Bacillus subtilis DegU acts as a positive regulator for *comK* expression

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Abstract ***Bacillus subtilis*** **ComK plays a critical role in competence development. We report that *B. subtilis degR*, a positive regulator for exoenzyme production, is negatively regulated by overproduced ComK caused by a *mecA* null mutation. To identify a positive regulator for *comK* expression in the *mecA* background, mutations that allowed the *degR* gene to be expressed were screened in Tn10 transposon insertion mutants. As a result, we identified *degU* insertion mutations as those having such a property. The *degU* mutation reduced *comK-lacZ* expression in a competence medium in both the wild-type and *mecA* cells in sporulation and competence media. These results indicate that the *degU* gene product acts as a positive regulator for *comK* expression even under the condition where the negative regulation of *comK* by MecA is released.**

Key words: *Bacillus subtilis*; Competence development; Exoenzyme production; *degS-degU*; ComK regulation; Transposon mutagenesis

1. Introduction

Bacillus subtilis is a Gram-positive soil bacterium and has many adaptive response systems such as competence development, degradative enzyme production and spore formation. The response systems are subject to well-tuned regulation. For degradative enzyme synthesis, a DegS-DegU two-component system plays an important role in transcription of genes encoding exoenzymes [1]. In addition, several DegU-dependent co-activators are known, and one such factor DegR, a 60-amino-acid protein, increases exoprotease production by stabilizing the phosphorylated form of DegU [2,3].

Competence development is the differentiation of a subpopulation of cells to those that have an ability to take up exogenous DNA into the cytoplasm [4]. This process involves a highly regulated signal transduction route. Various pieces of information leading to competence development converge to the competence transcription factor, ComK, which then directly activates late competence genes encoding apparatus for processing and taking up DNA [5–8]. It has been postulated that there are two regulatory pathways leading to ComK; one involves MecA/MecB and the other the Spo0A-AbrB [5,7,9]. The MecA/MecB is thought to be negatively regulated by a ComP-ComA two-component system through ComS [10–12], and a group of factors including DegU, SinR and AbrB [5,7]. Finally, *mec* null mutations cause apparent overproduction of ComK irrespective of the growth medium used [5,7]. Hahn et al. attributed the effect of the *mecA* mutation to a loss of cell type specific production of ComK in the competent cell population [13].

Regulatory systems of competence development and exoenzyme synthesis intersect at several regulatory proteins. For example, unphosphorylated DegU is needed for late competence gene expression in competence development [5,7].

We have recently observed that the expression of *degR* in a sporulation medium is negatively regulated by ComK; expression of *degR* is severely reduced in a *mecA* null mutant, and this inhibition was completely relieved by introduction of *comK* deficiency to the mutant (unpublished). To investigate further the regulation of *degR*, we searched for a mutation by transposon mutagenesis that permitted expression of *degR* in the *mecA* background.

We report in this paper that transposon insertion into *degU* relieved *degR* expression in the *mecA* mutant. Since overproduced ComK is responsible for the reduction of *degR* expression, we conclude that DegU exerts a positive effect on *comK* expression.

2. Materials and methods

2.1. Materials

Restriction enzymes were purchased from Toyobo, Co. (Tokyo, Japan). DNA ligation kit was bought from Takara Shuzo, Co. (Shiga, Japan). Bacterial strains are listed in Table 1.

2.2. Media and antibiotics

The media used were Luria-Bertani broth [14], Luria-Bertani agar medium [14], Antibiotic medium 3 (Difco Laboratories), Modified Competence medium [15] and Schaeffer's sporulation medium [16]. Concentrations of the antibiotics added to the media were 100 μg/ml for spectinomycin (Spc), 10 μg/ml for kanamycin (Km), 5 μg/ml for chloramphenicol (Cm) and 0.5 μg/ml for erythromycin (Em). X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added at a concentration of 100 μg/ml.

