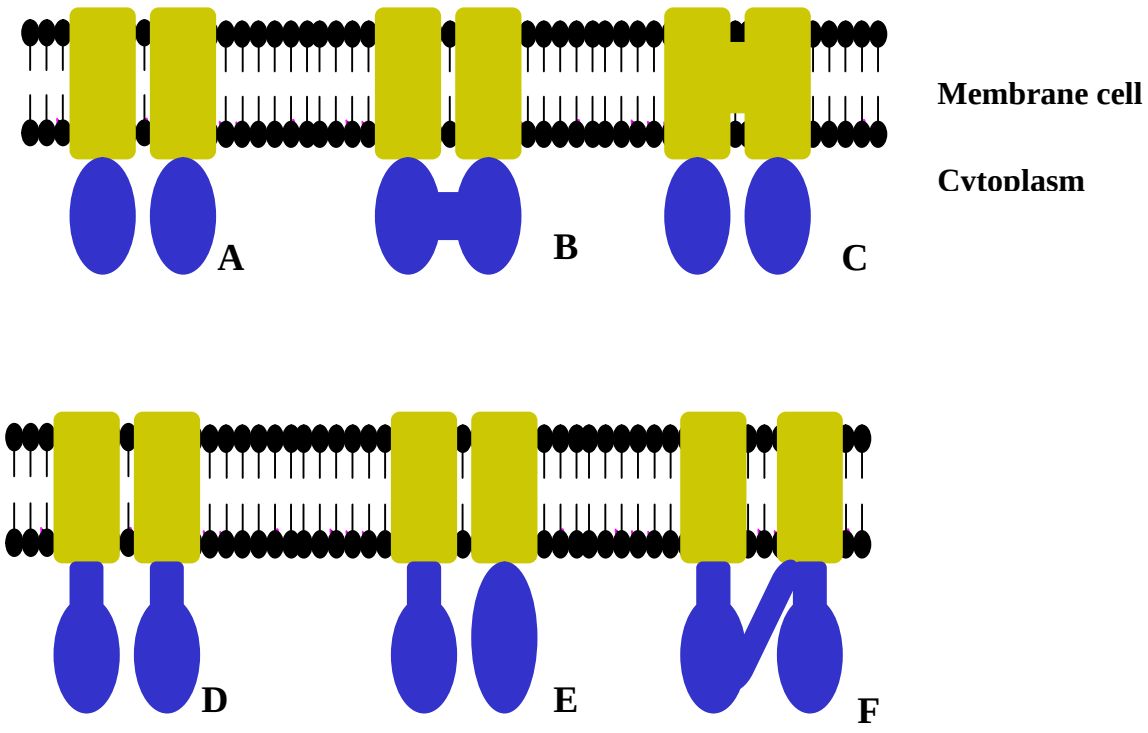

Figure 2. Domain organisation of ABC transporters. The four "core" domains are colored, the transmembrane domains (TMD) in yellow and the nucleotide binding domains (NBD) in blue. The domains can be associated in various ways: (A) four separated domain; (B) fused NDBs (NBD-NBD organization) with heterodimeric TMDs; (C) fused TMDs (TMD-TMD organization) with heterodimeric NBDs; (D) one NBD fused to one TMD (NBD-TMD or TMD-NBD; (E) one TMD fused to one NBD but also including separate TMD and separate NBD; and (F) all four domains in single protein, which is often the arrangement foud in ABC transporters from higher eukaryotes (Linton and Higgins, 1998).

ABC transport systems are now recognized as one of the largest families of proteins in particular organisms (Higgins, 1992). For example the genome of *E. coli* contains 80 genes containing the ABC signature, corresponding to 2% of the genome (Linton and Higgins, 1998). It has been reported that the ABC signature was detected in 41, 18, 16, 17, 54 and 17 genes in *H. influenzae, H. pylori, M. genetalium, Mycoplasma pneumoniae, Synechocystis* sp and *Methanococcus jannaschii,* respectively, and the ATP-binding protein of ABC transport system, which constitutes also around 2% of the entire protein content, is the largest paralogous protein family in each genome (Tomii and Kanehisa, 1998). About 4100 proteins are encoded in the *B. subtilis* genome, and 521 proteins are annotated as transporters, among which 191 proteins including 78 ATP binding proteins are speculated to be member of ABC transport systems. Furthermore comparison of the repertory of ABC transport systems with that of *E. coli* suggested that the extruder are over-represented in *B. subtilis*, due to an expansion of the subfamilies of antibiotic and drug resistance systems (Quentin *et al*., 1999).

I. 3. The functional link between two-component regulatory systems and ABC transporters

Genetic organization of two-component regulatory system genes revealed that they often locate adjacent to the transporter genes and regulate their expression (Stock *et al.*, 2000). Such a functional link between two component regulatory system and ABC transporter has been reported in several bacteria. In *Bacillus licheniformis*, that produces a peptide antibiotic, bacitracin, a two-component system, *bacRS*, senses the bacitracin and induces the expression of BcrABC transporter for self-resistance (Neumüller *et al*., 2001). In *E. coli*, the EvgAS and BaeRS two-component systems are governing the expression of multidrug resistance-transporters, YhiUV and MdtABCD, respectively (Nishino and Yamaguchi, 2002; Baramova and Nikaido, 2002; Nagakubo *et al*., 2002).

In *B. subtilis*, 40 operons are predicted to encode efflux transporter of ABC transporter family (Quentin *et al.*, 1999), and eight possible ABC efflux transporter operons, *ycbMN*, *natAB*, *yclIH*, *yfiLMN*, *yvfRS*, *ytsCD*, *yvcRS*, and *yxdLM*, locate next to genes encoding two-component regulatory system and probably constitute an operon with them (Figure 3) (Kunst, *et al*., 1997). Among the eight operons, *ytsAB-CD*, *yvcPQ-RS*, and *yxdJK-LM* operons are highly homologous to each other in sequence and arrangement of genes in the operon. Furthermore similar operons have been found to be widely conserved in low G+C gram-positive bacteria (Joseph *et al*., 2002). Therefore I focused on the three operons for functional characterization.

In this thesis, I report that the three operons are involved in the response to anitibiotics stresses in *B. subtilis*. As mentioned above, combinations of two-component system and ABC transporter for antibiotic resistance have been found in antibiotics producing bacteria, but this is the first report demonstrating the existence of the similar system in anitibiotics-non-producing bacteria. Furthermore, I found that the antibiotics stresses also induce different ECF sigma factors, whose activities are regulated by membrane embedded anti-sigma factor proteins. This finding indicate that *B. subtilis* has developed a complex signal transduction network to cope with the antibiotics stresses.

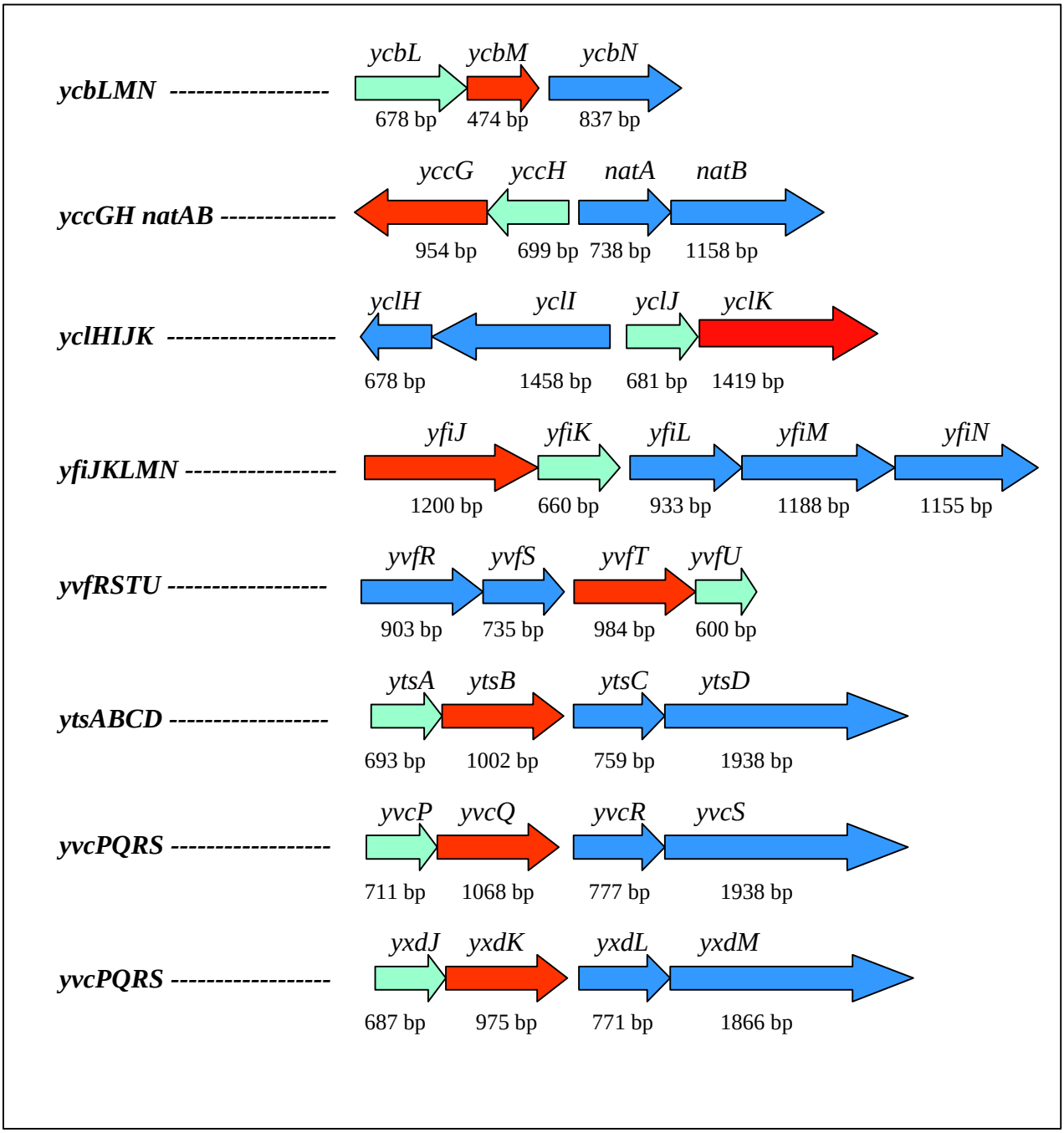

Figure 3. Genetic organization of eight ABC efflux transporters and two-component regulatory system in *B. subtilis*. The *ycbN*, *natAB*, *yclIH*, *yfiLMN*, *yvfRS*, *ytsCD*, *yvcRS* and yxdLM ABC transporter (green) , locate next to the genes encoding sensor kinase (blue) and response regulator (red) of two-component regulatory system.

II. MATERIALS AND METHODS

II. 1. Bacterial strains and growth media

Bacterial strains used in this study are listed in Table 1. *B. subtilis* and *E. coli* cells were cultured in Luria-Bertani (LB) medium (10 g of pancreatic digest of casein, 5 g of yeast extract, 5 g of NaCl per liter, pH 7.2). When required, antibiotics were added at the following conditions: ampicillin at 100 μg/ml, spectinomycin at 100 μg/ml, chloramphenicol at 20 μg/ml for *E. coli* and at 5 μg/ml for *B. subtilis*, kanamycin at 20 μg/ml for *E. coli* and at 10 μg/ml for *B. subtilis*, erythromycin at 100 μg/ml for *E. coli* and at 1 μg/ml for *B. subtilis*, and lincomycin at 12.5 μg/ml. All *B. subtilis* growth media were supplemented with L-tryptophan (required for the growth of parent strain, 168 *trpC2*; 50 μg/ml).

II. 2. Construction of plasmids and *B. subtilis* strains

Table 2 summaries relevant characteristics of the plasmids I constructed in this study. They were used to transform *B. subtilis* cells as indicated in Table 1. The primers for PCR amplification of *B. subtilis* chromosomal fragments used to construct the plasmids are listed in Table 3.

Multi-copy shuttle vectors replicating in *E. coli* and *B. subtilis*, pDG148 (Kobayashi *et al*., 2001) and pNO41 (Moriya *et al*., 1998), were used to overexpress response regulators in *B. subtilis* cells. Derivatives of pMutinT2 (Vagner *et al*., 1998) were used to construct insertional inactivation mutants of *B. subtilis* genes. Strains containing transcriptional fusion of the gradually deleted promoter sequence of transporter genes and the *lacZ* reporter gene at the *amyE* locus on the *B. subtilis* chromosome were constructed using pDL2 derivatives linearized by *Pst*I digestion (Fukuchi *et al.*, 2000). pUC118 was used to construct insertional antibiotic resistance gene, *spec*, used to replace the target gene was obtained from pDG1726

(Guerout-Fleury *et al*., 1995). The *yvcP* or *yvcR* gene inserted antibiotic resistance gene, *spec*, was cloned to pUC118 to generate pGY25 and pGY26. Transformation of *B. subtilis* cells to obtain the strains in Table 1 was done according to Moriya *et al.* (1998). To create *B. subtilis* cells having multiple mutations, chromosomal DNA of single mutants was used for transformation. Other *B. subtilis* strains used in this study were provided by Dr Ohki, Dr. Kobayashi and laboratory stock (Table 1).

To express response regulators (from the initiation to termination codon) in *E. coli*, as a fusion with the GST protein at the N-terminus, I used pGEX-4T-1 (Amersham Pharmacia Biotech).

II. 3. b- **galactosidase assay**

B. subtilis strains grown in LB medium at OD_{600} of 0.01, and incubated at 37^{o}C to OD_{600} of 0.5. Cells were collected by centrifugation and the activity of the ß-galactosidase was assayed according to Youngman *et al*. (1985). Harvested cells were suspended in 300 µl of the Z buffer (0.006M Na_2HPO_4, 0.04M NaH_2PO_4, 0.001M $MgSO_4$, and 0.001 M DTT). Subsequently 3 µl lysis solution (10 mg/ml lysozyme, 1 mg/ml DNase I in H_2O) was added to the suspension, followed by incubation at 37^{o}C for 30 min. Cell debris was separated by centrifugation (14.000 rpm at 4^{o}C for 2 min), and 240 µl of supernatant was removed. Then 50 µl of supernatant was incubated with 10 µl of MUG (4-Methylumbelliferyl-ß-D-galactoside, 0.4 mg/ml) at 28^{o}C for 60 min, followed by incubation at 95^{o}C for 5 minutes to stop the reaction. Amount of MUG hydrolyzed was determined by quantitating the fluorescence of hydrolyzed MUG using Labsystem Fluoroskan II spectrophotometre. One unit was defined as nmole of MUG hydrolyzed in 1 min per 1 mg of protein. Protein concentration was determined using Bio-Rad Protein Assay Kit. All results are the means of three assays.

II. 4. Northern blotting experiments

B. subtilis cells growing in LB medium were collected by centrifugation, and total RNA was extracted from the cells as described previously (Igo and Losick, 1986). Harvested cells were resuspended in a mixture of 0.55 ml of LETS buffer (100 mM LiCl, 10 mM EDTA, 10 mM Tris-HCl, pH 7.4, and 1% SDS), 0.5 ml of glass beads (0.7 mm, Asone) and 0.5 ml of phenol-chloroform-isoamyl alcohol (24:1:1), then vortexed at maximum speed for 4 minutes. Cell debris and glass beads were removed by centrifugation at 8,000 rpm for 10 minutes at 4^{o}C. Then, the supernatant was extracted with equal amount of phenol-chloroform-isoamyl alcohol again, and RNA was precipitated by 2.5 X volume of ethanol, after the addition of 15 μl of 4M LiCl. RNA pellets were collected by centrifugation at 14,000 rpm for 10 minutes, and washed twice with 70% ethanol. Dried pellets were resuspended in 150 μl H_2O, subsequently 450 μl of 3 M NaOAc (pH 5.6) was added, and kept at –30^{o}C overnight. RNA was collected by centrifugation at 14,000 rpm for 5 minutes, washed twice with 70% ethanol, dried, and dissolved in 50 μl of H_2O.

Aliquots containing 5 μg RNA were electrophoresed on a 1% formaldehyde-agarose gel and blotted onto the positively charged nylon membrane (Hybond N^{+}, Amersham Pharmachia) according to the procedure of Sambrook *et al*., (1989). Hybridization were performed at 68^{o}C overnight with digoxigenin-labeled RNA probes (10 ng) according to the manufacturer`s instructions (Boehringer Mannheim). Digoxigenin-labeled RNA probes were synthesized in vitro with T7 RNA polymerase. DNA templates for the reactions were obtained by PCR using primers having the T7 RNA polymerase promoter sequence (Table 3). Hybridization signals were detected using CSPD^{R}, ready–to-use (Roche) according to the manufacturer’s instruction.

II. 5. Purification of response regulator proteins

E. coli BL21(DE3)pLysS cells (Novagen) harboring the pGEX-*ytsA*, pGEX-*yvcP*, or pGEX-*yxdJ* were incubated at 30^{o}C in 100 ml LB medium containing ampicillin (100 μg/ml). When the culture reached to an OD_{600} of 0.7, IPTG was added to a final concentration of 1 mM. The cells were incubated for another 3 hours, then harvested by centrifugation, and the GST-YtsA, GST-YvcP, or GST-YxdJ protein were purified using the GST purification kit (Amersham Pharmacia Biotech). Harvested cells were suspended in phosphate-buffered saline (PBS:140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH7.3) and broken by sonication on ice using ultrasonic processor machine (Astrason) with total process time 4 minutes; pulse on 10 and pulse off 30 seconds. Cellular debris was removed by centrifugation at 8,000 rpm for 30 minutes at 4^{o}C, and the supernatants were applied to glutathione sepharose column (Amersham Pharmacia Biotech). The columns were washed with 10 ml of PBS buffer 3 times, then GST- fusion proteins were eluted by a 50 mM Tris-HCl (pH8.0) containing 10 mM gluthathione. The fractions containing GST-fusion proteins were stored at –80^{o}C after the addition of glycerol to 10% (v/v).

