J. Biochem. 2012;151(6):643–655 doi:10.1093/jb/mvs036

JB THE JOURNAL OF BIOCHEMISTRY

SwrA regulates assembly of *Bacillus subtilis* DegU via its interaction with N-terminal domain of DegU

Received January 23, 2012; accepted February 20, 2012; published online April 9, 2012

Mitsuo Ogura* and Kensuke Tsukahara

Institute of Oceanic Research and Development, Tokai University, 3-20-1 Orido-Shimizu, Shizuoka 424-8610, Japan

*Mitsuo Ogura, Institute of Oceanic Research and Development, Tokai University, 3-20-1 Orido-Shimizu, Shizuoka 424-8610, Japan. Tel: +543 34 0411 (Extn 2934), Fax: +543 34 9764, email: oguram@scc.u-tokai.ac.jp

The *Bacillus subtilis* response regulator DegU controls many physiological events including swarming motility and exoprotease production. Swarming motility is a multicellular movement of hyper-flagellated cells on a surface. The swarming motility regulator SwrA and DegU cooperatively drive transcription of *fla/che* encoding flagella components, chemotaxis constituents and motility-specific sigma factor, which is regarded as the primary event in the development of motility. We have identified *ycdA* involved in swarming motility, encoding a putative lipoprotein. We showed that the *ycdA* gene is positively regulated by DegU and SwrA. Mutational analysis of *ycdA–lacZ* revealed that SwrA changes the use of *cis*-acting sites for DegU. This suggested that SwrA operates the DegU-regulation mode through changes in the DegU assembly state. DegU binding to the *ycdA*–promoter region carrying an unusual arrangement of DegU-recognition sequences with low affinity was found to be stimulated by SwrA in electrophoretic mobility shift assay and DNase I footprinting. Yeast two- and three-hybrid analyses revealed that the N-terminal domain of DegU interacts with whole DegU, which is facilitated by SwrA. Together, these results demonstrate that SwrA can stabilize the binding of DegU to the *ycdA* promoter with low affinity. Thus, SwrA is a novel type of bacterial transcription factor in this regard.

Keywords: domain interaction/flagella/motility/multimerization/swarming/two-component system.

Abbreviations: BN–PAGE, blue native–polyacrylamide gel electrophoresis; CTD, C-terminal domain; EMSA, electro-mobility shift assay; IPTG, isopropyl β-D-thiogalactopylanoside; MC, modified competence; NTD, N-terminal domain; ORF, open reading frame; SD base, synthetic dropout base; YPD, yeast extract–peptone–dextrose.

Introduction

Soil microorganism *Bacillus subtilis* is a model Gram-positive bacterium and is showing biological phenomena of increasing interest, for example, recently identified properties including biofilm formation and swarming motility (*1*). Swarming motility is a multicellular movement of hyper-flagellated cells on a surface (*2*). This is different from swimming motility by a single cell in a liquid, although both processes require the rotation of flagella. The whole-gene network that regulates swarming motility remains unknown; however, some key transcription factors are known to play roles in the cascade, such as SwrA, DegU and alternative sigma factor sigmaD (Fig. 1). SwrA and DegU cooperatively drive the *fla/che* operon encoding flagella components, chemotaxis constituents and the alternative sigma factor sigmaD, which is regarded as the primary event in the development of motility (*3*, *4*). In *B. subtilis*, swarming motility requires a biosurfactant, surfactin, which is not produced by the 'laboratory'-strain due to loss of the functional *sfp* gene (*5*, *6*).

DegU is a response regulator belonging to the NarL subfamily and is activated by phosphorylation of single Asp site on its receiver domain by the cognate kinase DegS (*7*). DegU is known to control many genes and biological processes (*1*, *8–10*). In its low level of phosphorylation state, including the non-phosphorylation state, DegU activates *comK* (a master regulator of genetic competence) and the *fla/che* operon (*4*, *11*, *12*). In a high-level phosphorylation state, *degU* is itself activated and induces many target genes encoding extracellular degrading enzymes (*13*, *14*). In addition, high DegU-P level results in repression of motility (*10*, *15*). DegU is a DNA-binding protein that recognizes AT-rich octamers in a variety of arrangements (*4*). Moreover, DegU is regulated by protein–protein interactions, including the RapG-PhrG system. RapG inhibits DegU binding to DNA and the extracellular pentapeptide PhrG inhibits RapG activity (*16*). The other regulatory mechanism involves the degradation of phosphorylated DegU (DegU-P) by AAA^+ protease ClpCP, which modulates the *degU* autoactivation loop (*17*).

SwrA has been identified as a swarming motility regulator in the so-called 'wild strain' of *B. subtilis* and shows no similarity to known proteins (*5*, *18*). SwrA is required for *fla/che* operon expression and a suppressor mutation of the *swrA* defect (*soa*) occurs in the −10 sequence of the *fla/che* promoter, leading to enhancement of its basal transcription level (*3*). These observations raised the possibility that SwrA was a transcription factor of unknown function. The transcription of *swrA* seemed to be regulated by DegU-P and SwrA itself, but detailed analysis were not reported (*19*). SwrA has been also identified as a regulator of *pgsB* encoding the γ-polyglutamic acid

