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Extra-chromosomal DNA maintenance in *Bacillus subtilis*, dependence on flagellation factor FliF and moonlighting mediator EdmS

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ABSTRACT

Extra-chromosomal DNA maintenance (EDM) as an important process in the propagation and genetic engineering of microbes. *Bacillus subtilis* EdmS (formerly PgsE), a protein comprising 55 amino acids, is a mediator of the EDM process. In this study, the effect of mutation of global regulators on *B. subtilis* EDM was examined. Mutation of the *swrA* gene abolished EdmS-mediated EDM. It is known that *swrA* predominantly regulates expression of the *fla*/*che* operon in *B. subtilis*. We therefore performed EDM analysis using *fla*/*che*-deletion mutants and identified an EDM-mediated EDM cooperator in the *flgB*–*fliL* region. Further genetic investigation identified the flagellation factor FliF is a crucial EDM cooperator. To our knowledge, this is the first observation of the moonlighting function of FliF in DNA maintenance.

1. Introduction

Extra-chromosomal DNA maintenance (EDM) is an important biological process, which may help our understanding of molecular evolution and has applications in the genetic engineering and propagation of microbes [1,2]. Little is known about the genetic basis of EDM; however, it is ubiquitous among microorganisms.

Poly-γ-glutamate (PGA) has been detected in various organisms [3,4], though the operon responsible for PGA synthesis has only been identified in bacteria [5]. *Bacillus subtilis* possesses a chromosomally encoded PGA synthetic system that contains a cryptic protein of 55 amino acids, designated PgsE [3,6]. We previously reported that PgsE affects the Zn^{2+}-dependent enhancer-like activity observed during PGA synthesis [7].

Recently, we identified a candidate EDM mediator, resulting from the vector DNA-mediated transformation (or functionalization) of *B. subtilis* mutants. Unexpectedly, this inducer of EDM was found to be PsgE (from here on referred to as EdmS) [8,9]. It remains to be determined whether EDM in *B. subtilis* is dependent on this factor alone. Here we investigate EDM in more detail in *B. subtilis* and analyze the roles of the flagellation factor – FliF and the moonlighting mediator – EdmS.

2. Materials and methods

2.1. Bacterial strains

Escherichia coli JM109 competent cells were purchased from TaKaRa Bio. (Shiga, Japan) and were used to propagate plasmids. *B. subtilis* strains and mutants used in this study are listed in Supplementary Table S1.

2.2. DNA oligonucleotides and vectors

The oligonucleotides used for gene cloning and detection are provided in Supplementary Table S1. The following plasmid vectors were also used: a *Bacillus-E. coli* shuttle vector pWH1520 (MoBiTec, Göttingen, Germany), pEDMS1 (a pWH1520 derivative carrying the *B. subtilis edmS* gene) [8], and pEDMS2 (a pWH1520 derivative carrying the *B. subtilis edmS* and *fliF*genes). The methodology used for construction of the vectors is illustrated in Supplementary Fig. S1.

2.3. Transformation of B. subtilis

Vectors were isolated from the *E. coli* transformant cells using the alkali-SDS method [10], and *B. subtilis* strains were transformed using the standard competence method [11]. The *B. subtilis* transformed cells were then screened on a 2%-agar plate of Luria–Bertani (LB) medium [10] containing appropriate antibiotic(s),

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these included chloramphenicol (10 μg ml^{-1}), kanamycin (10 μg ml^{-1}), and/or tetracycline (Tc; 12.5 μg ml^{-1}).

2.4. Vector-maintenance tests

Vector-maintenance tests were performed according to published procedures [8], and the maintenance ratios (R_m) were determined using the following equation.

$$R_m(\%) = \frac{\text{Score of the colony number formed on Tc – supplemented LB plate}}{\text{Score of the colony number formed on Tc – free LB plate}} \times 100$$

3. Results

In *B. subtilis*, global regulators, such as ComA (predominantly for competence development) [12], DegSU–DegQ (predominantly for proteolysis) [13], SwrA (predominantly for swarming and *sigD* expression) [14], and σ^D (predominantly for flagellation and chemotaxis) [15], are responsible for PGA production (Fig. 1). To investigate the potential effect of these global regulators on the process of EDM, *B. subtilis* regulator mutants were transformed with the pEDMS1 vector and R_m sensitive transformants were selected. Surprisingly, the EDM effect was absent in a *swrA* mutant (designated W619) derived from *B. subtilis* natural isolate ATCC6051 (Table 1). It has previously been reported that the *swrA* genes of the laboratory strain 168 and its derivatives (TF20, TF29, and DEGQd) were disrupted spontaneously [16]. Interestingly, the influence of the *sigD* mutation on EDM induction in the natural isolate of *B. subtilis* differed from that in the laboratory strain (Fig. 2). These findings suggested that in the laboratory strain σ^D activates the weaker, auxiliary promoter of the *fla/che* (flagellation and chemotaxis) operon [17,18] and eventually operates as an anaplerotic effector for SwrA. A gene involved in EDM was therefore likely to be located in the *fla/che* region of the *B. subtilis* genome.