II. 6. DNAse I footprinting experiments

DNA probes for DNAse I footprinting experiments were prepared by PCR using primers listed in Table 3. One of the primer pair was previously labeled with T4 polynucleotide kinase (Takara shuzo) and [γ–^{32}P] dATP. Labeled PCR products were purified with the Nick Spin Columns (Pharmacia Biotech). Binding reactions were performed by incubation of 300,000 cpm labeled DNA fragments (6-9 fmole) with various amounts (5 – 15 pmole) of GST-fusion proteins in a 50 μl volume (50 mM PIPES, 200 mM NaCl, 4 mM $MgCl_2$, 4 mM DTT, 0.5% Tween-20, 10% glycerol, and 5 μl poly (dI-dC) at 25^{o}C for 30 minutes. Then, DNase I (0.14 U; Takara Shuzo) was added to the binding reactions and

incubated at 25°C for 1 minute, and the reactions were stopped by adding 100 μl of 20 mM EDTA. DNA fragments were extracted by phenol-chloroform-isoamylalcohol (24:1:1), followed by ethanol precipitation. The pellets were dissolved in 4 μl stop solution (0.01% bromophenol blue, 0.01% xylene cyanol and 1 mM EDTA in 90% formamide), and 3 μl of the solution was loaded onto 6% polyacrylamide gels. After electrophoresis, radioactive bands were detected with Imaging Plates and a BAS2500 scanner (Fuji Photo Film). DNA ladders used as size markers were created with the appropriate end-labeled primer and a cycle sequencing kit (TaKaRa Biomedicals).

II. 7. Primer extension experiments

The GY59 primer was labeled with [γ-^{32}P]-ATP (Amersham Pharmacia Biotech) and T4 polynucleotide kinase (Takara Shuzo). The RNA sample (10 μg) was incubated with the end-labeled primer (0.5 pmol) for 60 min at 60°C. Reverse transcription was carried out for 60 min at 42°C using a SUPERSCRIPT reverse transcriptase according to the manufacturer's instruction (GIBCO BRL). The cDNA products were then electrophoresed through an 8% polyacrylamide urea gel and radioactive bands were detected with Imaging Plate and BAS2500 scanner (Fuji Photo Film). DNA ladders for use as size markers were created with the same end-labeled primer and a cycle sequencing kit (Takara Shuzo). Primers, GY58 and GY59, were used to amplify the template DNA for the sequencing reaction.

II. 8. Assay of bacitracin-resistant phenotype

Bacitracin (Sigma, 70 Umg^{-1}) resistance was assayed in LB medium, as described in an previous report (Ohki *et al.*, 2003). To calculate the relative resistance for various mutants, the concentration of bacitracin leading to 50% inhibition of growth (IC_{50}) was expressed as a ratio relative to the IC_{50} value in the control strain.

II. 9. Plate assay for induction of the transporter promoters by antibiotics

The BSGY08, BSGY16, and BSGY24 cells containing P*ytsC-lacZ*, P*yvcR-lacZ*, and P*yxdL-lacZ* fusion at the *amyE* locus, respectively, were grown on LB medium at 37^{o}C to mid logarithmic phase (OD_{600}=0.5). One ml of culture was added to 10 ml of melted LB agar (0.8%) containing 100 μg/ml X-gal, and poured onto 2% solidified LB agar containing 100 μg/ml X-gal. Sterilized-filter paper disks (8 mm in diameter, Advantec) containing 50 μl of antibiotics solution (2 mg/ml) were placed on the lawn of bacteria. The plates were incubated at 37^{o}C overnight, and induction of the expression of P*ytsC-lacZ*, P*yvcR-lacZ*, or P*yxdL-lacZ* fusion was examined by the color of cells around the filters.

Table 1. Bacterial strains used in this study

Strain	Relevant genotype*	Source
B. subtilis 168	*trpC2*	Pasteur stock
BSGY01	*trp*C2 *amyE::*P*ytsC* (-57 to + 52)-*lacZ cat*	pGY01 X 168
BSGY02	*trp*C2 *amyE::*P*ytsC* (-68 to + 52)-*lacZ cat*	pGY02 X 168
BSGY03	*trp*C2 *amyE::*P*ytsC* (-78 to + 52)-*lacZ cat*	pGY03 X 168
BSGY04	*trp*C2 *amyE::*P*ytsC* (-88 to + 52)-*lacZ cat*	pGY04 X 168
BSGY05	*trp*C2 *amyE::*P*ytsC* (-106 to + 52)-*lacZ cat*	pGY05 X 168
BSGY06	*trp*C2 *amyE::*P*ytsC* (-149 to + 52)-*lacZ cat*	pGY06 X 168
BSGY07	*trp*C2 *amyE::*P*ytsC* (-204 to + 52)-*lacZ cat*	pGY07 X 168
BSGY08	*trp*C2 *amyE::*P*ytsC* (-247 to + 52)-*lacZ cat*	pGY08 X 168
BSGY09	*trp*C2 *amyE::*P*yvcR* (-12 to + 51)-*lacZ cat*	pGY09 X 168
BSGY10	*trp*C2 *amyE::*P*yvcR* (-32 to + 51)-*lacZ cat*	pGY10 X 168
BSGY11	*trp*C2 *amyE::*P*yvcR* (-51 to + 51)-*lacZ cat*	pGY11 X 168
BSGY12	*trp*C2 *amyE::*P*yvcR* (-79 to + 51)-*lacZ cat*	pGY12 X 168
BSGY13	*trp*C2 *amyE::*P*yvcR* (-93 to + 51)-*lacZ cat*	pGY13 X 168
BSGY14	*trp*C2 *amyE::*P*yvcR* (-109 to + 51)-*lacZ cat*	pGY14 X 168
BSGY15	*trp*C2 *amyE::*P*yvcR* (-132 to + 51)-*lacZ cat*	pGY15 X 168
BSGY16	*trp*C2 *amyE::*P*yvcR* (-159 to + 51)-*lacZ cat*	pGY16 X 168
BSGY17	*trp*C2 *amyE::*P*yxdL* (-96 to +4)-*lacZ cat*	pGY17 X 168
BSGY18	*trp*C2 *amyE::*P*yxdL* (-148 to +4)-*lacZ cat*	pGY18 X 168
BSGY19	*trp*C2 *amyE::*P*yxdL* (-159 to +4)-*lacZ cat*	pGY19 X 168
BSGY20	*trp*C2 *amyE::*P*yxdL* (-169 to +4)-*lacZ cat*	pGY20 X 168
BSGY21	*trp*C2 *amyE::*P*yxdL* (-179 to +4)-*lacZ cat*	pGY21 X 168
BSGY22	*trp*C2 *amyE::*P*yxdL* (-198 to +4)-*lacZ cat*	pGY22 X 168
BSGY23	*trp*C2 *amyE::*P*yxdL* (-253 to +4)-*lacZ cat*	pGY23 X 168
BSGY24	*trp*C2 *amyE::*P*yxdL* (-297 to +4)-*lacZ cat*	pGY24 X 168
BSGY25	*trp*C2 *amyE::*P*yvcR* (-159 to + 51)-*lacZ cat*, *yvcP*::*spec*	pGY25 X BSGY16
BSGY26	*trp*C2 *amyE::*P*yvcR* (-159 to + 51)-*lacZ cat*, *yvcR*::*spec*	pGY26 X BSGY16

Table 1. (continued)

YTSAd	*ytsA*::pMutin2	Dr. Ohki
YTSBd	*ytsB*::pMutin2	Kobayashi *et al*., 2003
YTSCd	*ytsC*::pMutin2	Kobayashi *et al*., 2003
YTSDd	*ytsD*::pMutin2	Kobayashi *et al*., 2003
ywoA::*tet*	*ywoA*::*tet*	Dr. Ohki
YTSCd/*ywoA*::*tet*	*ytsC*::pMutin2, *ywoA*::*tet*	Dr. Ohki
YTSDd/*ywoA*::*tet*	*ytsC*::pMutin2, *ywoA*::*tet*	Dr. Ohki
SigI::*cat*	*sigI*::*cat*	Lab stock

*Number in parentheses indicate the nucleotide numbers correspond to translation initiation site of each gene

Table 2. Plasmids used in this study

Plasmid	Vector	Insert*	Primer
pNO41-*ytsA*	pNO41	entire coding region of *ytsA*	GY40, GY41
pNO41-*yvcP*	pNO41	entire coding region of *yvcP*	GY42, GY43
pNO41-*yxdJ*	pNO41	entire coding region of *yxdJ*	GY44, GY45
pGEX-*ytsA*	pGEX-4T-1	entire coding region of *ytsA*	GY46, GY47
pGEX-*yvcP*	pGEX-4T-1	entire coding region of *yvcP*	GY48, GY49
pGEX-*yxdJ*	pGEX-4T-1	entire coding region of *yxdJ*	GY50, GY51
pGY01	pDL2	110 bp fragment of the promoter region of *ytsC* (-57 to + 52)	GY01, GY09
pGY02	pDL2	121 bp fragment of the promoter region of *ytsC* (-68 to + 52)	GY02, GY09
pGY03	pDL2	131 bp fragment of the promoter region of *ytsC* (-78 to + 52)	GY03, GY09
pGY04	pDL2	141 bp fragment of the promoter region of *ytsC* (-88 to + 52)	GY04, GY09
pGY05	pDL2	159 bp fragment of the promoter region of *ytsC* (-106 to + 52)	GY05, GY09
pGY06	pDL2	202 bp fragment of the promoter region of *ytsC* (-149 to + 52)	GY06, GY09
pGY07	pDL2	257 bp fragment of the promoter region of *ytsC* (-204 to + 52)	GY07, GY09
pGY08	pDL2	300 bp fragment of the promoter region of *ytsC* (-247 to + 52)	GY08, GY09
pGY09	pDL2	063 bp fragment of the promoter region of *yvcR* (-12 to +51)	GY10, GY18
pGY10	pDL2	084 bp fragment of the promoter region of *yvcR* (-32 to +51)	GY11, GY18
pGY11	pDL2	103 bp fragment of the promoter region of *yvcR* (-51 to +51)	GY12, GY18
pGY12	pDL2	131 bp fragment of the promoter region of *yvcR* (-79 to +51)	GY13, GY18
pGY13	pDL2	145 bp fragment of the promoter region of *yvcR* (-93 to +51)	GY14, GY18
pGY14	pDL2	161 bp fragment of the promoter region of *yvcR* (-109 to +51)	GY15, GY18
pGY15	pDL2	184 bp fragment of the promoter region of *yvcR* (-132 to +51)	GY16, GY18
pGY16	pDL2	211 bp fragment of the promoter region of *yvcR* (-159 to +51)	GY17, GY18
pGY17	pDL2	101 bp fragment of the promoter region of *yxdL* (-96 to + 4)	GY19, GY27
pGY18	pDL2	153 bp fragment of the promoter region of *yxdL* (-148 to + 4)	GY20, GY27
pGY19	pDL2	164 bp fragment of the promoter region of *yxdL* (-159 to + 4)	GY21, GY27
pGY20	pDL2	174 bp fragment of the promoter region of *yxdL* (-169 to + 4)	GY22, GY27
pGY21	pDL2	184 bp fragment of the promoter region of *yxdL* (-179 to + 4)	GY23, GY27
pGY22	pDL2	203 bp fragment of the promoter region of *yxdL* (-198 to + 4)	GY24, GY27

Table 2. (continued)

pGY23	pDL2	258 bp fragment of the promoter region of *yxdL* (-253 to + 4)	GY25, GY27
pGY24	pDL2	302 bp fragment of the promoter region of *yxdL* (-297 to + 4)	GY26, GY27
pGY25	pUC118	entire coding region of *yvcP* with an insertion of *spec* gene	GY42, GY43
pGY26	pUC118	entire coding region of *yvcR* with an insertion of *spec* gene	GY60, GY61

*Number in parentheses indicate the nucleotide numbers correspond to translation initiation site of each gene

Table 3. Primers used in this study

Primer	Sequence*	Restriction Enzymes	Location in *B. subtilis* chromosome
Construction of promoter deletion mutants			
GY01	5`-cgccgtgaattcGGAAAAGCCCGGCATTCC-3`	*Eco*RI	3111228-3111210
GY02	5`-ctttgagaattcGCCGTATCGAAGGAAAAG-3`	*Eco*RI	311239 -311221
GY03	5`-ctttgagaattcTTTTTTGTTCGCCGTATC-3`	*Eco*RI	3111249-3111231
GY04	5`-ctttgagaattcCATGCTTTTCTTTTTTGT-3`	*Eco*RI	3111259-3111241
GY05	5`-acatgtgaattcCGTGTGACGAAAATGTCA-3`	*Eco*RI	3111277-3111258
GY06	5`-gaacaggaattcCATTAACCTTTCCAATACGG-3`	*Eco*RI	3111320-3111300
GY07	5`-aaaagcgaattcGCCGTTATTGATCCATATTG-3`	*Eco*RI	3111376-3111356
GY08	5`-ccatgagaattcGTCAACTGGCATGGGGCTG-3`	*Eco*RI	3111418-3111399
GY09	5`-cacttccctggatccTCAGCTTGTTTCCATAAC-3`	*BamH*I	3111117-3111135
GY10	5`-ctttgagaattcGGAGGAGCAGAACATGAACG-3`	*Eco*RI	3565348-3565328
GY11	5`-ctttgagaattgCTTTTTATAATAAAGAAAAAG-3`	*Eco*RI	3565368-3565347
GY12	5`-ctttgagaattcATGGGAGGATGCTGACTTCC-3`	*Eco*RI	3565387-3565368
GY13	5`-ctttgagaattcTTGGGGAGCGGAATTGCAAG-3`	*Eco*RI	3565415-3565396
GY14	5`-ctttgagaattcACAGCATTGTAAGATTGGGG-3`	*Eco*RI	3565429-3565410
GY15	5`-ctttgagaattcCACCCTCGTGAATGTGAC-3`	*Eco*RI	3565445-3565428
GY16	5`-ctttgagaattcATGACCATCGTGTTTTCAAG-3`	*Eco*RI	3565468-3565441
GY17	5`-ctttgagaattcGGAATCAAAAGAAGGGGC-3`	*Eco*RI	356546-3565449
GY18	5`-gcaggcagatctCGATAGGTTCGTTGTTTGCAACACG-3`	*Bgl*II	3565305-3565329
GY19	5`-ttccccgaattcACATGGATTTATGTCAAGGG-3`	*Eco*RI	4070052-4070032
GY20	5`-tttcttgaattcATGTAAGGCTGCGTTAAG-3`	*Eco*RI	4070330-4070313
GY21	5`-ctttgagaattcTTCTTACAAAAATGTAAG-3`	*Eco*RI	4070341-4070324
GY22	5`-ctttgagaattcCGATTTTCATTTCTTACA-3`	*Eco*RI	4070125-4070107
GY23	5`-ctttgagaattcGACATCAGTTCGATTTTC-3`	*Eco*RI	4070135-4070117
GY24	5`-cggtgggaattcGCTCGTCTCCCGGTGAAGG-3`	*Eco*RI	4070154-4070135
GY25	5`-tctacggaattcGGCCTCCATCTCGTCAAAG-3`	*Eco*RI	4070209-4070190
GY26	5`-ttgaccgaattcACACGGGTGAAAACGGGCG-3`	*Eco*RI	4070253-4070234
GY27	5`-ttcaagcaggatccCCATGAAAATCCTCCTTATG-3`	*BamH*I	4069951-4069971

Table 3. (continued)

Northern blot experiments			
GY28	5`-tgccgcggatccGTTTCAGCAGGCTTTGATGC-3`	*BamH*I	3112193-3112174
GY29	5`-taatacgactcactatagggcgaGCATCGCTGTTAATGGAG-3`	-	3111844-3111861
GY30	5’-aacaagaagcttAAACAGGAAGTGCTGAAGGG-3`	*Hind*III	3111083-3111064
GY31	5’-taatacgactcactatagggcgaGCAACTTCCTCAAACTTGCG-3`	-	3110755-3110774
GY32	5`-attattggatccTCATTATTAGGGATAGGGTC-3`	*BamH*I	3566413-3566394
GY33	5`-taatacgactcactatagggcgaCCCATTGGTTGGTGAAGGTG-3`	-	3566056-3566075
GY34	5`-attcaaaagcttGAACAATATCATACCAAGCG-3`	*Hind*III	3565305-3565285
GY35	5’-taatacgactcactatagggcgaGCGTATCCGCCAGTTCATCC-3`	-	3564908-3564928
GY36	5`-catgaaaagcttTCTCCGGTCTCATGCAGT-3`	*Hind*III	4071291-4071274
GY37	5`-taatacgactcactatagggcgaTATACGTCACCCGCGCG-3`	-	4070957-4070973
GY38	5’-ataaaaaagcttAAGGACAAGTGTCCTATCAG-3`	*Hind*III	4070138-4070119
GY39	5’-taatacgactcactatagggcgaCGAGCTTTGCCGCGATGCCG-3`	-	4069788-4069807
BceSF	5`-TTCAGGATTTCAGTCAGGTGCTG-3`	-	3112850-3112857
BceSR	5`-CATTGCTGACGAGGTTCTGCTCC-3`	-	3112525-3112540
BceRF	5`-TGCTGTTCATTGCTTTTGTC-3`	-	3112189-3112208
BceRR	5`-GGGATTTCAGTGCTTTTTCT-3`	-	3111834-3111853
BceAF	5`-CTGAATAAACAGGAAGTGCTGA-3`	-	3111100-3111121
BceAR	5`-GCTTCCTTTTTTGATAGTTTCG-3`	-	3110811-3110832
BceBF	5`-TTTATTTCGCCTTTGTCACGCT-3`	-	3110327-3110340
BceBR	5`-GCAGTTTCGCATCCGCTTTGACA-3`	-	3109980-3110002
BcrCF	5`-GAACACACAAAGCAGAAAACA-3`	-	3757735-3757755
BcrCR	5`-AAAAGCCCAAAAATGACAAGC-3`	-	3757966-3757986
YkoZF	5`-GCAAAGACGGTTTCATCCGT-3`	-	1411332-1411351
YkoZR	5`- taatacgactcactatagggcgaGGATAGCGGCCAATTCTTCA-3`	-	1411791-1411772
Cloning of response regulator genes into pNO41			
GY40	5’-gtataagcgggatccGGCGTTTTGCTATACACTTG-3’	*BamH*I	3112980-3112961
GY41	5`-cgataaggggatccTTAATCATAGAACTTGTCCTC-3’	*BamH*I	3112236-3112256
GY42.	5’-agaaaaaaggatccTTACTATTCCGCACATGC-3’	*BamH*I	3567243-3567226
GY43	5’gatgagatggatccTTAACATTCCGCTTCATCC-3’	*BamH*I	3566452-3566470
GY44	5’-catacgccggatccGATTATGATGATGATGGCAGCAG-3’	*BamH*I	4072028-4072013
GY45	5’-cgggagaaggatccTCATGACTGCGCCCTCAGC-3’	*BamH*I	4071299-4071317

