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Positive regulation of *Bacillus subtilis sigD* by C-terminal truncated LacR at translational level

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Abstract DegR is a positive regulator for degradative enzyme synthesis in *Bacillus subtilis*. The *degR* gene is transcribed by RNA polymerase containing σ^D, and the level of its expression is low in a *mecA*-deficient mutant. In a search for suppressors of the *mecA* effect through mini-Tn*10* transposon mutagenesis, a *lacR* mutation designated *lacR288* was discovered. The *B. subtilis lacR* gene encodes the repressor for *lacA* which specifies β-galactosidase, and therefore, inactivation of the *lacR* gene results in overproduction of the enzyme. In the *lacR288* mutant, however, the expression of *lacA* was at a negligible level, indicating that the repressor activity was not destroyed by the mutation. The putative gene product of the *lacR288*-containing gene is a 288-amino acid protein lacking the C-terminal 42 amino acids of intact LacR and carries no extra amino acids derived from the transposon sequence. The suppression by *lacR288* of the decreased *degR* expression in the *mecA* background was found to be caused by an increase in the σ^D level as shown by Western blot analysis. Furthermore, the increase was due to post-transcriptional regulation of *sigD*, the gene encoding σ^D, as revealed by using both transcriptional and translational *sigD-lacZ* fusions. The *lacR288* mutation had no effect on the stability of the σ^D protein. Based on these results we conclude that the *lacR288* mutation stimulates *sigD* expression at the translational level.

Key words: *lacR*; *lacR288* mutation; *sigD* regulation; Translational regulation; *Bacillus subtilis*

1. Introduction

Bacillus subtilis σ^D is an alternative sigma factor involved in transcription of the genes for motility [1] and certain autolysin enzymes [2,3], *ywcG* encoding a protein related to energy metabolism [4], and *degR* [5], a regulatory gene involved in degradative enzyme synthesis [6,7]. These genes constitute the σ^D regulon.

B. subtilis carries the gene for β-galactosidase that is encoded by *lacA* [8]. In laboratory conditions *lacA* is repressed by LacR, which belongs to a group of DNA-binding repressors including GalR and LacI, and most of the members of this group contain a helix-turn-helix structure at their N-terminal regions [9].

Competence development in *B. subtilis* is regulated mainly by the level of the competence transcription factor ComK [10,11], which is inactive when associated with MecA and ClpC [12–14]. Inactivation of *MecA* results in overproduction of ComK [10,11,15], leading to inhibition of the σ^D-dependent *degR* expression [16]. Recently Liu and Zuber [17] demonstrated that ComK positively regulates the transcription of the *flgM*-containing operon that encodes an anti-σ^D factor FlgM which binds to σ^D [18–21]. Thus the expression of the σ^D regulon is negatively regulated by ComK.

Expression of *degR* is prevented by the overproduction of ComK in a *mecA*-deficient mutant [16]. We expected, therefore, that factors involved in the process leading to the expression of *degR* could be found by employing a system in which the recovery of *degR* expression is easily detected. We used a transposon mini-Tn*10* [22] for this purpose and discovered two genes, *degU* and *med*, which were shown to be positive regulators of *comK* in the *mecA* background [23,24]. In this paper we report analyses of a newly isolated transposon mutation *lacR288*, which caused an increase in the cellular level of σ^D at the translational level.

2. Materials and methods

2.1. Strains and plasmids

Strains used in this study are shown in Table 1. Construction of the transcriptional *sigD′-lacZ* fusion was done by digestion of the PCR fragment produced by using the sigD-H (5′-GTAAGCTTGATATGCTGATAGAAGCGG-3′) and sigD-Bg (5′-GTAGATCTCTAAACGAGGCGTAGGTATC-3′) primers with *Hin*dIII and *Bgl*II, followed by cloning of the resultant fragment between the *Hin*dIII and *Bam*HI sites of pMutin2 [25]. The constructed plasmid pSigDZ was transformed into strain CU741 by Campbell-type recombination. Disruption of the *lacA* gene was carried out as follows. First, a PCR fragment containing *lacA* was prepared with the lacA-E (5′-GTGAATTCAAGGAGGAGAATGTGATGTC-3′) and lacA-B (5′-GTGGATCCATATCGAGCGGAGCATCAGC-3′) primers, digested with *Hin*dIII and *Bam*HI, and inserted between the *Hin*dIII and *Bgl*II sites of pDH88 [26]. Second, the *Sma*I fragment carrying the tetracycline resistance gene from pBEST309 [27] was inserted into the *Sma*I site of the *lacA* gene. The inactivated *lacA* gene in the resultant plasmid pLacA was introduced into the chromosome by a double crossover event. Plasmid pEX lacking the *sigD* gene of pSigD [18] was created by digestion of the latter plasmid with *Sal*I and *Sph*I, followed by treatment with T4 DNA polymerase and DNA ligase. The *comK′-′lacZ* fusion was obtained from Dubnau [10].

