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In addition to the two-component regulatory system, DegS-DegU, five other genes degR, degQ, senS, tenA, and proB positively regulate the production of *Bacillus subtilis* exoproteases when they are present on a multicopy plasmid. To study the extent of involvement of these genes in exoprotease production in a single copy state and possible relationship among these genes, strains carrying single or multiple disruption mutations in these loci were constructed, and the expression of aprE'-lacZ was analyzed. From such studies, the following results were obtained with respect to the regulation of aprE expression. i) Disruption mutations were divided into two groups; one (degQ and degR) significantly reduced aprE expression, while the other (senS, tenA, and proB) had little effect on it. ii) SenS was involved in temporal regulation. iii) The combined effects of some of the disruption mutations tested were not necessarily additive. iv) The extent of the negative effects generated by the mutations depended on the medium used.

**Key words:** *Bacillus subtilis; degS-degU; degR; degQ; SenS; tenA; proB; alkaline protease*

*B. subtilis* cells produce and secrete extracellular proteases when they enter the stationary phase because of nutrient starvation, high cell density, and so forth.1 The timing and extent of the expression of the extracellular protease genes are strictly regulated,2,3 and this regulation is exerted mainly by a two-component regulatory system, DegS-DegU, in which the sensor kinase DegS accepts environmental stimuli, autophosphorylates on its own histidine residue, and transfers the phosphate to the aspartate residue of the cognate response regulator DegU.4,5 In addition, several positive and negative factors have been shown to be involved in the regulation. The factors such as SinR,6 Hpr,7 and AbrB7 work as negative regulators and directly repress the transcription of aprE,8-10 the structural gene for alkaline exoprotease. Repression by AbrB is relieved by the major transition state regulator Spo0A.11 The pat locus negatively regulates exoprotease production in a multicopy state.12 On the other hand, five other genes, proB,13 degR,14-16 degQ,17,18 senS,19 and tenA20 have been cloned based on their effects in increasing production of extracellular degradative enzymes when such genes are carried on a multicopy plasmid, and among these genes the proB, degR, degQ, and tenA genes have been reported to have their effects in a DegS-DegU dependent manner.13,20-22

The proB gene encodes γ-glutamyl kinase, which is the first enzyme in the proline biosynthetic pathway. Its presence on a multicopy plasmid stimulates aprE'-lacZ expression and this stimulation is further increased synergistically in the presence of multicopy degR.13 DegR is a 60-amino acid peptide with sequence similarity to the N-terminal region of DegS.13,14 It has been shown that DegR has its positive effect through the stabilization of phosphorylated DegU.22 The expression of degR is subject to regulation by the competence/stress related regulatory gene products, MeC/A/MecB, and is dependent on a chemotaxis specific σ factor, σH.23,35 DegQ is a small protein composed of 46-amino acids.18 The expression of degQ is regulated by the ComP-ComA and DegS-DegU two-component systems and various nutrient starvation signals.21 On the basis of these phenomena and the dependence of the effects of DegQ and DegR on the DegS-DegU system, it is conceivable that some environmental signals that stimulate the production of the exocellular proteases are transmitted to the DegS-DegU system at least in part via expression of degR and degQ.

The products of the five genes described above may be called the accessory proteins since disruption of the respective chromosomal loci does not result in reduction of the amount of the secreted proteases.16,17,19,20 More recent work found, however, that disruption of the chromosomal degQ gene causes a significant decrease of the expression of the extracellular levansucrase gene, sacB.24 On the other hand, disruption of the chromosomal proB locus partially inhibits the effect of multicopy degR on increasing aprE expression.13 It therefore appears that the so-called accessory genes may be involved in the regulation of aprE expression in a single copy state. These considerations led us to study the effects of gene disruption of these positive factors on the regulation of aprE and to examine whether any additional effects would be observed in strains carrying combinations of disruptions in these genes.