Table 3. (continued)

Cloning of response regulator genes into pGEX-4T-1			
GY46	5’-aatattcaattgGTGTATCGGATTTTGCTTGTG-3`	*Mun*I	3567195-35671974
GY47	5’-aatattctcgagTTAACATTCCGCTTCATCCTTCCG-3`	*Xho*I	3566482-3566506
GY48	5`-attattgaattcTTGTTTAAACTTTTGCTGATT-3`	*Eco*RI	3112963-3112942
GY49	5`-aatattgtcgacTTAATCATAGAACTTGTCCTC-3`	*Sal*I	3112268-3112289
GY50	5`-aatattgaattcTTGAATAAAATCATGATTGTG-3`	*Eco*RI	4071762-4071742
GY51	5`-aatattgtcgacTCATGACTGCGCCCTCAGCTG-3`	*Sal*I	4071073-4071094
DNAse I footprinting experiments			
GY52	5’-TTGGCAAAAAAAGCAGCGGCG-3`	-	3111364-3111343
GY53	5`-TCAGCTTGTTTCCATAAC-3`	-	3111117-3111135
GY54	5`-TGAAAGAGGCAACTGGAATGG-3`	-	3565539-3565519
GY55	5’-GTATGTTTTCGATAGGTTCG-3’	-	3565267-3565286
GY56	5`-CCAAGAATCTACGGGAATC-3`	-	4070454-4070436
GY57	5`-CGATAGGTTCGTTGTTTGCAACACG-3`	-	3565305-3565329
Primer extension experiments			
GY58	5`-GGA ATGTACTTTATATGGTGTATTTGTGCA-3`	-	3112137-3112108
GY59	5`-CCCTTTTCAATGAGAATATCGATGCCCTTCAGC-3`	-	3111040-3111072
Construction of pGY25 and pGY26 plasmids			
GY42	5’-agaaaaaaggatccTTACTATTCCGCACATGC-3’	*BamH*I	3567243-3567226
GY43	5`-gatgagatggatccTTAACATTCCGCTTCATCC-3’	*BamH*I	566452-3566470
GY60	5`-ggaggatccATGAACGTGTTGCAAACAACGAAC-3’	*BamH*I	3565305-3565282
GY61	5`-ggaggatccCGGGCGATAGTTCTCAAATTC-3’	*BamH*I	3564529-3564549

*Lower-case indicate artificial sequence and underlined characters indicate artificial recognition site of restriction enzymes.

III. RESULTS

III. 1. YtsA, YvcP and YxdJ egulators induce the expression of ABC transporter genes in the same operon

III. 1. 1. Transcriptional analysis of *ytsABCD, yvcPQRS,* and y*xdJKLM* operon

The *B. subtilis* genome sequence revealed three possible operons, *ytsABCD*, *yvcPQRS*, and *yxdJKLM*, highly homologous to each other in sequence and arrangement of genes in the operon (Figure 4). Each operon comprises two gene pairs encoding a two component regulatory system and an ABC transport system, respectively, located in tandem separated by an intergenic region less than 150 bp.

To confirm operonic structure of these genes, I performed Northern blot analysis using probes specific for each gene on *ytsABCD, yvcPQRS,* and *yxdJKLM* operons. *B. subtilis* 168 cells were grown in LB medium at 37^{o}C and harvested for RNA extraction at times shown in Figure 5A. Northern blot experiments revealed that the transcription of *ytsABCD* and *yxdJKLM* was composed of two transcriptional units. The longer transcripts (4.7 kb) were detected by probes for all genes in each operon (Figure 5B and 5D), while the shorter transcripts corresponding to the first two genes encoding response regulator and sensor kinase on each operon. On the other hand, only one transcript covering the whole operon was detected for *yvcPQRS* (Figure 5C). These result demonstrated that indeed *ytsABCD, yvcPQRS* and *yzdLKLM* constitute operons. Transcripts of three operons were detected in the log phase of bacterial growth, while their signals disappeared after the entry into the stationary phase, suggesting that three operons are constitutively expressed depending on RNA polymerases containing major sigma factor, SigA.

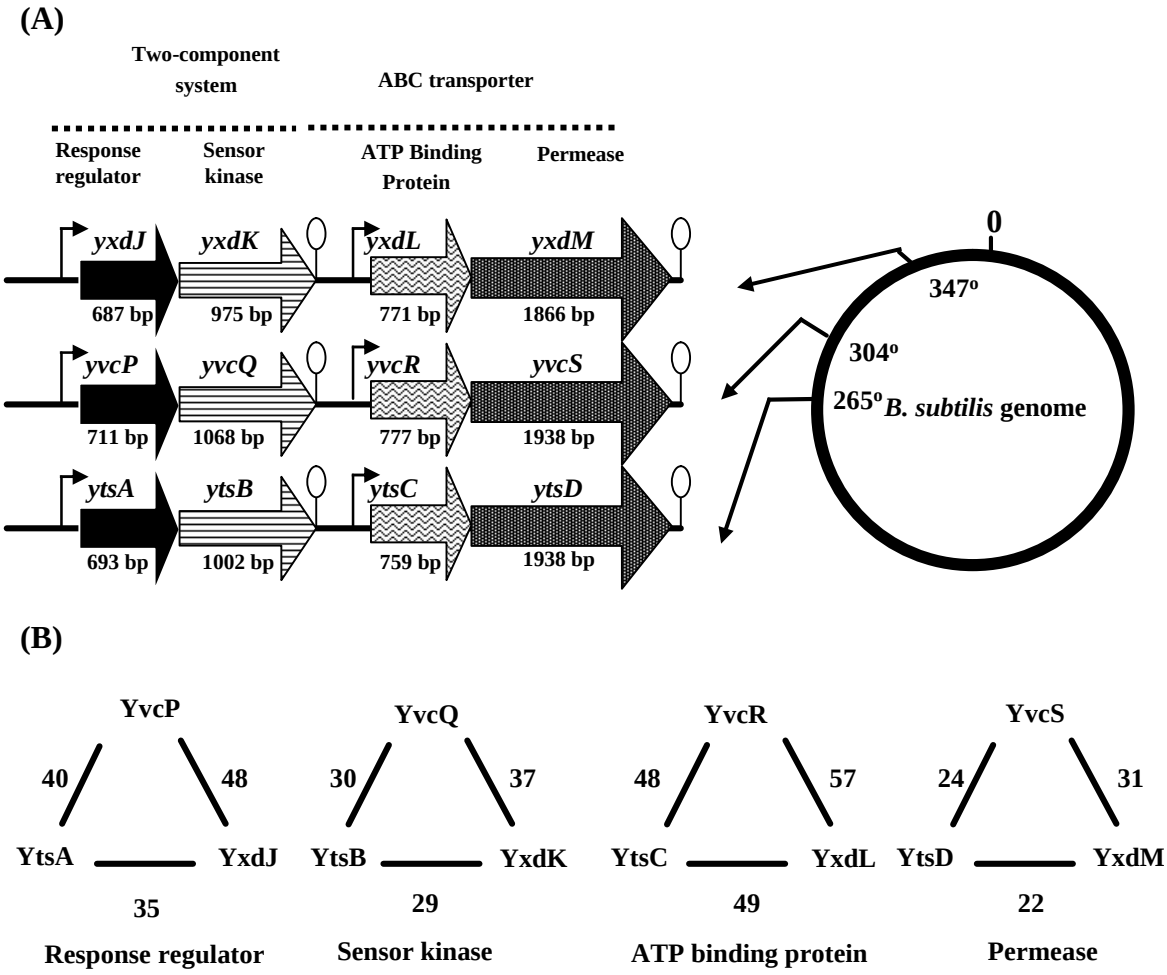

Figure 4. Location and genetic organization of *ytsABCD, yvcPQRS,* and *yxdJKLM* operon. (A) Thin arrows indicate promoters and a circle on a stem indicates a transcriptional termination signal. (B) Amino acid sequence identity (%) of the corresponding genes in the three operons.

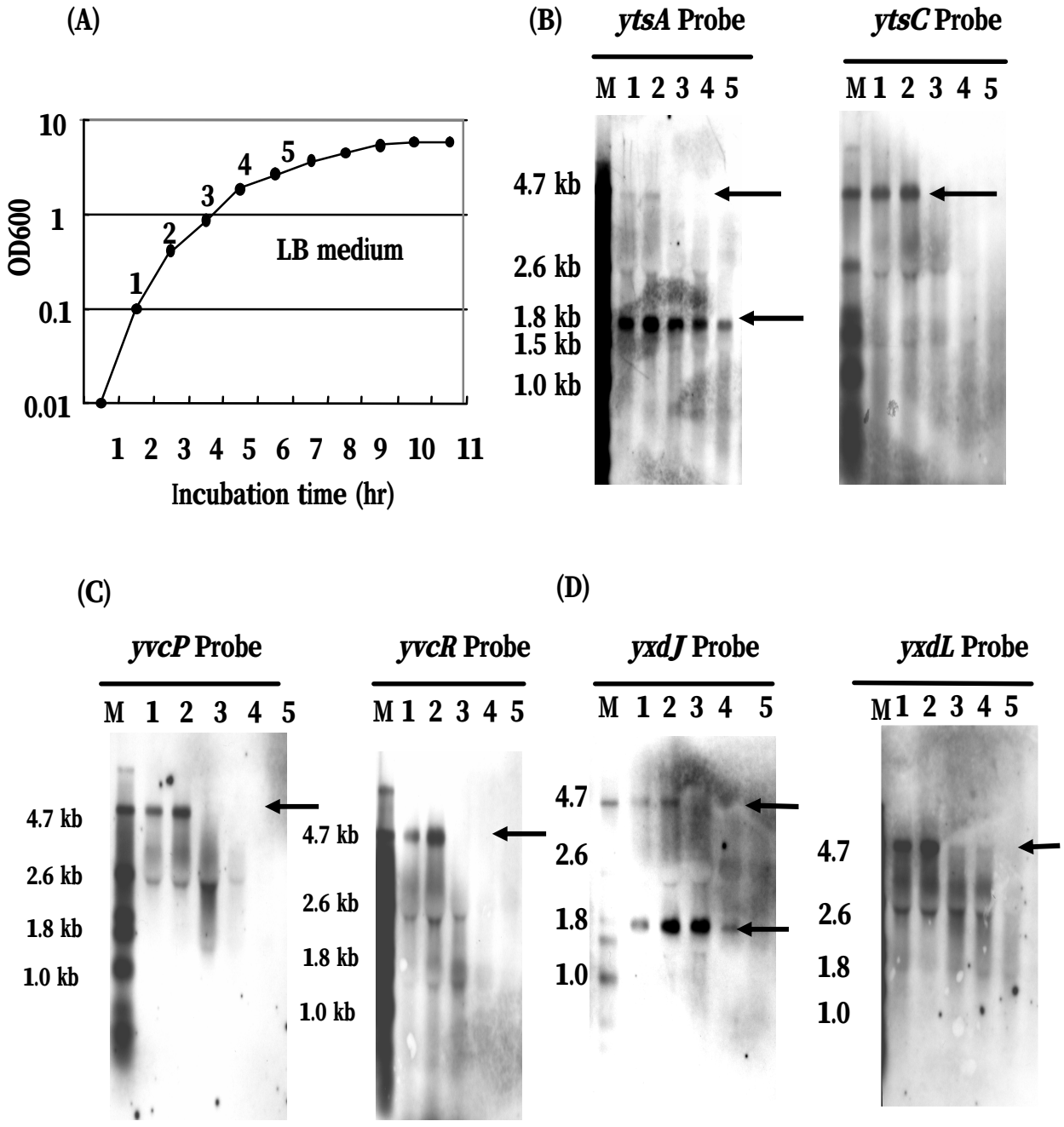

Figure 5. Transcriptional analysis of *ytsABCD, yvcPQRS,* and *yxdJKLM* operons. (A). Sampling time of *B. subtilis* cells subjected for Northern blot analysis. (B)-(D) Northern blot analysis with: *ytsA*-, *ytsC*-, *yvcP*-, *yvcR*-, *yxdJ*-, and *yxdL*-specific RNA probes. Transcript detected by Northern blot analysis are indicated by arrows. Each lane contained 5 μg tota RNA. M: molecular weight marker. Lane 1 - 5: RNA from cells harvested at time indicated in panel (A).

III. 1. 2 Overexpression of response regulators induce the transcription of ABC transporter genes in the same operon

The two-component regulatory systems on the *ytsABCD, yvcPQRS,* and *yxdLKLM* operons are not essential for cell growth as indicated by availability of null mutants of the response regulator or sensor kinase gene. In order to identify genes under the control of YtsA, YvcP and YxdJ response regulators, I constructed *B. subtilis* strains overexpressing each regulator. On this experiment, *ytsA, yvcP* and *yxdJ* were cloned on a multicopy shuttle plasmid pNO41, and introduced into the *B. subtilis* 168 cell. Preliminary analysis of the effect of YvcP overexpression on the total protein profile of *B. subtilis* cells by two-dimensional gel electrophoresis revealed induction of one spot, which was shown to be YvcR by the MALDI TOF spectrometry (data not shown). This result suggested that each response regulator would induce the downstream ABC transporter genes.

We examined this possibility by Northern blot analysis shown in Figure 6. As expected, transcription of the ABC transporter genes, but not of the two-component systems themselves, was induced by overexpression of the upstream response regulator gene. Furthermore, although amino acid identity among of three response regulators are high each other (Figure 3B), no cross induction of genes in other operons, were observed (data not shown). These results are in agree with results of systematic microarray analysis of *B. subtilis* two-component systems by Kobayashi *et al.* (2001), indicating that overexpression of YtsA, YvcP, and YxdJ protein in the background of inactivation of the cognate sensor kinase gene (*ytsB, yvcQ* or *yxdK*) induce the transcription of the downstream ABC transporter genes (*ytsCD, yvcRS,* or *yxdLM*).

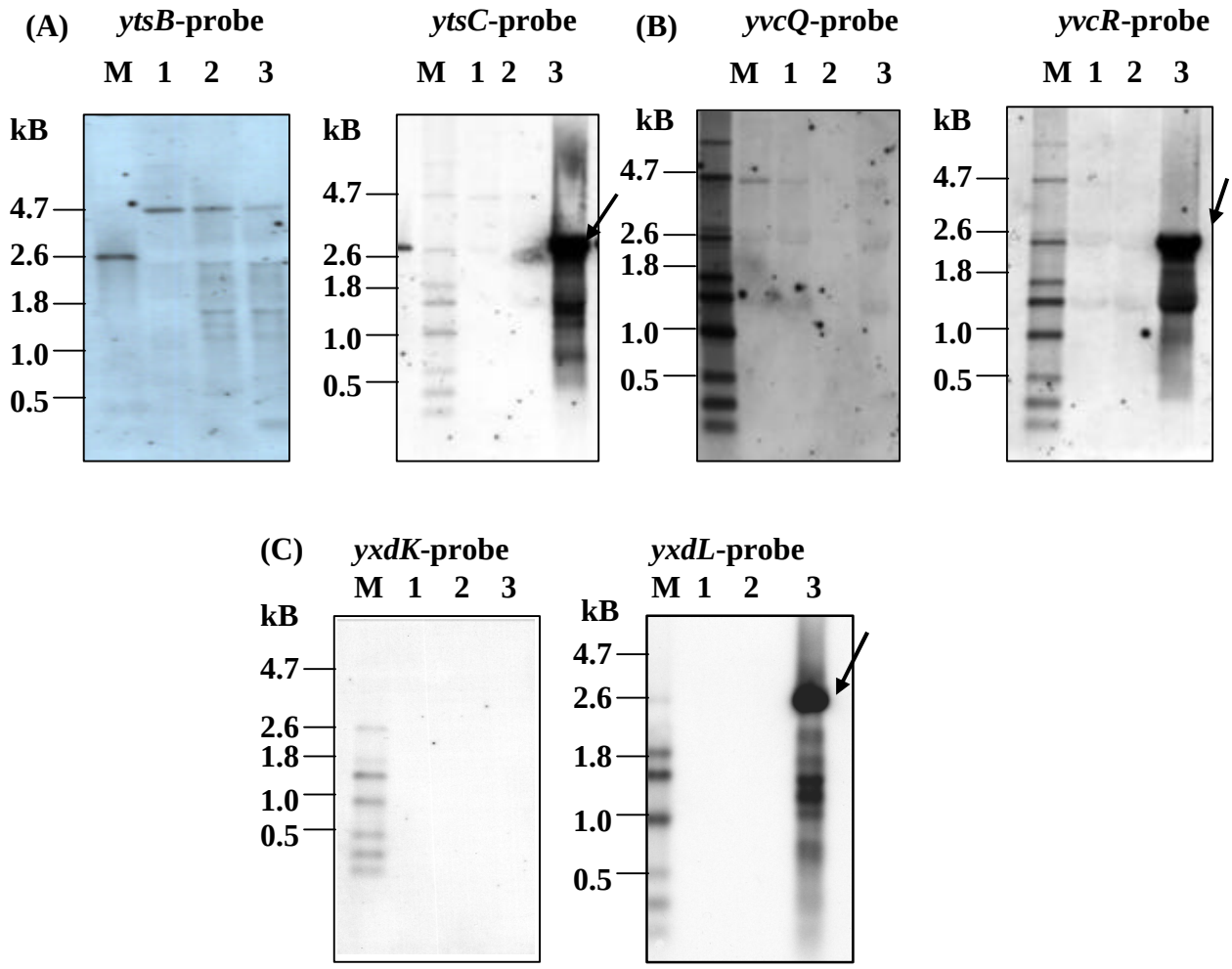

Figure 6. Figure 5. Overexpression of response regulator proteins induced the transcription of the ABC transporter genes in the same operon. *B. subtilis* wild type cells (lane 1) and cells harboring pNO41 (lane2), pNO41-*ytsA* (panel A, lane 3), pNO41-*yvcP* (panel B, lane 3), and pNO41-*yxdJ* (panel C, lane 3) were grown to OD600 of 0.5. Then, total RNA was purified from each cell and subjected to Northern blot analysis with probes specific to *ytsB* and *ytsC* (panel A), *yvcQ* and *yvcR* (panel B), and *yxdK* and *yxdL* (panel C). Each lane contained 5 μg of total RNA. An RNA marker (Boehringer Mannheim) was loaded in lane M and marker sizes are indicated on the left of the blots.