2.3. Transposon insertion mutagenesis

Transposon insertion was essentially carried out according to the procedure of Dartois et al. [17]. The mini Tn10-delivery vector pIC333 specifying resistance against Spc and Em (Spc^r Em^r) [18] was introduced into ODM40 by co-transformation with DNA carrying *mecA*::Km^r. A series of 10 1-ml precultures were made at 28°C from 10 different Em^r colonies. After inoculation of the preculture into the Luria-Bertani medium and subsequent incubation for 4 h at 28°C, the growth temperature was shifted to 37°C to inactivate temperature-sensitive replication of the plasmid, and the cells were incubated for a further 4 h. Tn10-carrying cells were selected on Luria-Bertani agar plates containing Spc, Km, Cm and X-gal. Since the transposon carries the replicon from ColE1 origin [18], the DNA fragments flanking the transposon insertion sites were retrieved by digestion of the total DNAs from the candidate *B. subtilis* cells with *Eco*RI or *Hin*dIII, followed by ligation and transformation into *E. coli* JM103. Spc^r cells were selected, and the plasmids contained in transformants were directly used as the templates for DNA sequencing.

2.4. Sequence determination

Sequences were determined on both strands using an ABI 377 DNA Sequencer and Dye Terminator Cycle Sequencing Kit from Perkin Elmer.

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2.5. β-Galactosidase assay

Cells were grown in Schaeffer's sporulation medium or Modified Competence medium as described in the figure legends and processed as detailed previously [19]. Results shown in the figures are typical experiments among those conducted two to three times.

3. Results

3.1. Transposon insertion mutagenesis

We have recently reported that *mec* null mutations markedly reduced the expression of *degR* in a sporulation medium, i.e. the ComK protein overproduced in the *mecA* background inhibited the expression of *degR* (unpublished). A typical example is shown in Fig. 1. ComK is a critical competence transcription factor, and diverse signals for competence development are converged to ComK through various regulatory molecules including MecA, Spo0A and DegU [5,7,8]. Regulatory mutants in which *degR* expression has been released from repression due to *mecA* deficiency, therefore, may contribute to a profound understanding of *comK* regulation. To screen such a mutant, ODM40ma (*mecA*::Km^r *degR'-'lacZ*) carrying a mini Tn10-delivery vector pIC333 (Spc^r and Em^r) [18] was used. ODM40 forms white colonies on a Luria-Bertani agar plate containing X-gal because the expression of the *degR'-'lacZ* fusion is reduced by the *mecA* mutation. Among approx. 40000 colonies from 3 independent experiments we found 34 blue colonies. After single colony isolation, total DNAs from the 34 clones were isolated, and the sequences flanking the Tn10 transposon were isolated as described in Section 2. Sequence determination around the Tn10 transposon insertion sites revealed that 32 clones carried Tn10 in the *degU* ORF at two different sites. One is between codons 38 and 39 and the other between codons 41 and 42 of *degU* ORF (designated as *degU38* and *degU41*, respectively). The remaining two clones carried new loci, and the characterization of them will be described elsewhere.

3.2. The degU mutation restores degR'-'lacZ expression reduced in the mecA background

In order to determine the extent to which the *degU* mutation allowed the *degR'-'lacZ* fusion to be expressed in the *mecA* mutant in sporulation medium, we measured β-galactosidase activities produced in strains MU38 (*mecA*::Km^r *degR'-'lacZ degU38*) and MU41 (*mecA*::Km^r *degR'-'lacZ degU41*). Under conditions where *mecA* deficiency reduced the expression of *degR'-'lacZ* markedly, the Tn-10 insertion into the *degU* locus in mutants MU38 and MU41 increased the β-galactosidase activities to 48 and 66% of the control level, respectively, and the profiles of β-galactosidase synthesis in the mutants were similar to that of the parent strain, ODM40 (Fig. 1). It has been observed previously that *degU* disruption alone does not affect the expression of *degR'-'lacZ*, and we confirmed that this is also the case for the *degU38* and *41* mutations (data not shown).

