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DNA microarray analysis of *Bacillus subtilis* DegU, ComA and PhoP regulons: an approach to comprehensive analysis of *B.subtilis* two-component regulatory systems

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ABSTRACT

We have analyzed the regulons of the *Bacillus subtilis* two-component regulators DegU, ComA and PhoP by using whole genome DNA microarrays. For these experiments we took the strategy that the response regulator genes were cloned downstream of an isopropyl-β-D-thiogalactopyranoside-inducible promoter on a multicopy plasmid and expressed in disruptants of the cognate sensor kinase genes, *degS*, *comP* and *phoR*, respectively. The feasibility of this experimental design to detect target genes was demonstrated by the following two results. First, expression of *lacZ* fusions of *aprE*, *srfA* and *ydhF*, the target genes of DegU, ComA and PhoP, respectively, was stimulated in their cognate sensor kinase-deficient mutants upon overproduction of the regulators. Secondly, by microarray analysis most of the known target genes for the regulators were detected and, where unknown genes were found, the regulator dependency of several of them was demonstrated. As the mutants used were deficient in the kinase genes, these results show that target candidates can be detected without signal transduction. Using this experimental design, we identified many genes whose dependency on the regulators for expression had not been known. These results suggest the applicability of the strategy to the comprehensive transcription analysis of the *B.subtilis* two-component systems.

INTRODUCTION

Recent advances in genomics have revealed the presence of many two-component regulatory systems in various organisms including prokaryotes, lower eukaryotes and plants, indicating that these signal transduction systems have versatile roles in many cellular functions (1–3). In *Bacillus subtilis*, 37 sensor kinases and 34 response regulators have been found, and among them 30 kinase–regulator pairs reside consecutively in the chromosome (4,5). The roles of most of the systems, however, are still unknown. It is generally thought that many types of information are processed and appropriate responses are made via these two-component systems so that organisms can adapt to changing environmental conditions (3). A typical two-component system is composed of a sensor kinase and its cognate response regulator. The catalytic part of the kinase phosphorylates its own histidine residue by responding to the input of a signal, and the phosphoryl group is then transferred to a conserved aspartate residue on the cognate response regulator, which acts as a transcription factor in most cases. Many kinases also have a phosphatase activity for the cognate phosphorylated regulator, although some response regulators have an intrinsic phosphatase activity (3,6).

The *B.subtilis* DegS–DegU two-component system regulates many cellular processes including exoprotease production, competence development and motility (7). Phosphorylated DegU (DegU-P) stimulates transcription of *aprE* and *nprE* encoding the major extracellular proteases, and inhibits expression or the activity of an alternative sigma factor SigD (7–9), whereas unphosphorylated DegU enhances the transcription of *comK* encoding the competence transcription factor by binding to its promoter region (7,10). Therefore, DegU is regarded as a molecular switch controlling the expression of two alternative sets of genes. It has been demonstrated that salt stress affects expression of *aprE*, *sacB* (levansucrase) and *wapA* (wall-associated protein) via DegS–DegU (11,12), but how DegU-P exerts its effect on target gene expression remains unknown.

The ComP–ComA two-component system is activated by cell density signals (13,14). Phosphorylated ComA (ComA-P), the activated form of the regulator, binds to the promoter region of the *srfA* operon (14) encoding the enzyme complex catalyzing the synthesis of a lipopeptide antibiotic surfactin and also the competence regulatory factor ComS, which lies within and out-of-frame with the *srfAB* gene (7). In addition, ComA stimulates the expression of *degQ*, *rapA* and *rapC* (13).

The PhoP–PhoR system regulates expression of the Pho regulon that is induced by phosphate starvation (15). The Pho regulon includes the structural genes *phoA* and *phoB* for

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the two major alkaline phosphatases, *phoD* for alkaline phosphatase–phosphodiesterase, the operons *pstSACB1B2*, *tagAB* and *tagDEF*, and *tuaABCDEFGH*, which are involved in high-affinity phosphate transport, teichoic acid synthesis and teichuronic synthesis, respectively, and *phoPR* itself (16–18). Recently the *glpQ* and *ydhF* genes were shown to belong to the Pho regulon (16). The consensus sequence for the binding of phosphorylated PhoP (PhoP-P) has been determined (19).

The recently developed DNA microarray technique is a powerful tool for transcriptome analysis of the entire genome, as a large amount of information is obtained at a time, and has been successfully applied to transcriptional analysis of several bacteria including *B.subtilis* (20–23). With respect to the microarray analysis of the *B.subtilis* two-component system, studies have been reported for two response regulators, ResD and Spo0A (20,21). In both cases, a global change in gene expression has been observed in strains bearing disruption of *resD* and *spo0A*.

To deduce the functional roles of all the *B.subtilis* two-component systems, knowledge of their target genes obtained by a global method such as microarray analysis will be of great help. For such studies, disruption of the regulator gene followed by microarray analysis is certainly the method of choice. However, this strategy may not be applicable to the cases where expression of the target genes is very low and, therefore, the effect of regulator gene disruption is ambiguous. Furthermore, the fact that the signals inducing most of the two-component systems are unknown makes it impossible to identify target genes by stimulating the cells with the signals. One way to overcome these potential problems would be that the regulator gene product is amplified in the cell, so that expression of the target genes is forced to be enhanced or repressed, and thus, the sensitivity of the microarray analysis may be increased. Following this expectation we applied the amplification method for regulators DegU, ComA and PhoP whose targets are known in some detail. We show in this study that overexpression of the response regulator genes indeed resulted in stimulation of target gene expression in the strains carrying disruption of their cognate sensor kinase genes. Therefore, this method may be potentially applicable to identify possible target genes of two-component regulatory systems.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture media

All the strains and plasmids used in this study are listed in Table 1. Either Schaeffer's sporulation medium or Luria–Bertani (LB) medium was used for β-galactosidase assays and the isolation of total cellular RNA. *Escherichia coli* cells for DNA manipulation were grown in liquid or agar LB medium. The concentrations of the antibiotics used in this study were described previously (9,24).