III. 1. 3. The *cis*-acting sequence required for the ABC transporter expression

Results described above indicated the existence of second promoter sequences upstream of the ABC transporter genes. To further analyze the regulation of ABC transporter gene expression by the upstream two-component system, I determined the *cis*-acting regions necessary for the induction of ABC transporter transcription. A series of strains having a transcriptional fusion with progressively shorter ABC transporter promoter sequences and the *lacZ* reporter gene at the *amyE* locus on the *B. subtilis* chromosome were constructed. Then, I monitored the effect of response regulator overexpression in these strains, by using pNO41-*ytsA*, pNO41-*yvcP*, and pNO41-*yxdJ* as transfected plasmids (Figure 7, 8 and 9). The β-galactosidase (LacZ) activities of the cells harboring pNO41 plasmid were at background levels for all the constructs. However, LacZ activity was strongly induced by the presence of pNO41-*ytsA* in cells containing at least 107 bp of the upstream region of the *ytsCD* translation start site. In contrast, deletion of a further 18 bp from the 107 bp sequence completely abolished the induction (Figure 7). Similarly *yvcR* and *yxdL* promoter activities were strongly induced by the overexpression of upstream response regulator, YvcP and YxdJ, respectively, in cells containing at least 110 bp or 160 bp upstream region of the *yvcR* or *yxdL* translation initiation site, and the inductions became the background level with further deletion of 16 bp of the *yvcR* promoter sequence or 11 bp of the *yxdL* sequence (Figure 8 and 9). These results indicated that sequences between -106 to -88 bp, -109 to -93 bp, and -159 to -148 bp upstream of *ytsC*, *yvcR* and *yxdL* initiation codon, respectively, are essential for the induction of *ytsCD*, *yvcRS*, and *yxdLM* transcription by the upstream response regulator protein.

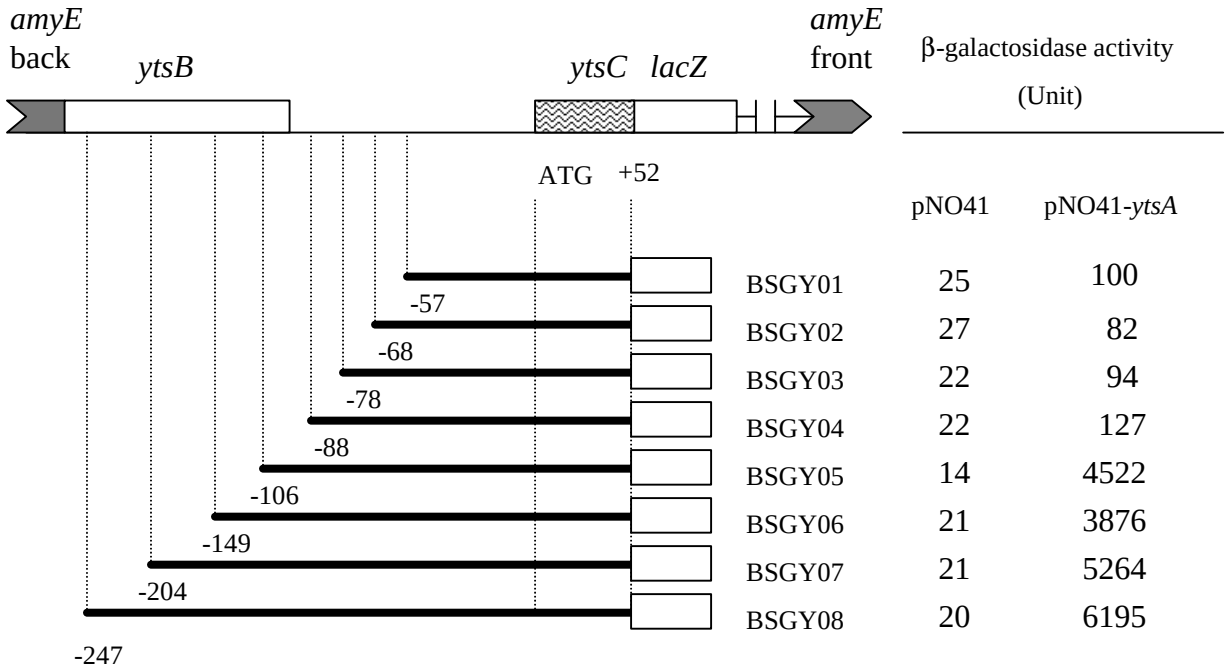

Figure 7. The *cis*-acting sequence required for induction of the *ytsC* expression by the YtsA response regulator. Various sizes of promoter region of *ytsC* was transcriptionally fused to the *lacZ* gene, and integrated into the *amyE* locus of *B. subtilis* chromosome. The YtsA response regulator overexpressing plasmid (pNO41-*ytsA*) was introduced into these strains, grown at 37°C to OD_{600} of 0.4 and β-galactosidase activities were measured.

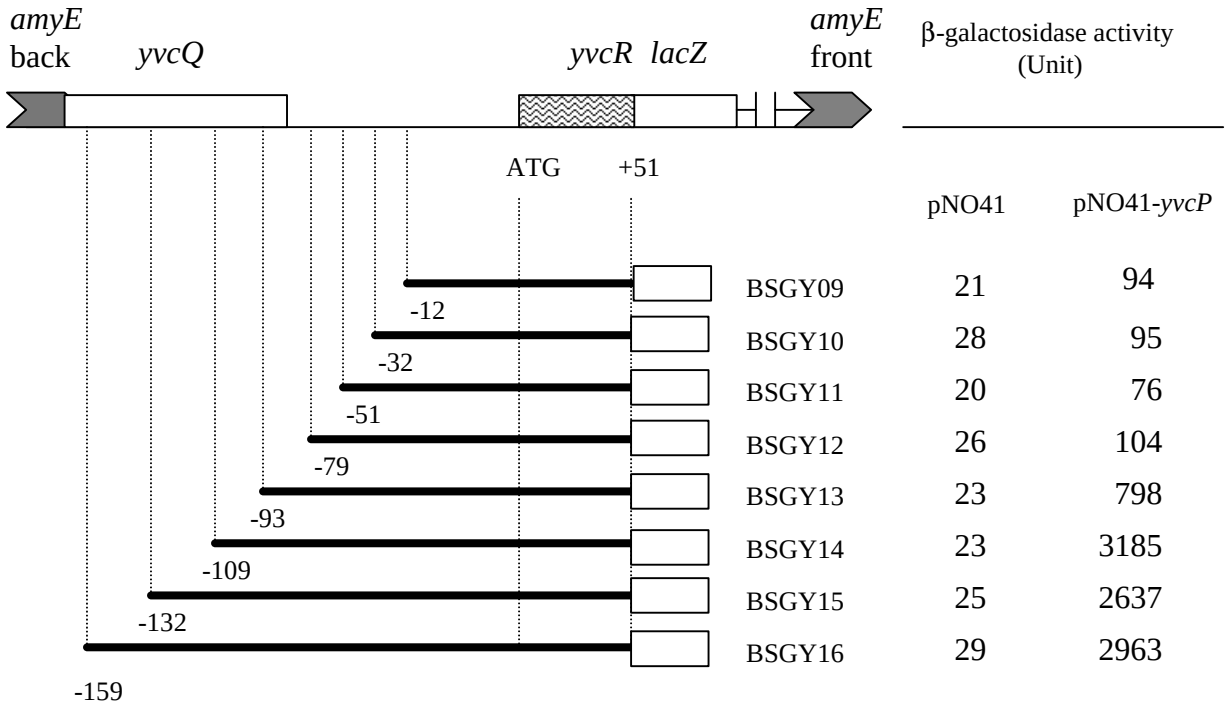

Figure 8. The *cis*-acting sequence required for induction of the *yvcR* expression by the YvcP response regulator. Various sizes of promoter region of *yvcR* was transcriptionally fused to the *lacZ* gene, and integrated into the *amyE* locus of *B. subtilis* chromosome. The YvcP response regulator overexpressing plasmid (pNO41-*yvcP)* was introduced into these strains, grown at 37°C to OD_{600} of 0.4 and β-galactosidase activities were measured.

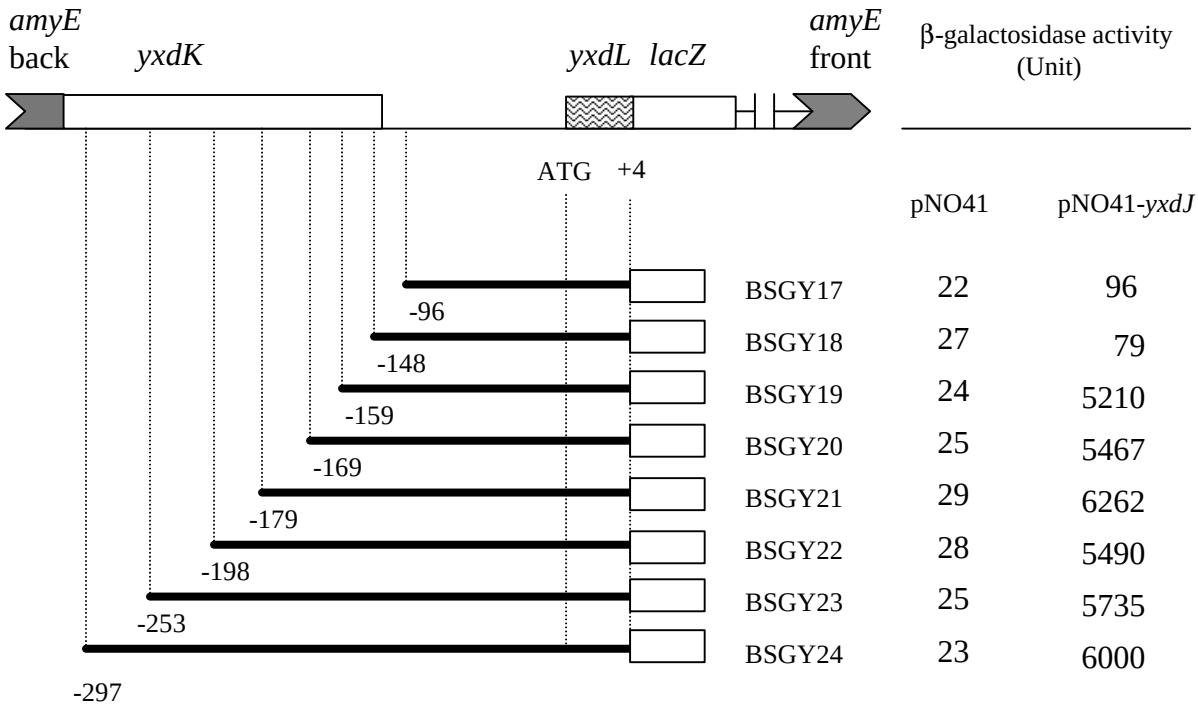

Figure 9. The *cis-acting* sequence required for induction of the *yxdL* expression by the YxdJ response regulator. Various sizes of promoter region of *yxdL* was transcriptionally fused to the *lacZ* gene, and integrated into the *amyE* locus of *B. subtilis* chromosome. The YxdJ response regulator overexpressing plasmid (pNO41-*yxdJ*) was introduced into these strains, grown at 37ºC to OD_{600} of 0.4 and β-galactosidase activities were measured.

III. 1. 4. The response regulator proteins bind directly to the upstream region of the ABC transporter promoter

To prove that response regulator proteins indeed binds to the *cis-acting* sequence required for induction of ABC transporter expression, each response regulator protein was fused to GST at the N-terminus, expressed in *E. coli*, and purified to homogeneity by glutathione-sepharose column (Figure 10). Then, I performed DNase I footprinting experiments to demonstrate the direct binding of regulators to ABC transporter promoter region. DNA fragments encompassing each of the ABC transporter promoter regions (279 bp fragment from -226 to 52 bp relative to the *ytsC* initiation codon, 273 bp from -233 to 39 bp relative to the *yvcR* initiation codon, and 277 bp from -272 to 4 bp relative to the *yxdL* initiation codon) were radioactively labeled at either end, and incubated with increasing amounts of the purified GST-regulator proteins, followed by DNase I partial digestion. The results shown in Figures 11, 12, and 13 demonstrated that the *cis*-regulatory regions required for induction of the ABC transporter gene transcription in the promoter deletion experiments were indeed protected by GST-regulator proteins in both coding and non-coding strands. Regulatory elements for induction of *ytsCD*, *yvcRS*, and *yxdLM* transcription are summarized in Figure 11C, 12C and 13C, respectively. I identified inverted repeat sequences, AAGCgTGTGACgaaatGTCACAtGCTT, AATgTgACAgcatTGTaAgATT, and CTTACAaaaaTGTAAG in the regions protected from DNase I as a possible recognition sequence for YtsA, YvcP, and YxdJ, respectively. Consistent with no cross induction in the regulator overexpression experiments, similarities among these biding sequences were low. Taken together, my results indicated that expression of the *ytsAB*, *yvcRS*, and *yxdLM* ABC transporter genes are controlled by the response regulators encoded in the same operon, and suggested that these regulators would be activated by the accompanied sensor kinases in some environmental condition.

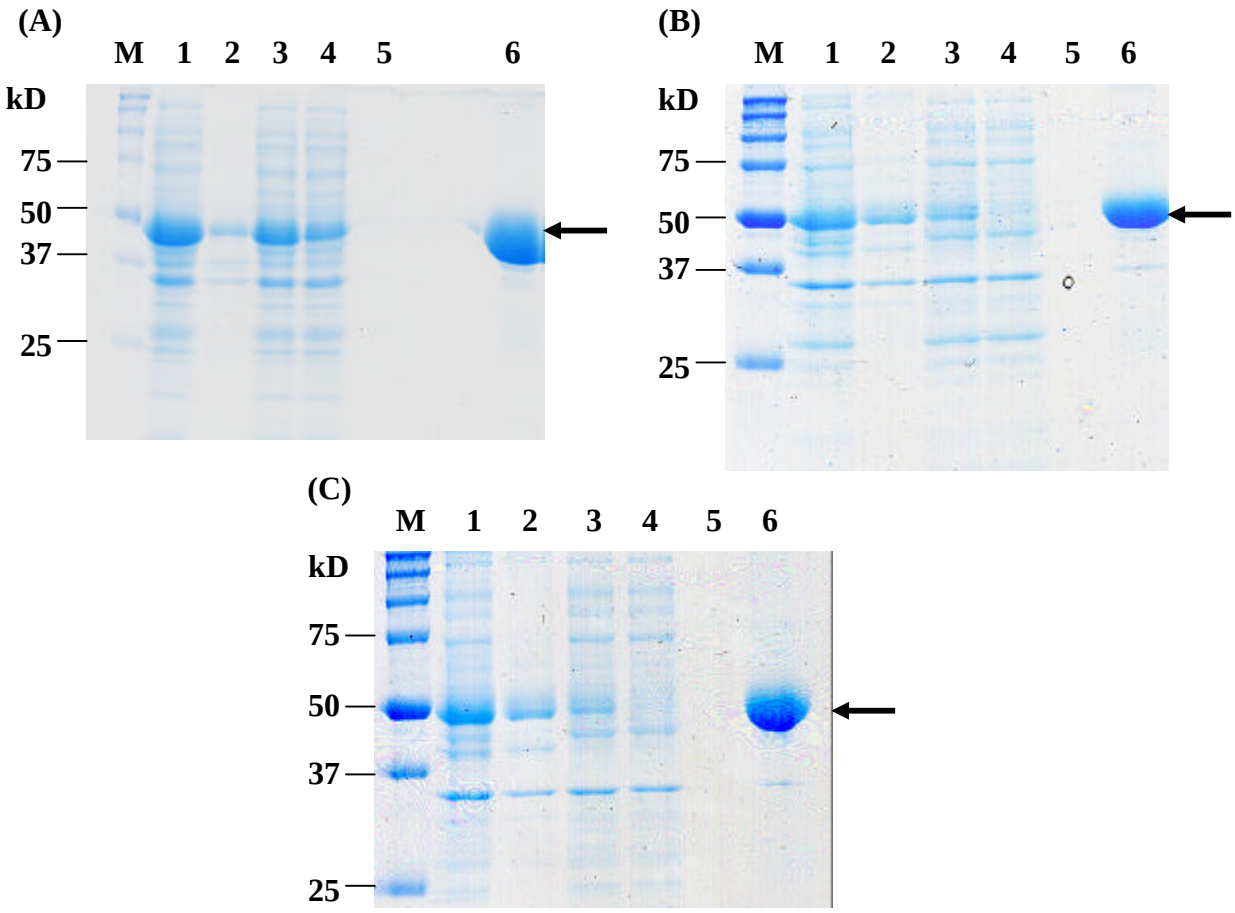

Figure 10. Purification of GST-YtsA, GST-YvcP and GST-YxdJ proteins. GST fused response regulator proteins were purified using glutathione sepharose column as described in MATERIALS AND METHODS. Proteins in each purification step of GST-YtsA (A), GST-YvcP (B) and GST-YxdJ (C) were separated by 10% SDS-PAGE and stained with coomassie brilliant blue. Lanes M, 1, 2, 3, 4, 5 and 6 contained molecular weight standards (Bio-Rad), crude extract, pellet, supernatant, column flowthrough, column wash, and column equate, respectively.

