Functional analysis of the stability determinant AlfB of pBET131, a mini plasmid of Bacillus subtilis (natto) plasmid pLS32

Running title: Functional analysis of DNA-binding protein AlfB

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Bacillus subtilis plasmid pBET131 is a derivative of pLS32, which was isolated from a natto strain of Bacillus subtilis. The DNA region in pBET131 that confers segregational stability contains an operon consisting of three genes, of which alfA encoding an actin-like ATPase and alfB are essential for plasmid stability. In this work the alfB gene product and its target DNA region were studied in detail. Transcription of the alf operon initiated from a σ^A-type promoter was repressed by the alfB gene product. Overproduction of AlfA was inhibitory to cell growth, suggesting that the repression of the alf operon by AlfB is important for maintaining appropriate levels of AlfA. An electrophoretic mobility-shift assay and foot-printing analysis with purified His-tagged AlfB showed that it bound to a DNA region containing three tandem repeats of 8-bp AT-rich sequence (here it is designated parN), which partially overlaps the –35 sequence of the promoter. A sequence alteration in the first or third repeat did not affect the AlfB binding and plasmid stability, whereas that in the second repeat resulted in inhibition of these phenomena. The repression of alfA-lacZ expression was observed in the constructs carrying a mutation in either the first or third repeat but not in the second repeat, indicating a correlation between plasmid stability, AlfB binding and repression. It was also demonstrated by the yeast two-hybrid system that AlfA and AlfB interact with each other and among themselves. From these results it was concluded that AlfB participates in partitioning pBET131 by forming a complex with AlfA and parN, the mode of which is typified by the type II partition mechanism.
Faithful distribution to daughter cells is essential for plasmids to be inherited in dividing cells, and this is achieved by the mechanisms including active partitioning, post-segregational killing, and multimer resolution (15, 32). Low-copy-number plasmids are known to carry active partitioning mechanisms (17, 50), which are composed of a centromere-like site and two partitioning genes, one being an ATPase and the other a protein that binds to the centromere-like site. Active partitioning apparatus can be classified into two types on the basis of the ATPase (13): type I as exemplified by the *Escherichia coli* F and P1 plasmids contains a Walker-type ATPase motif (4, 5, 7, 14, 18, 31), while type II signified by *E. coli* R1 encodes an actin-like ATPase (10, 13, 22).

Three inheritance mechanisms have also been reported for gram-positive bacteria. Multimer resolution systems, which effect random distribution of plasmid copies in the cell, have been reported for plasmids from *Clostridium perfringence*, *Streptococcus pyogenes* and *Enterococcus faecalis* (12, 37, 43). With respect to the plasmid maintenance system classified as the killer-antidote system, several examples have been reported, including a restriction-modification system (24), a toxin-antitoxin system (53) and a determinant involving two small RNA molecules (49). Stability determinants involving the active partitioning mechanism have also been reported for several plasmids from gram-positive bacteria. Plasmids from *Lactococcus lactis*, *Streptococcus pyogenes* and *Enterococcus faecalis* carry proteins with sequence similarities to the type I ATPases (8, 9, 11, 23, 36). The partition system encoded by a staphylococcal plasmid, pSK1, is unusual in that the *parA*
gene product that has no sequence similarity to known type I or type II ATPases exerts the stabilization function without the involvement of other factors (41). Less well characterized determinants involving a small DNA region and a protein SpbA have been reported for a plasmid from Bacillus thuringiensis (26). Another novel partition mechanism has been found recently, in which partitioning is executed by a tubulin-like protein (25).

In addition, a type II actin-like ATPase was found to be involved in partitioning a B. subtilis plasmid, pBET131, whose replication origin was derived from a B. natto plasmid, pLS32 (3, 45). pBET131 carrying 11 genes including the replication origin region is present in the cell at 1 to 2 copies per chromosome (46). It is less stable than pLS32 in both the original strain B. subtilis (natto) IAM1163 and B. subtilis 168 (T. Tanaka, unpublished data), but much more stable than pBET131 derivative lacking the plasmid stability region (3).