Typical profiles of β-galactosidase activities for the mutant strains grown in SM are shown in Fig., and the average peak values from four to five experiments in both SM and LB are depicted in Table, except for the senS-carrying strains, which showed maximal activities at T9 (see below). To minimize the differences in the culture conditions among the strains tested, selection with antibiotics was omitted during the culture except for the addition of chloramphenicol (5 μg/ml) to ensure the presence of the aprE'-lacZ fusion in the chromosome. After all the samples were taken from the time course experiments, the cells in the remaining cultures were spread on LB agar plates containing 5 μg/ml of chloramphenicol. One hundred colonies thus obtained showed the expected antibiotic resistance patterns, indicating that the disruptions remained present during the growth without antibiotics selection. Among the five strains carrying single mutations, those that carry disruptions of degQ and degR showed low levels of aprE'-lacZ expression in SM (21% and 34% compared with the wild type value, respectively) (Fig. (a) and Table). Similar results were obtained for the cells grown in LB (7% and 14%, respectively), except that the peaks of β-galactosidase activities in LB were greatly retarded (data not shown). For comparison, aprE expression in a strain carrying a degU mutation is also shown. The extents of reduction by both the degR and degQ mutations were similar to that by the degU mutation, indicating that both gene products participate in aprE expression significantly. The effect of the degQ mutation observed is also consistent with the involvement of DegQ in the expression of sacB.29 Disruption of proB reduced aprE expression slightly in SM (Fig. (a) and Table), but a significant stimulation effect was observed in LB (Table). We have previously reported that dis-
The host strain carrying the mutations is *B. subtilis* TT715 *leuC7 trpc2 aprE'-psKD1*. Cells were cultured in Schaefer's sporulation medium [28] and the β-galactosidase activities (Miller units) were measured at the indicated times. Numbers on x-axis represent the growth time in hours after the end of the vegetative growth (T0).

Construction of mutant strains: The *degE:Em* unit was used by Yang et al. [20]. A *degQ* disruption was constructed by insertion of the blasticidin S resistance gene cassette from pBEST402 [20] into the *recA* site in the *degQ* coding region [20]. A *neo* gene cassette was inserted into the *recA* site within the *proB* ORF to disrupt the function of the *proB* [32]. To inactivate *tenA*, the same *Abtl* fragment that was used for disruption of *tenA* by Pang et al. [36] was first cloned into a pUC18 derivative carrying phiK10 [35] and then the resulting plasmid was introduced into the chromosome by Campbell-type recombination. To disrupt *senS*, an artificial *SenS* site was inserted at the 7th codon of the *senS* ORF by using PCR-mediated mutagenesis, followed by insertion of the *acr* gene from pC150 [31] into the *SenS* strain. TT715 is a derivative of TT715 carrying *degU:neo* mutation. Strains carrying more than two gene disruptions were constructed by transformation of the single mutants with DNA isolated from the other mutants. Attempts to isolate strains carrying the double mutations *senS proB, senS degR*, and *degQ proB* were not successful. All the strains used in this study are derivatives of *B. subtilis* TT715, which carries an *aprE'-lacZ* fusion (Chr.). [35].

![Fig. Expression of *aprE'-lacZ* Translational Fusion in Various Mutants.](image)

**Fig.** Expression of *aprE'-lacZ* Translational Fusion in Various Mutants.

The disruption of *proB* did not cause any change of *aprE* expression in SM using the *B. subtilis* MT33 strain [15] derived from DB104. [31] This discrepancy with respect to the disruption of *proB* could be due to a strain difference between MT33 and TT715. A *tenA* disruption showed little reduction in *aprE* expression in both SM and LB (Fig. (a) and Table). The *senS* strain had a different profile of *aprE'-lacZ* expression in SM compared to those of the other mutants; there was no peak in the profile, and the β-galactosidase activity steadily increased during the incubation period up to T9, while the wild type and the other four strains carrying a single mutation showed a peak between T2 and T3 (Fig. (a)). One interpretation of this effect is that *SenS* plays a role in the temporal regulation of *aprE* expression. It is known that multicycopy *senS* can move the peak of *aprE'-lacZ* expression to a position 1 hour earlier than that of the control strain, [20] and an observation in concert with this notion. Another interpretation is that the *senS* disruption may have caused a delay in spore formation, resulting in a slower increase in *aprE* expression, since *aprE* expression is tightly regulated by *abdB* which is under the control of *Spo0A*. [30]

However, clarification of the mechanism underlying this observation requires further study.