2.2. Medium and others

Cells were grown in Schaeffer's sporulation medium [28]. β-Galactosidase activities were measured as described previously [29].

3. Results

3.1. Isolation of lacR288 mutation that suppresses the inhibitory effect of mecA on degR expression

A transposon mutant in which *degR′-′lacZ* expression was recovered was isolated after growing strain ODM40mak (*degR′-′lacZ mecA*) harboring the mini-Tn*10* delivery vector pIC333 [22]. By out-cloning of the DNA region that carried the transposon, a plasmid designated pLacR was obtained.

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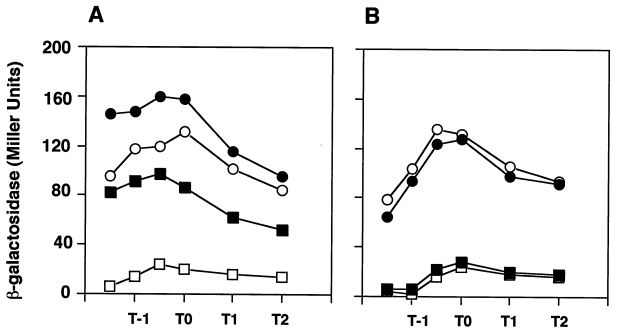

Fig. 1. Effect of the *lacR288* (A) and Ω*(lacR::spc)83* (B) mutations on *degR′-′lacZ* expression in *mecA*-deficient mutants. Numbers on the *x*-axis represent the growth time in hours relative to the end of the vegetative growth (*T*0). A: All strains carry *degR′-′lacZ*. ○, ODM40 (*mecA*$^+$); □, ODM40mak (*mecA*); ■, MM42 (*mecA lacR288*); ●, ODM402 (*mecA*$^+$ *lacR288*). B: All strains carry both *degR′-′lacZ* and *lacA*. ○, ODM403 (*mecA*$^+$); □, ODM405 (*mecA*); ■, ODM406 (*mecA* Ω*(lacR::spc)83*); ●, ODM404 (*mecA*$^+$ Ω*(lacR::spc)83*.

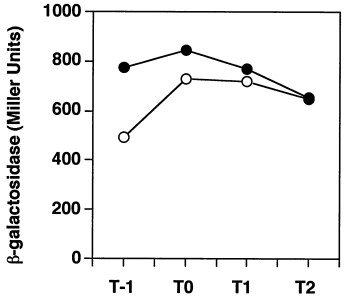

Fig. 2. Effect of the *lacR288* mutation on the expression of *comK′-′lacZ* translational fusion. Numbers on the *x*-axis represent the growth time in hours relative to the end of the vegetative growth (*T*0). ○, OCM106 (*comK′-′lacZ mecA lacR*$^+$); ●, OCM107 (*comK′-′lacZ mecA lacR288*).

When it was linearized and introduced into the chromosome of strain ODM40 (*degR′-′lacZ*), followed by transformation of the resultant strain with *mecA*-containing DNA, the transformants formed blue colonies on a X-gal-containing plate, indicating that the observed phenotype was indeed caused by the transposon insertion mutation. A sequence analysis revealed that Tn*10* had inserted into codon 288 of *lacR*, which is composed of 330 codons. An in-frame TGA stop codon had been created at the transposon insertion site, showing that the putative protein, LacR288, specified by *lacR288* was a truncated protein without extra amino acids at its C-terminal end derived from the transposon.

The level of β-galactosidase directed by the *degR′-′lacZ* fusion was greatly reduced in the *mecA* background (ODM40-mak) as reported previously [23], whereas it was restored to 80% of the wild type level in the strain bearing both the *mecA* and *lacR288* mutations (MM42) (Fig. 1A). Strain ODM402 bearing *lacR288* alone showed a slight increase in the expression of *degR′-′lacZ* (Fig. 1A). These results show that a deletion of the C-terminal 42 amino acids from LacR caused an increase in the expression of *degR*.