It was shown previously that the B. subtilis chromosome was dissected into two subgenomes when pBET131 was inserted into a DNA region in the chromosome surrounded by two homologous DNA sequences, but one of the subgenomes carrying the pBET131 origin oriN was rapidly lost or integrated into the other subgenome when only the oriN region was used, indicating the importance of the stability region for the stable maintenance of the oriN-driven subgenome (19, 20). Thus, in order to make oriN more useful for cloning purposes or genome analysis, it will be useful to characterize the region conferring the segregational stability to oriN. Becker et al have shown that two consecutive genes, alfA and alfB, on pBET131 confer segregational stability on the plasmid and that AlfA is an ATPase that forms actin-like filaments in B. subtilis cells (3). A partition mechanism that involves a putative actin-like protein has also been
reported for the *Staphylococcus aureus* plasmid pSK41 (40). Since the partition involving type II ATPase is less well studied for plasmids of gram-positive bacteria, it will be of interest to characterize the partition mechanism of pBET131 and compare the results with those of the gram-negative counterparts. Recent studies on the AlfA structure and dynamics have shown that the AlfA filament is distinct from the ParM actin-like filament of plasmid R1 in that the former constitutes a more twisted helix and does not show dynamic instability (3, 35). It was also suggested by those authors that segregation by AlfA is significantly different from that exerted by ParM.

In this report I describe characterization of the partition mechanism of pBET131 in vivo and in vitro by focusing on the functional characteristics of AlfB.

**MATERIALS AND METHODS**

**Materials.** Restriction enzymes were purchased from Toyobo Co., Takara Co., and New England Biolabs. T4 DNA ligase was from New England Biolabs. DNase I and T4 DNA polymerase were purchased from Takara Co. and Roche, respectively. Synthetic oligonucleotides were commercially prepared by Tsukuba Oligo Service Co. and described in Table S1 in the supplemental material. NuSieve 3:1 agarose was from Cambrex Bio Science Rockland, Inc. Matchmaker GAL4 Two-hybrid System 3, YPD medium, minimal SD base and Dropout (DO) supplements lacking Leu/Trp or Ade/His/Leu/Trp were purchased from Clontech. Protein molecular weight markers (2,500-17,000 Da) were obtained from Sigma.
Bacterial strains, and strain construction. The bacterial and yeast strains used in this study are listed in Table 1. The strains carrying the alfA-lacZ fusions were constructed by linearization of plasmid pOF5LA2 with ScaI, followed by insertion into the amyE locus of strain CU741.

Medium and antibiotics. Schaeffer sporulation (39) and Luria-Bertani (LB) media were used for growing B. subtilis and both E. coli and B. subtilis strains, respectively. Chloramphenicol (Cm), Tetracycline (Tc) and Neomycin (Nm) were added at concentrations of 5 µg/ml, 10 µg/ml and 15 µg/ml, respectively. Ampicillin (Ap) was added at 100 µg/ml for E. coli culture. YPD medium supplemented with 0.2% adenine hemisulfate was used for growing the yeast strain AH109. For the selection of AH109 transformants, the SD minimal medium added with either the –Leu/-Trp DO supplement or the -Ade/-His/-Leu/-Trp DO supplement was used. X-α-Gal (5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside) was added at a concentration of 80 µg/ml. The media for growing yeast were prepared according to the procedure recommended by Clontec.

Construction of plasmids. The procedures to construct the plasmids used in this study are described in Supplemental material.

Primer extension analysis. Primer extension was performed with an AMV RT cDNA Synthesis Kit obtained from Takara Co. The reaction mixture contained 10 µg of RNA and a primer, ORF5Bio3, biotinylated at the 5’ end. The reaction product was run in a sequencing gel, together with sequencing ladders prepared by using the same primer and pBET131 as a
template. RNA was isolated as described previously (52).

β-galactosidase activities. Cells were grown overnight in LB medium containing appropriate antibiotics, and the culture medium (50 µl) was transferred to 50 ml of Schaeffer sporulation medium containing the same antibiotics. IPTG (Isopropyl-1-thio-β-D-galactopyranoside) was added at a concentration of 0.1 mM to the Schaeffer medium when necessary. Samples (1 ml) were withdrawn, and the cells collected by centrifugation. β-galactosidase activities were measured as described previously (33).

Purification of Histidine (His)-tagged AlfB. E. coli JM103 carrying pQEORF6 was grown overnight in LB medium containing Ap, and the culture medium was transferred to the same fresh medium at a concentration of 1%. After the cell density reached 70 Klett units (red filter), IPTG was added at a final concentration of 2 mM. His-tagged AlfB protein was purified by a Ni²⁺-nitrilotriacetic acid silica column as described previously (44).