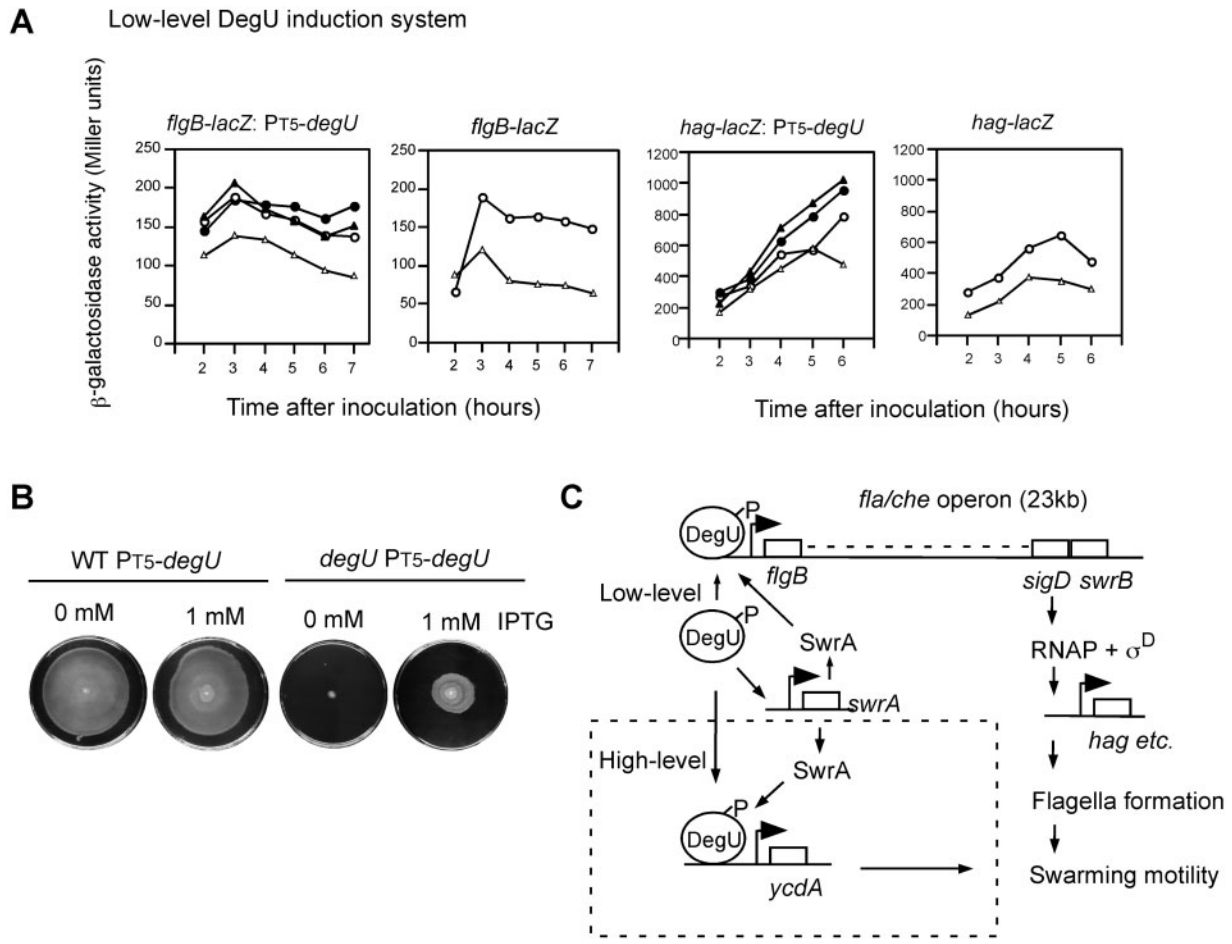

Fig. 1 Effects of low-level induction of *degU* on flagella gene expression and swarming motility. (A) β-Galactosidase analysis of the expression of *flgB-lacZ* and *hag-lacZ*. Cells were grown in LB medium. 'P_{T5}-degU' indicates a low-level induction *degU* system (addition of 1 mM IPTG, filled symbols; no IPTG, open symbols). Symbols indicate the following strains: open circle (OAM434, *degU*$^+$), open triangle (OAM435, *degU*$^-$) in the left panel, open circle (OAM436, *degU*$^+$), open triangle (OAM437, *degU*$^-$) in the mid-left panel, open circle (OAM438, *degU*$^+$), open triangle (OAM439, *degU*$^-$) in the mid-right panel and open circle (OAM440, *degU*$^+$), open triangle (OAM441, *degU*$^-$) in the right panel. (B) Swarming assay of OAM587 (*degU*$^+$) and OAM588 (*degU*$^-$). (C) Schematic representation of the genetic control system underlying swarming motility. Each constituent of the 23-kb-long *fla/che* operon is omitted (dotted line). Low-level phosphorylation of DegU activates *fla/che* expression with SwrA. A newly identified pathway requiring a high-level induction system for DegU, that is, high-level DegU-P, is depicted in the dotted box. Recently, it has been reported that the anti-sigmaD gene, *flgM*, is positively regulated by DegU-P (*28*).

synthesis enzyme, which is subject to direct regulation by DegU-P (*20*–*22*). To date, no other genes have been identified that are regulated directly by SwrA. Hence, none of the other genes in the DegU-regulon is known to be regulated by SwrA. Like *sfp*, *swrA* is mutated in the 'lab'-strain of *B. subtilis*, leading to a loss of swarming activity (*5*, *18*, *23*).

We here report the identification of a new gene, *ycdA*, which is required for full swarming motility. Mutational analysis of *ycdA*–*lacZ* fusions revealed that SwrA changes which of the six *cis*-acting sites for DegU are used in the *ycdA*-controlling region. This suggested that SwrA operates the DegU-regulation mode through changes in the DegU assembly state. We observed an enhancement of DegU-P binding to *ycdA* in the presence of SwrA in *in vitro* DNA-binding experiments. In yeast two- (Y2H) and three-hybrid (Y3H) analyses, an interaction between the N-terminal domain of DegU (DegUNTD) with full-length DegU was observed, which was stimulated by SwrA. This suggests that SwrA facilitates and stabilizes the domain interaction of DegU.

Materials and Methods

Materials and general method

One-step competence medium (MC and ref. *7*), sporulation medium (*24*) and Luria–Bertani (LB Lennox, Difco) medium were used in the experiments. Antibiotic concentrations were used as described previously (*25*). Synthetic oligonucleotides were commercially prepared by the Tsukuba Oligo Service (Ibaraki, Japan) and are listed in Supplementary Table S1. Matchmaker GAL4 Two-hybrid System 3, yeast extract–peptone–dextrose (YPD) medium and minimal Synthetic Dropout (SD) base were purchased from Clontech. All the strains and plasmids used in this study are listed in Table I and Supplementary Table S2. Construction procedures for each plasmid and strain are described in the Supplementary Data.

Table I. Strains and plasmids.

Strain/plasmid	Relevant genotype	References
Bacillus subtilis strain		
168[a]		Lab-stock
OAM434	*thrC::swrA*(Sp^r) *amyE::*P_{T5}-*degU*(Sp^r) *flgB-lacZ*(Tc^r) pDG148 (*Km^r lacI*)	This study
OAM435	*thrC::swrA*(Sp^r) *amyE::*P_{T5}-*degU*(Sp^r) *flgB-lacZ*(Tc^r) *degU* (Cm^r) pDG148 (Km^r *lacI*)	This study
OAM436	*thrC::swrA*(Sp^r) *flgB-lacZ*(Tc^r)	This study
OAM437	*thrC::swrA*(Sp^r) *flgB-lacZ*(Tc^r) *degU* (Cm^r)	This study
OAM438	*thrC::swrA*(Sp^r) *amyE::*P_{T5}-*degU*(Sp^r) *hag-lacZ*(Tc^r) pDG148 (Km^r *lacI*)	This study
OAM439	*thrC::swrA*(Sp^r) *amyE::*P_{T5}-*degU*(Sp^r) *hag-lacZ*(Tc^r) *degU* (Cm^r) pDG148 (Km^r *lacI*)	This study
OAM440	*thrC::swrA*(Sp^r) *hag-lacZ*(Tc^r)	This study
OAM441	*thrC::swrA*(Sp^r) *hag-lacZ*(Tc^r) *degU* (Cm^r)	This study
OAM587	*thrC::swrA*(Sp^r) *amyE::*P_{T5}-*degU*(Sp^r) pDG148 (Km^r *lacI*)	This study
OAM588	*thrC::swrA*(Sp^r) *amyE::*P_{T5}-*degU*(Sp^r) *degU* (Cm^r) pDG148 (Km^r *lacI*)	This study
OAM589	*thrC::swrA*(Sp^r)	This study
OAM590	*thrC::swrA*(Sp^r) *degU* (Km^r)	This study
OAM591	*thrC::swrA*(Sp^r) *ycdA–lacZ* (Em^r *ycdA* is disrupted)	This study
YCDAd	*ycdA–lacZ* (Em^r *ycdA* is disrupted)	(*8*)
OAM140	*ycdA–lacZ* (Em^r *ycdA* is disrupted) *degU* (Km^r)	(*8*)
OAM594	*thrC::swrA*(Sp^r) *ycdA–lacZ* (Em^r *ycdA* is disrupted) *degU* (Km^r)	This study
OAM595	*ycdA–lacZ* (Em^r *ycdA* is disrupted) *abrB* (Sp^r) *abh* (Km^r)	This study
OAM596	*ycdA–lacZ* (Em^r *ycdA* is disrupted) *abrB* (Sp^r) *abh* (Km^r) *degU* (Cm^r)	This study
OAM597	*amyE::ycdA–lacZ* (Cm^r, −149 to +179 relative to the transcription start site)	This study
OAM598	*thrC::swrA* (Sp^r) *amyE::ycdA–lacZ* (Cm^r, −149 to +179 relative to the transcription start site)	This study
OAM599	*amyE::*P_{T5}-*swrAtruncated yciC* (*lacI* Em^r-*lacZ::*Tc^r)	This study
Plasmid		
pDG148	Amp^r Km^r *lacI,* Shuttle vector of *B. subtilis* and *E. coli*	(*40*)
pDG148-ycdA	Amp^r Km^r *lacI* Pspac-*ycdA*	This study
pC194	Cm^r	(*41*)
pCRV	Cm^r *degQ*	(*42*)
pIS284	Amp^r Cm^r *amyE*	I. Smith
pIS284-ycdA-wt	pIS284 carrying the *ycdA* region (−149 to +179 relative to the transcription start site)	This study
pGADT7	Amp^r *LEU* GAL4-activation domain, Shuttle vector of *S. cerevisiae* and *E. coli*	Clontech
pGBKT7	Km^r *TRP* GAL4-DNA-binding domain, Shuttle vector of *S. cerevisiae* and *E. coli*	Clontech
Pbridge	Amp^r *TRP* GAL4-DNA-binding domain Pmet, Shuttle vector of *S. cerevisiae* and *E. coli*	Clontech
pDG1730	Amp^r Sp^r Em^r *amyE*	(*43*)
pDG-P_{T5}-degU	pDG1730 carrying P_{T5}-*degU*	(*15*)
pDG1730-His-swrAt	pDG1730 carrying P_{T5}-His-*swrA*truncated	This study
pTYB-degU	Amp^r P_{T7}-*degU*-intein	(*15*)
pHis-degU	pQE8 carrying His-*degU*	(*14*)
pET-degS	pET28b carrying His-*degS*	(*15*)
pQE8	Amp^r P_{T5}-His	Qiagen
pHis-swrAt	pQE8 carrying *swrA* (N-terminal six residues truncated)	This study

[a]All strains are derivatives of 168 carrying *trpC2* and defective in *swrA* due to a frame-shift mutation.