Materials

Synthetic oligonucleotides were commercially prepared by Espec Oligo Service (Ibaraki, Japan). PCR fragments were prepared by PJ2000 (Perkin-Elmer Cetus). Nucleotide sequencing was carried out using a 377 DNA Sequencer and a Dye Terminator Cycle Sequencing Kit (Applied Biosystems).

Plasmid construction

DNA regions encompassing the structural genes for *degU*, *comA* and *phoP* and their SD sequences were amplified by PCR using oligonucleotide pairs DegUF1 and DegUR1, ComAF3 and ComAR1, and PhoPF1 and PhoPR1, respectively (Table 2). The amplified DNA fragments were digested with *Hin*dIII and *Sal*I, and then cloned into pDG148 (25) digested with the same restriction enzymes, resulting in plasmids pDG148-degU, pDG148-comA and pDG148-phoP, respectively. The nucleotide sequences in the cloned DNA regions were confirmed by sequence determination of the entire regions.

Growth condition and RNA isolation

Strains TT7291 and OSM103 carrying pDG148-degU and pDG148-comA, respectively, were grown overnight in LB medium. Two milliliters of the overnight cultures were inoculated into 100 ml of Schaeffer's medium contained in two 500 ml Erlenmyer flasks, and cells were grown at 37°C to a Klett unit of ~50. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to one of the flasks at a concentration of 1 mM, and the cells were harvested after 2 h (~30 min after the end of log phase). For strain MH5913 carrying pDG148-phoP, LB medium was used for cell growth, as the strain did not grow well in Schaeffer's medium. Total RNA was isolated from the cells essentially as described previously (22).

Preparation of fluorescently-labeled cDNA

Fluorescently-labeled cDNA probes used for hybridization to microarrays were prepared by a two-step procedure: cDNA for total RNA was aminoallyl-labeled by reverse transcription with specific primers in the presence of aminoallyl-dUTP, followed by fluorescence-labeling of the resultant aminoallylated cDNA with *N*-hydroxysuccinimide-activated Cy3 or Cy5. The procedure was performed according to the manufacturer's protocol (Atlas glass fluorescent labeling kit; Clontech) with a slight modification. RNA (50 µg) and a mixture of 4050 primers that were complementary to mRNAs (0.5 pmol each) and used for the preparation of microarrays were mixed with human transferrin receptor (hTFR) mRNA and its complementary hTFR primer (0.5 pmol). hTFR mRNA synthesized *in vitro* was kindly supplied by Takara Shuzo (Shiga, Japan), and used as a positive control for microarray analysis. To this mixture was added Tris–acetate (pH 8.4, 50 mM final concentration), potassium acetate (75 mM), magnesium acetate (8 mM), dithiothreitol (10 mM), 4 µl of 10× dNTP Mix (dNTP and aminoallyl-dUTP) of the labeling kit, RNaseOUT (40 U; Life Technology, Inc., Rockville, MD) and Thermoscript (30 U; Life Technology, Inc.), and the final volume was made 40 µl. Reverse transcription for aminoallyl-labeling of cDNA was carried out at 60°C for 1 h, and then continued for another 1 h after the addition of 30 U Thermoscript. RNA was digested by the addition of 10 U RNaseH, followed by incubation at 37°C for 5 min. To inactivate Thermoscript, the reaction mixture was incubated for 5 min at 85°C. The Cy3- or Cy5-fluorescently-labeled cDNA was prepared exactly following the protocol supplied by the manufacturer, and was finally dissolved in 12 µl of distilled water. This preparation was stable for several months when stored at –20°C in the dark.

Table 1. *Bacillus subtilis* strains and plasmids used in this study

Strain/plasmid	Relevant phenotype and description	Reference or source[a]
Strains		
TT7291	*trpC2 leuC7*Δ*degS aprE'-'lacZ* (Cm^r)	53
OKB167	*trpC2 pheA1* Δ*comQXPA* (Em^r)	48
LAB358	*trpC2 pheA* SPβ *c2del2*::Tn917::*srfA-lacZ* (Cm^r)	54
OSM102	*trpC2* SPβ *c2del2*::Tn917::*srfA-lacZ* (Cm^r) Δ*comQXPA* (Em^r)	This study
OSM103	*trpC2* Δ*comQXPA* (Em^r)	This study
YDHFd	*trpC2 ydhF*::pMYDHF (*lacZ*, Em^r)	This study
OAM137	*trpC2 ydhF*::pMYDHF (*lacZ*, Em^r) *phoPR*::Tc^r	This study
MH5913	*trpC2 pheA1 phoPR*::Tc^r	F. M. Hulett
JJ10	*trpC2 amyE*::*bpr-lacZ* (Cm^r)	K. Ochi
OAM138	*trpC2 amyE*::*bpr-lacZ* (Cm^r) *degU*::Km^r	This study
YUKLd	*trpC2 yukL*::pMYUKL (*dhbF-lacZ*, Em^r)	JAFAN
OAM139	*trpC2 yukL*::pMYUKL (*dhbF-lacZ*, Em^r) *degU*::Km^r	This study
YCDAd	*trpC2 ycdA*::pMYCDA (*ycdA-lacZ*, Em^r)	JAFAN
OAM140	*trpC2 ycdA*::pMYCDA (*ycdA-lacZ*, Em^r) *degU*::Km^r	This study
BFS1211	*trpC2 rapF*::pMutin4ywhJ (*rapF-lacZ*, Em^r)	MICADO
OAM141	*trpC2 rapF*::pMutin4ywhJ (*rapF-lacZ*, Em::Tc^r)	This study
OAM142	*trpC2 rapF*::pMutin4ywhJ (*rapF-lacZ*, Em::Tc^r) Δ*comQXPA* (Em^r)	This study
YYCPd	*trpC2 yycP*::pMYYCP (*yycP-lacZ*, Em^r)	JAFAN
OAM143	*trpC2 yycP*::pMYYCP (*yycP-lacZ*, Em^r) *phoPR*::Tc^r	This study
BFS436	*trpC2 yjdB*::pM2yjdB (*yjdB-lacZ*, Em^r)	MICADO
OAM144	*trpC2 yjdB*::pM2yjdB (*yjdB-lacZ*, Em^r) *phoPR*::Tc^r	This study
Plasmids		
pDG148	Kanamycin resistance	26
pDG148-degU	pDG148 carrying *degU*	This study
pDG148-comA	pDG148 carrying *comA*	This study
pDG148-phoP	pDG148 carrying *phoP*	This study
pEm::Tc	Tetracycline resistance	K. Asai

[a]JAFAN, http://bacillus.genome.ad.jp/. MICADO, http://locus.jouy.inra.fr/cgi-bin/genmic/madbase/progs/madbase.operl.