Binding of His-tagged AlfB protein to DNA. Binding of His-tagged AlfB to DNA and subsequent analysis by agarose gel electrophoresis were performed by the method described previously (44). The reaction mixture contained 15 to 20 ng of 290-bp DNA fragments to be tested, 170 ng of RsaI fragments of pUC19 (51) and various amounts of His-tagged AlfB. The reaction mixtures were left at room temperature for 40 min and then directly added to the wells formed in 3.5% NuSieve 3:1 agarose for electrophoretic mobility-shift assay (EMSA).
**DNase I foot printing.** PCR fragments from positions 2871 to 3330 and from positions 3131 to 2700, which were biotin-labeled at the 5’ ends, were prepared by using primers 2871Bio plus BET3330R (for coding strand) and 3131Bio plus 2700F (for noncoding strand), respectively. Sequencing ladders were prepared by using the same biotinylated primers and the plasmid pBET131 as a template. The binding reaction was performed as described above in a total volume of 40 µl at room temperature for 40 min, followed by the addition of 3.5 µl of 10x concentrated DNase I buffer (Roche) and 1.4 µl of DNase I (1 unit/ml). After the reaction mixture was incubated at 37°C for 2 min, the DNase I-treated DNAs were prepared for gel electrophoresis by the procedure described by Tsukahara and Ogura (47).

**Segregational stability of plasmids.** Constructed plasmids in *E. coli* JM103 were transformed into strain CU741, and the plasmid stability was determined as follows. Cells grown overnight at 37°C in LB medium containing Cm and Tc were inoculated into LB medium without the antibiotics, and grown for 10 generations. The cells before and after the incubation were spread on LB plates without the antibiotics and incubated overnight. Among the colonies formed, 150-200 were picked and examined for resistance to both Cm and Tc by toothpick transfer onto LB plates with or without the antibiotics.

**Yeast two-hybrid system.** Matchmaker GAL4 Two-hybrid System 3 obtained from Clontech was used for testing protein-protein interactions. *Saccharomyces cerevisiae* strain AH109 was made competent and transformed with plasmids as described in the Clontech’s manual.
RESULTS

Plasmid stability region in pBET131. Becker et al have reported that among the 11 open reading frames of pBET131 (Fig. 1A) only the alfA and alfB genes are required for the maintenance of the plasmid (3), and these results have been confirmed (T. Tanaka, unpublished data). The open reading frames alfA, alfB and orf7 apparently constitute an operon, since the last codons of alfA (275 codons) and alfB (93 codons) overlap the initiation codons of alfB and orf7 (51 codons), respectively (46).

Transcription initiation site of the alf operon. As part of characterization of the control region of the alf operon, we first determined the transcription initiation site. RNA was isolated from the CU741 cells carrying pBET131 and used as the template for a reverse transcriptase reaction. It was shown that the transcription starts at position 3061 (Fig. 2A), which is 39 nucleotides upstream of the initiation codon of alfA (Fig. 2B). Putative –35 (TTTACG) and –10 (TATAAT) regions which are likely to be recognized by σ^A-RNA polymerase were identified upstream of the transcription initiation site (Fig. 2B).

Confinement of the DNA region responsible for plasmid stability. The E. coli plasmids such as F, P1 and R1 carry a cis sequence with which one of the par proteins interacts (4, 6, 11, 15, 50). As an attempt to narrow down the cis DNA region necessary for the stable maintenance of pBET131 (Fig. 1A), three plasmids carrying deletions upstream of alfA
were constructed. Plasmid pBIF2965 is a derivative of pBET131 carrying a deletion from the BamHI site to position 2964, while pBIF6561 is a derivative of pBIF2965 carrying a deletion from positions 3033 to 3060, where most of the promoter region for the \textit{alf} operon is located (Fig. 1B, 2B). Plasmid pBIC6561 carries the P\textit{spac} promoter (16) upstream of position 2965 of pBIF6561. It was shown that pBIC2965 but not pBIF6561 was as stable as pBET131 (Fig. 1B). These results show that the region upstream of position 2965 is not required for plasmid stability and that the promoter for the \textit{alf} operon is necessary as expected. When the P\textit{spac} promoter was placed upstream of position 2965 of pBIF6561, the resultant plasmid pBIC6561 (Fig. 1B) became stable, indicating that the DNA region between positions 2965 and 3032 is sufficient to stabilize the plasmid as long as the \textit{alf} operon is transcribed.