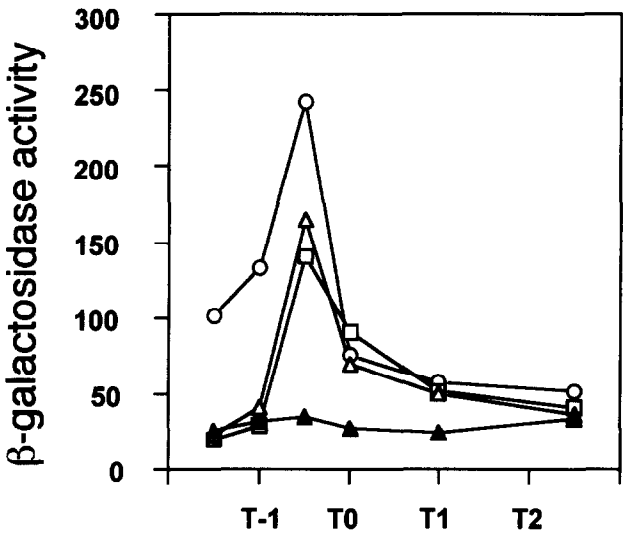

Fig. 1. Inhibition of *degR* expression by *mecA* deficiency, and restoration of the expression by Tn10 insertion mutations *degU38* and *degU41*. Cells were grown in Schaeffer's sporulation medium, and β-galactosidase activities (Miller units) were determined as described previously [19]. Numbers on *x*-axis represent the growth time (in h) relative to the end of vegetative growth (T0). (○) ODM40, (▲) ODM40ma, (□) MU38, (△) MU41.

The expression of *degR* is dependent on the σ^D factor [20], and therefore it was possible that the transposon insertion into the *degU* gene activated the *fla/che* operon containing the structural gene for σ^D [21], resulting in the expression of *degR'-'lacZ* in the *mecA* mutant. However, we found that the *degU* mutations did not affect *sigD'-'lacZ* expression significantly (data not shown). Tokunaga et al. have reported that the expression of *sigD'-'lacZ* in a *degU* deficient mutant is two-thirds of the control level [22], a result essentially compatible with ours.

Table 1
Bacterial strains used in this study

Strain	Relevant phenotype and description	Reference or source
CU741	*trpC2 leuC7*	[30]
ODM40	*trpC2 leuC7 amyE*::(*degR3'-'lacZ*(Cm^r))	[20]
QB4650	*trpC2 mecA*::Km^r	F. Kunst
ODM40ma	*trpC2 leuC7 amyE*::(*degR3'-'lacZ*(Cm^r))*mecA*::Km^r	QB4650→ODM40
MU38	*trpC2 leuC7 amyE*::(*degR3'-'lacZ*(CM^r)*mecA*::Km^r *degU38*::Tn10(Spc^r)	This work
MU41	*trpC2 leuC7 amyE*::(*degR3'-'lacZ*(CM^r)*mecA*::Km^r *degU41*::Tn10(Spc^r)	This work
8G33	*trpC2 comK'-'lacZ*(Km^r)	[6]
OCM100	*trpC2 leuC7 comK'-'lacZ*(Km^r)	8G33→CU741
CU741ma	*trpC2 leuC7 mecA*Spc^r	Unpublished
OCM102	*trpC2 leuC7 comK'-'lacZ*(Km^r) *mecA*::Spc^r	CU741ma→OCM100
TT711	*trpC2 leuC7 degU*::Cm^r	[31]
OCM104	*trpC2 leuC7 comK'-'lacZ*(Km^r) *degU*::Cm^r	TT711→OCM100
OCM105	*trpC2 leuC7 comK'-'lacZ*(Km^r) *degU*::Cm^r *mecA*::Spc^r	CU741ma, TT711→OCM100