Hybridization and microarray analysis

DNA microarrays were prepared as described previously (22). They contained 4005 genes excluding those for rRNA and tRNA, but did not contain 45 genes including *degQ* and *tuaA* due to a problem in amplification of DNA by PCR. The hybridization and microarray analyses were performed as described previously (22) except that the microarrays were washed in 2× SSC and 0.1% SDS after pre-hybridization, and in 0.5× SSC and 0.01% SDS at 48°C for 5 min after hybridization.

RESULTS

Specific expression of target genes by induction of response regulator genes in strains bearing a disruption of the cognate sensor kinase genes

Our strategy to identify the target genes of response regulators was to amplify the regulator proteins and examine the expression levels of the chromosomal genes on a microarray (see Introduction). We chose DegU, ComA and PhoP as the model response regulators for a global analysis of the two-component system in *B.subtilis*, because a part of their target genes have been identified in each case. We placed the regulator genes *degU*, *comA* and *phoP* under the control of the IPTG-inducible Pspac promoter in pDG148, constructing pDG148-degU, pDG148-comA and pDG148-phoP, respectively (Materials and Methods), and then tested the effect of IPTG-induction on gene expression. To monitor the expression of the target genes of DegU, ComA and PhoP, fusions *aprE-lacZ*, *srfA-lacZ* and *ydhF-lacZ* were used, respectively. Cells were grown to mid-log phase in Schaeffer's sporulation medium except for the cells carrying pDG148-phoP for which LB medium was used, and expression of the regulator genes was induced (for details see Materials and Methods). As shown in Figure 1, the expression levels of *aprE-lacZ*, *srfA-lacZ* and *ydhF-lacZ* were very low in the *degS*-, *comP*- and *phoR*-deficient mutants, respectively,

Table 2. Oligonucleotides used in this study

Name	Sequence
DegUF1	5′-GTAAAGCTTGACCGAATGCTAGAGTATATAG-3′
DegUR1	5′-GTAGTCGACTAGTAAAAGGCAAGTCTCC-3′
ComAF3	5′-GTAAAGCTTGAGTGAGTAAAAGGGAGGAAAAC-3′
ComAR1	5′-GTAGTCGACGCATCGTTCCGCTGTGTT-3′
PhoPF1	5′-GTAAAGCTTAATAGAGAAATAGGATGTCGGG-3′
PhoPR1	5′-GTAGTCGACACCAGAATCATACAGACAACG-3′
dhbF1	5′-AGCAGTCTTTTTCGCTGGAT-3′
dhbF2	5′-TAATCTCCAGGTTCAGGAAC-3′
murD1	5′-ATGTTGCAGTCAATGATCAA-3′
murD2	5′-GCTTCGCCGTTAAACATAATC-3′

whereas the addition of IPTG greatly increased the expression levels in those strains. When the same experiments were performed with strains carrying the wild-type kinase genes, no enhancing effect on the target genes was observed (data not shown). These results indicate that overexpression of the regulator genes enhanced the target genes without signal transduction through the sensor kinases and mimicked the signal input that results in phosphorylation and activation of the cognate regulators in the wild-type strain.

We applied the above experimental strategy to identify possible target genes of the response regulators by microarray analysis. RNAs were isolated from the cells grown with and without IPTG addition, and subjected to cDNA synthesis and the microarray procedures (see Materials and Methods). We took the ratios of >4.5- and ≤0.25-fold as the criteria of stimulation and inhibition of gene expression by the regulators, respectively. The DNA microarray data are available on the web site: http://www.genome.ad.jp/kegg/expression.

Global analysis of regulons of two-component regulators by DNA microarray

DegU regulon. The results obtained with RNA from TT7291 (Δ*degS*) carrying pDG148-degU are shown in Table 3. The *aprE*, *nprE* and *ispA* genes have been shown to be the targets of phosphorylated DegU (7), and in good agreement with this the transcription levels of these genes were found to be 12.7-, 9.5- and 8.3-fold higher in the IPTG-induced cells, respectively. Transcription of the *nprB* gene encoding an exoprotease was stimulated as expected (7). Table 3 also shows that expression of many genes/operons whose relationship to DegU had been unknown was either stimulated or decreased by overproduced DegU. In fact DegU affected the expression of ~2.8% of the *B.subtilis* genes (116 genes) based on our criteria. In order to test whether the genes found in this experiment are indeed under DegU regulation, we examined expression of several genes by *lacZ* fusion or northern analysis in CU741 (*degU*$^+$) and its *degU*-knockout strain. The expression of *bpr-lacZ*, *yukL-lacZ* and *ycdA-lacZ* was found to be decreased in *degU* strains as shown in Figure 2. It should be noted that the former *dhbF* (fold ratio, 4.5), *yukL* and *yukM* are now in the large *dhbF* gene and form a gene cluster of siderophore synthesis with the upstream *dhbA*, *C*, *E* and *B* genes (26,27). Furthermore, the expression level of *dhbA* was much lower in the *degU* mutant as shown by northern analysis (Fig. 3A). Among the genes that we tested for DegU dependency, we could not see much difference in expression for the genes *ywfD*, *yvdA*, *yraJ* and *yitN*, because β-galactosidase activities in *degU*$^+$ and *degU* strains were too low or too close to each other. It seems that the *lacZ* fusion assay is less sensitive than the microarray assay and also has its limitation possibly due to the difference in stability of natural and fusion-gene mRNAs.