Measurements of β-galactosidase activities were performed as described previously (*25*).

Swarming motility assay and microscopic observation

The swarming motility assay was carried out by inoculating fresh colonies with toothpicks onto the centre of a 0.7% LB agar plate containing appropriate antibiotics and 20 µl of surfactin solution (Sigma, 10 mg/ml in 0.04 N NaOH), which was desiccated in a clean bench for 5 min. After 6–8 h incubation at 37°C, the plates were left for 12 h at room temperature and then photographed. For staining of flagella, cells were collected by scraping the surface near the edge on the swarm assay plate and suspended in 10 µl water on a glass plate. After drying in a clean bench, 10 µl of Ryu solution was added to the sample followed by cover glass (*26*). Microscopy and image processing described previously (*27*).

Production and purification of proteins

Escherichia coli cells were grown to mid-log phase. His-DegU was induced in *E. coli* M15 (pRep4) cells carrying pHis-degU by the addition of IPTG (0.5 mM) at 30°C for 6 h. Chitin-binding domain- and intein-fused DegU was induced in *E. coli* BL21(DE1) cells carrying pTYB-degU by IPTG addition (0.5 mM) at 20°C for 20 h. His-DegU and DegU were purified using previously described methods (*4*, *17*). His-DegS was induced in BL21 (DE1) cells carrying pET-degS by the addition of 0.05 mM IPTG at 20°C for 20 h. The harvested cells were processed using previously described methods (*17*), except that buffer A [25 mM MOPS–KOH (pH 7.0), 500 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT] was used. One millilitre of 50% Ni-NTA resin (Qiagen) equilibrated in buffer A was added to the soluble fraction of the cell lysate, which was then shaken gently for 30 min at room temperature. The resultant suspension was packed in a mini-column (washed first with 30 ml of buffer A). His-DegS was step-wise eluted with buffer A containing 0.1 through 0.5 M imidazole. The fraction was dialysed against buffer A containing 10% glycerol. His-SwrA was induced in *E. coli* M15 (pRep4) cells carrying pHis-swrAt by IPTG addition (0.5 mM) at 30°C for 6 h. The purification procedure for

His-SwrA was similar to that used for His-DegS, except that the KCl concentration was 100 mM. Aliquots of the purified proteins were stored at −80°C.

EMSA and DNase I footprint assay

The probes were prepared by PCR amplification using an appropriate forward primer and the biotinylated primer, ycdA-R1-bio. The indicated amounts of His-DegS and DegU were incubated with each DNA probe (20 fmol) in 12 µl of a buffer comprising [25 mM MOPS-KOH (pH 7.0), 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 3% glycerol] and containing appropriate amount of poly[dIdC] (Amersham) and 0.1 mM ATP for 10 min at 28°C. After the addition of 2 µl of a loading buffer (*17*), the samples were applied onto a 4.5% polyacrylamide gel and electrophoresis was performed in a 0.1× TBE buffer at 4°C. The EMSA using His-DegU was performed as described previously (*16*). For footprint assay, the reaction mixture was similarly prepared to EMSA except for using 0.1 pmol probe DNA. After incubation for 10 min at 28°C, 0.05 U of DNase I (Takara) was added and incubated for 3 min at 28°C. After addition of 25 µl of TE buffer, phenol–chloroform–isoamyl alcohol (25:25:1) extraction was performed followed by ethanol precipitation with carrier tRNA. The samples were applied onto a 6% polyacrylamide gel containing 7 M urea and electrophoresis was performed in a TBE buffer. The detection of biotin-labelled DNA has been described previously (*25*).

Y2H and Y3H assays

The Matchmaker GAL4 Two-hybrid System 3 (Clontech) was used for testing protein–protein interactions. *Saccharomyces cerevisiae* strain AH109 was made competent and transformed with plasmids as described in the Clontech manual. The Y3H assay, which was performed in AH109-carrying derivatives of pBridge and pGADT7, was performed on an appropriate SD-based medium without Met according to the protocol at the Clontech website.

Results

Full swarming motility requires an unidentified gene regulated by high level DegU-P

We have developed two types of *degU* induction system, low level and high level, which utilize the same P_{T5}-*degU* cassette (*17*). These systems differ in the *lacI* gene number, i.e., the low-level system is regulated by multicopy *lacI* (pDG148) whereas the high-level induction is mediated by a single copy of the gene. The low-level induction system of *degU* could not induce the expression of most of the DegU-regulon genes, but could activate the *fla/che* operon (Fig. 1A). As a result of the induction of *sigD* in the *fla/che* operon, the expression of the *hag* gene-encoding flagellin was induced. These results were observed in the *swrA*-active background. It should be noted that although the optimal expression of *fla/che* and *hag* is dependent on the *degU* gene, significant basal expression was observed (Fig. 1A and Refs *4*, *28*). It was anticipated that swarming motility would be sustained by this low-level induction of *degU* because *fla/che* and *hag* expression were completely recovered to wild-type levels. The swarming activity, however, was not fully recovered by the addition of IPTG under *swrA*-active conditions on the 0.7% agar plate containing surfactin (Fig. 1B). This observation raised the possibility that full induction of swarming motility might require not only *fla/che* expression but also unidentified gene(s) regulated by high-level DegU-P.

The ycdA gene is required for swarming motility

The DegU-regulon is composed of more than 120 genes including so-called 37 function-unknown 'y-genes', which are positively regulated by DegU-P (*8*). Thus, we screened these disruptants of y-genes for swarming-deficiency characteristics. We found that the disruptant of the *ycdA* gene was defective in swarming motility and that this defect was fully recovered by IPTG-induced *ycdA* on a multicopy plasmid (Fig. 2A). This complementation test indicated that the *ycdA* gene is required for swarming motility. Furthermore, we observed flagella on the strains on the swarming plate by both flagella-staining and phase contrast microscopy (Fig. 2B). The *swrA*-active cells were highly flagellated as reported previously and no cells without flagella were observed (*18*). The *swrA*-inactive strain is not motile and flagellated cell number was <60% of the observed cells. In addition, the number of flagella/single cell was much smaller than that of the *swrA*-active cells. The *swrA*-active and *degU*-deficient cells are not motile and ~45% of the observed cells showed a chained cell shape, which is the SigD-OFF phenotype (*3*). The remaining SigD-ON cells were less-flagellated compared with the *swrA*-active cells. In the $ycdA^-$ $swrA^+$ cells, no chained cells were observed and the cells appeared comparable with *swrA*-active cells in terms of flagellation, suggesting that the *ycdA* gene might function downstream of flagellation in the regulatory cascade of swarming motility (Fig. 1C).

Expression of ycdA encoding a putative lipoprotein

The *ycdA* gene constitutes a monocistronic operon and encodes a putative lipoprotein (Fig. 3A). To test the temporal expression pattern of *ycdA*, we used a *ycdA–lacZ* transcriptional fusion and examined the associated β-galactosidase activity levels in the sporulation medium, because the activities in LB liquid medium was very low (data not shown). In the *swrA*-deficient background, *ycdA* expression peaked at T7. This late induction is exceptional among the well-known DegU-regulon genes. In the *swrA*-active background, the expression of *ycdA* peaked and showed 3-fold enhanced activity at T4, strongly suggesting that the *ycdA* gene is positively regulated by SwrA (Fig. 3B, left). Irrespective of the *swrA* state, the *degU* disruption eliminated *ycdA* expression, confirming the DegU dependency of this gene. The *ycdA* gene is the third member of an SwrA-activated genetic unit, in addition to the *fla/che* and *pgsB* operons. The *degS* deletion also abolished *ycdA* expression indicating that it is activated by DegU-P (data not shown).