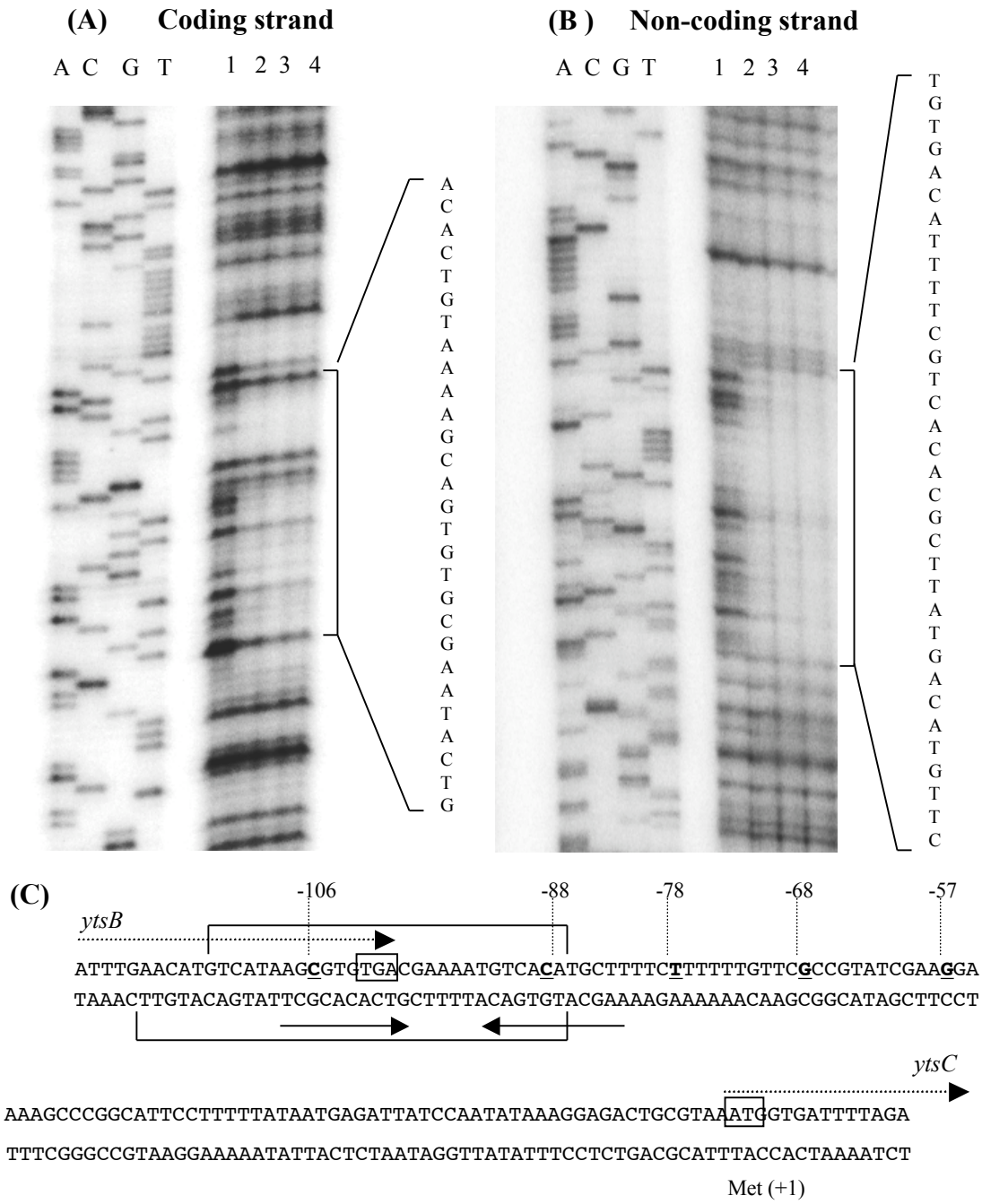

Figure 11. DNAse I footprinting analysis of GST-YtsA binding to the *ytsCD* promoter region. A 279 bp fragment encompassing -226 to +52 bp relative to the *ytsC* translation initiation site was labeled radioactively at either end and used as coding (A) and non-coding (B) strand probe. A constant amount of probe (300,000 c.p.m., 9.25 fmol) was incubated with different amount of GST-YtsA. Protein amounts in lanes 1-4 are 0, 5, 10, 15 pmol respectively. Lanes A, C, G and T contain sequencing ladder. Sequences of the maximum protected region in the coding and non-coding strands are shown. (C) The nucleotide sequence of the promoter region of *ytsCD* is depicted. The initiation codon of *ytsC* and termination codon of *ytsB* are boxed. Coding region of *ytsB* and *ytsC* are indicated by dashed arrows. Number (relative to the *ytsCD* translation initiation site) at the top of bold underlined characters indicate the end-point of promoter sequences used in deletion analysis (Figure 6). DNAse I-protected areas (A and B) are specified by brackets. Thick arrows signify an inverted repeat in the protected region.

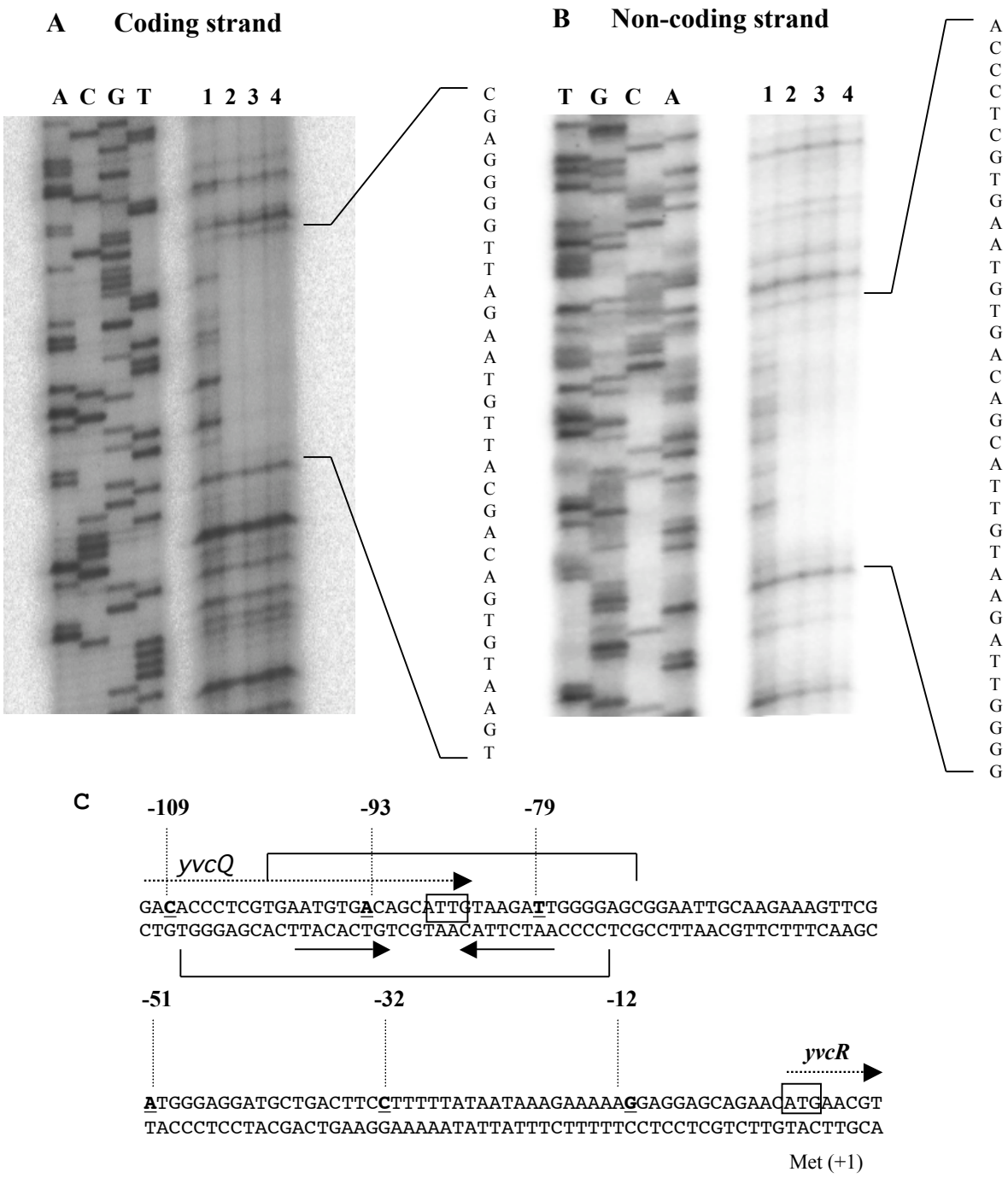

Figure 12. DNAse I footprinting analysis of GST-YvcP binding to the *yvcRS* promoter region. A 273 bp fragment encompassing -233 to +39 bp relative to the *yvcR* translation initiation site was labeled radioactively at either end and used as coding (A) and non-coding (B) strand probe. A constant amount of probe (300, 000 c.p.m., 6.75 fmol) was incubated with different amount of GST-YvcP. Protein amounts in lanes 1-4 are 0, 5, 10, 15 pmol respectively. Lanes A, C, G and T contain sequencing ladder. Sequences of the maximum protected region in the coding and non-coding strands are shown. (C) The nucleotide sequence of the promoter region of *yvcRS* is depicted. The initiation codon of *yvcR* and termination codon of *yvcQ* are boxed. Coding region for *yvcQ* and *yvcR* are indicated by dashed arrows. Number (relative to the *yvcRS* translation initiation site) at the top of bold underlined characters indicate the end-point of promoter sequences used in deletion analysis (Figure 7). DNAse I-protected areas (A and B) are specified by brackets. Thick arrows signify an inverted repeat in the protected region.

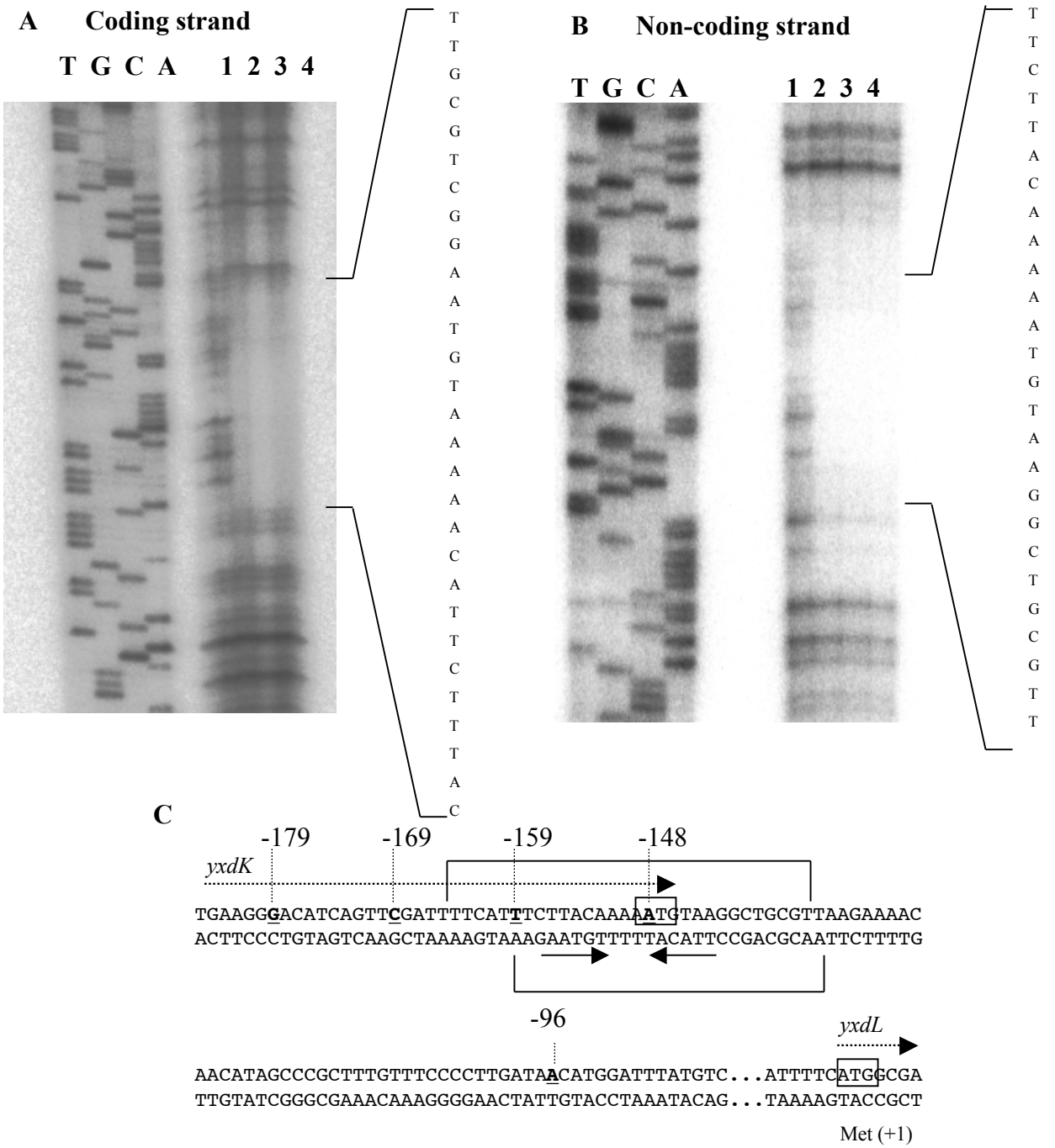

Figure 13. DNAse I footprinting analysis of GST-YxdJ binding to the *yxdLM* promoter region. A 277 bp fragment encompassing -272 to +4 bp relative to the *yxdL* translation initiation site was labeled radioactively at either end and used as coding (A) and non-coding (B) strand probe. A constant amount of probe (300, 000 c.p.m., 8.75 fmol) was incubated with different amount of GST-YxdJ. Protein amounts in lanes 1-4 are 0, 5, 10, 15 pmol respectively. Lanes A, C, G and T contain sequencing ladder. Sequences of the maximum protected region in the coding and non-coding strands are shown. (C) The nucleotide sequence of the promoter region of *yxdLM* is depicted. The initiation codon of *yxdL* and termination codon of *yxdK* are boxed. Coding region of *yxdK* and *yxdL* are indicated by dashed arrows. Number (relative to the *yxdLM* initiation translation site) at the top of bold underlined characters indicate the end-point of promoter sequences used in deletion analysis (Figure 8). DNAse I-protected areas (A and B) are specified by brackets. Thick arrows signify an inverted repeat in the protected region.

III. 2. The *ytsABCD* operon is involved in bacitracin resistance

III. 2. 1. Inactivation of *ytsA, ytsB, ytsC* or *ytsD* reduced the bacitracin resistance of *B. subtilis* cells

During a systematic search of transporters involved in drug efflux among 40 possible ABC efflux transporter operons of *B. subtilis* in Dr. Ohki's Laboratory in Kyorin University, disrupted mutants of either *ytsC* (YTSCd) or *ytsD* (YTSDd) were found to show hypersensitivity to bacitracin (Table 4). Bacitracin is a dodecapeptide antibiotic synthesized non-ribosomally in some strains of *Bacillus* (Johnson *et al*., 1945; Azevedo *et al*., 1993) and has potent activity against Gram-positive bacteria, through forming a complex mediated by a metal ion (Zn^{2+}) with the lipid C_{55}-isoprenyl pyrophosphate (IPP) (Scogin *et al*., 1980; Stone and Strominger, 1970), which is a carrier of peptidoglycan unit or a disaccharide with pentapeptide across the membrane. Bacitracin, by binding IPP, inhibits the conversion of IPP to C_{55}-isoprenyl phosphate, which is catalysed by a membrane associated pyrophosphatase (Siewert and Strominger, 1967) I further characterized this phenotype in collaboration with Dr. Ohki.

Table 4. Bacitracin sensitivity of various mutants. Relative resistance was determid by dividng the IC_{50} for mutant strains by the IC_{50} for wild type strain 168. IC_{50} for strain 168 is 39.9 U ml^{-1}

Strain	Genotype	Relative resistance
168		1
YTSAd	*ytsA*:: pMutin2	0.013
YTSBd	*ytsB*:: pMutin2	0.009
YTSCd	*ytsC*:: pMutin2	0.007
YTSDd	*ytsD*:: pMutin2	0.007

The *ytsC* and *ytsD* genes constitute an operon. Therefore, it is necessary to confirm that the bacitracin-sensitive phenotype of YTSCd was due to the inactivation of the *ytsC* gene but not due to the polar effect of the pMutin insertion on the downstream *ytsD* expression. In the disruptants, the downstream genes are located under the *spac* promoter on the pMutin2 plasmid, which is induced by the addition of IPTG (Vagner and Ehrlich, 1998). Northern hybridization analysis using total RNA isolated from YTSCd grown in the presence or absence of 0.5 mM IPTG demonstrated that a transcript detected with a probe specific to *ytsD* increased 25 fold by the addition of IPTG. However, no increase in bacitracin resistance was observed by the addition of IPTG, indicating that YtsC is required, in addition to YtsD, for bacitracin resistance (data not shown).

As described previously, the YtsA response regulator induced the *ytsCD* transcription, probably through activation by the YtsB sensor kinase. Indeed, the disruption of either *ytsA* (YTSAd) or *ytsB* (YTSBd) also caused a decrease in bacitracin resistance to a level similar to that observed in *ytsC* and *ytsD* mutants (Table 4). In YTSAd, the addition of IPTG resulted in more than 10 fold increase in the amount of 1.7 kb transcript detected with a probe specific to *ytsB*, but had no effect on bacitracin resistance (data not shown), indicating that the bacitracin sensitive phenotype of YTSAd was due to the *ytsA* gene defect but not due to the polar effect on the downstream located *ytsB* gene. Furthermore, the bacitracin-hypersensitive phenotype of *ytsB* mutant (YTSBd) suppressed by introduction of the YtsA overproducing plasmid, pDG148*ytsA* (Kobayashi *et al*., 2001).

These results collectively suggested that *ytsC* and *ytsD* constitute subunits of a bacitracin efflux transporter and that *ytsA* and *ytsB* are involved in the regulation of bacitracin resistance. Therefore, we named *ytsC* and *ytsD* as *bceA* and *bceB* (bacitracin efflux), and *ytsA* and *ytsB* as *bceR* and *bceS*, and I will use this nomenclature hereafter.

Table 5. Bacitracin resistance of wild type and YTSAd (*bceR*-disrupted strains) harboring a plasmid carrying *bceR*. Relative resistance was determined by dividing the IC_{50} for mutant strains by the IC_{50} for wild type strain 168. IC_{50} for strain 168 was 40 U l^{-1}.