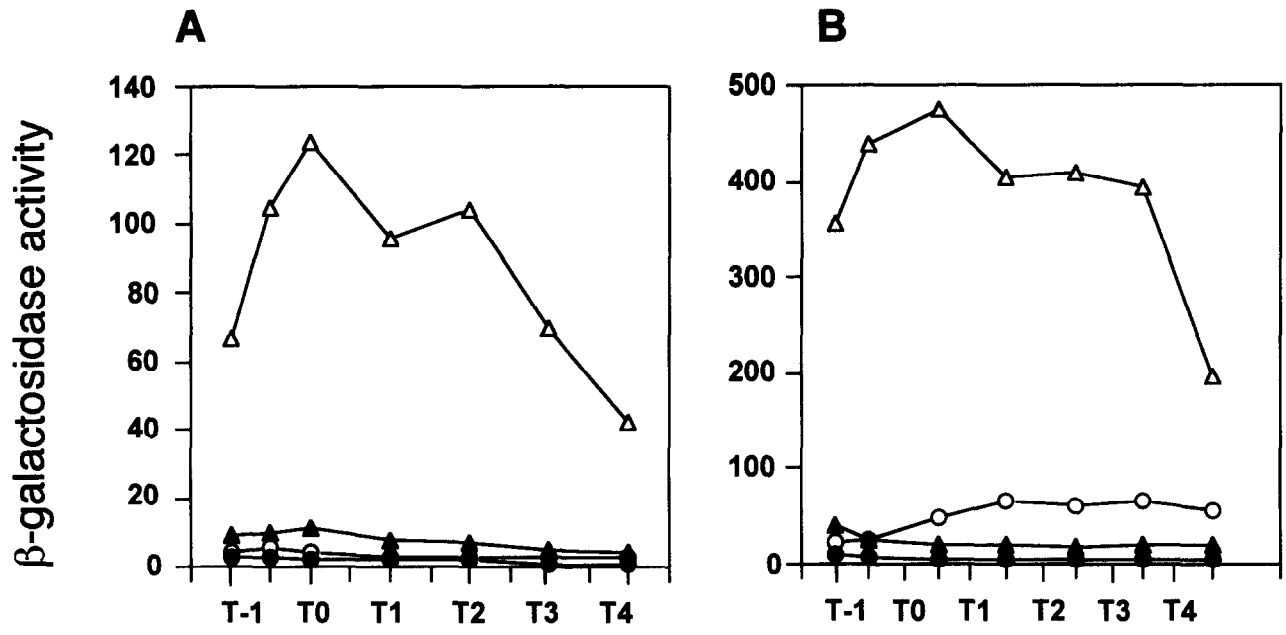

Fig. 2. Effect of *mecA* and/or *degU* null mutations on the expression of *comK-lacZ*. Growth condition and measurement of β-galactosidase activities are identical to those in the legend to Fig. 1 except for the culture media used. β-Galactosidase activities are shown in Miller units. Numbers on *x*-axis represent the growth time (in h) relative to the end of vegetative growth (T0). Media used in experiments were Schaeffer's sporulation medium (SM) and modified competence medium (MC) for experiments A and B, respectively. (○) OCM100, (△) OCM102, (●) OCM104, (▲) OCM105.

3.3. degU deficiency decreases comK-lacZ expression in the mecA background

The results described so far could be interpreted to show that *degU* deficiency down-regulated the expression of *comK* in the *mecA* background, resulting in the restoration of *degR* expression. To test this notion, we constructed a strain carrying *comK-lacZ* together with *degU* and *mecA* mutations (strain OCM105), and the profile of β-galactosidase activities in the strain was examined. The *degU* mutation used for this experiment was generated previously by insertion of a *cat* cassette between codons 54 and 55 in the *degU* ORF. We used this *degU* mutation to avoid overlap of antibiotic resistance markers, and we confirmed that it had identical properties to those of the Tn10 insertion mutations. It has been demonstrated that the expression of *comK-lacZ* is not detectable in a sporulation medium [6], and this was also the case for this strain (Fig. 2A). The presence of the *mecA* mutation enhanced *comK-lacZ* expression markedly in agreement with the previous results [5,7], and by further introduction of the *degU* mutation this expression level was reduced to a level close to that in the control strain (about 8% of the activity observed in the *mecA* strain; Fig. 2A).