The other up-regulated genes whose functions are already known include those for energy production (*atpB*, *E*, *F*, *H*, *A*, *G*, *D*, *D* and *C*; 28), polyketide biosynthesis (*pksP*, *M*, *L*, *K* and *G*; 29), cell wall biosysnthesis (*murD*, *mraY*; 30), pyruvate dehydrogenase (*pdhA*; 31) and translation (*frr* and *tsf*; 5).

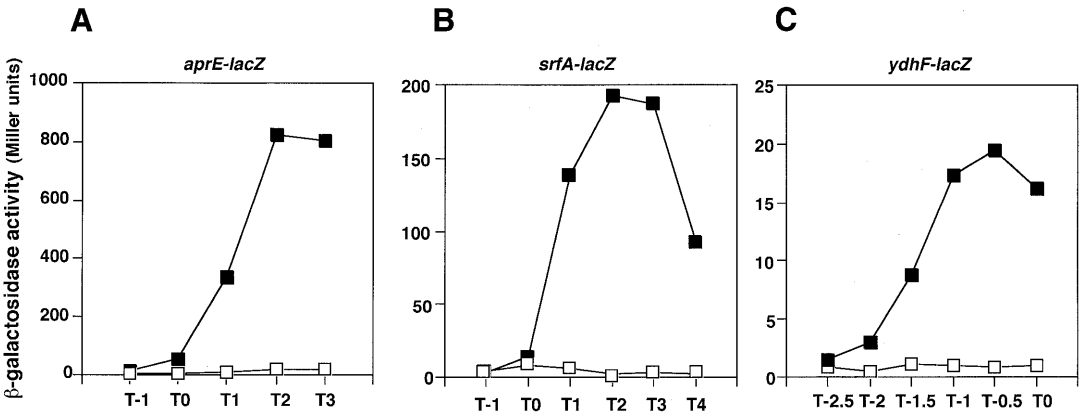

Figure 1. Effect of overexpression of response regulator genes on target gene expression. Cells were grown as described in Materials and Methods, except that the total culture volume was 50 ml. After the addition of IPTG (1 mM) at T-1, T-1 and T-2.5 for the *aprE-lacZ*, *srfA-lacZ* and *ydhF-lacZ* experiments, respectively, samples were withdrawn at the indicated times and processed as previously described (24). The numbers on abscissa indicate the growth time in hours relative to the end of vegetative growth (T0). Open and closed symbols indicate the β-galactosidase activities in the cells grown without and with the addition of IPTG, respectively. (**A**) TT7291 carrying pDG148-degU. (**B**) OSM102 carrying pDG148-comA. (**C**) OAM137 carrying pDG148-phoP.

Table 3. Microarray analysis of the DegU regulon[a]

Gene	Ratio[b]	Description
yybF	5.7	unknown; similar to antibiotic resistance protein
yxiD	9.3	unknown
ywfB	5.4	unknown
ywfC	10.2	unknown
ywfD	11.1	unknown; similar to glucose 1-dehydrogenase
ywfE	7.9	unknown
ywfF	10.8	unknown; similar to efflux protein
ywlA	8.4	unknown; similar to unknown proteins from *B.subtilis*
ywlB	6.2	unknown
atpB	5.8	ATP synthase (subunit a)
atpE	6.3	ATP synthase (subunit c)
atpF	6.8	ATP synthase (subunit b)
atpH	6.7	ATP synthase (subunit delta)
atpA	7.5	ATP synthase (subunit alpha)
atpG	7.6	ATP synthase (subunit gamma)
atpD	8.1	ATP synthase (subunit beta)
atpC	7.0	ATP synthase (subunit epsilon)
ywqH	8.6	unknown
ywqI	7.1	unknown; similar to unknown proteins from *B.subtilis*
ywqJ	10.7	unknown; similar to unknown proteins from *B.subtilis*
ywqK	10.0	unknown
degU	6.6	two-component response regulator
yvpA	15.7	unknown; similar to pectate lyase
yvdA	7.0	unknown; similar to carbonic anhydrase
yuiI	9.5	unknown
dhbA	8.2	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase
dhbC	6.5	isochorismate synthase
dhbE	6.8	2,3-dihydroxybenzoate-AMP ligase
dhbB	6.6	isochorismatase
yukM	5.7	unknown; similar to antibiotic synthetase
yukL	4.8	unknown
yukE	6.2	unknown
yukD	7.1	unknown
yukC	14.0	unknown
yueB	4.9	unknown
yueC	4.8	unknown
ytvB	11.6	unknown
yraI	16.0	unknown; similar to unknown proteins from *B.subtilis*
yraJ	21.2	unknown; similar to unknown proteins from *B.subtilis*
ypjH	6.2	unknown; similar to lipopolysaccharide biosynthesis-related protein
pksP	4.6	polyketide synthase
ppsM	5.7	polyketide synthase
pksL	5.2	polyketide synthase
pksK	10.2	polyketide synthase
pksG	10.7	involved in polyketide synthesis
frr	6.7	ribosome recycling factor
smbA	7.1	uridylate kinase
tsf	7.6	elongation factor Ts
bpr	7.9	bacillopeptidase F
murD	5.0	UDP-*N*-acetylmuramoylalanine-D-glutamate ligase
mraY	5.5	phospho-*N*-acetylmuramoyl-pentapeptide transferase
nprE	9.5	extracellular neutral metalloprotease
ispA	8.3	major intracellular serine protease
pdhA	4.6	pyruvate dehydrogenase (E1alpha subunit)
nprB	5.2	extracellular neutral protease B
yitP	22.1	unknown; similar to unknown proteins
yitO	32.5	unknown; similar to unknown proteins from *B.subtilis*
yitN	52.3	unknown; similar to unknown proteins from *B.subtilis*
yitM	36.4	unknown; similar to unknown proteins from *B.subtilis*
yhfS	5.6	unknown; similar to acetyl-CoA C-acetyltransferase
aprE	12.7	extracellular alkaline serine protease (subtilisin E)
yfjA	7.9	unknown
yfjB	4.8	unknown
yfjC	13.3	unknown
yfjD	7.9	unknown; similar to unknown proteins from *B.subtilis*
yfkN	8.3	unknown; similar to 2′,3′-cyclic-nucleotide 2′-phosphodiesterase
yflE	5.2	unknown; similar to anion-binding protein
ycdC	4.6	unknown
ycdA	26.9	unknown
yxnB	0.18	unknown
yxbA	0.19	unknown
yxbB	0.24	unknown; similar to unknown proteins
yxbc	0.19	unknown
hutM	0.19	histidine permease
hutG	0.20	formiminoglutamate hydrolase
hutI	0.17	imidazolone-5-propionate hydrolase
hutU	0.21	urocanase
hutH	0.18	histidase
licB	0.20	PTS lichenan-specific enzyme IIB component
licC	0.25	PTS lichenan-specific enzyme IIC component
epr	0.25	minor extracellular serine protease
acdA	0.21	acyl-CoA dehydrogenase
ywtD	0.23	unknown; similar to murein hydrolase
yviF	0.20	unknown; similar to unknown proteins from *B.subtilis*
hag	0.16	flagellin protein
fliS	0.23	flagellar protein
yvzB	0.21	unknown; similar to flagellin
yusL	0.22	unknown; similar to 3-hydroxyacyl-CoA dehydrogenase
yusK	0.24	unknown; similar to acetyl-CoA C-acyltransferase
yusJ	0.21	unknown; similar to butyryl-CoA dehydrogenase