To further investigate the DNA sequence necessary for plasmid stability, the region between the BamHI and SnaBI (position 3032) sites of pBET131 was replaced with a synthetic sequence spanning positions 2978 to 3032 (Fig. 1C and 2B). The resultant plasmid pBIAW2 showed stability similar to the level exhibited by pBET131 (Fig. 1C). On the other hand, pBIBM6 lacking the synthetic DNA was found to be unstable (Fig. 1C), indicating the importance of this DNA region. It was noted that this region contains three tandem repeats with a consensus sequence, 5’-T(A)TTATTTA-3’, which are separated each other by four nucleotides (Fig. 2B). To examine whether the repeats are involved in plasmid stability, the nucleotide sequences of the repeats were changed and the segregational stability of the resultant plasmids were examined. The results showed that the stability was not affected when the first repeat was changed to a sequence, 5’-ATATATAT-3’ (pBIBM19), while the same
sequence change in the second repeat resulted in plasmid instability (pBIBM24) (Fig. 1C). The nucleotide alterations in both the first and second repeats by the same sequence caused instability of the plasmid (pBIBM8) as expected, and the mutation in the third repeat from 5’-ATTATTTA-3’ to 5’-TAATTTGA-3’ did not affect plasmid stability (pBIBM34) (Fig. 1C). These results can be interpreted to indicate that either two consecutive repeats have to be present adjacently or the second repeat alone is sufficient for the stable maintenance of pBET131. However, since the sequences of the first and the second repeats are the same and the presence of this sequence was not sufficient to stabilize the plasmid (pBIBM24), it is unlikely that the second repeat alone provides the stability function.

The existence of A-T tracts in the repeat region suggested static bending of DNA, but no such bent DNA was observed as shown by polyacrylamide gel electrophoresis run at both low and high temperatures (34) (T. Tanaka, data not shown).

Repression of alf operon expression by AlfB. Becker et al showed that deletion of alfB resulted in inhibition of cell growth (3). One possible explanation for this phenomenon is that excessive levels of AlfA are detrimental to cell growth and that the expression of alfA may be controlled by AlfB. To test these possibilities, the alfA gene alone or both the alfA and alfB genes were placed downstream of the IPTG-inducible Pspac promoter carried on pDG148 (42), resulting in plasmids pDGEN51 and pDGEX561, respectively (Fig. 3; see also Materials and Methods in the supplemental material). In addition the SacI site in alfA (Fig. 1A) was filled with DNA polymerase and ligated to disrupt the alfA gene in the two
plasmids, resulting in pENSA51 and pEXSA564, respectively (Fig. 3). In these constructs, the 5’ terminus of the cloned DNA region is the SnaBI site located at position 3033 within the –35 region (Fig. 1A and 2B), and thus the expression of the downstream genes solely depends on the Pspac promoter. When the promoter was activated by the addition of IPTG (0.05 mM), the host cells carrying pDG51 or pDGEX561 but not pENSA51 or pEXSA564 failed to form colonies on Nm-containing plates (T. Tanaka, data not shown), indicating that overproduced AlfA leads to growth inhibition, irrespective of the presence or absence of AlfB.

Next, the expression of alfA was examined. The DNA region between the BglII site in orf4 and the SacI site in alfA (Fig. 1A), which contains the upstream region of alfA and its N-terminal region, was inserted before the promoter-less E. coli lacZ gene in pIS284, and the resultant alfA-lacZ fusion was placed at the amyE locus of CU741 (strain LA213). The expression levels of alfA-lacZ were quantified in the cells carrying pENSA51, pEXSA564 and their vector pDG148. It was found that the expression of alfA-lacZ was unaffected by overexpression of truncated alfA (pENSA51) as compared to the control level exhibited by the pDG148-containing cells, whereas it was greatly reduced by overexpression of alfB on pEXSA564 (Fig. 4). It should be noted that alfA-lacZ expression was not affected by overexpression of alfA as shown by using pDGEN51 under the condition where the concentration of IPTG added had little growth effect on the host strain (T. Tanaka, data not shown). These results show that AlfB works as a repressor of the alf operon.