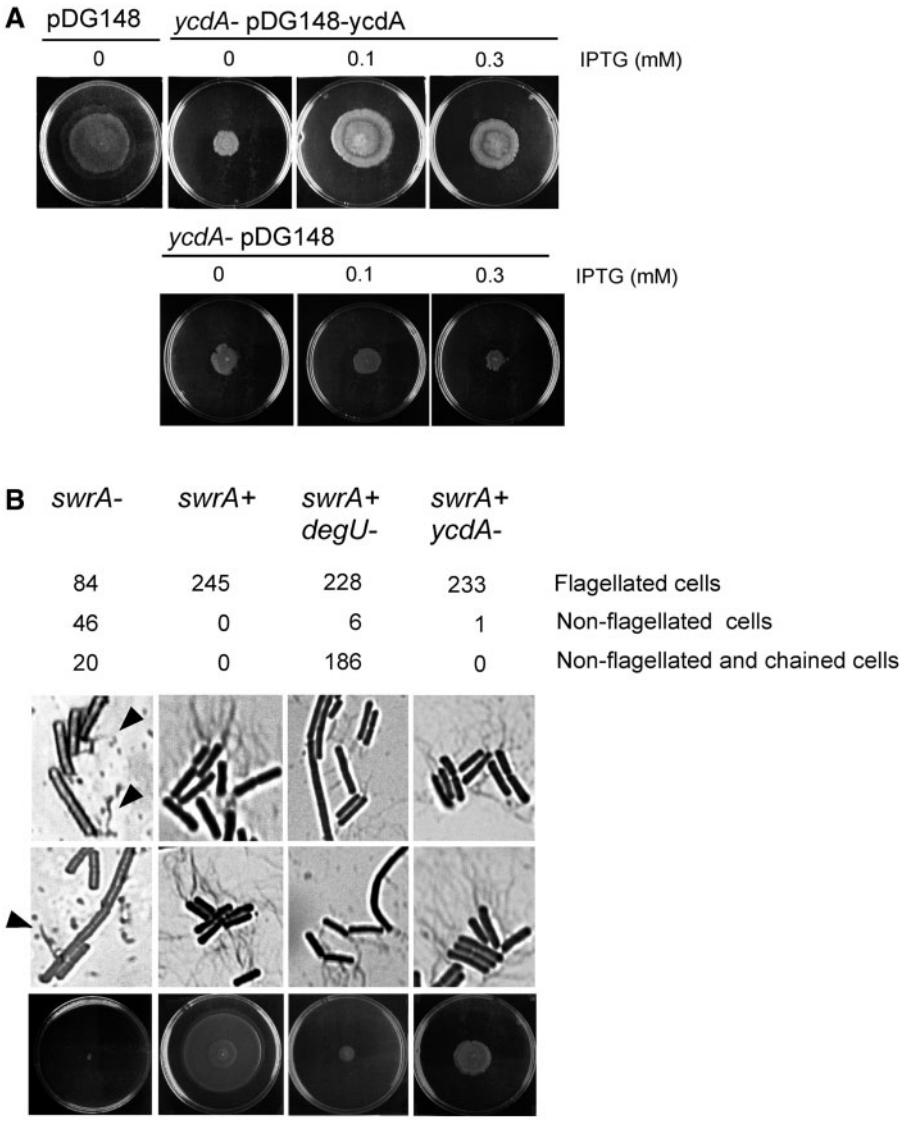

Fig. 2 Swarming motility analysis of *ycdA* disruptants. (A) Complementation test of the swarming activity of *ycdA* disruptants via the IPTG-controlled *ycdA* gene on a multicopy plasmid. 'pDG148', '*ycdA*$^-$ pDG148-ycdA' and '*ycdA*$^-$ pDG148' indicate OAM589 carrying pDG148, OAM591 carrying pDG148-ycdA and OAM591 carrying pDG148, respectively. (B) Upper and middle panels show micrographs of stained flagella of each strain. Arrowheads indicate flagella. Bottom panels show swarming assays of the strains. Cell numbers of the each type of the cell are shown.

Next, we examined whether stimulation of DegU phosphorylation affects the expression of *ycdA*. In the experiment, multicopy *degQ*, which is known to stabilize phosphorylated DegS-DegU (*29*), was introduced into the *ycdA–lacZ* strain. The stabilization of DegU-P resulted in early and enhanced expression of *ycdA–lacZ*, but the peak expression observed in the *swrA*-active strain at T4 disappeared and sustained the late expression at T6 (Fig. 3B, middle). This observation strongly suggested that the function of SwrA in the regulation of *ycdA* differs from the enhancement of DegU-P concentration.

Recently, it has been reported that AbrB, which is a global-type repressor in Gram-positive bacteria, and its homologue Abh directly and negatively regulate *ycdA* expression (*30*). To confirm this using *ycdA–lacZ*, disruptions of the *abrB* and *abh* genes were introduced into the *ycdA–lacZ* strain. The strain bearing these two mutations showed a similar expression profile to the strain carrying a multicopy *degQ* (Fig. 3B, right). This suggested that the elevated DegU-P concentration affects AbrB/Abh levels or stimulates activity or expression of AbrB-antagonist AbbA (*31*). The elevated expression of *ycdA–lacZ* in the *abrB/abh* double mutant was abolished by the introduction of a *degU* disruption, suggesting that DegU would function not only in the occlusion of repressors but as a transcriptional activator.

Effect of SwrA on DegU-P binding to ycdA in EMSA

Since DegU-P and SwrA are involved in the regulation of *ycdA*, we first investigated whether DegU-P binds to the *ycdA* regulatory region using EMSA. Incubation of DegU-P with a ycdA probe did not result in the formation of distinct shift bands (Fig. 4A), which is consistent with the very low and retarded expression of

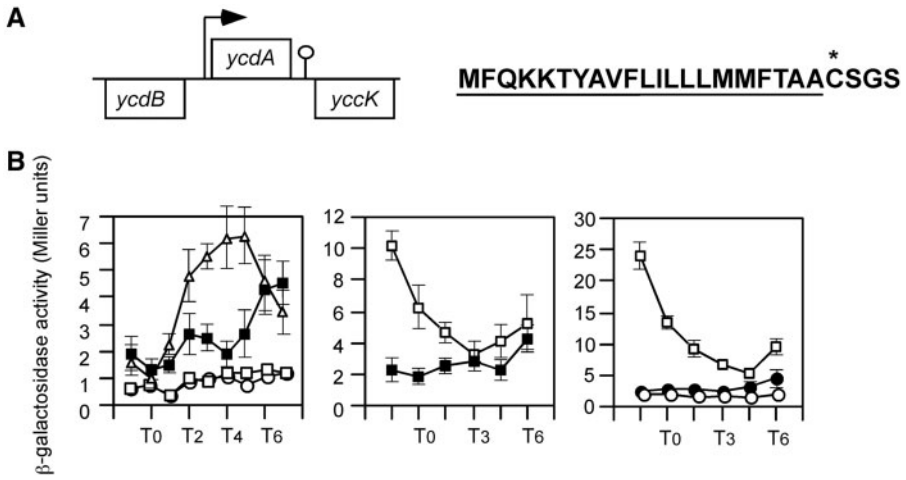

Fig. 3 *ycdA* genomic region and expression profile. (A) Schematic depiction of the *ycdA* region (left). A promoter and a terminator are shown. The N-terminal sequence of YcdA is shown in the right. The underline and asterisk indicate a signal sequence and putative lipidation site, respectively. (B) Effects of *swrA* mutation, stimulation of DegU phosphorylation and the *abrB*/*abh* double mutation on the expression of *ycdA–lacZ*. All strains carrying *ycdA–lacZ* (Em^r) were grown in sporulation medium. T1, 1 h after the end of the exponential growth phase. Symbols indicate the following strains: Left—open triangle, OAM591 ($swrA^+$); closed square, YCDAd ($swrA^-$); open circle, OAM594 ($swrA^+$ *degU*); open square, OAM140 ($swrA^-$ *degU*). Middle—open square, YCDAd carrying pCRV ($swrA^-$ $degQ^+$); filled square, YCDAd carrying pC194 ($swrA^-$ vector). Right—open square, OAM595 ($swrA^-$ *abrB*/*abh*); filled circle, YCDAd ($swrA^-$); open cirlce, OAM596 ($swrA^-$ *abrB*/*abh degU*). Error bars indicate standard deviations. Independent experiments were carried out at least three times.