It was shown by Daniel et al. [8] that inactivation of *lacR* results in overexpression of intrinsic *lacA*. Therefore, the possibility arose that the *lacR288* mutation we isolated caused overexpression of *lacA* and the β-galactosidase activity we observed was derived from *lacA* but not from *degR′-′lacZ*. This possibility was, however, excluded by the observation that the levels of *degR′-′lacZ* expression were almost the same in *lacR288 mecA* strains carrying either the intact or disrupted *lacA* gene (data not shown).

These results show that the *lacR288* gene product retains the activity as the repressor of *lacA* and has a suppressive effect on the *mecA* inhibition of *degR′-′lacZ*.

Table 1
B. subtilis strains and plasmids used in this study

Strain or plasmid	Relevant phenotype and description	Reference or source
Strains		
CU741	*trpC2 LeuC7*	[29]
ODM40	*trpC2 leuC7 amyE::(degR3′-′lacZ*(Cm^r))	[5]
ODM40mak	*trpC2 leuC7 amyE::(degR3′-′lacZ*(Cm^r)) *mecA*::Km^r	[24]
MM42	*trpC2 leuC7 amyE::(degR3′-′lacZ*(Cm^r)) *mecA*::Km^r *lacR288*(Tn*10*(Sp^r))	This work
ODM402	*trpC2 leuC7 amyE::(degR3′-′lacZ*(Cm^r)) *lacR288*(Tn*10*(Sp^r))	This work
ODM403	*trpC2 leuC7 amyE::(degR3′-′lacZ*(Cm^r)) *lacA*::Tc^r	This work
SG83	*trpC2* Ω*(lacR*::Sp^r)83	[8]
ODM404	*trpC2 leuC7 amyE::(degR3′-′lacZ*(Cm^r)) Ω*(lacR*::Sp^r)83 *lacA*::Tc^r	This work
ODM405	*trpC2 leuC7 amyE::(degR3′-′lacZ*(Cm^r)) *mecA*::Km^r *lacA*::Tc^r	This work
ODM406	*trpC2 leuC7 amyE::(degR3′-′lacZ*(Cm^r)) *mecA*::Km^r Ω*(lacR*::Sp^r)83 *lacA*::Tc^r	This work
OCM106	*trpC2 leuC7 amyE::(comK-lacZ*(Cm^r))	This work
OCM107	*trpC2 leuC7 amyE::(comK-lacZ*(Cm^r))*lacR288*(Tn*10*(Sp^r))	This work
OLM100	*trpC2 leuC7 lacR288*(Tn*10*(Sp^r))	This work
OLM101	*trpC2 leuC7 lacA*::Tc^r	This work
ODS200	*trpC2 leuC7 sigD′-′lacZ*(Cm^r)	[5]
ODS201	*trpC2 leuC7 sigD′-′lacZ*(Cm^r) *lacR288*(Tn*10*(Sp^r))	This work
ODS202	*trpC2 leuC7 sigD-lacZ*(Cm^r)	This work
ODS203	*trpC2 leuC7 sigD-lacZ*(Cm^r) *lacR288*(Tn*10*(Sp^r))	This work
Plasmids		
pIC333	Em^r mini-Tn*10* (Sp^r)	[22]
pLacR	pUC19 carrying *lacR288* (Tn*10*(Sp^r))	This work
pLacA	pDH88 carrying entire *lacA* in which Tc resistance gene is inserted	This work
pSigDZ	pMutIn2 carrying upstream and N-terminal portion of *sigD*	This work
pSigD	pDG148 carrying *sigD*	[18]
pEx	pDG148 derivative	This work

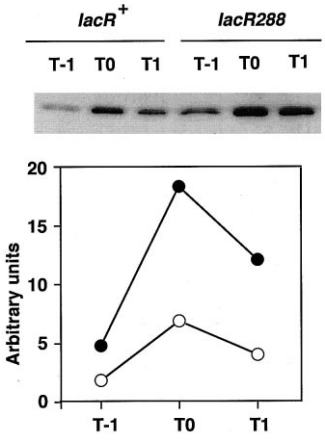

Fig. 3. Quantitation of σ^D by Western blot analysis. Protein concentrations were adjusted by densitometric scanning of the protein bands on SDS-PAGE gels after staining with Coomassie brilliant blue. Detection of σ^D and anti-σ^D interaction was carried out by BM Chemiluminescence Western Blotting Kit (Rabbit/Mouse) (Boehringer Mannheim). The lower panel shows the quantitative analysis of the σ^D bands as determined by densitometric analysis. ○, CU741 (*lacR288*$^+$); ●, OLM100 (*lacR288*).