If the effects of the five gene disruptions are independent, then it is expected that some combinations of the mutations would result in a drastic decrease of *aprE* expression because of possible additive effects. Among the combinations tested, such an effect was observed only for *tenA proB* (Fig. (b) and Table) and in part for *tenA degR* in SM (Fig. (d) and Table). The *senS* effect described above was also observed in the *tenA senS* and *degQ sens* double mutants and even in the *degQ senS tenA* triple mutant (Fig. (c)). The *degQ tenA* strain showed an expression level similar to that found in the *degQ* strain (Fig. (a), (d) and Table). The values in the *degQ degR* mutant were slightly higher than those in their ancestral *degQ* and *degR* strains in SM (Fig. (a), (d) and Table), and addition of *tenA* to *degQ degR* further enhanced the expression of *aprE'-lacZ* in SM (Fig. (d) and Table), resulting in the recovery of *aprE'-lacZ* expression to the wild type level. A similar increasing effect of *tenA* was observed for the *degQ sens tenA* strain in which the level of *aprE'-lacZ* expression was increased 2-fold compared with that in the *degQ sens* strain (Fig. (c) and Table). The increasing effect of *tenA*, however, was not observed in LB as shown in Table, i.e., the *degQ* and *degR* strains showed 7% and 6% of the control value, respectively (Table). In contrast to these positive effects of the *tenA* mutation, the level of *aprE'-lacZ* expression in the *proB degR* mutant was not changed significantly by the addition of the *tenA* mutation (Fig. (b) and Table).

It can also be seen that the peaks of *aprE'-lacZ* expression broadened and tended to be delayed as the number of mutations...
introduced were increased (Fig. (b) and (d)).

The results of this study may be summarized as follows concerning the regulation of aprE expression, although the exact mechanisms underlying the phenomena remain unknown. i) What are called positive accessory factors were divided into two groups; one group (DegQ and DegR) is involved in positive regulation in a single copy state, while the others (SenS, TenA and ProB) are not. ii) The SenS protein may be involved in temporal regulation of aprE. iii) The mutation effects were not necessarily additive; a typical example was for the degQ degR strain in which a higher activity than those in their original degQ or degR strains was observed. These results suggest an unknown mechanism by which an effect of one mutation is compensated for by another mutation. iv) The mutation effects on aprE expression depended on the medium used. The SM medium which is poorer in nutrients than the LB medium may be more suitable for tighter growth-stage regulation including the programming of sporulation, and therefore, the major differences in the effects of mutations between the two media could be due to the timing and magnitude of the expression of the regulatory genes. It is also possible that some disruption mutations affected the function of major regulatory factors such as Spo0A and resulted in a fluctuation of aprE expression. In fact a delay in sporulation was observed for a tenA strain.

Acknowledgments. We thank H. Chinushi, D. Murakami, and Y. Oshiro for their technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan, and RIKEN Biodiesel Research.

References

<p>| Table β-Galactosidase Activity of aprE-lacZ Fusion Observed in Various Regulatory Mutants* |
|---------------------------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Medium</th>
<th>Genotype</th>
<th>SM</th>
<th>LB</th>
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<tr>
<td>Control</td>
<td>170±19</td>
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<td>657±128</td>
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<td>degU</td>
<td>49±10</td>
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<tr>
<td>ProB</td>
<td>106±20</td>
<td>62</td>
<td>1965±168</td>
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<td>degR</td>
<td>57±9</td>
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<td>91±22</td>
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<td>degQ</td>
<td>37±7</td>
<td>21</td>
<td>45±11</td>
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<tr>
<td>tenA</td>
<td>108±24</td>
<td>64</td>
<td>626±145</td>
</tr>
<tr>
<td>senS</td>
<td>128±7</td>
<td>75</td>
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</table>

* The numbers shown are the averages of the peak values for the strains not carrying the senS mutation or of the maximal values (at T9) for those carrying the senS mutation. SM and LB show the media of Shaeffer and Luria-Bertani, respectively.