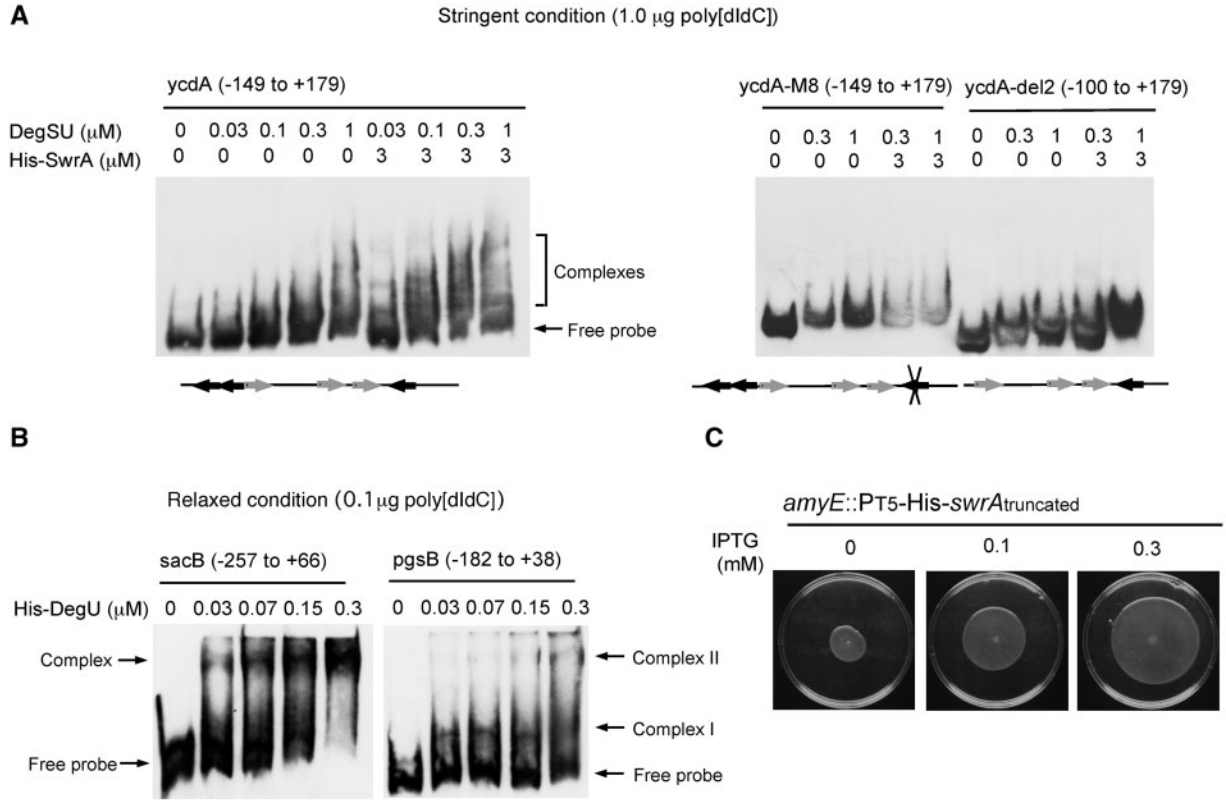

Fig. 4 Effects of SwrA on DegU binding to *ycdA* and different affinity for DegU in *sacB* and *pgsB*. (A) EMSA using the ycdA probe and DegU-P. 'DegSU' indicates a solution containing His-DegS and intact DegU (1:5 molar ratio). The indicated molar shows DegU concentration. Under the each panel a schematic structure of the probe, where arrows indicate the *cis*-sites for DegU (see Fig. 5), is shown. In both (A) and (B), the numbers in parenthesis indicate the probe-spanning region relative to the transcription start site. (B) EMSA using His-DegU. The probes used are indicated. (C) *In vivo* confirmation of swarming ability directed by His-SwrA without its N-terminal six amino acid residues. The swarming activity of OAM599 was assayed by adding IPTG.

ycdA–lacZ in the $swrA^-$ strain (Fig. 3B). To investigate the role of SwrA *in vitro*, we first attempted to clone the entire *swrA* ORF into several expression vectors, but this was not successful. We thus cloned a truncated *swrA* ORF lacking the first six codons into pQE8. To examine its functionality, this construct, P_{T5}-*swrA* truncated, was introduced into a *B. subtilis* $swrA^-$ strain carrying *lacI*. IPTG addition gradually

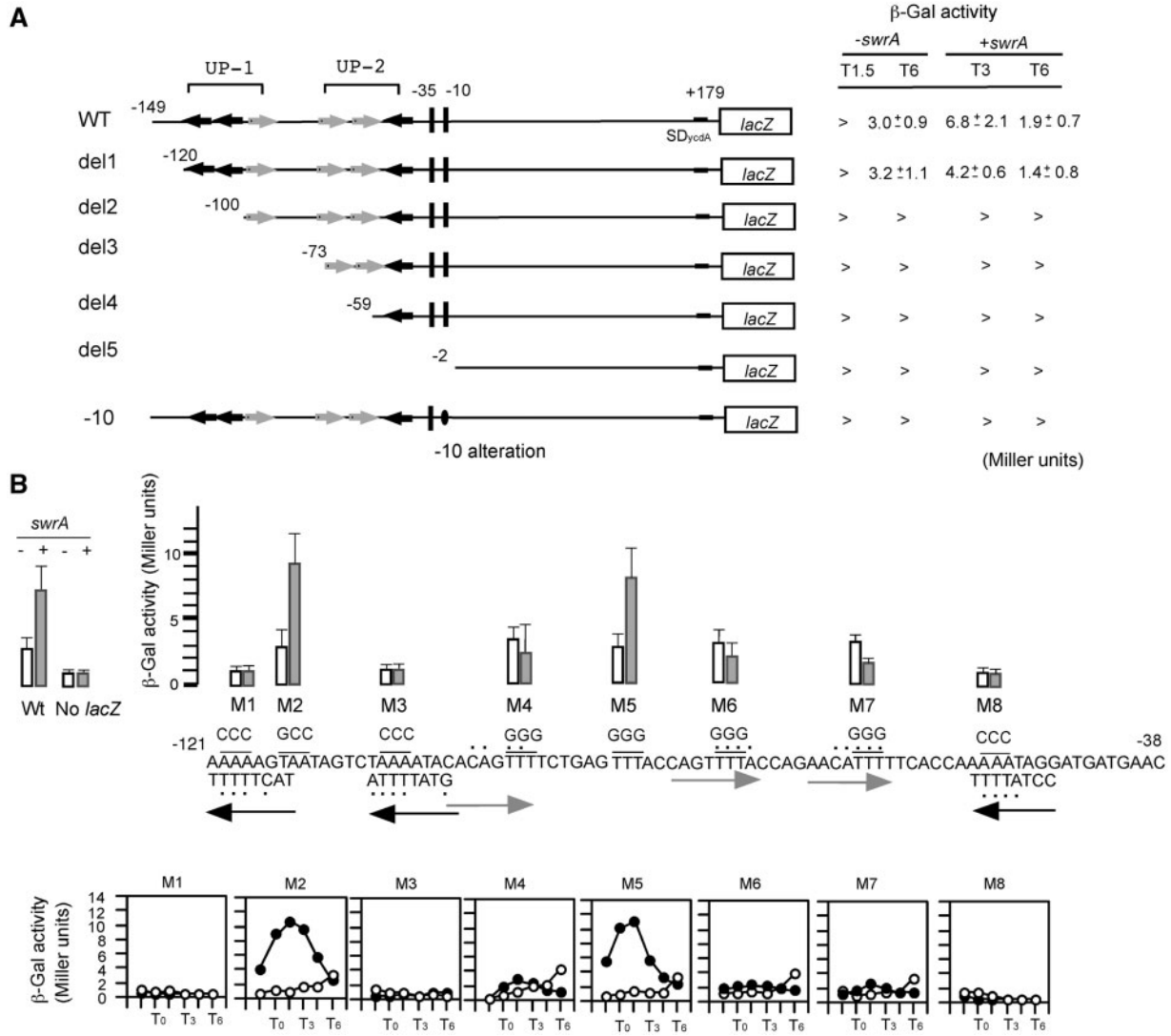

Fig. 5 Effects of deletion and site-directed mutagenesis on *ycdA–lacZ* expression. Cells were grown in sporulation medium. All strains are derivatives of OAM597 (*swrA*−) and OAM598 (*swrA*$^+$) and are listed in Supplementary Table S2. Grey and black arrows denote DegU-recognition half sites active in only the *swrA*$^+$ background and active in both *swrA*$^+$ and − backgrounds, respectively. Error bars indicate standard deviations. Independent experiments were carried out at least three times. (A) Schematic diagram of deletions and promoter mutations. Averages of the β-galactosidase activities are shown at two growth points with standard deviations. '>' indicates the activity less than the background β-galactosidase activity from a strain with no *lacZ* fusion. Numbers above the ends of the horizontal lines indicate each deletion point relative to the transcription start site. (B) Site-directed mutagenesis of *ycdA–lacZ*. The nucleotide sequence of the *ycdA* control region is shown and the octamer sequences associated with arrows indicate DegU-recognition half sites, where small black points denote the nucleotides matching the consensus sequence. The changed nucleotides are shown above the target sequences in each mutant. Open and grey vertical bars represent the peak β-galactosidase activity in wild-type, each mutant and strain with no fusion in both the *swrA*$^+$ and − backgrounds. Below the sequences, the β-galactosidase activity profile of each mutant in both the *swrA*$^+$ (filled circle) and − (open circle) backgrounds is indicated.

induced swarming motility of the strain, demonstrating the functionality of the truncated *swrA* gene product (Fig. 4C). Thus, this truncated protein was purified from *E. coli* and used for EMSA. SwrA itself did not bind to the ycdA probe as expected as it lacks any known DNA-binding domain (ref. *3* and Supplementary Fig. S1). The addition of SwrA to the reaction mixture containing DegU-P resulted in the generation of two and at least four shifted bands at the lowest and highest DegU-P concentrations used, respectively (Fig. 4A). These observations suggested that SwrA might change the mode of interaction of DegU-P with the ycdA probe.