To identify the EDM cooperator-encoding gene(s) from this locus, we examined EDM induction in *fla/che*-deletion mutants from

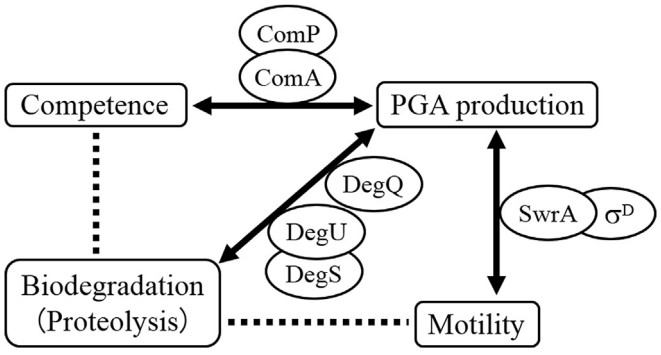


Fig. 1. Global regulators participating in *B. subtilis* PGA production.

Table 1
Effects of the mutation of global regulators on *B. subtilis* EDM in the presence of the pEDMS1 (*edmS* gene-present) and pWH1520 (*edmS* gene-absent) vectors.

Strains	Mutation	Vectors	Maintenance ratios (%)[a]	
			Generations	
			10	50
ATCC6051		pEDMS1	98 ± 2	98 ± 2
		pWH1520	60 ± 10	10 ± 4
W619	Δ*swrA*	pEDMS1	80 ± 5	>1
		pWH1520	38 ± 12	>1
W648	*degS*⁻	pEDMS1	92 ± 2	76 ± 4
		pWH1520	65 ± 20	12 ± 2
Marburg 168		pEDMS1	88 ± 8	80 ± 10
		pWH1520	55 ± 15	5 ± 5
DEGQd	Δ*degQ*	pEDMS1	82 ± 8	72 ± 12
		pWH1520	45 ± 10	5 ± 5
TF20	Δ*comA*	pEDMS1	90 ± 5	76 ± 14
		pWH1520	72 ± 8	15 ± 5
TF29	Δ*degU*	pEDMS1	85 ± 10	82 ± 8
		pWH1520	70 ± 10	24 ± 16

[a] Values are given as means ± SEM of data from three independent assays.

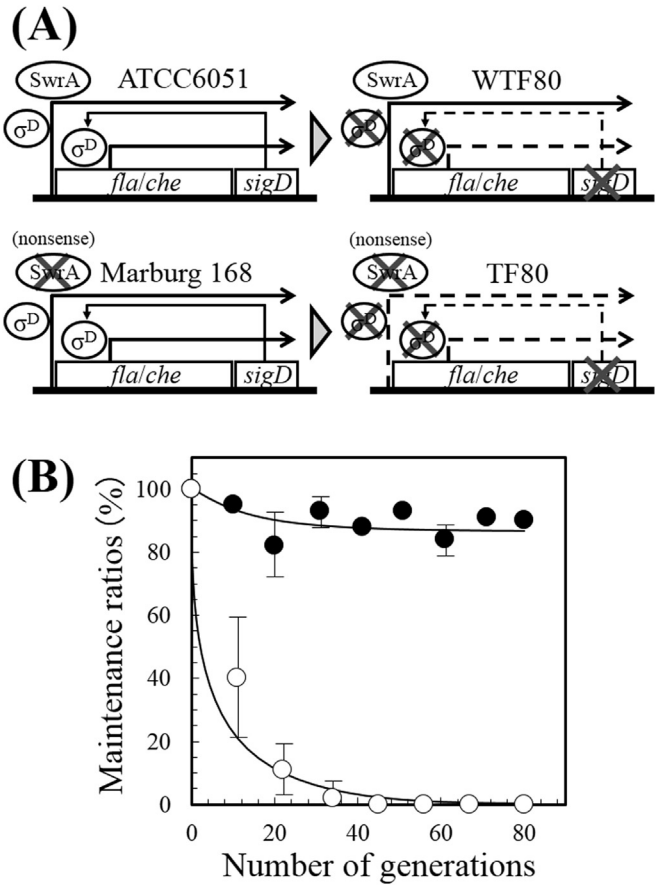
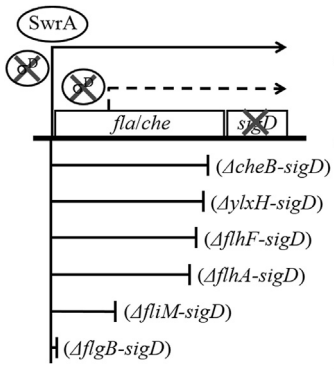


Fig. 2. Distinct expression modes of the *fla/che* operon in some strains of *B. subtilis* (A) and EDM effects of the WTF80 (*closed circles*) and TF80 (*open circles*) mutants for the pEDMS1 vector (B). In (A), the crosses indicate the genes that were disrupted, and the solid and broken arrows indicate the ON or OFF expression (or induction) of the *fla/che* operon, respectively. (B) Error bars, SEM ($n = 3$).