Table 3. *Continued*

Gene	Ratio[b]	Description
mcpA	0.19	methyl-accepting chemotaxis protein
ytzE	0.25	unknown; similar to transcriptional regulator
ysfD	0.04	unknown; similar to glycolate oxidase subunit
ysfC	0.05	unknown; similar to glycolate oxidase subunit
yomZ	0.22	unknown
yobO	0.18	unknown; similar to phage-related pre-neck appendage protein
yoeB	0.20	unknown
flgE	0.25	flagellar hook protein
fliJ	0.24	flagellar protein required for formation of basal body
fliI	0.24	flagellar-specific ATP synthase
fliH	0.23	flagellar assembly protein
fliG	0.24	flagellar motor switch protein
fliF	0.18	flagellar basal-body M-ring protein
fliE	0.14	flagellar hook-basal body protein
flgC	0.15	flagellar basal-body rod protein
flgB	0.14	flagellar basal-body rod protein
ylqB	0.11	unknown
mcpC	0.18	methyl-accepting chemotaxis protein
ykwB	0.20	unknown; similar to unknown proteins from *B.subtilis*
rapA	0.16	response regulator aspartate phosphatase
yhfV	0.20	unknown; similar to methyl-accepting chemotaxis protein
acoC	0.19	acetoin dehydrogenase E2 component
acoB	0.19	acetoin dehydrogenase E1 component
acoA	0.14	acetoin dehydrogenase E1 component
yfmT	0.18	unknown; similar to benzaldehyde dehydrogenase
ybdO	0.14	unknown
ybdN	0.19	unknown

[a]RNA was isolated from *B.subtilis* TT7291 (pDG148-degU) grown with and without IPTG, and used for microarray analysis (see Materials and Methods).
[b]Indicates the ratios of the signal intensities observed for the samples from IPTG-induced cells to those from uninduced cells.

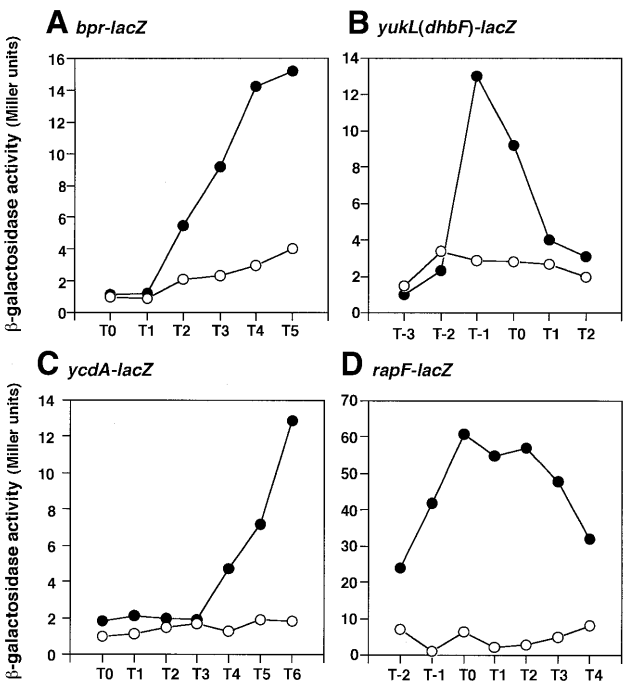

Figure 2. Effect of disruption of *degU* and *comA* on expression of *bpr-lacZ*, *yukL-lacZ*, *ycdA-lacZ* and *rapF-lacZ*, respectively. Cells were grown in Shaeffer's medium, and the samples were taken at the indicated times for the determination of β-galactosidase activities. Open and closed symbols indicate the β-galactosidase activities in disruption mutants of *degU* (**A**–**C**) and *comA* (**D**), and those in the wild-type strains, respectively.

In contrast to the DegU regulation described so far, *murD* transcription was found to be increased by *degU* deficiency as shown by northern analysis (Fig. 3B), apparently indicating that *murD* is regulated by DegU in both positive and negative ways. In addition, 39 genes whose functions are unknown were found to be positively regulated by DegU (Table 3).