3.2. The effect of lacR288 mutation on expression of comK and cellular level of the σ^D protein

Based on the principle for the screening of suppressors, it is possible that an isolated mutation affects the expression of either *comK* or *sigD* (see Section 1). We thus tested the effect of the *lacR288* mutation on the expression of a *comK'-'lacZ* translational fusion. Fig. 2 shows that the *lacR288* mutation did not affect the expression of this fusion significantly. We therefore conclude that the target of the *lacR288* mutation is not *comK*.

Next we determined the σ^D level in the cells carrying the *lacR288* mutation by Western blot analysis using anti-σ^D antibody. As shown in Fig. 3, the level of σ^D in the *lacR*$^+$ strain reached the peak at *T*0, which is in accordance with the observation that *sigD* expression becomes highest at around *T*0 [2,5]. A similar expression pattern was seen in the *lacR288* strain, but the σ^D level was about threefold higher.

If the enhanced expression of *sigD* by the *lacR288* mutation is responsible for the restoration of *degR'-'lacZ* expression in the *mecA*-deficient mutant, then overexpression of *sigD* should overcome the inhibitory effect of *mecA*. To test this possibility we constructed ODM40 derivatives carrying either pSigD in which the *sigD* gene is placed downstream of the IPTG-inducible *spac* promoter [18] or its derivative, pEX, in which the entire *sigD* gene has been deleted. Plasmid pSigD is based on pUB110. Table 2 shows that in fact overexpression of *sigD* overcame the *mecA* inhibition of *degR'-'lacZ* in an IPTG-dependent manner. Addition of IPTG at as low as 0.01 mM was shown to have some suppressive effect on *mecA*, and at the highest concentration (0.3 mM) *degR'-'lacZ* expression was increased about three times (Table 2). These results are in agreement with the above observation that there was a threefold increase in the σ^D level in the *lacR288* mutant. From these results we conclude that the enhancing effect of the *lacR288* mutation on *degR'-'lacZ* expression is due to the enhanced expression of the *sigD* gene.

Table 2
Suppression of the inhibitory effect of *mecA* on *degR'-'lacZ* by overexpression of *sigD*

Host strain	β-Galactosidase activity					
	IPTG	0	0.01	0.03	0.1	0.3 (mM)
	plasmid					
ODM40	pEX	26.6	–[a]	–	○	23.5
ODM40	pSigD	23.1	25.2	29.7	42.7	47.3
ODM40ma	pEX	6.6	–	–	–	7.0
ODM40ma	pSigD	11.4	13.2	14.0	28.0	30.4

Cells were grown in Schaeffer's sporulation medium. The β-galactosidase activities expressed in Miller units are the peak values observed at either *T*−0.5 or *T*0. IPTG was added when the cell growth reached 0.3 at OD_{600}.
[a]Experiments were not carried out.

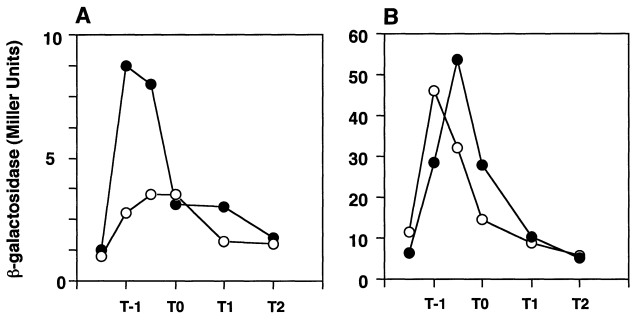

Fig. 4. Effect of the *lacR288* mutation on the expression of translational and transcriptional fusions of *sigD* to *lacZ*. A: Translational fusion: ○, ODS200 (*sigD'-'lacZ lacR*$^+$); ●, ODS201 (*sigD'-'lacZ lacR288*). B: Transcriptional fusion: ○, ODS202 (*sigD'-'lacZ lacR*$^+$); ●, ODS203 (*sigD'-'lacZ lacR288*).

The expression of *degR'-'lacZ* was low in the experiments in Table 2 as compared with those shown in Fig. 1. Similar low level expression of *degR'-'lacZ* was observed when another pUB110 derivative was used (data not shown), although the reason is not known at present.