Strain	IPTG	Relative resistance [a)]
168	-	1
YTSAd/pDG148	-	0.006
	+	0.012
YTSAd/pDG148*bceR*(*ytsA*)	-	0.007
	+	2.01

III. 2. 2. Induction of the *bceAB* transcription by bacitracin in culture medium

Northern blot experiments using total RNA isolated from wild type cells detected a 1.7 kb band with probe specific for *bceR* or *bceS* and a 2.7 kb band with probe specific for *bceA* or *bceB* (Figure 5B). As expected, addition of 10 U ml^{-1} bacitracin in culture medium resulted in increase of the level of *bceAB* mRNA more than 50-fold. On the other hand, the level of *bceRS* mRNA was rather decreased under the same growth condition (Figure 14).

I determined the 5' end of the *bceAB* transcript by primer extension analysis using a primer complementary to the sequence 55 to 97 bp downstream from the initiation codon of *bceA*. Using total RNA from *B. subtilis* 168 cells grown in the presence of 10 U ml^{-1} bacitracin and from 168 cells harboring pDG148*bceR* grown in the presence of IPTG, the transcription initiation site was detected at 25 bp upstream of the initiation codon for *bceA* (Figure 15), accompanied by a -10 consensus sequence for σ^A, TATAAT, located at 7 bp upstream of the transcription start site. On the other hand, no clear -35 sequence could be identified at the appropriate position from the -10 sequence, as is often the case for promoters regulated by transcriptional activators (Figure 15B).

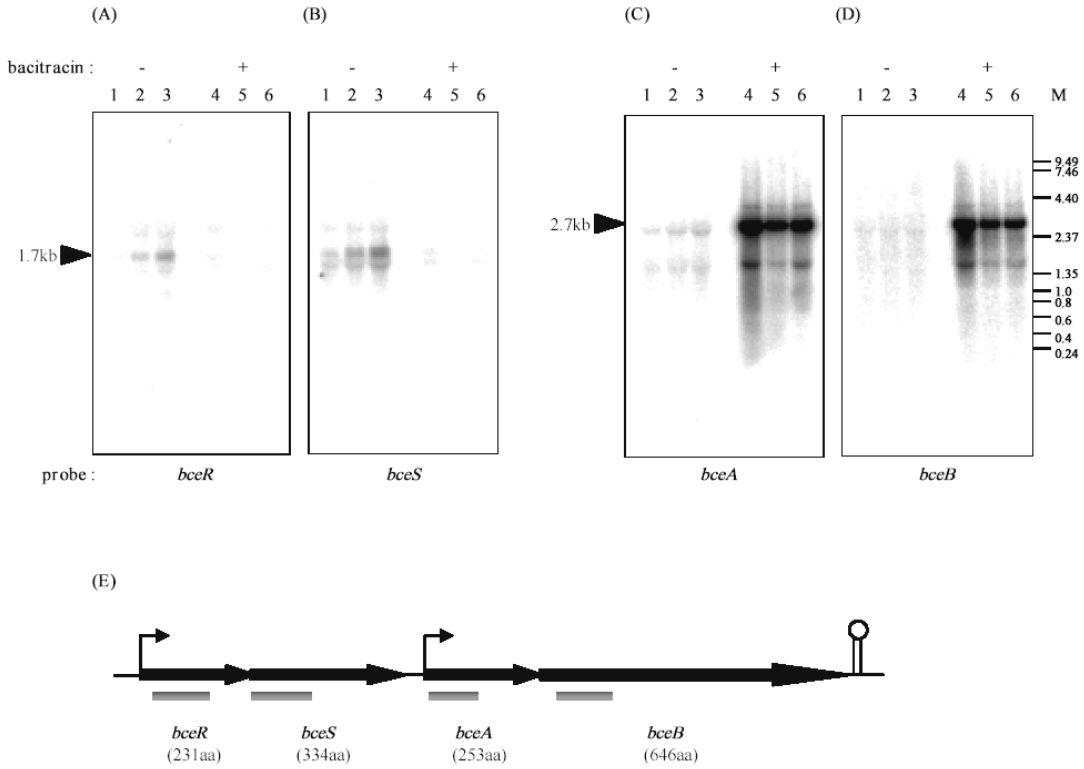

Figure 14. Induction of *bceAB* operon expression by preincubation with 10 U ml^{-1} bacitracin An overnight culture of *B. subtilis* 168 grown in LB medium was inoculated into LB medium (OD_{530}, 0.05) with or without 10 U ml^{-1} bacitracin. Samples were withdrawn during early (OD_{530}, 0.5; lanes 1 and 4), middle (OD_{530}, 1.2; lanes 2 and 5), and late (OD_{530}, 2.0; lanes 3 and 6) log phase growth. Isolation of total RNA and Northern hybridization were carried out as described in Experimental procedures. ^{32}P-labeled *bceR*, *bceS*, *bceA*, and *bceB* probes were used as indicated. Arrows indicate 1.7-kb *bceRS* (panels A and B) and 2.7-kb *bceAB* transcripts (panels C and D). Lanes 1 to 3, LB medium; lanes 4 to 6, LB medium containing 10 U ml^{-1} bacitracin; M, molecular size standard (Novagen Perfect RNA^{TM} Markers 0.1-1 kb, Invitrogen RNA Ladder 0.24-9.5 kb). (E) Thick arrows indicate open reading frames (ORF) of *bceR*, *bceS*, *bceA* and *bceB*. Thin arrows indicate promoters and a circle on a stem indicates a transcriptional termination signal. Boxes under ORFs indicate the probes used for Northern hybridization

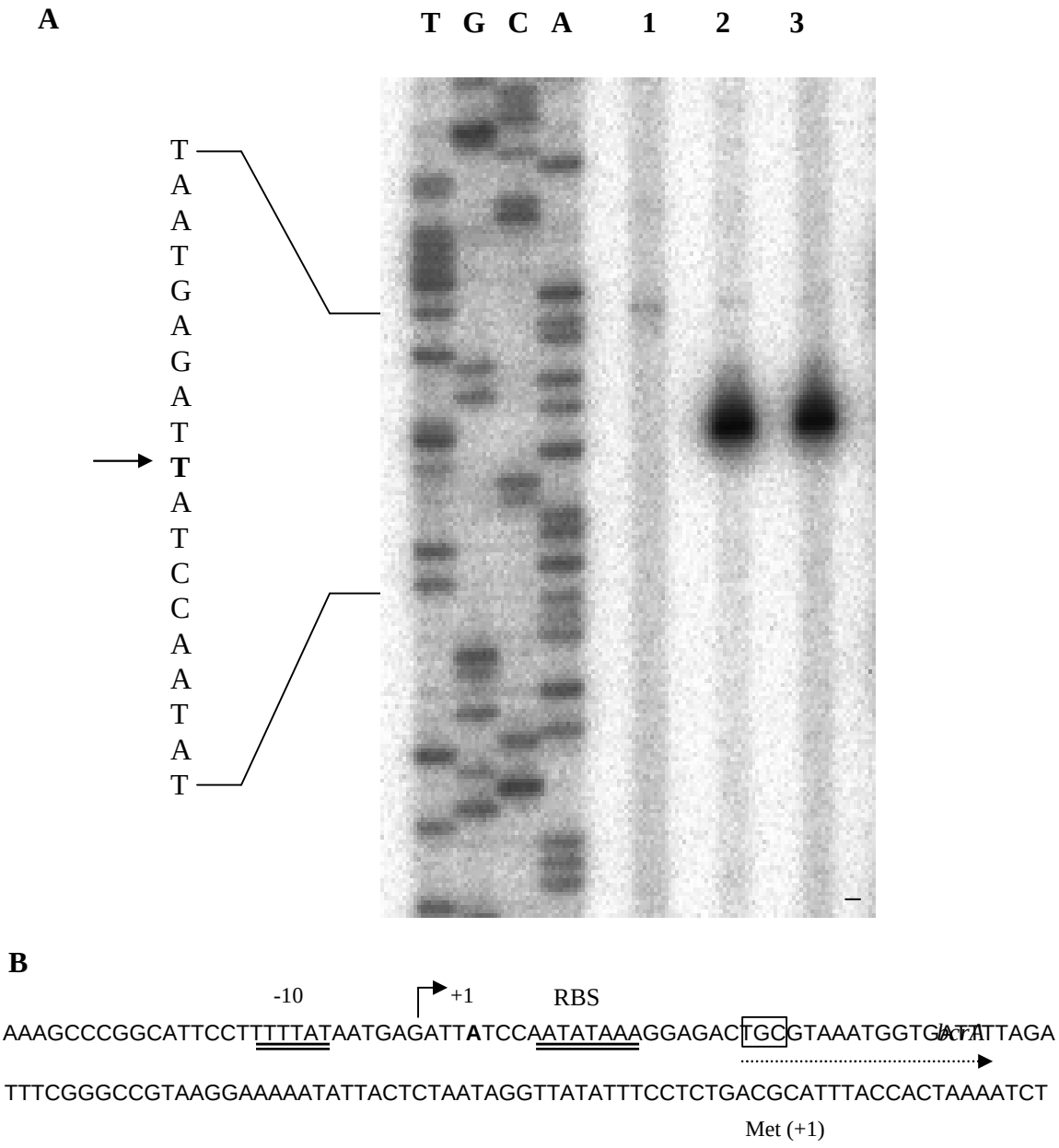

Figure 15. Primer extension analysis on *bceAB* promoter region. (A). Determination of the *bceAB* transcription start site by primer extension. Total RNA was prepared from *B. subtilis* 168 cells grown in LB medium (OD_{600} of 0.5) without (lane 1) and with (lane 2) bacitracin, and from 168 cells harbouring pDG148-*bceR* (lane 3) grown in LB medium with IPTG (OD_{600} of 0.5) and used for primer extension analyses. Sequencing ladder (Lane T, G, C and A) were generated with the primer used for the reverse transcriptase reaction. The sequence and transcriptional start site (indicated by an arrow) are shown. (B). The nucleotide sequence of the promoter region for *bceAB* transcription is depicted. The bent arrow indicates the transcription start site detected using primer extension.The initiation codon of *bceA* is boxed. Coding region for *bceA* indicated by dashed arrows. The ribosome binding sequence (RBS) for *bceA* and possible -10 sequence of *bceAB* promoter are double underlined.

I confirmed that the BceR binding region described previously is also necessary for induction of *bceAB* transcription by bacitracin. The BSGY01 to BSGY08 strains were grown with or without 10 U ml^{-1} of bacitracin to early log phase and β-galactosidase activities were compared with those BceR overexpressing cells (Table 6). The β-galactosidase activities were strongly induced in the strain containing at least 107 bp of the upstream region of the *bceA* translation initiation site by the addition of bacitracin to the culture medium. Consistent with the induction by BceR (YtsA), deletion of the further 18 bp from the 107 bp sequence completely abolished the induction by bacitracin (Table 6).

These results strongly suggested the following signal transduction mechanism. Bacitracin in culture medium is detected by the BceS sensor kinase, which activates the BceR response regulator probably by phosphorylation. Then activated BceR binds to the upstream region of the *bceAB* promoter, to induce the BceRS transporter that will efflux bacitracin to protect cells from the antibiotics.

Table 6. The b**-galactosidase activities of *B. subtilis* strains containing various sizes of P*bceA*(*ytsC*)-lacZ fusion cultivated in the presence of bacitracin.** BSGY01 to BSGY08 were grown in LB medium containing 10U/ml bacitracin to OD600=0.4. LacZ activities were measured. LacZ activities in the strains harboring pNO41-*bceR* (*ytsA*) are also include for comparison.

Strains	Insert*		β-galactosidase activity (unit)	
			pNO4-*bceR*(*ytsA*)	Bacitracin
BSGY01	110 bp fragment of promoter region of *bceA*	(-57 to + 52)	100	25
BSGY02	121 bp fragment of promoter region of *bceA*	(-68 to + 52)	82	35
BSGY03	131 bp fragment of promoter region of *bceA*	(-78 to + 52)	94	38
BSGY04	141 bp fragment of promoter region of *bceA*	(-88 to + 52)	127	28
BSGY05	159 bp fragment of promoter region of *bceA*	(-106 to + 52)	4522	4975
BSGY06	202 fragment of bp promoter region of *bceA*	(-149 to + 52)	3876	4808
BSGY07	257 fragment of bp promoter region of *bceA*	(-204 to + 52)	5264	4050
BSGY08	300 fragment of bp promoter region of *bceA*	(-247 to + 52)	6195	3234

* Nucleotide numbers relative to the initiation codon of *bceA*

III. 2. 3. Two independent pathways for bacitracin resitance in *B. subtilis*

Previous works reported that disruption of the *B. subtilis ywoA* gene encoding a homologue of the presumed MSD subunit of the *B. licheniformis* bacitracin transporter, YwoA, also resulted in hypersensitivity to bacitracin, and that expression of *ywoA* is dependent on the ECF σ factors, σ^M and σ^X (Cao and Helmann, 2002; Ohki *et al.*, 2003). If *ywoA* together with *bceA* and *bceB*, constitute the bacitracin efflux transporter, its expression may also be controlled by the BceRS two-component system. However, Northern hybridization analysis showed that *ywoA* mRNA expression in both wild type and *ytsB* mutant (BCESd) strains harboring pDG148*bceR* were not affected by the addition of IPTG (Figure 16), indicating that the *ywoA* expression is independently regulated on the BceRS two-component system.

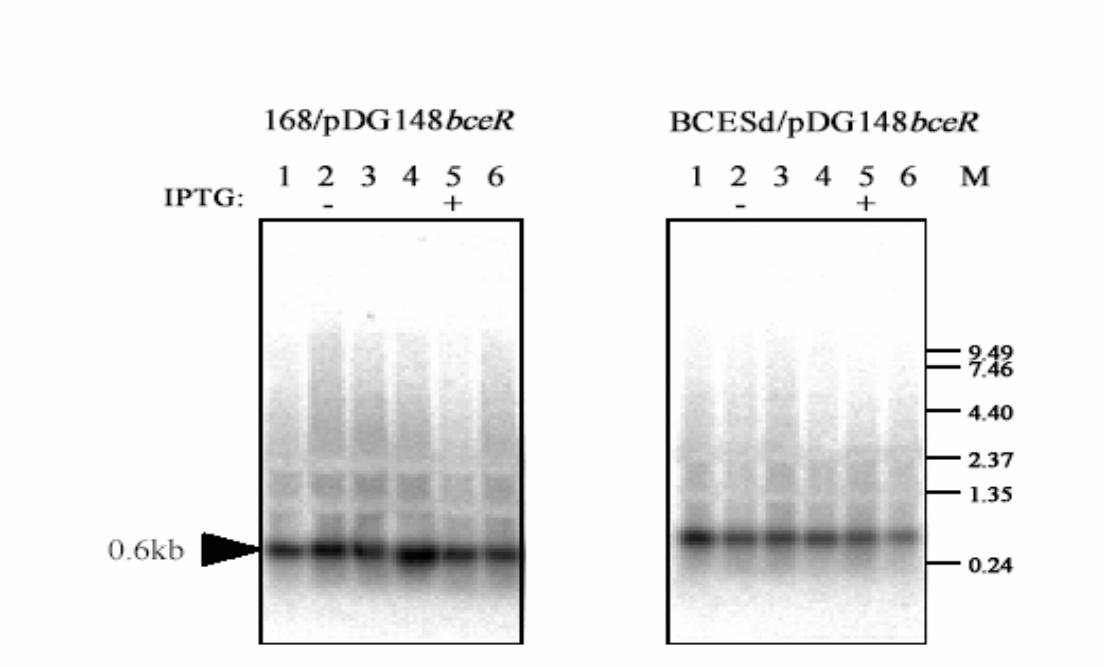

Figure 16. Expression of *ywoA* gene in wild type and *bceS*-disrupted mutant strains harboring a plasmid carrying *bceR*. *B. subtilis* 168 and *ytsB* mutant (BCESd) strains harboring pDG148*bceR* were grown in LB medium with or without 0.5 mM IPTG to early (OD_{530}, 0.5; lanes 1 and 4), middle (OD_{530}, 1.2; lanes 2 and 5), and late ($O.D._{530}$, 2.0; lanes 3 and 6) log phase. Total RNA (5 μg) was loaded per lane. ^{32}P-labelled *ywoA* probe was used. The arrow indicates a 0.6 kb band, which corresponds to *ywoA* transcripts.

We examined whether an absence of YwoA in addition to the absence of BceA or BceB has an additive effect on bacitracin resistance by creating double mutants of YtsCd/*ywoA*::*tet* and YtsDd/*ywoA*::*tet*. As shown in Table 7, bacitracin resistance of double mutants were further decreased compared to the single mutants. We also examined the bacitracin resistance in a double mutant *ywoA*::*tet*/*bceR*::*cat.* Bacitracin resistance decreased to one tenth of that in *bceR*::*cat* (data not shown). These results indicated that YwoA and BceAB are involved in different process of bacitracin resistance in *B. subtilis.*

Table 7. Bacitracin sensitivity of *bceA* (*ytsC*)/*ywoA* and *bceB* (*ytsC*)/*ywoA* Double mutants. Relative resistance was determined by dividing the IC50 for mutant strains by dividing the IC50 for *ywoA*::t*et*

Strain	Relative resistance
ywoA::*tet*	1
YTSCd	0.28
YTSDd	0.28
YTSCd/*ywoA*::*tet*	0.04
YTSDd/*ywoA*::*tet*	0.05

The *bcrC* gene of *B. licheniformis* has been assumed to encode an membrane spanning domain (MSD) subunit of the bacitracin efflux transporter regardless of the low amino acid sequence homology to known MSD subunits of ABC transporters. This assumption is largely based on the fact that *bcrC* constitutes an operon with the *bcrA* and *bcrB* genes encoding typical nucleotide binding domain and MSD subunits, respectively, of an ABC transporter (Neumüller *et al*., 2001). However, our results suggest that these proteins contribute independently to bacitracin resistance, although both are absolutely required. From the amino acid sequence we could reasonably assume that BcrA and BcrB

are subunits of the bacitracin efflux transporter. On the other hand, YwoA is now recognized to belong to the PAP2 superfamily of phosphatases in the Pfam database (Bateman *et al*., 2002) and is annotated as a membrane-associated phospholipid phosphatase in the COG database (http://www.ncbi.nlm.nih.gov/cgi-bin/COG/). The *sigM* regulon was induced by stresses to the membranes, salts and antibiotics (Horsburgh *et al.*, 1999, Cao *et al.*, 2002). It could be speculated that YwoA protein expression is induced by membrane stresses, in turn modifying the membrane structure. The modified membrane might alter the accessibility of bacitracin to the target, ^{55}C-isoprenyl pyrophosphate.