We found that overproduction of DegU resulted in reduced expression of the genes for histidine degradation (*hutM*, *G*, *I*, *U* and *H*; 32), the PTS system for lichenan (*licB* and *licC*; 33), fatty acid metabolism (*acdA*; 5), chemotaxis-motility (*hag*, *fliS*, *mcpA* and *C*; 34), a response regulator aspartate phosphatase (*rapA*; 13) and fermentation (*acoC*, *B* and *A*; 35), and those located within the 5′-portion of the large *fla-che* operon (*flgE*, *fliJ*, *I*, *H*, *G*, *F*, *E*, *flgC* and *B*; 34). It is interesting to note that the transcription of the minor exoprotease gene, *epr* (36), was repressed, in contrast to the positive regulation of other protease gene expression by DegU. In addition, transcription of 20 unknown genes was found to be repressed by DegU (Table 3).

The previous and the current experimental results with *aprE*, *nprE*, *ispA*, *bpr*, *yukL*, *ycdA* and *dhbA*, together with the DegU effect on the expression of the SigD-driven chemotaxis-motility genes, support the validity of our experimental approach to identify target candidates of DegU.

ComA regulon. Results of a microarray analysis obtained by overexpression of *comA* in a *comPA* disruptant are shown in Table 4. The transcription of the *srfA* operon genes, *srfAA*, *AB*, *AC* and *AD*, which are known to be directly regulated by ComA-P (14), was greatly increased (7.7–43-fold). Expression of *rapA* has been reported to be regulated by ComA-P (13), and indeed we found the gene overexpressed in our analysis. ComA-P also regulates *degQ* and *rapC* (13), but they are not listed in Table 4, as the *degQ* DNA had not been spotted on the microarray grid (see Materials and Methods) and the fold expression of *rapC* (2.8-fold) did not meet our criterion (>4.5-fold expression level).

The expression of *rapF* was increased as shown by the microarray analysis, and we confirmed the result by using a *rapF–lacZ* fusion in *comA* cells (Fig. 2D). The *rapF* gene encodes a putative phosphatase gene for an unknown response regulator, which suggests that the target response regulator of RapF is under the control of ComA. A further microarray analysis of *rapF* would extend the network of ComA

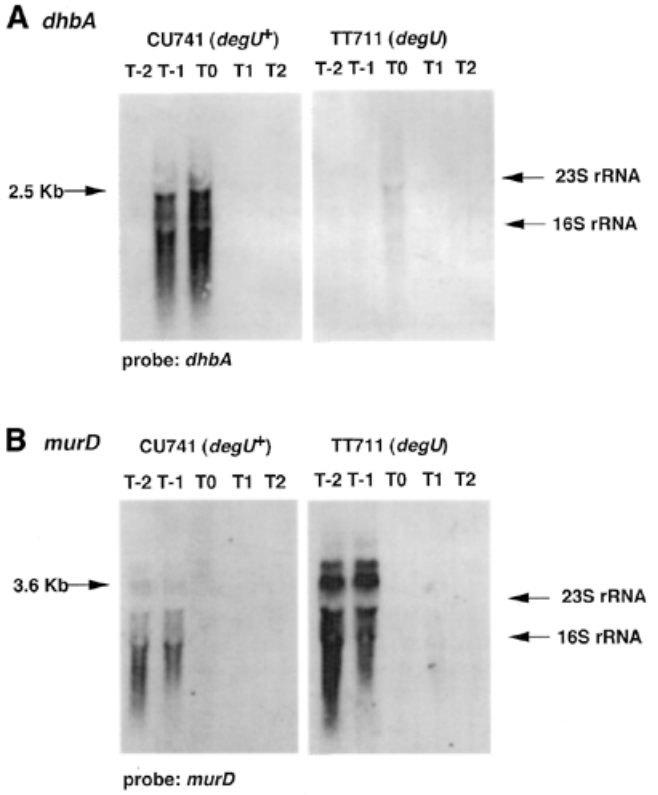

Figure 3. Northern analysis of *dhbA* (**A**) and *murD* (**B**) expression. RNAs were isolated from 20 ml cultures at the indicated times as described in Materials and Methods, and 10 µg of RNA was subjected to gel electrophoresis. Specific RNA was detected with DIG-labeled probes prepared by PCR using primer sets dhbF1 and dhbR1, and murD1 and murD2 for *dhbA* and *murD*, respectively.

regulation. As expected from a computational search for ComA binding sites (13), the expression of *pel* encoding pectate lyase (37) was increased by overproduced ComA. These results together with those of the known genes described above show again that most of the target genes of ComA can be detected by our strategy.

The genes *sacV* (a transcriptional regulator of the levansucrase gene *sacB*; 38) and *sunA* (sublancin 168 lantibiotic antimicrobial precursor peptide; 5) were identified as possible new members of the ComA regulon. We note that the genes *yddI* through *sacV* are overexpressed, although the extents of enhancement were variable. They are arranged on the *B.subtilis* map in the order of *sacV*, *ydcO*, *P*, *Q*, *R*, *S*, *T*, *yddA*, *B*, *C*, *D*, *F*, *G*, *H* and *I* (5). The fold ratios for *yddE* and *yddJ* that reside in or at the terminus of this group of genes were 3.9 and 3.6, respectively. These results suggest that the genes constitute a large operon. We note that *yqaT*, *sunA-yopZ-yoqL* and the genes *yddI* through *sacV* are constituents of the skin element, phage SPβ and prophage2, respectively (5).

PhoP regulon. For the microarray analysis of *phoP* expression, cells were grown in LB medium, i.e. a condition in which no phosphate starvation was exerted. Overexpression of *phoP* in *phoPR* cells stimulated the known target genes *phoA*, *phoB*, *ydhF*, *phoD*, *tuaB, C*, *D*, *E*, *F*, *G*, *H*, *pstS*, *A*, *C*, *B1*, *B2, glpQ* and *phoR* (Table 5), indicating that overproduced PhoP in the absence of PhoR can stimulate PhoP target genes without phosphate starvation. The expression of the *yycP*, *glnQ* and *yjdB* genes was stimulated by overproduced PhoP. We tried to confirm PhoP dependency for the two genes *yycP* and *yjdB*, but the cells carrying a *yycP-lacZ* (YYCPd) or *yjdB-lacZ* (BFS436) fusion showed very low levels of β-galactosidase activity, which precluded an accurate estimation of expression. However, the strains gave blue colonies on low-phosphate medium plates, whereas the colonies of their *phoP* mutants (OAM143 and OAM144) exhibited no blue color, indicating that the expression of the genes is PhoP-dependent (data not