DegU-recognition of cis-sequences in the ycdA control region

Since DegU directly binds to the *ycdA* upstream region, we examined the effects of deletions and point mutations of the control region on *ycdA* expression to identify the DegU-recognition sequence. To discuss the effects of *cis*-elements on transcription, a determination of the transcription start site is critical. With respect to the *ycdA* gene, a recent genome-wide determination of the transcription start sites of transcriptional units identified the *ycdA* transcription start site and an extremely long untranslated region on its mRNA (*32*). The deduced sigmaA-type −10 and −35

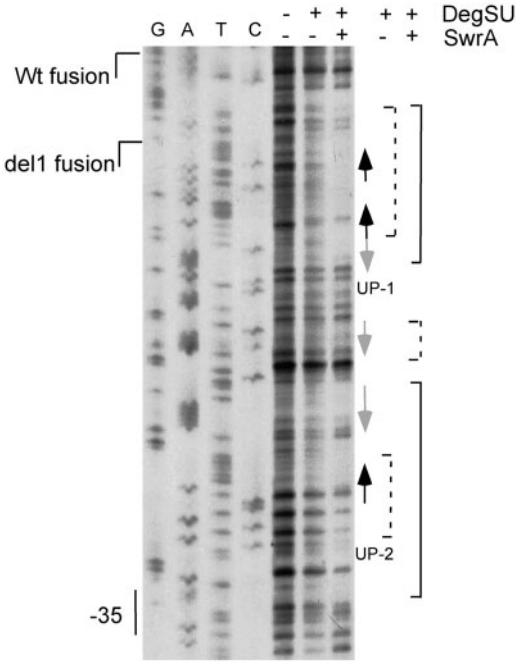

Fig. 6 DNase I footprint assay of ycdA probe. The probe was the same one used for EMSA using the wild-type DNA. 'DegSU' indicates a solution containing His-DegS and DegU (1:5 molar ratio) and DegU concentration was 0.1 μM. SwrA concentration was 3 μM. The reaction mixture contained 0.1 μg of poly[dIdC]. Grey and black arrows denote DegU-recognition half sites active in only the *swrA*$^+$ background and active in both *swrA*$^+$ and − backgrounds, respectively. The brackets show the protected regions and the dotted ones do weakly protected regions. The 5′-endpoints of *lacZ* fusions are shown. Sequence ladder was prepared with the same biotinylated primer used for probe generation.

elements are TAAAAT and TTCAAT, respectively, and the nucleotide change in the −10 sequence abolished *ycdA* expression in both *swrA*-active and -inactive backgrounds, indicating that SwrA does not change recognition of the core promoter (Fig. 5A). In addition, the del5 fusion, which lacks the promoter, did not sustain any transcription, suggesting that the detected promoter alone directs *ycdA* expression. The deletion experiment further revealed that the −120 to −100 region, containing three putative *cis*-elements for DegU, is critical for *ycdA* expression. To further elucidate the region that contains the *cis* sites for DegU, site-directed mutagenesis was performed for the candidate *cis*-sequences (Fig. 5B). In the *swrA*-inactive background, a direct repeat with a long spacing (6 nt) and one half-site of DegU-binding *cis*-sequence are located at the far-upstream and promoter–proximal regions, respectively (corresponding to the M1, M3 and M8 regions). The effects of the mutations were more easily visible in the plate culture (Supplementary Fig. S2). On the other hand, in the *swrA*-active background, the activity of more *cis*-acting sites [UP-1 (M1, M3 and M4 regions) and UP-2 (M6, M7 and M8 regions)] for DegU was detected in an unusual arrangement. Both core regulatory regions are composed of three *cis*-sites (Fig. 5B), and the odd number of *cis*-sites would not be suitable for binding of a dimer of a transcription factor. These observations raised the possibility that SwrA may affect the assembly state of DegU on the target DNA.

DegU-P was not able to bind to the DNA probes corresponding to del2 and M8 even in the presence of SwrA in EMSA (Fig. 4A, right). These results are consistent with those of the mutational *lacZ* fusion analysis. When DegU-P was incubated with the M8 probe in the presence of SwrA, the amounts of free probe were decreased, but any shifted band was not observed. This might be due to generation of unstable DNA–protein complex, leading to disperse of the signal. Together, the results indicate that the observed shifted bands in EMSA using the wild-type probe were specific to the *cis*-acting sites on the *ycdA* regulatory region.

Next, to determine DegU-binding sites in *ycdA*, we performed DNase I footprinting experiment using the ycdA probe. Without SwrA, DegU-P protected UP-1 and UP-2 from DNase I attack weakly and partially, while with SwrA, DegU-P protected both regions strongly (Fig. 6). In the latter case, DegU-P protected the wider region along UP-1 and UP-2. The observation is in good agreement with the detection of *cis*-sites for DegU-P binding in the Lac-assay (Fig. 5).

Comparison of the cis-site arrangements for DegU among the DegU-regulon genes

To date, several DegU *cis*-sites have been determined using mutational analyses of *lacZ* fusions and *in vitro* DNA-binding experiments such as DNase I footprinting and EMSA (Fig. 7). The only non-phosphorylated DegU-activated gene is known to be *comK*, whose *cis*-site arrangement is head-to-head. In contrast, all known *cis*-site arrangements for DegU-P-activated genes are head-to-tail. We note that site-1 for *flgM* is tail-to-tail as in the case of *pgsB*, but its *swrA* dependency has not yet been reported. Finally, the genes activated by DegU-P and SwrA have two types of arrangement; type 1 is a combination of three *cis*-sites, and type 2 is tail-to-tail. This comparison suggested that arrangements of the *cis*-site may determine the SwrA dependency of a particular gene.

Examination of the DNA-binding affinity of differently arranged cis-sites

A previous report has shown that the CTD of *E. coli* NarL efficiently binds to a probe with a head-to-head arrangement, whereas this protein binds to probes with the head-to-tail and tail-to-tail arrangements with a poor efficiency (refs. *33, 34*; note that the designation of the arrangements in this report differs from that used in our current study). We speculated that these properties might be observed in DegU, because DegU belongs to the NarL family of DNA-binding proteins. To examine this, we used the sacB probe with head-to-tail arrangement and the pgsB probe with tail-to-tail arranged sites. In addition, the expression of *pgsB* but not *sacB* is dependent on SwrA (Supplementary Fig. S3 and ref. *22*). To detect possible differences in the maximal binding, His-DegU was used in EMSA, because it has a higher affinity for DNA without phosphorylation, and perhaps positively charged poly-His enhances DegU-binding