**Binding of AlfB to the upstream region of the alfA promoter.** The
repressor activity of the AlfB protein suggested that it might bind to a specific region upstream of *alfA*. To test this possibility, the AlfB protein histidine-tagged at the N-terminus was prepared (Fig. 5A) and used for gel shift analysis. A series of DNA fragments of 290 to 298 bp, referred to as “290-bp” fragment, starting from positions 2965, 2992, 3009, 3021 and 3032 (Fig. 5B, see also Fig. 2B) were prepared by PCR, and incubated with the His-tagged AlfB protein. To this reaction mixture were also added three Rsal-digested pUC19 fragments, which served as an internal control for testing non-specific DNA binding to the AlfB protein. Under the condition where there was little effect on the mobility of the pUC19-derived DNA bands (shown by the dots in Fig. 5B), band shifting was observed for the 290-bp fragments starting from positions 2965, 2992 and 3009 (Fig. 5B, a, b and c), whereas no shifted band was observed for those starting from positions 3021 and 3032 (Fig. 5B, d and e), indicating that the 5’ boundary of the AlfB binding site exists downstream of position 3009. Similarly, the 3’ boundary of the binding region was examined using the 290-bp fragments ending at positions 2992, 3009, 3021, 3032, 3044 and 3052. The results showed that shifted bands were seen for the fragments carrying the 3’ termini at positions 3021, 3032, 3044 and 3052 (Fig. 5B h, i, j and k), indicating that the 3’ boundary is located upstream of position 3021. It is noted that in both experiments the 290-bp DNA fragments contained two adjacent repeats in the cases where the DNA binding by AlfB was observed.

Footprinting analysis. To gain more insight into the binding site of AlfB, DNaseI footprinting was performed. It was shown that the DNA region spanning positions 2985 to 3038 was protected from DNase I digestion in
the nontemplate strand, whereas the region from positions 2984 to 3036 was protected in the template strand (Fig. 6). The three tandem repeats are within the protected region, and the -35 sequence of the alf promoter resides in the 3’ end of this DNA region (Fig. 2B).

Involvement of tandem repeat sequences in the binding of AlfB. To further investigate the nature of the binding of AlfB to the upstream region of alfA, we tested AlfB binding to the 290-bp fragments spanning positions 2979 to 3263, which carry wild-type or altered sequences in the repeats. The PCR fragments were prepared by using wild-type pBIAW2 and its mutants pBIBM19, pBIBM24, pBIBM34 and pBIBM8 (Fig. 1C) as the templates. The results shown in Fig. 7 indicate that as the concentration of AlfB was increased, the intensities of the shifted bands increased for the DNA fragments derived from the stable plasmids pBIAW2, pBIBM19 and pBIBM34, which carry wild-type repeats, sequence alterations in the first and third repeat, respectively, whereas no band shifting was observed for those derived from the unstable plasmids pBIBM24 and pBIBM8 carrying alterations in the second repeat and both the first and second repeats, respectively.

The result with pBIBM24 shows that AlfB is unable to bind the 290-bp fragment if the DNA region carries the sequence alteration in the middle of the three consecutive repeats, indicating that neither the individual single repeat at the first or third position nor the presence of two repeats with the middle repeat carrying the sequence alteration support AlfB binding. This is in accordance with the finding that two repeats were present consecutively when AlfB bound to the 290-bp fragment successfully (Fig. 5). Another possibility remains unanswered in
these data that the binding requires only the second repeat. However, since
the first and second repeat share the same sequence (Fig. 2B) and the first
repeat alone or together with the third repeat could not support AlfB
binding as described above, this possibility may be ruled out.

Hereafter, the region containing the three tandem repeats is designated
parN.

**Requirement of the tandem sequences for AlfB repression.** We next
investigated whether the three tandem repeats are necessary for repression
of the alf operon. To do this, alfA-lacZ fusions were constructed in which
the three tandem repeat sequences were altered: strain WILA2 carried the
wild-type sequence, whereas strains LMLA2, MDLA13 and RMLA11
carried altered sequences in the first repeat, in both the first and second
repeats and in the third repeat, respectively. The introduced sequence
alterations were the same as those used for the construction of pBIAW2,
pBIBM19, pBIBM8 and pBIBM34, respectively. Plasmid pEXSA564 was
introduced into these strains, and the β-galactosidase activities were
estimated in the presence and absence of IPTG. It was found that alfA-lacZ
expression was repressed by the addition of IPTG in strains WILA2,
LMLA2 and RMLA11 but not in MDLA13 (Table 2). The variations in
the β-galactosidase activities seen without the addition of IPTG may be
due to the sequence alterations in the repeat sequences, which might affect
the alf operon transcription. Apparently, the higher activity in strain
RMLA11 was due to the sequence alteration, which resulted in a -35
sequence closer to the consensus sequence of the σA-type promoter (from
5′-TTTACG-′3 to 5′-TTGACG-′3). The result with strain MDLA13 also
shows that the transcription level of the alf operon was similar to that in
strain WILA2 (Table 2), indicating that the instability of pBIBM8 carrying sequence alterations in the first and second repeats was not caused by a defect in transcription of the alf operon. These results show that the repression of alfA expression is correlated with the binding of AlfB in that where alfA-lacZ was repressed, AlfB bound to the parN region (Fig. 7).