Since the expression of *comK* was not observed in the sporulation medium, we next performed the same experiments in a competence medium. As shown in Fig. 2B, *comK* expression could be observed in this medium. It has already been shown that a *degU* null mutation severely reduces the expression of *comK* in a competence medium [5,7], and this was confirmed in this experiment (Fig. 2B). The *mecA* mutation caused an enhancement of *comK* expression, and this enhanced expression was greatly reduced by the *degU* mutation. These results together with those shown in Fig. 2A indicate that *degU* is an direct activator of *comK* expression and that the negative effect of the *degU* mutation on *comK* expression cannot be bypassed by the *mecA* mutation.

4. Discussion

We have previously shown that *degR* expression is inhibited by overproduced ComK caused by *mecA* deficiency (unpublished). We showed in this study that Tn10 insertion into *degU* partially restored *degR* expression diminished by the *mecA* deficiency (Fig. 1). This partial restoration is most likely due to reduced synthesis of ComK, since disruption of *comK* led to the complete recovery of *degR* expression in a *mecA* null mutant (unpublished) and the *degU* disruption by the transposon resulted in a reduction of *comK* expression (Fig. 2). From these observations we conclude that DegU is a positive regulator of *comK* expression. It has already been demonstrated that the expression of *comK* requires unphosphorylated DegU [5,7] and that the *degU* requirement for the expression of the late competence gene *comG* is bypassed by a *mecA* null mutation [23]. We have confirmed the latter observation by demonstrating that full expression of *comG-lacZ* does not require DegU in the presence of the *mecA* null mutation (data not shown). The reduced level of ComK in the *mecA degU* deficient mutant is apparently contradictory to the full expression of *comG*, since *comG* expression depends on ComK. How might this contradiction be explained? It is possible that a small amount of ComK is still produced in the cell carrying *mecA degU* deficiency, since complete recovery of *degR* expression was not attained in the *mecA degU* double mutants (Fig. 1). Furthermore, it was shown that in the *mecA degU* double mutant *comK* expression was still observed to a reduced but significant level as compared to *comK* expression in the *mecA*$^+$ *degU* strain (Fig. 2). We presume that the reduced level of ComK would be sufficient for sustaining the expression of *comG* in the *mecA* background. In this respect Hahn et al. noted that MecA may inhibit inappropriate activation of ComK and that inactivation of MecA would result in differentiation of all the cells to a competent state but not

in overproduction of ComK in the competent cell population [13]. Thus, although the ComK level is low in the *mecA degU* mutant cells, the amount of ComK would be sufficient for the expression of *comG*, since there is no MecA protein available for inhibition or sequestration of ComK protein [24]. Another interpretation would be that the *degU* mutation might cause alteration of expression or activity of an unknown regulatory molecule involved in the expression of late competence genes so that the requirement of ComK is bypassed.

In addition to many genes involved in competence development, several genes including *nucA*, *dinR* and *sacB* are also regulated by ComK [25,26], although the reason why they are under ComK regulation is not clear. It has been suggested by Msadek et al. that the post-exponential growth phase responses such as spore formation, competence development and degradative enzyme synthesis are mutually exclusive [27]. We have shown that *degR* plays a positive role in the expression of alkaline protease gene [28], and therefore the down-regulation of *degR* by the competence transcription factor ComK is in line with the above notion.

After completion of the manuscript, we noticed that Hahn et al. showed by using a *mecA*$^+$ *degU* mutant in which *comK* is expressed from an inducible promoter that DegU does not appear to act via Mec [29]. The conclusion drawn from this different approach coincides with our data.

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