Strains	Maintenance Ratios (%) Generations 10	50
W935	96±4	88±8
W934	98±2	92±4
W933	88±8	80±10
W932	92±2	90±5
W931	90±5	86±4
W834	45±20	>1

Fig. 3. Schematic diagram of the construction of the *B. subtilis fla/che*-deletion mutants and their EDM effects in the presence of the pEDMS1 vector. The description of the expression of the *fla/che* operon is the same as that described in Fig. 2A. Values are given as the means ± SEM of data from three independent assays.

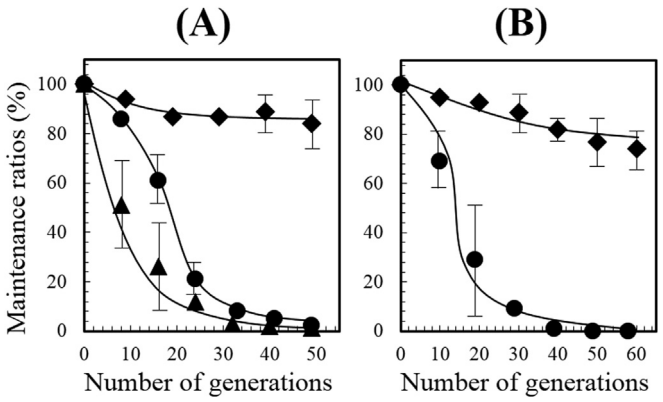


Fig. 4. Absence and restoration of EDM function in the *fliF* mutant W954 (A) and the *fla/che*-null mutant W834 (B) from *B. subtilis* ATCC6051. Vectors, pWH1520 (*triangles* in A); pEDMS1 (*circles* in A and B); pEDMS2 (*diamonds* in A and B) (*see* Supplementary Fig. S1 for details). Error bars, SEM ($n = 3$).

B. subtilis ATCC6051. As shown in Fig. 3, deletion of the *flgB–fliL* region abolished EDM. Within this region are some open reading frames encoding proteins involved in the flagellum hook-basal body complex [19], including an important flagellation factor designated FliF [20]. Next, a *B. subtilis fliF* mutant (W954) was subjected to EDM analysis using a pEDMS1 vector (Fig. 4A). The results indicated that the EDM effect was lost in the absence of the FliF factor. To investigate this further, we constructed a pEDMS2 vector in which the EdmS and FliF factors were coproduced in the presence of xylose (Supplementary Fig. S1). Interestingly, the functions of the *fliF* mutant W954 (Fig. 4A) and the *fla/che*-null mutant W834 (Fig. 4B) were restored following the introduction of the pEDMS2 vector, suggesting that FliF is a crucial EDM cooperator.

4. Discussion

Our investigations into the unique EDM system of *B. subtilis* have identified two crucial factors, FliF and EdmS. Among over 30 structural genes in the *B. subtilis fla/che* operon, the *fliF* gene was found to be essential for the EDM process (Fig. 4). This finding indicated that FliF has two independent functions, namely FliF-dependent EDM and flagellation, similar to EdmS that also displays two distinct functions, namely EdmS-dependent EDM and PGA production [8]. In this case, the “one gene, one protein (or function)” hypothesis is not applicable. Instead, the theory of moonlighting proteins [21], in which one gene does not necessarily encode only one protein with a single role [22–25], becomes accepted as an alternative hypothesis. To our knowledge, this is the first observation of the moonlighting role of FliF in DNA maintenance.

The EDM factors – FliF and EdmS – do not possess any of the consensus (or characteristic) motifs associated with DNA-binding proteins [26–28] or resemble any factors involved in the maintenance, replication, segregation, or copy-number control of plasmids [29–34]. Furthermore, these genes are chromosomally located in *B. subtilis* and are not carried on any natural plasmids identified to date [35]. These findings therefore indicate the identification of a novel biological system for EDM.

A recent study reported that the *Bacillus megaterium capE* (or *edmS*) gene (DDBJ accession no., AB544060) complemented the EDM phenotype in *B. subtilis*, while the *edmS* gene from *B. subtilis* complemented the EDM phenotype in *B. megaterium* cells [9]. This suggested that *edmS* genes of the EDM system are transmissible between microbes, and can function in different host backgrounds, providing an interesting insights into the molecular mechanisms of EDM. These indicate a fundamental means for adaptation to the environment (or evolution) by some microbes by the uptake and incorporation of foreign EDM genes, and this may be demonstrated by the fact that the FliF orthologues are ubiquitous among prokaryotes.

Conflict of interest

The authors declare no conflict of interest

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.152.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.152.

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