Table 4. Microarray analysis of the ComA regulon[a]

Gene	Ratio	Description
rapF	11.9	response regulator aspartate phosphatase
comA	34.9	two-component response regulator
yuxO	17.6	unknown; similar to unknown proteins
yqaT	13.0	unknown; similar to phage-related terminase large subunit
sunA	7.6	sublancin 168 lantibiotic antimicrobial precursor peptide
yopZ	4.9	unknown
yoqL	5.2	unknown
rapA	6.1	response regulator aspartate phosphatase
pel	7.5	pectate lyase
yddI	33.5	unknown
yddH	6.9	unknown; similar to transposon protein
yddG	13.5	unknown
yddF	4.6	unknown
yddD	5.8	unknown
yddC	64.0	unknown
yddB	32.6	unknown; similar to transposon protein
yddA	8.1	unknown
ydcT	26.3	unknown; similar to unknown proteins from *B.subtilis*
ydcS	33.5	unknown; similar to unknown proteins from *B.subtilis*
ydcR	12.9	unknown; similar to transposon protein
ydcQ	27.3	unknown; similar to transposon protein
ydcP	9.5	unknown; similar to transposon protein
ydcO	14.0	unknown
sacV	32.5	transcriptional regulator of the levansucrase gene
srfAD	7.7	surfactin synthetase/competence
srfAC	42.7	surfactin synthetase/competence
srfAB	22.6	surfactin synthetase/competence
srfAA	26.1	surfactin synthetase/competence
yckB	14.5	unknown; similar to amino acid ABC transporter
atpD	0.25	ATP synthase (subunit beta)
secY	0.23	preprotein translocase subunit
mfd	0.21	transcription-repair coupling factor
yabH	0.24	unknown; similar to unknown proteins

[a]RNA was isolated from *B.subtilis* OSM103 (pDG148-comA) as described in the legend to Table 3.

Table 5. Microarray analysis of the PhoP regulon[a]

Gene	Ratio	Description
yycP	10.5	unknown
tuaB	76.8	biosynthesis of teichuronic acid
tuaC	47.9	biosynthesis of teichuronic acid
tuaD	50.2	biosynthesis of teichuronic acid (UDP-glucose 6-dehydrogenase)
tuaE	49.7	biosynthesis of teichuronic acid
tuaF	46.4	biosynthesis of teichuronic acid
tuaG	29.3	biosynthesis of teichuronic acid
tuaH	48.1	biosynthesis of teichuronic acid
phoP	9.3	two-component response regulator
phoR	7.2	two-component sensor histidine kinase
glnQ	7.5	glutamine ABC transporter (ATP-binding protein)
pstS	72.6	phosphate ABC transporter (binding protein)
pstA	102.3	phosphate ABC transporter (permease)
pstC	91.2	phosphate ABC transporter (permease)
pstB1	78.1	phosphate ABC transporter (ATP-binding protein)
pstB2	94.2	phosphate ABC transporter (ATP-binding protein)
yjdB	6.1	unknown
phoA	14.4	alkaline phosphatase A
phoB	116.2	alkaline phosphatase III
ydhF	24.7	unknown; similar to unknown proteins from *B.subtilis*
phoD	6.9	phosphodiesterase/alkaline phosphatase
glpQ	4.8	glycerophospharyl diester phosphodiesterase
ydbH	0.13	unknown; similar to C4-dicarboxylate transport protein

[a]RNA was isolated from *B.subtilis* MH5913 (pDG148-phoP) as described in the legend to Table 3.

shown). Furthermore, it was found that the expression of *yycP* was low-phosphate-inducible, whereas no such specificity was observed for *yjdB* (data not shown).

Although it has been reported that *tagAB*, *tagDEF* and *resA-BCDE* are repressed by PhoP (17,18), we could not confirm this under the condition we adopted in this study.

DISCUSSION

The *lacZ* fusion experiments (Fig. 1) show that overproduction of the response regulators DegU, ComA and PhoP stimulate the expression of the genes *aprE*, *srfA* and *ydhF*, respectively, in their cognate sensor gene disruptants, whereas there was no such stimulation in the wild-type strains (data not shown). These results provided the basis of our experimental strategy to identify possible targets of two-component regulatory systems, and indeed we identified many genes that were proved to be under the regulation of DegU, ComA and PhoP. Several explanations could be envisaged for the successful expression of the target genes in the sensor gene disruptants but not in the wild-type cells. It may be possible that without stimuli the sensor protein would serve as a phosphatase for its overproduced cognate response regulator. The *E.coli* sensor kinase KdpD is thought to be activated by a physiological signal acting to inhibit the phosphatase activity intrinsic to the sensor protein (39). In the *B.subtilis* DesK–DesR system, the overproduced DesR regulator stimulates target gene expression in the absence of the DesK kinase, and it was suggested that DesK works as a phosphatase of phosphorylated DesR unless a stimulus (temperature shift down) comes into the cell (40). The other explanation would be that an overproduced response regulator is inhibited by its cognate sensor kinase. It has been demonstrated that the *E.coli* UhpA response regulator is inhibited by its cognate sensor kinase UhpB in the absence of stimulation by UhpC possibly through binding and sequestration of UhpA by inactive UhpB (41). Regardless of the precise mechanism underlining these phenomena, overproduction of regulators in the absence of the cognate sensor kinases results in 'constitutive' expression of the target genes and eliminates the need for physiological signal input. This is important for studying two-component regulatory systems, for most of which the nature of the inducing signal is unknown.

The experimental results that overproduction of DegU, ComA and PhoP in cognate sensor disruption mutants enhanced the expression of the target genes indicate that the overproduced response regulators behave like phosphorylated regulators. Two explanations could be conceivable. First, the response regulators would be phosphorylated by a non-partner kinase or low molecular-weight phosphate donors, for example, acetyl phosphate (42), leading to activation of the target gene expression. In fact, ComA can be phosphorylated by acetyl phosphate *in vitro* (43). Secondly, an elevated concentration of the response regulator in the cell might result in multimer formation of the response regulator, which is proposed to activate some response regulators (6,44,45). To unravel the mechanism underlying this phenomenon, in-depth analysis is needed.