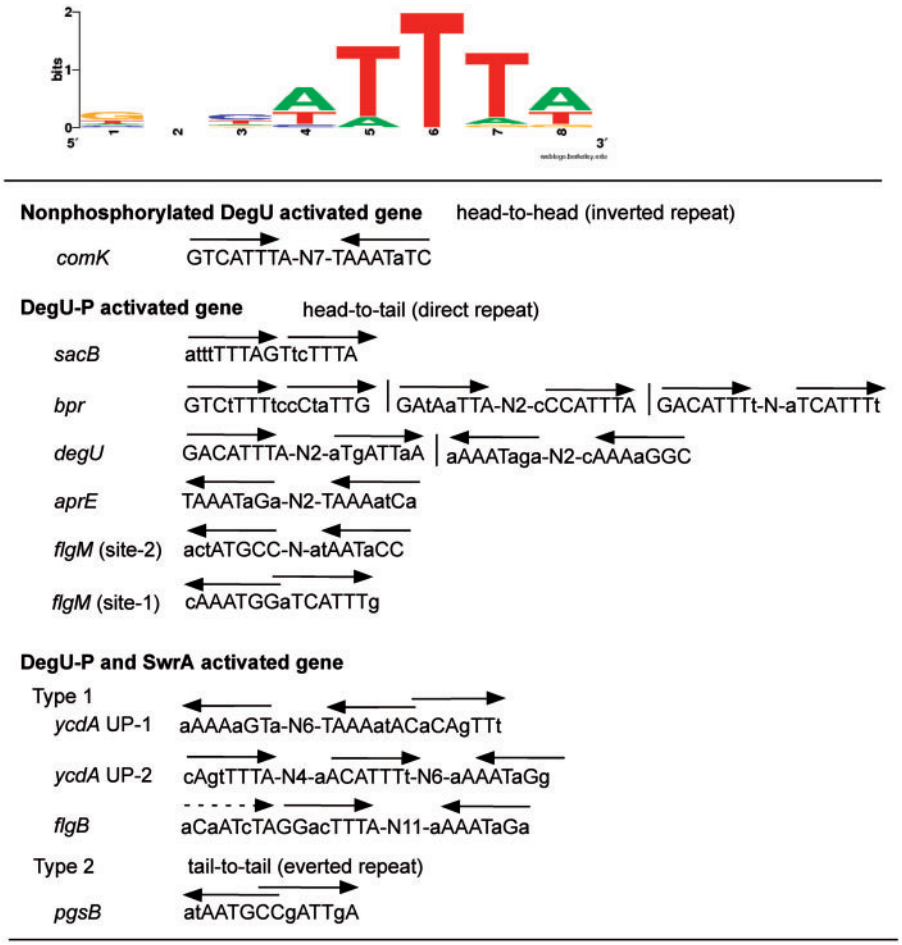

Fig. 7 DegU-recognition sequences. The consensus sequence of the DegU-recognition sequence generated with Weblogo (*38*) is shown on top. The capital characters in each half site indicate nucleotides matching the consensus. These depictions are based on the mutational analysis in the *lacZ* fusion expression and *in vitro* DNA-binding experiments. The data are also obtained from ref. (*39*) (*comK* and *aprE*), ref. (*4*) (*sacB* and *flgB*), ref. (*14*) (*bpr*), ref. (*22*) (*pgsB*), ref. (*17*) (*degU*) and ref. (*28*) (*flgM*). With respect to *flgB*, the dotted arrow denotes the sequence within the region protected by His-DegU in DNase I footprinting.

electrostatically. In addition, relaxed conditions, that is, employing low amounts of the non-specific DNA antagonist poly[dIdC], were also used. His-DegU bound more efficiently to the sacB probe than to the pgsB probe thus suggesting the differential binding affinity dependent on the *cis*-site arrangement (Fig. 4B). The shifted bands are specific to the DegU-recognition sequences shown in Fig. 4B, because the deletion of the *cis*-sites from the probes abolished these shifted bands (Supplementary Fig. S4 for *sacB*; ref. *22* for *pgsB*).

Y2H analysis of the interactions among whole DegU and DegU domains

To examine the possibility that SwrA may change the assembly state of DegU, it was important to know whether the NTD and CTD regions of DegU interact with each other in a stand-alone state and in combination with other domains. We used an Y2H assay and detected a stable interaction between isolated NTDs; between NTD and whole DegU (Fig. 8A). An interaction between whole DegUs was observed faintly due to an unknown reason.

Interaction of SwrA and NTD stimulates the interaction of DegU and NTD

If SwrA changes the assembly state of DegU, we speculated that it would interact with DegU and/or its domains. To examine this possibility, Y2H assays were employed (Fig. 8B). We noted that the combination of SwrA and whole DegU inhibited yeast cell viability, resulting in no colony formation from the initial transformant even in a medium containing histidine and adenine (data not shown). The combination of SwrA and NTD facilitated blue-colony formation, indicating that SwrA interacts with NTD. The specificity of the detected interaction was reinforced by the observation that SwrA did not interact with the NTD of ComA, which is also a member of the NarL family (*1*). Next, we adopted Y3H analysis to examine the possibility that SwrA facilitates the interaction of DegU with DegU itself or with its NTD. This system is composed of Y2H and an expression system controlled by the presence of methionine (Fig. 8A, right). The lack of methionine activated Pmet-*swrA*, leading to expression of SwrA in yeast cells carrying derivatives of pGAD and pGBK for Y2H. The

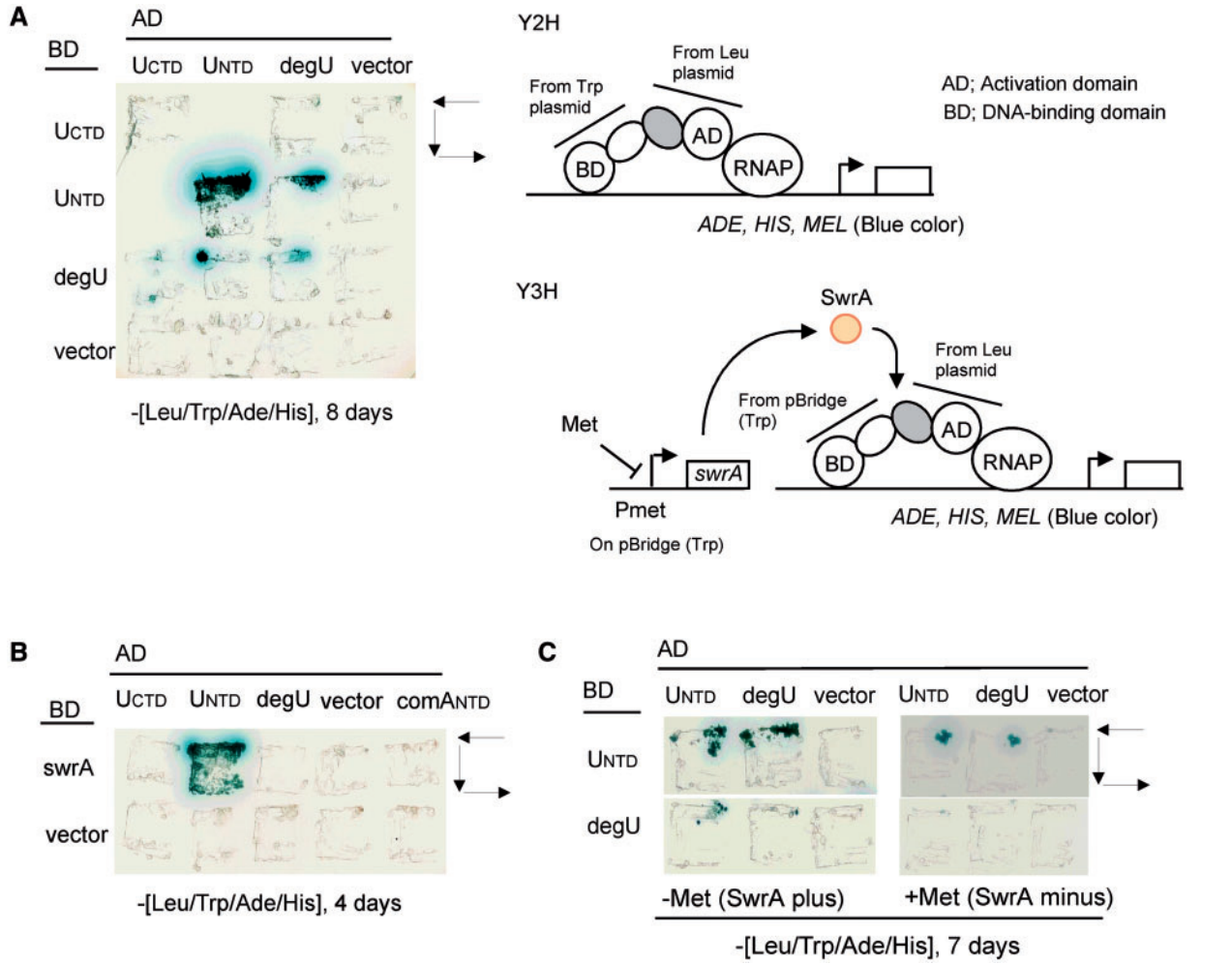

Fig. 8 Interaction analysis between DegU domains and between DegU domains and SwrA. In Y2H and Y3H, each yeast strain carrying the indicated pGAD (AD) and pGBK derivatives (BD) was streaked for single-colony isolation on the indicated SD plate containing X-α-gal (80 μg/ml). The direction of streak is shown along the panels. The incubation days are also shown. (A) Interaction between DegU domains. The photograph of the plate is shown on the left. A schematic representation of the principles of Y2H and Y3H is shown on the right. The bent arrows and T-bar indicate promoters and repression effects, respectively. Three genes (*ADE, HIS* and *MEL*) are activated by synthetic GAL4. Yeast growth without leucine and tryptophan necessitates that the yeast cells carry both plasmids. The expression of Pmet-*swrA* is controlled in the presence of 1 mM methionine in the SD plate. (B) Interaction between SwrA and DegU domains. (C) Examination of the effect of SwrA on the interaction between DegU domains. The results of Y3H are shown.