**Interaction of AlfA and AlfB as shown by yeast two-hybrid system.**

AlfA belongs to a new family of actins and forms actin-like filaments inside the cell (3). It is known that the E. coli plasmid R1 partitions with the help of a partition complex consisting of the actin-like protein, ParM, and the ParR protein bound to the centromere-like parC region (15, 29). To study if the partition system of pBET131 also involves interaction between AlfA and AlfB, the yeast Gal4 two-hybrid system was used. The yeast strain AH109 used as the host carries three reporters, ADE2, HIS3 and MEL1, which are under the control of distinct GAL4 upstream activating sequences and TATA boxes, and these features help to eliminate false positives (2, 21). The strain shows the phenotype Ade⁻ His⁻ Trp⁻ and Leu⁺, and the vector plasmids pGBK7 (vector) and pGAD7 (vector) complement the Leu⁺ and Trp⁻ phenotypes, respectively. The interaction of a protein fused to the GAL4 DNA-binding domain in pGBK7 with a protein fused to the GAL4 activation domain in pGAD7 renders the host cell growth independent of histidine and adenine. In addition, the protein-protein interaction causes the secretion of α-galactosidase encoded by MEL1, whose activity can be measured on a plate containing X-α-Gal (1). The alfA and alfB genes were cloned in pGBK7 and pGAD7 by transformation of E. coli JM103, resulting in pGBA3 (alfA) and pGAB142 (alfB), respectively. The constructed plasmids and the vectors were
transformed into AH109 in various combinations, and the colonies formed on the -Leu, -Trp (-LT) selection plates were patched on both the -LT and -AHLT (lacking Leu, Trp, Ade and His) plates containing X-α-Gal. It was found that all the transformants carrying pGBK7 (vector) or its derivative pGBA3 (alfA) together with pGAD7 (vector) or its derivative pGAD142 (alfB) showed growth on the -LT plate, but the cells transformed with pGBA3 (alfA) and pGAB142 (alfB) turned blue (Fig. 8A, upper panel). Furthermore, on the -AHLT plate only the cells carrying these plasmids grew and turned blue (Fig. 8A, lower panel).

We next studied the interaction of the AlfA and AlfB proteins among themselves. To do this, the alfA and alfB genes were cloned in the vectors pGAD7 and pGBK7, respectively, resulting in pGA51 (alfA) and pGB64 (alfB). The AH109 cells transformed with pGBA3 and pGA51, both of which carried the alfA gene, and those transformed with pGB64 and pGAB142, both of which carried the alfB gene, grew and turned blue on both the -LT and -AHLT plates containing X-α-Gal, whereas neither the transformants carrying pGA51 (alfA) and pGBK7 (vector) nor those carrying pGB64 (alfB) and pGAD7 (vector) formed colonies on the -AHLT plate (Fig. 8B).

The following control experiments were performed to eliminate false positives (T. Tanaka, data not shown). The plasmids carried in the blue colonies on the -AHLT plates were isolated and transformed into E. coli JM103, from which individual plasmids were isolated and checked for integrity. These plasmids were then used for transformation of AH109 as described above, resulting in identical phenotypes. Furthermore, the combinations of pGBA3 (alfA) or pGAB142 (alfB) with unrelated fusion plasmids pGAD7-T or pGBK7-53 (Table 1), respectively, did not
support the growth of AH109 on the –AHLT plate. These results show specific interactions of the AlfA and AlfB proteins.

**DISCUSSION**

It was shown previously that the partition mechanism of the mini-pLS32 plasmid pBET131 involves an actin-like ATPase, AlfA (3). The current study reports a functional study of ParB. It was shown that AlfB binds to the *parN* region upstream of the *alf* operon consisting of three tandem repeats and that this binding most likely requires two consecutive repeats (see above). It was also shown that only the stable plasmids contain the *parN* region to which AlfB binds (Fig. 1 and 5), suggesting strongly that the binding of AlfB to *parN* is prerequisite to the segregational stability of pBET131. Furthermore, there was a correlation between AlfB binding to *parN* and the repression of the *alfA* expression (Table 2). These results are consistent with the notion that specific binding of AlfB to *parN* results in both plasmid stabilization and repression of *alfA* expression. Since overproduction of AlfA (see above) or deletion of *alfB* (3) results in growth defect, one role of AlfB is to keep *alf* operon expression below a certain level so that appropriate amounts of AlfA and AlfB are produced.