3.3. LacR regulates expression of sigD post-transcriptionally

To know how the elevated level of the σ^D protein is achieved, we carried out epistatic analyses using both translational and transcriptional fusions of *lacZ* to *sigD*. It was found that the expression of a *sigD'-'lacZ* translational fusion was enhanced approximately two times in the *lacR288* mutant (Fig. 4A) as compared with that in the wild type strain. In contrast to this result the expression of the *sigD'-'lacZ* transcriptional fusion was not affected significantly by the *lacR288* mutation (Fig. 4B). Furthermore, it was shown that the stability of σ^D in vivo was not affected by the *lacR288* mutation (data not shown). From all of these results, we conclude that LacR288 is a translational regulator of the *sigD* gene.

3.4. Intact LacR does not regulate degR'-'lacZ expression

The results described thus far show that LacR288 effects positive translational regulation on *sigD*. This would in turn suggest that LacR is a negative regulator of *sigD*. If this was the case, a *lacR* disruption mutation, *Ω(lacR::spc)83*, should also suppress the *mecA* effect on *degR'-'lacZ*. As shown in Fig. 1B, the *Ω(lacR::spc)83* mutation had no effect on *degR'-*

'lacZ expression in a *lacA*-deficient background, indicating that LacR itself does not play any role in the regulation of *degR*.

4. Discussion

We described in this paper a mini-Tn*10* transposon mutation, *lacR288*, which suppressed the inhibitory effect of *mecA* deficiency on *degR*. It appears that LacR288 has dual functions. First, it may have a DNA-binding ability and functions as a repressor for transcription of *lacA* as does the intact LacR [8,9], since the *lacR288* mutation retained the ability to repress the expression of *lacA*. In contrast, Daniel et al. [8] observed more than 100 times higher activity of LacA in a *B. subtilis* JH642 derivative carrying the *lacR*-deficient mutation Ω*(lacR::spc)83*. This difference is not due to the strain difference, since a similar level of enhancement was observed when we used a derivative of our standard strain CU741 carrying the same Ω*(lacR::spc)83* mutation (data not shown), indicating that the *lacR288* and Ω*(lacR::spc)83* mutations are different in terms of the phenotypes that they show. Second, LacR288 functions as a translational activator of the *sigD* gene, which is contained in the *fla/che* operon [30].

It is not known whether LacR288 acts on translation of *sigD* directly or indirectly. The possibility, however, could be ruled out that LacR288 would derepress expression of an unknown gene(s) that is required for translation of the *sigD* gene, since the truncated LacR288 protein still has the repressor activity for *lacA* (and therefore DNA-binding activity), and it might be unlikely that the protein has lost the repressor activity for this hypothetical gene.

Apparently intact LacR itself does not regulate *sigD*, since a disruption by the Ω*(lacR::spc)83* mutation had no effect on the expression of either *sigD* or *degR*. It could be speculated, however, that LacR has an activity to regulate translation of *sigD* through its N-terminal domain and that the C-terminal domain of LacR inhibits this function. *B. subtilis* LacR shows the highest homology to *Escherichia coli* EbgR in amino acid sequence [8]. The N-terminal regions of the LacI-GalR family proteins have been shown to have a DNA-binding activity, while the middle regions carry both dimerization and effector-binding activities. The role of the C-terminal regions is not known except for LacI [9]. Intramolecular inhibition of a certain function by another region of the same protein is exemplified in the case of σ^{70} [31]. Moreover, several proteins with a helix-turn-helix structure including *E. coli* LacI have been shown to bind 10Sa RNA [32]. By analogy with these findings we infer that LacR also has an RNA-binding activity and that truncation of a certain C-terminal region of LacR results in alteration of the specificity for RNA. Thus, LacR288 lacking the C-terminal 42 amino acids could work as a translational regulator of *sigD* possibly through binding to its mRNA. That a specific DNA-binding protein also works as a translational regulator is not unprecedented. For example, NtrC, a DNA-binding protein, has been shown to work as a translational activator of the *nifR3* operon in *Rhodobacter capsulatus* [33]. Moreover, *B. subtilis* SinR, a sequence-specific DNA-binding protein, regulates the expression of *comS* at the translational level; the *comS* gene is located in the middle of the 27-kb *srf* mRNA and directs a positive regulator of ComK [14,34,35]. In eukaryotes, the bcd homeodomain protein in *Drosophila* binds to mRNA and functions at the translational level [36]. The precise mechanisms underlying these observations, however, remains to be elucidated.

The biological implication of the derepression of *sigD* by LacR288 remains unknown. However, the fact that a new function was identified in a LacR mutant may be important from an evolutionary point of view, since the observation that a hidden function was found in LacR might be extended to the other members of the LacI-GalR group.

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