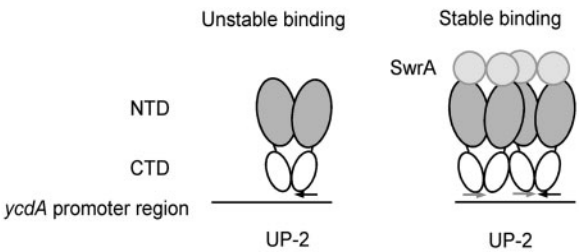

Fig. 9 Model of SwrA effect on DegU binding to *ycdA*. SwrA stabilizes DegU binding to *ycdA*, which would lead to use of the new *cis*-sites (grey arrow). Left, in an *swrA*–background, right, in an *swrA*$^{+}$ background. We note that this stoichiometry is one of the plausible models. An odd number of recognition sites could not efficiently accommodate dimers of transcription factors. UP-2 (Fig. 5) is composed of the type-1 arrangement (Fig. 7). With respect to UP-1, a binding mode of the DegU dimer to the direct repeat is unknown, thus it was not depicted.

interaction of DegU and its NTD was stimulated by SwrA in the both AD and BD sides in addition to the interaction of NTDs (Fig. 8C).

DegU is a dimer in solution

We next analysed the DegU multimerization state *in vitro* using blue native–polyacrylamide electrophoresis (BN–PAGE). In this experiment, DegU formed a dimer without phosphorylation (lane 1, right panel in Supplementary Fig. S5) and DegS formed a tetramer (lane 3). Upon phosphorylation of DegU, a very thin band was observed by BN–PAGE (lane 2). Thus, a phosphorylation effect on DegU multimerization was not clearly observed under the condition used.

Conclusion

We observed an enhancement of DegU-P binding to *ycdA* in the presence of SwrA in *in vitro* DNA-binding experiments. In Y2H and Y3H analyses, an interaction

between DegUNTD with full-length DegU was observed, which was stimulated by SwrA. Furthermore, a strong interaction between SwrA and DegUNTD but not ComANTD was also detected, which strongly suggests that SwrA facilitates and stabilizes the domain interaction of DegU. Together, these results strongly suggested that SwrA can stabilize the weakened binding caused by unusual arrangement of the recognition motifs.

Discussion

In our present study, *ycdA* was identified as a novel swarming motility gene. This gene encodes a putative lipoprotein, which localizes at the cell surface. At present, there are no data that show whether YcdA plays a role in the regulatory cascade of swarming motility or is a structural constituent of the swarming motility machinery. The regulation of swarming motility is poorly understood, but it involves several key steps including transcription of the *fla*/*che* operon and post-translational control of sigma D activity (Fig. 1C and ref. *28*). It is at least known that the *ycdA* gene is not involved in *fla*/*che* transcription and flagella biogenesis, because *fla*/*che* transcription and flagella were observed in the $swrA^+$ $ycdA^-$ cells at similar levels to the $swrA^+$ cells. Thus, YcdA might function at some downstream process of flagella formation (Fig. 1C). The fact that *ycdA* is expressed in the *swrA*-inactive background in the stationary phase raises the possibility of a role of the *ycdA* gene that is separate from swarming motility. Indeed, the YcdA protein was detected from the mature spore in a proteome analysis (*35*).

The regulation of *ycdA* is complex, because SwrA, DegU, AbrB and Abh are directly involved. Gene disruption of any of these regulators drastically changes the expression profile of *ycdA*. Stabilization of DegU-P by multicopy *degQ* results in a similar expression profile to that caused by the *abrB*/*abh* double mutation. AbrB/Abh binding to the *ycdA* region has been identified by genome-wide ChAp-chip analysis (*30*). According to these results, the binding region of these repressors is very broad, that is, up to 1.05 kb. Thus, it is possible that DegU-P may interact with AbrB/Abh on the *ycdA* control region in the genome, which may lead to occlusion of these repressors. In cells carrying multicopy *degQ* or *abrB*/*abh*, the expression of *ycdA* reached its minimum during the early stationary phase (around T3), suggesting the existence of another unknown layer of *ycdA* regulation.

One direct repeat with exceptional long spacing between half-sites and a single half-site for DegU recognition are used in the *swrA* background. Both elements are separated by a distance of 44 nt. DegU-P dimers on these two regions might interact with each other by DNA looping leading to stabilization of DegU-P binding to *ycdA*. The observation that no distinct band was detected in EMSA using the ycdA probe without SwrA is consistent with the expected thermodynamic restrictions of the possible long-range DNA looping. In addition, although SwrA promotes the generation of DegU–DNA complexes in this *in vitro* assay, it remains unknown to which DNA region DegU binds, leading to the band shift. At least, the deletion of the UP-1 region and the mutation of the M8 region abolished complexes (Fig. 4A), suggesting critical roles of the both regions in the DNA binding of DegU-P to *ycdA*. Compared to the distinct protection pattern by DegU-P with SwrA in the footprint assay, the relatively weak binding pattern was observed in EMSA with both proteins. In fact, when using the highest concentration of DegU-P with SwrA, the unbound probe was observed in EMSA (Fig. 4A). This difference would be caused by the different nature of two assays. In the footprint assay, short-time interaction of DegU-P with the target DNA (5 min) resulted in generation of a protection pattern, whereas in EMSA the nucleoprotein complexes should be detected after 2–3 h electrophoresis.

According to our Y2H analysis, DegUNTD forms a dimer without phosphorylation, and thus the dimer formation of DegU would be mediated by DegUNTD. This is consistent with the former observations of other response regulators (*36*). Moreover, in the Y2H assays an interaction of the whole-DegU proteins were detected very weakly due to an unknown reason. The NTD dimer structure and the DNA-binding CTD structure are both symmetrical (*33*, *36*). DegU dimers would likely form in a symmetrical fashion like some of the other NarL-family regulators. The DegU dimer therefore binds to a symmetrically arranged sequence (head-to-head; Fig. 7). To date, however, the known DegU-P recognition sequences are arranged in asymmetrical head-to-tail sequences, except for the SwrA-regulated genes. It has been unclear why a probable symmetrical dimer can bind to asymmetrical head-to-tail sequences.

With respect to the type 1 arrangement for the SwrA-regulated genes, it is noteworthy that an odd number of recognition sites could not efficiently accommodate the dimers of transcription factors (Figs 7 and 9). If the symmetric DegU dimer occupies the head-to-head sequence, a dimer could not stably bind to a single motif without SwrA. Compared with the sacB probe (head-to-tail *cis*-sites), *pgsB* showed low affinity for DegU-P perhaps due to the tail-to-tail arrangement (Fig. 4B). In this case, one dimer may bind to a single binding half-site, and then the other would bind to the other half-site. *In toto*, the arrangement of *cis*-sites would lead to the altered association of DegU dimer. These modes of DNA binding would be unstable, and thus to stabilize this DNA–protein complex, SwrA would be required. It should be noted that SwrA dependency might be simply caused by low affinity of the *cis*-elements rather than *cis*-sites arrangement.

The arrangement of DegU-recognition sequences could work as a platform to determine the DegU assembly state, which is a determinant of the SwrA requirement. In other words, SwrA works as a stabilization factor for DegU binding. Although the regulated assembly of a transcription factor has been widely observed among many organisms including prokaryotes and eukaryotes (*37*), the factor that facilitates the assembly has not been known in prokaryote

to our knowledge. Thus, SwrA is a novel type of bacterial transcription factor in this regard.

Supplementary Data

Supplementary Data are available at *JB* Online.

Acknowledgements

We thank K. Kobayashi for communicating unpublished results and technical advice on flagella staining. We are grateful also to S. Ishikawa for generously providing *abrB* and *abh* strains. We thank T. Tanaka for technical advice on the Y2H experiments and for critical reading of the paper. We are indebted to K. Fukui, M. Murakawa and T. Ohsawa for performing the Lac-assay in Fig. 1 and construction of pHis-SwrAt and pDG1729-swrA.

Funding
Grant-in-aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflict of Interest

None declared.

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