Becker et al showed that, after replication, pBET131 enters the forespore before septum formation (3). They also showed that AlfA filaments extend to the cell pole, and a pBET131 derivative carrying the *oriN* and *parN* regions is translocated to the prespore that is formed at an extreme end of the mother cell. AlfA has a partial amino acid sequence similarity to ParM encoded on *E. coli* plasmid R1 (3). A model has been presented for the plasmid maintenance of R1, according to which the ParR
protein binds to the centromere-like sequence parC, forming a partitioning complex, and ATP-bound ParM molecules attach to the complex via ParR and push the plasmid copies toward the cell poles as they grow in filaments in the cell (6, 27, 28, 29, 30).

The experiments with a yeast two-hybrid system revealed that the AlfA and AlfB proteins interact not only with each other but also among themselves (Fig. 8). The interaction between the AlfA molecules was expected from the previous observation that they form filaments in the cell (3). On the other hand, the interaction between AlfA and AlfB, and the formation of a complex between AlfB and parN suggest the possibility of formation of a ternary complex among them. An attempt to detect binding between a parN-containing DNA fragment and partially purified His-tagged ParA was unsuccessful (T. Tanaka, unpublished data), suggesting that parN may not interact directly with AlfA. Thus, it is possible that another role of ParB is to interact with ParA for partition.

Taken together, and by analogy with the partition mechanism of plasmid R1, it is likely that AlfB and parN form a DNA-protein complex, to which AlfA binds successively to form actin-like filaments, presumably pushing the AlfB-parN complex toward the cell pole. Although the overall partition mechanism of pBET131 may be similar to that of R1, they are different in details: parR carries ten 10-bp repeats (38) as opposed to three 8-bp repeats in parN, the AlfA filaments are more stable than the ParM filaments, and AlfA forms highly twisted, helical filaments (35).

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REFERENCES


Fig. 1. Localization of the DNA region responsible for the segregational stability of pBET131. Plasmid stability was determined as described in Materials and Methods, and is shown as % plasmid loss per generation. (A) Restriction map of pBET131. The DNA region between the BamHI and HindIII sites was derived from pLS32 (46). The open arrows show the open reading frames on pBET131. Abbreviations: Ba, BamHI; Bg, BglII; Cl, ClaI; Cs, Csp45I; Hi, HindIII; Nh, NheI; Nr, NruI; Sa, SalI; Sc, SacI; Sn, SnaBI; Xh, XhoI. (B) Derivatives of pBET131 carrying modifications in the upstream region of alfA. The rectangle and the dotted lines show the Pspac promoter and the deleted region between positions 3033 and 3060, which contains part of the promoter for the alf operon (see Fig. 2B), respectively. The three arrows depict the tandem repeat sequences located between positions 3000 and 3031. (C) Derivatives of pBET131 carrying synthetic 55-nucleotide sequences between the BamHI and SnaBI (position 3032) sites of pBET131. The arrows and crosses show the tandem repeats and nucleotide changes, respectively.

Fig. 2. Determination of the transcriptional start site of the alf operon. (A) Primer extension analysis. The transcriptional start point and the putative –10 sequence are shown by the asterisk and bar, respectively. Numbers on the left show the coordinates starting from the BamHI site of pBET131 (Fig. 1A; 46). (B) The nucleotide sequence starting from the Csp45I site (position 2843) to the 7th codon of alfA on pBET131. The numbers with the bent arrows above and below the sequence show the 5′ and 3′ ends of the DNA fragments, respectively, that were used for gel shift analysis. The three arrows, bars and rectangle depict the tandem repeats, promoter and Shine-Dalgarno sequences, respectively.
Fig. 3. Derivatives of pDG148 carrying alfA and alfB downstream of the IPTG-inducible Pspac promoter. The solid bars depict the DNA regions carried in the plasmids. The filled triangles show the SacI sites that were blunt-ended with T4 DNA polymerase and ligated. For the abbreviations of the restriction enzymes, see the legend to Fig. 1. oriB, replication origin for B. subtilis; oriE, replication origin for E. coli.