III. 3. Enduracidin induced the transcription of the *yvcRS* transporter gene

III. 3. 1. Search for antibiotics that induce the *yvcRS* or *yxdLM* transcription

We demonstrated that *bceRSAB* (*ytsABCD)* has a role in bacitracin resistance in *B. subtilis*. This finding strongly suggested that the *yvcPQRS* and *yxdJKLM* operons are also involved in resistance to antibiotics. Therefore I tested various kinds of peptide antibiotics produced by gram-positive bacteria for the ability to induce the expression of the *yvcR* or *yxdL* gene (Table 8). *B. subtilis* strains having P*bceA-lacZ*, P*yvcR-lacZ*, or P*yxdL-lacZ* fusion at the *amyE* locus were grown on LB medium containing 100 μg/ml X-gal, and effects of antibiotics on the expression of the *lacZ* reporter gene were examined as described in MATERIALS AND METHODS. As a result, I found that enduracidin, a cyclodepsipeptide isolated from *Streptomyces fungicidicus* that inhibits the transglycosylation step in cell wall synthesis, specifically induces the expression of P*yvcR-lacZ* (Table 8). In line with my previous results, bacitracin induced strongly the expression of P*bceA-lacZ* also in this assay. Interestingly, bacitracin also induced expression off P*yvcR-lacZ* and the induction was dependent on the YtsA regulator protein (data not shown). I could not find antibiotic that induces the expression of the P*yxdL-lacZ* fusion.

Table 8. Survey of peptide antibiotics that induce the *bceA*, *yvcR* or *yxdL* promoter.

Peptide antibiotic	*B. subtilis* strains			Peptide antibiotic	*B. subtilis* strains		
	P*bceA-lacZ*	P*yvcR-lacZ*	P*yxdL-lacZ*		P*bceA-lacZ*	P*yvcR-lacZ*	P*yxdL-lacZ*
Actinomycin	-	-	-	MAPI	-	-	-
Bacitracin	+++	+	-	Nisin	-	-	-
Bestati	-	-	-	Pepstatin A	-	-	-
Chymostatin	-	-	-	Phleomycin	-	-	-
Distamycin	-	-	-	Polylysin	-	-	-
Duramycin	-	-	-	Subtilisin A	-	-	-
Elastatinal	-	-	-	Surfactin	-	-	-
Enduracidin	-	+++	-	Thiostrepton	-	-	-
Gramicidin	-	-	-	Valinomycin	-	-	-
Iturin A	-	-	-	Vancomycin	-	-	-
Leupeptin	-	-	-	Virginiamycin	-	-	-

+: induction positive (blue colony)

-: induction negative (white colony)

Next I examined the induction of the *yvcRS* expression by enduracidin with Northern hybridization experiments. A probe specific to *yvcR* was hybridized to blots of total RNA from wild type and *yvcP*::*spec* strains, after exposing to 0.01 μg and 0.2 μg/ml of enduracidin in the culture medium. As shown in Figure 17, the 2.7 kb *yvcRS* transcript, was induced transiently by the enduracidin addition. The transcription of *yvcRS* was detected at a low concentration of enduracidin (0.01 μg/ml), while the addition twenty-fold higher concentration of the antibiotic (0.2 μg/ml) increased the *yvcRS* expression level.

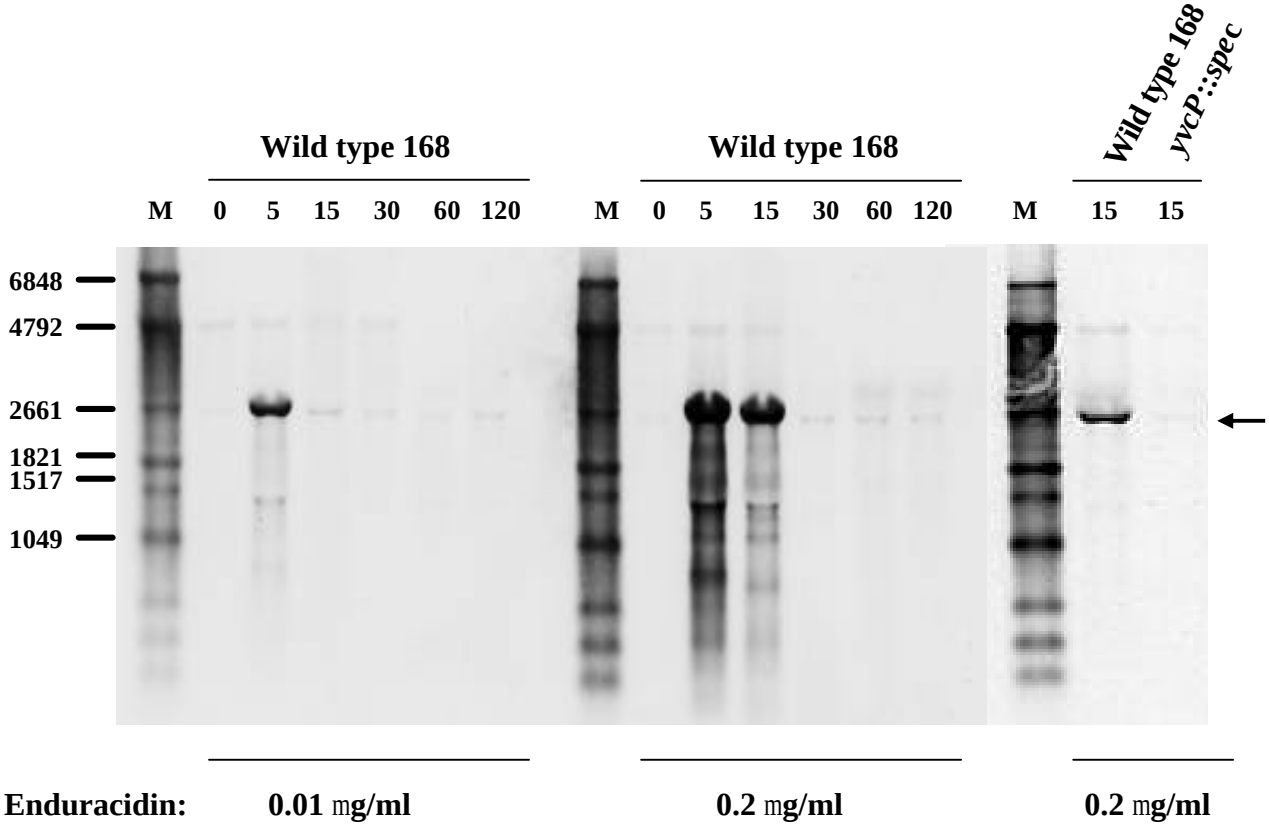

Figure 17. Northern blot analysis of total RNA from wild type and *yvcP::spec* cells using *yvcR*-specific probe. The strains were grown in LB medium at 37°C to OD_{600} of 0.4. Enduracidin was added to the culture medium in final concentration of 0.01 or 0.2 μg/ml, subsequently cells were harvested at different time after addition of enduracidin as indicated (in minutes). Each lane contained 1 μg of total RNA. An RNA marker (Boehringer Mannheim) was loaded in lane M and marker sizes are indicated on the left of the blots. Transcript detected by Northern blot analysis are indicated by arrow.

Furthermore the disruption of *yvcP* abolished the induction of *yvcRS* transcription. In addition, the *cis*-acting sequence required for the induction of *yvcRS* transcription by the YvcP overexpression (Figure 8) was also essential for the enduracin dependent induction of *yvcRS* (Table 9). These results demonstrated that enduracin induces the YvcRS transporter expression, although transiently, through the action of the YvcPQ two-component system.

Table 9. The b**-galactosidase activities of *B. subtilis* strains containing various sizes of P*yvcR*-lacZ fusion in the presence of enduracidin.** BSGY09 to BSGY16 were grown in LB medium to OD600=0.4. Enduracidin was added in medium culture to a final concentration of 0.2 μg/ml for 15 minutes. LacZ activities were measured. LacZ activities in the strains harboring pNO41-*yvcP* are also including for comparison.

Strain	Insert*		β-galactosidase activity (unit)	
			pNO4-*yvcP*	Enduracidin
BSGY09	63 bp fragment of promoter region of *yvcR*	(-12 to +51)	94	8
BSGY10	84 bp fragment of promoter region of *yvcR*	(-32 to +51)	95	26
BSGY11	103 bp fragment of promoter region of *yvcR*	(-51 to +51)	76	9
BSGY12	131 bp fragment of promoter region of *yvcR*	(-79 to +51)	104	14
BSGY13	145 bp fragment of promoter region of *yvcR*	(-93 to +51)	798	15
BSGY14	161 bp fragment of promoter region of *yvcR*	(-109 to +51)	3185	1455
BSGY15	184 bp fragment of promoter region of *yvcR*	(-132 to +51)	2637	1387
BSGY16	211 bp fragment of promoter region of *yvcR*	(-159 to +51)	2963	1473

* Nucleotide numbers in parentheses are relative to the translation initiation site of *yvcR*

III. 3. 2. The YvcRS transporter protein participates in activation of the YvcPQ two component system

To further study the YvcPQ two-component system, I introduced the *yvcP* or *yvcR* deletion into the *B. subtilis* strain containing the P*yvcRS-lacZ* fusion at the *amyE* locus. In

agreement with the result of Northern blot analysis, the induction of the LacZ reporter activity by enduracidin was abolished by the YvcP regulator inactivation (Figure 17). Interestingly I found that deletion of the *yvcR* gene also abolished the induction of the P*yvcRS-lacZ* expression (Figure 18), indicating that the YvcRS transporter protein is necessary for signal transduction of the YvcPQ two-component system. This phenomenon may be related to an atypical structure of the YvcQ kinase, in which two transmembrane helix are located closely in the N-terminus 60 aa sequence and essential region exposed to the outside exists. This unique structure is common in BceS, although I have not yet examined the role of the BceAB transporters in the signal recognition by the BceRS two-component system.

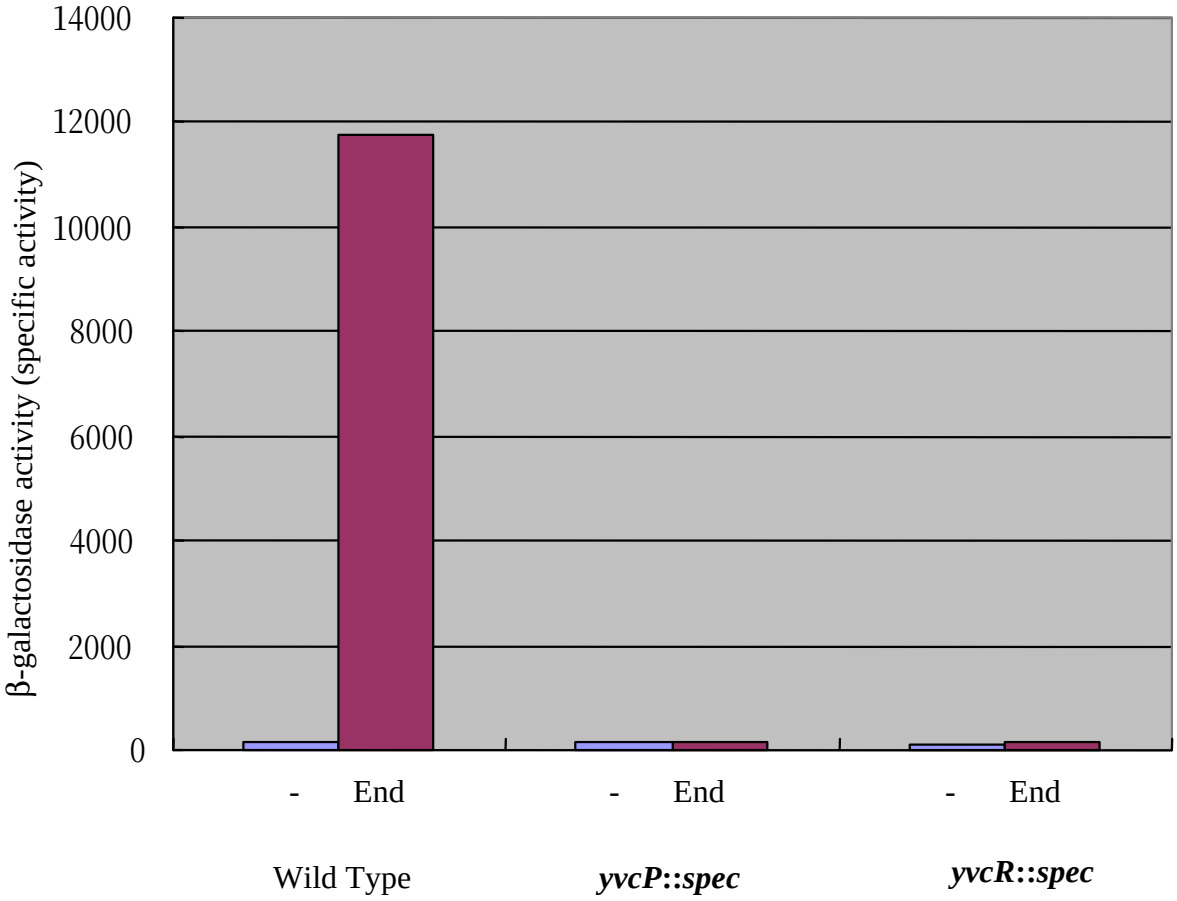

Figure 18. YycR is necessary for induction of the *yvcPQ* expression. *B. subtilis* strains having P*yvcR-lacZ* fusion at the *amyE* locus in wild type, *yvcP*::*spec* or *yvcR*::*spec* background were grown in LB medium to OD600=0.4. Cells were further incubated in the absence (-) or presence of 0.2 μg/m enduracidine (End) for 15 min, and β-galactosidase activities were measured.

III. 3. 3. The *sigI* transcription is induced by enduracidin and the *sigI* disruption increase the enduracidin sensitivity of *B. subtils* cells

I have demonstrated that enduracidin induces the expression of the *yvcRS* transporter through the action of the *yvcPQ* two component system. However the induction is transient and the inactivation each of the *yvcPQRS* gene or overexpression of YvcP regulator protein resulted in no change in the enduracidin sensitivity of *B. subtilis* cells (data not shown). Two defense systems, BceRS dependent and SigM-dependent, are involved in bacitracin resistance. Therefore I examined a possibility that ECF sigma factors also involved in enduracidin resistance, by measuring the enduracidin sensitivity of deletion mutants of each ECF sigma gene. As a result, I found that ECF σ factor, σ^I, is involved in enduracidin resistance. Disruption of *sigI* significantly increased the enduracidin sensitivity compared to wild type cells (Figure 19). The construction of a double mutant of *yvcP* and *sigI* did not result in increasing enduracidin sensitivity compared to the *sigI* single mutant, suggesting two independent mechanisms in response to enduracidin. Northern blot analysis revealed that two-kinds of the *sigI* transcripts were induced by enduracidin (Figure 20). The longest 2.6 kb transcript was also detected by the *ykoX* and *ykoY* probes (data not shown). This transcript is not detected in the *yvcP* or *yvcR* inactivated cells and induced independently on the enduracidin by the overproduction of YvcP, indicating the presence of the YvcP dependent promoter in front of *ykoX*. However, inactivation of *ykoX* or *ykoY* did not change the enduracidine sensitivity of cells, in agreement with no effect of the *yvcPQ* inactivation on it. In addition to the 2.6 kb transcript, several transcripts ranging 2.4 – 1 kb were detected in the presence of bacitracin, and these transcripts are detected in the *ykoX* or *ykoY* mutants. As *ykoX* and *ykoY* probes did not detected these transcripts, I speculate that there is a SigI-dependent promoter in front of

sigI and the *sigI* transcription is auto activated by enduracidin. By unknown reason overexpression of YvcP in wild type cells reduced the amount of these transcripts. These results suggested that major enduracidine resistance determinant(s) of *B. subtilis* is under the control of SigI

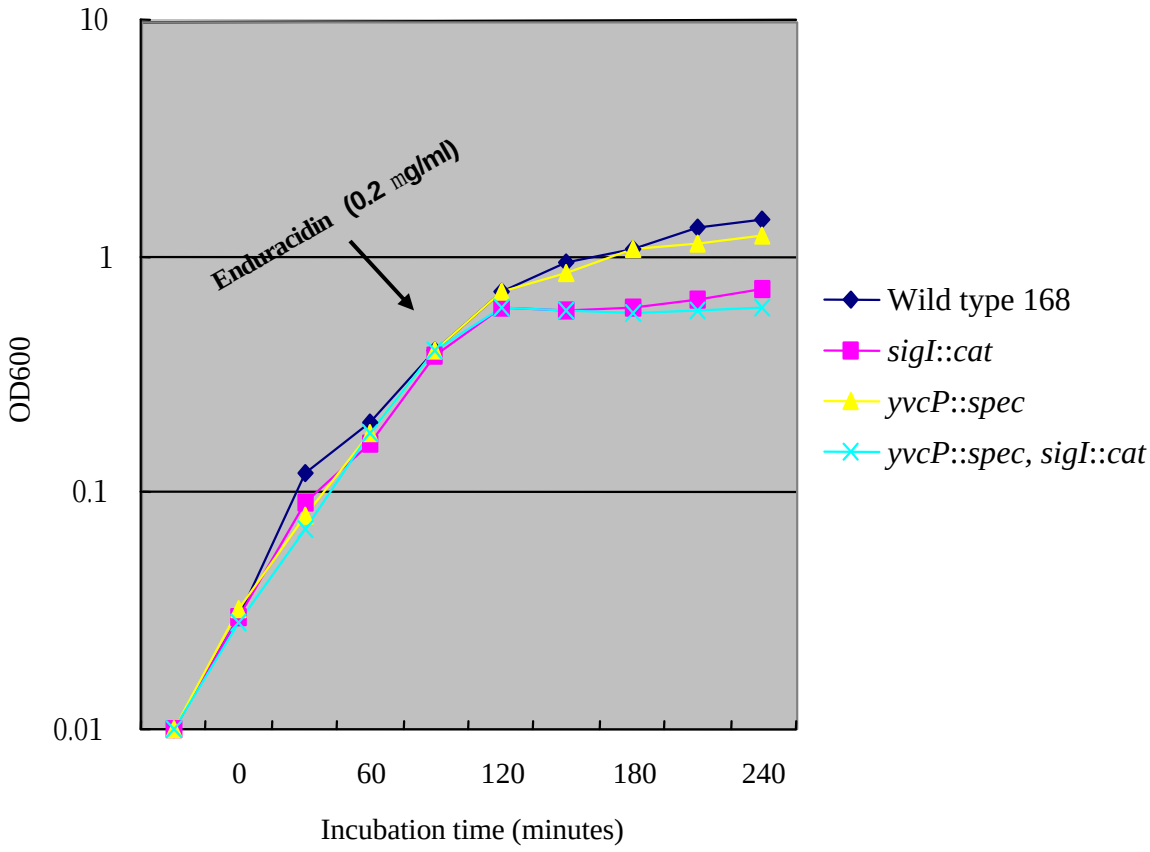

Figure 19. Growth of the *yvcP* and *sigI* mutants in the presence of enduracidin. The *B. subtilis* strains indicated in the figure were cultured in LB medium. At OD600=0.4, enduracidine was added to a final concentration of 0.2 µg/ml.