Expression of the genes for degradative enzyme synthesis and competence development has been shown to be positively but differentially regulated by the DegU response regulator: expression of the former genes is stimulated by phosphorylated DegU, while that of the latter genes is by unphosphorylated DegU (7). Likewise, it may be conceivable that a certain gene is negatively regulated by unphosphorylated DegU, whereas it is positively regulated by phosphorylation of DegU. Based on the assumption that overproduction of DegU mimics the phosphorylated form of DegU as described above, the putative *atp* and *murD-mraY* operons are likely to belong to this category, as their expression was stimulated by both multicopy *degU* (Table 3) and *degU* disruption (Fig. 3 and M.Ogura and T.Tanaka, unpublished result). On the other hand, expression of *dhbA* was stimulated by multicopy *degU* (Table 3), and inhibited in a *degU* disruptant as revealed by northern analysis (Fig. 3), suggesting that *dhbA* is positively regulated by DegU-P like *aprE*.

Several genes were found to be grouped in the DegU regulon, although they were not rigorously proved by *lacZ* fusion or northern analysis. They include the putative *atpIBE-FHAGDC* and *murE-murD-mraY* operons and the genes relating to polyketide synthesis (*pksG* through *pksR*). However, not all the constituents of these operons were detected by the microarray analysis. For example, *atpI* and *murE* in the *atp* and *murE-murD-mraY* operons, respectively, and *pksH*, *I*, *J*, *N*, *P* and *R* for the probable *pksGHIJKLMNPR*

operon were not included. One reason for this is due to our rigorous criterion (expression ratios of >4.5-fold). In fact, induction ratios (fold) of these genes were as follows: *atpI*, 2.8; *murE*, 4.2; *pksH*, 2.4; *I*, 4.6; *J*, 1.7; *N*, 2.3; *P*, 4.7; *R*, 1.4. It is interesting to note that, in addition to degradative enzyme synthesis and competence development, DegU may participate in cell wall synthesis, energy production, siderophore formation, protein translation and antibiotic synthesis, although implications of these observations are not clear at present.

In this microarray analysis, we could not detect genes whose expression was expected to be high in the DegU-overproducing cells; for example, the *amyE* gene belonging to the DegU regulon (7). This is probably because expression of the *amyE* gene is very low in strain CU741 used in this study for an unknown reason (M.Ogura and T.Tanaka, unpublished results). Others include *sacB* (7), *sacX* (46), *xynD* (7) and *bglS* (7). One possible reason is that these four genes are also under the regulation of another gene(s); for example, *sacB* expression requires the addition of sucrose in medium.

Elevation of the phosphorylation level of DegU has been shown to inhibit motility function through the inhibition of either transcription of *sigD* (8), which resides in the large *fla-che* operon (34), or the function of SigD (9). In concert with this, transcription of the *hag* and *fliS* genes that belong to the SigD regulon was inhibited (Table 3). The inhibition of SigD may block the transcription of the entire *fla-che* operon (47). This agrees with the observation that transcription of the genes related to chemotaxis and motility was repressed (Table 3).

It was found that transcription of *rapA* was repressed by overproduction of DegU. We note that transcription of the other *rap* genes was also slightly repressed: *rapB*, 0.39-fold; *rapE*, 0.34-fold; *rapF*, 0.27-fold; *rapC*, 0.27-fold.

In competence development through the ComP–ComA regulatory system, the extracellular ComX factor triggers ComP-dependent phosphorylation of ComA (13). Our microarray analysis revealed the target genes for ComA in a strain lacking the entire *comQXPA* region (48). Although the known target genes such as the *srfA* operon genes and *rapA* were identified in this global analysis of ComA, we failed to find the known genes, *rapC* (13) and *rapE* (49). This is due to low induction ratios of *rapC* (2.8-fold) and a low basal transcription level of *rapE* whose induction ratio was 8.0-fold.

In the microarray analysis of PhoP, we detected most of the genes reported to be under Pho-P regulation. However, transcription of the *tagAB* and *tagDEF* was not affected by overproduction of PhoP, although these operons are known to be repressed by Pho-P (16,17). This is probably because the *tag* operons are not repressed under phosphate-replete conditions (17). Although not detected in the current microarray analysis, the *resABCDE* and *ykoL* genes are under positive regulation of PhoPR. It has been shown that the addition of glutamate or growth in Schaeffer's sporulation medium supplemented with glucose greatly reduces the PhoPR dependency of these genes (18,50), respectively. As we used LB medium for this experiment, it is likely that the failure to detect the genes is due to a nutritional effect in the medium used. The *tuaA* gene whose expression is also expected to be stimulated was not spotted on the microarray plate.

The *B.subtilis* genome sequencing revealed that this organism possesses 37 sensor kinases and 34 response regulators of the two-component regulatory systems (4,5). Among them, nearly two-thirds remain to be characterized. Although we could not detect several genes by this system probably due to the reasons described above, the current approach will allow us to detect most of the target candidates of many two-component regulatory systems functionally uncharacterized. In these cases too, quantitative analyses such as *lacZ* fusion or northern experiments on the candidate genes detected may be necessary. We note that most of the kinase-regulator genes reside in the *B.subtilis* chromosome in pairs (5), so that we can mimic an unknown signal(s) to cause phosphorylation of an uncharacterized regulator by overexpression of the regulator gene and simultaneous disruption of the neighboring cognate sensor gene. We have already applied this strategy to analysis of uncharacterized *B.subtilis* two-component systems and successfully identified putative target genes for many regulators (K.Kobayashi, M.Ogura, H.Yamaguchi, K.-I.Yoshida, N.Ogasawara, T.Tanaka and Y.Fujita, unpublished observation). Obviously the essential two-component system, *yycF–yycG*, cannot be applied to this experimental approach (51,52).

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