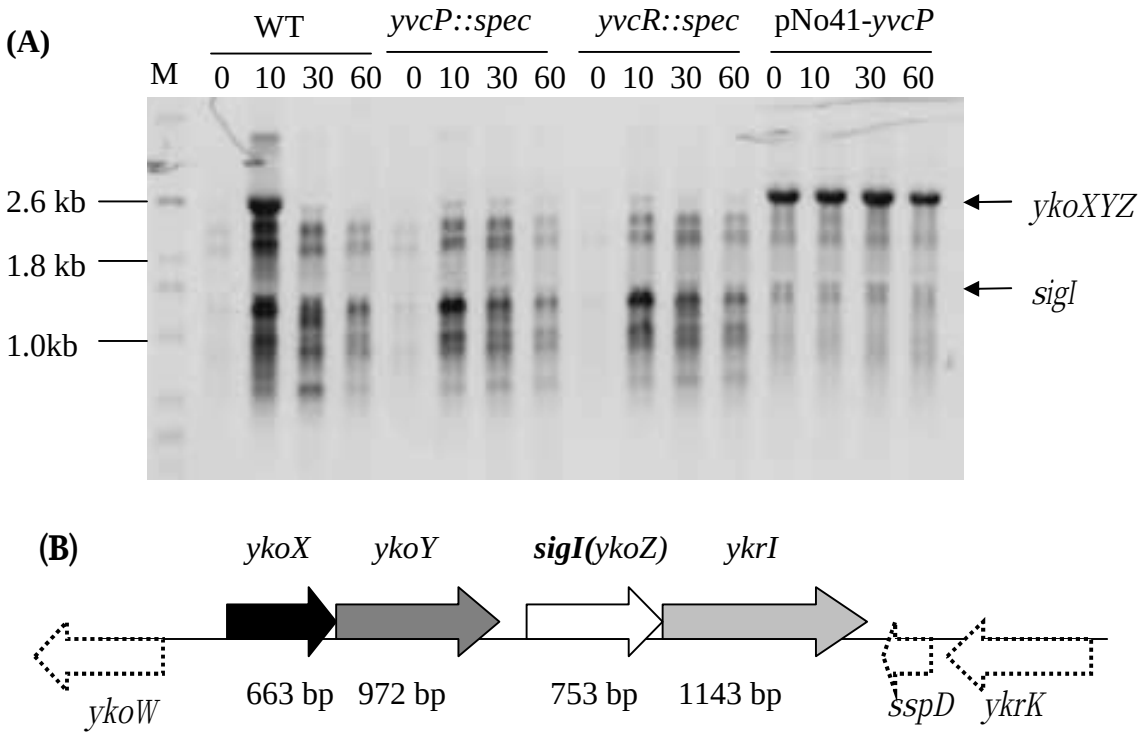

Figure 20. Northern blot analysis using *sigI*-specific probe. (A) Wild type, *yvcP::spec*, *yvcR::spec* and wild type cells harboring pNO41-*yvcP* were grown in LB medium to OD600=0.4. Then enduracidine was added to a final concentration of 0.2 μg/ml. Cells were harvested for Northern blot analysis at 0, 10, 30 and 60 min after the enduracidin addition. Lane M contained size markers. (B) The schematic representation of gene organization of the *sigI* region.

VI. DISCUSSION

In this thesis, I have demonstrated that the *bceRS* two-component system and *bceAB* ABC transporter are involved in bacitracin resistance in *B. subtilis*. Although there is no direct experimental evidence, it is reasonable to assume that the decrease in bacitracin resistance observed with *bceA* and *bceB* mutants is due to a defect in bacitracin excretion. I also demonstrated that the sensor kinase BceS responds to extracellular bacitracin and activates the response regulator BceR that binds specifically to the upstream region of the *bceAB* operon to induce transcription of *bceAB*. Tsuda *et al*. (2002) recently reported that disruption of the homologous genes in *Streptcoccus mutans* resulted in a bacitracin-sensitive phenotype. Disruption of the *mbrA* and *mbrB* genes, which encode the nucleotide binding protein (NBD) and membrane spanning domain (MSD) subunits, respectively, of the ABC transporter, resulted in an approximately 100-fold increase in bacitracin sensitivity. Furthermore, the *mbrC* and *mbrD* genes located immediately downstream of *mbrAB*, encode a response regulator and sensor kinase, respectively, of a two-component regulatory system. Disruption of either *mbrC* or *mbrD* also causes a decrease in bacitracin resistance similar to that observed in *mbrA* and *mbrB* mutants.

In addition to *bceRS-bceAB*, the *B. subtilis* genome contains two paralogous gene clusters, *yvcPQ-RS* and *yxdJK-LM*. These gene clusters are highly similar in both gene organization and sequence. I found that enduracidin induces the expression of the *yvcRS* transporter through the action the *yvcPQ* two component system, although the induction is transient and the inactivation of each of the *yvcPQRS* genes or overexpression of YvcP regulator protein resulted in no change in the enduracidin sensitivity of cells. Therefore, it is not clear whether *yvcRS* acts as enduracidin exporter or not. However, involvement of YvcR in signal transduction by YvcPQ suggests an interaction between enduracidin and the YvcRS transporter.

Bacitracin, a dodecapeptide antibiotic produced by some strain of *B. licheniformis* and *B. subtilis* (Azevedo *et al*., 1993; Johnson *et al*., 1945), inhibits dephosphorylation of C_{55}-isoprenyl pyrophosphate (IPP), which is a carrier of peptidoglycan unit (Siewert and Strominger, 1967), while enduracidin, a cyclodepsipeptide isolated from *S. fungicidus* (Higashide *et al*., 1968), inhibit the transglycosylation step in cell wall synthesis. These facts suggest that *yxdJK-LM* would also involve in response to antibiotics that inhibit cell wall synthesis.

Two component system and ABC transporter gene cluster involved in antibiotics resistance

In peptide antibiotic-producing bacteria, the biosynthetic operons for the antibiotics are frequently associated with genes encoding two-component regulatory systems as well as with genes encoding efflux transporters for self-protection (Ruyter *et al*., 1996; Altena *et al*., 2000; Neummüller *et al*., 2001; Stein *et al*., 2002). In *B. subtilis* ATCC6633, which is a subtilisin producer, the genes of the *spaRK* two-component regulatory system are located adjacent to the gene cluster encoding the subtilisin propeptide synthesis and the modifying enzymes of subtilisin, as well as to genes encoding the ABC transporter that is responsible for immunity against subtilisin (Klein and Entian, 1994). SpaRK has been shown to regulate the expression of these genes (Stein *et al*., 2002). In *Lactococcus lactis*, the genes of the NisRK two-component regulatory system are responsible for the regulation of the *nisFEG* gene, which encodes an ABC transporter involved in the nisin extrusion (Ruyter *et al*., 1996). In *B. licheniformis*, expression of *bcrABC* genes located downstream of the bacitracin biosynthetic operon, *bacABC*, is regulated by the BacRS two-component regulatory system (Neumüller *et al*., 2001). Disruption of *bacRS* leads to increased bacitracin sensitivity as well as to an increase in cellular BcrA. From this result, Neumüller *et al*. inferred that BacR

is a negative regulator of *bcrABC* transporter gene expression. Guder *et al*. (2002) reported that mersacidin-producing *Bacillus sp* has two-component regulatory system MrsR2/K2 and ABC transporter MrsFGE involved in self resistance mechanism. The disruption of *mrsR2/K2* decreased the transcription of *mrsFGE* and increased the mersacidin sensitivity of the cells.

This study is the first report showing the existence of similar systems in antibiotic non-producing bacteria. *B. subtilis* BceRS two-component regulatory systems and BceAB ABC transporters are distantly related in structure to the bacitracin exporter, BcrA and BcrB, and the regulatory system, BacR and BacS, of the self-protecting system of the bacitracin producer *B. licheniformis*. The *B. subtilis* membrane spanning domain (MSD) protein is 666 aa in length with 10 transmembrane helices (TMHs), while the *B. licheniformis* MSD protein is 208 aa in length with 6TMHs (predicted by the SOSUI program, http://sosui.proteome.bio.tuat.ac.jp/sosuimenu0.html). The *B. subtilis* nucleotide binding protein (NBD) protein (253 aa) is smaller in molecular size than the equivalent *B. licheniformis* protein (306 aa), and the standard BLAST search at NCBI using either sequence as probe did not detect the other. The sensor kinases and response regulators in the two bacterial systems are similar in size and architecture. The sensors in *B. subtilis* (231 aa) and *B. licheniformis* (238 aa) have an atypical domain organization: two closely spaced TMHs in the N-terminus 60 aa connected to the C-terminus kinase domain with a linker sequence of approximately 50 aa. Both *B. subtilis* and *B. licheniformis* regulators (334 and 348 aa, respectively) belong to the OmpR family of DNA binding proteins. However, sequence similarity between the kinases and regulators was not significant according to the NCBI BLAST search.

Two component system and ABC transporter gene clusters homologous to those studied here are widely distributed in bacterial genomes of the *Bacillus*/*Clostridium* group (Joseph *et al*., 2002); 4, 1, 1, 1, 2, 3, and 1 cluster(s) in *Bacillus halodulans*, *Oceanobacillus iheyensis*, *Listeria innocua*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium acetobutylicum*, and *Clostridium perfringens*, respectively. In addition, a cluster of reversed order, ABC transporter genes followed by two-component regulatory genes, was found in *Streptococcus agalactiae*, *S. mutans*, and *L. lactis.*

Various peptide antibiotics are synthesized in various bacteria of the *Bacillus*/*Clostridium* group. These organisms also evolved defense systems to antibiotics produced by themselves or others. Clusters of two-component systems and ABC transporter genes in the *Bacillus*/*Clostridium* group that are similar to those studied here would work as a defense against peptide antibiotics, because no possible genes for antibiotic production are associated with them. Identification of the substrates that these molecules can sense and exclude will be important for our understanding of the evolution of bacterial communities.

Multiple pathways for the resistance to antibiotics

In addition to response through two-component systems, I found that the antibiotic stress also induces the ECF sigma factor regulons. It should be noted that their activity is generally regulated by anti-sigma factors embedded in the membrane, like sensor kinases of two-component system (Yoshimura *et al*., 2004).

Ohki *et al.* (2003) and Cao and Helmann (2002) reported that disruption of the *bcrC* gene in *B. subtilis* 168 results in hypersensitivity to bacitracin and that transcription of *bcrC* is dependent on the ECF σ factors, σ^M and σ^X. Here I demonstrated that disruption of the *bceRSAB* system also causes the bacitracin hypersensitive phenotype, and the both systems contribute independently to the bacitracin resistance of the *B. subtilis* cells. In addition, I

demonstrated that enduracidin in the culture medium not only induced the transcription of *yvcRS* transporter through *yvcPQ* two-component regulatory system, but also induced the transcription of *sigI* gene, that encodes ECF sigma factor σ^I. Furthermore disruption of the *sigI* gene increased the enduracidin sensitivity of cells, indicating that gene(s) under the control of SigI is the major enduracidin resistance determinant, although I have not yet succeeded to identify it (them).

Very recently, Mascher *et al*. (2003) reported the comprehensive regulatory network of the cell wall stress response of *B. subtilis* by using global transcriptome analysis. They demonstrated that the vancomycin treatment induces σ^B-dependent general stress responses regulon, two ECF sigma, σ^M and σ^W, regulons and the YvqCE two-component system together with their target genes, *yvqIH*. On the other hand, bacitracin specifically induced the *bceRS-AB* genes, in addition to σ^B and σ^M regulons and the *yvqCEIH* genes which were common with the vancomycin treatment. It has been demonstrated also in our laboratory that the YvqCE two-component system induces the *yvqIH* expression and the inactivation of *yvqIH* resulted in the enduracidin hypersensitivity (Andriansjah, unpublished data).

These results indicate that *B. subtilis* has developed a complex system, composed of two-component signal transduction system, ECF sigma factors and general stress sigma factor σ^B, to cope with the stresses of antibiotics produced by other microorganisms, as schematically illustrated in Figure 21. Microorganisms have developed various ways to resist the toxic effects of antibiotics and other drugs: (i) an enzyme that inactivates the antimicrobial agent; (ii) an alternative enzyme that is inhibited by the antimicrobial agent; (iii) a mutation in the antimicrobial agent target which reduces binding of the antimicrobial agent; (iv) posttranscriptional or posttranslational modification of the antimicrobial agent's target, which reduces binding of the antimicrobial agent; (v) reduced uptake of the antimicrobial agent; (vi) active efflux of antimicrobial agent; and (vii) overproduction of

the target of antimicrobial agent (Fluit *et al*., 2001). Such multiple systems would be induced through different signal transduction mechanisms. Further analysis of these systems will provide clues to unders tand how microorganisms gain resistance to antibiotics produced by other microorganism.

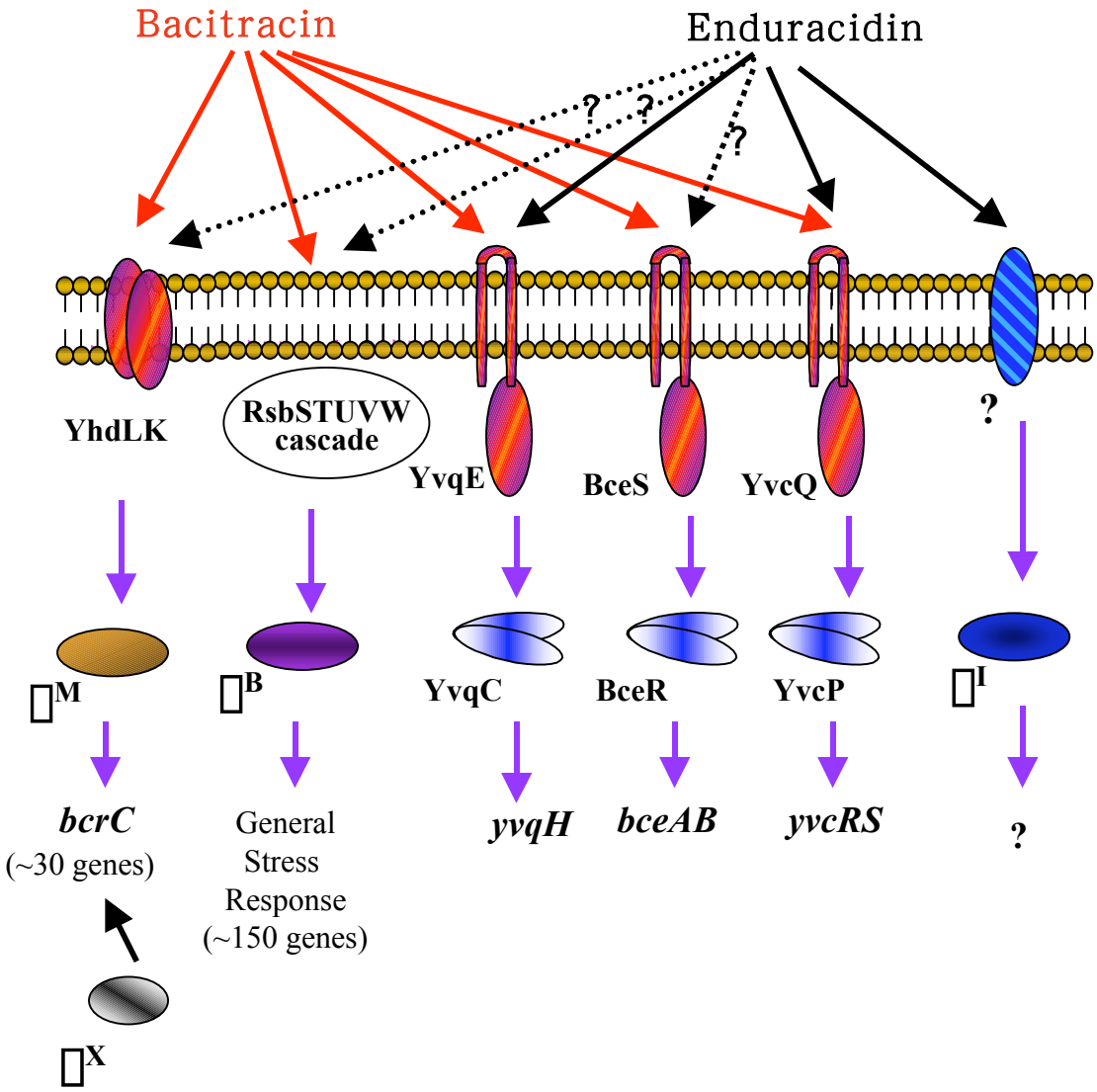

Fig 21. Responses to bacitracin and enduracidin in the *B. subtilis* cells. Solid arrows indicate known signal transduction pathways and dotted arrows with question mark presumed pathways.

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Bismillaahirramaanirrahiim

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Nara, January 2004

Giyanto

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