Fig. 4. Repression of alfA expression by AlfB. The cells of strain LA213 (amyE::alfA-lacZ) carrying the plasmids were cultured in the presence of Cm, Nm and IPTG, and the β-galactosidase activities were determined as described in MATERIALS and METHODS. Structure of the plasmids is shown in Fig. 3. ○, pDG148; ●, pENSA51; □, pEXSA564.

Fig. 5. Purification of His-tagged AlfB (A) and determination of the 5’ (left panel) and 3’ (right panel) boundaries of the AlfB binding site by using EMSA on fragments bearing various deletions upstream of alfA (B). (A) SDS polyacrylamide gel electrophoresis of His-tagged AlfB. Lanes: 1, purified sample; 2, molecular weight markers (from the top in kDa; 16,950, 14,440, 10,600, 8,160, 6,210, 2,520). (B) The 290-bp fragments used are shown below the panel (see also Fig. 2B for exact positions). The arrowheads and brackets to the left of the panels indicate the 290-bp DNA fragments at their original and shifted positions, respectively. The other three DNA bands indicated by the dots are the RsaI fragments of pUC19 (from the top in bp; 1769, 676, and 241). + and – show the presence and absence of His-tagged AlfB in the reaction mixture. The HaeIII fragments of φX174 DNA were run as size markers (M). The arrows in the scheme depict the tandem repeat sequences. The reaction conditions are described
in Materials and Methods except that 0.3 µg of His-tagged AlfB was used.

The DNA fragments were prepared by using the PCR primers (shown in Table S1) as follows: a, BET2965F and BET3255R; b, BET2992F and BET3290R; c, BET3009F and BET3299R; d, BET3021F and BET3315R; e, BET3032F and BET3330R; f, BET2702F and BET2992R; g, BET2718F and BET3009R; h, BET2731F and BET3021R; i, BET2742F and BET3032R; j, BET2754F and BET3044R; k, BET2762F and BET3052R.

Fig. 6. DNase I footprinting analysis of the binding region of His-tagged AlfB. DNA fragments labeled with biotin at either position 2871 (left) or position 3131 (right) were subjected to footprinting analysis as described in MATERIALS and METHODS. The open triangles above the panels show the increment of His-tagged AlfB (from left to right; 0, 0.3, 0.9 and 2.7 µg). The reaction conditions are described in MATERIALS and METHODS. The arrows and brackets denote the tandem repeats and the protected regions from DNase I, respectively. The numbers to the left of each panel show the nucleotide positions from the BamHI site of pBET131 (see Fig. 1A and 2B).

Fig. 7. Binding of His-tagged AlfB to the 290-bp wild-type and mutant DNA fragments containing the tandem repeat region. Various 290-bp DNA fragments were prepared using primers PBR358 and 3263R2, and the plasmids pBIBW2, pBIBM19, pBIBM24, pBIBM34 and pBIBM8 as the templates. The reaction conditions are described in MATERIALS and METHODS except that the reaction mixture contained 0 µg (lanes 2, 6, 10, 14, 18), 0.1 µg (lanes 3, 7, 11, 15, 19), 0.2 µg (lanes 4, 8, 12, 16, 20) and 0.3
µg (lanes 5, 9, 13, 17, 22) of His-tagged AlfB. Lanes 1 and 22: size markers (HaeIII fragments of φX174 DNA). The arrow and bracket to the left of the panel indicate the 290-bp DNA fragments at their original and shifted positions, respectively.

Fig. 8. Yeast two-hybrid system displaying the interaction between AlfA and AlfB (A) and among themselves (B). The AH109 transformants were grown overnight in minimal SD medium supplemented with a dropout (DO) supplement lacking Leu and Trp, centrifuged and resuspended in SD medium. The suspensions were patched on X-α-gal-containing SD plates with DO supplements lacking either Leu and Trp (-LT) or Ade, His, Leu and Trp (-AHLT). (A) Lanes: 1, AH109 carrying pGBA3 (alfA) and pGAB142 (alfB); 2, pGBA3 (alfA) and pGADT7 (vector); 3, pGBK7 (vector) and pGAD142 (alfB); 4, pGBK7 (vector) and pGADT7 (vector).

(B) Lanes: 5, AH109 carrying pGBA3 (alfA) and pGA51 (alfA); 6, pGAB142 (alfB) and pGB64 (alfB); 7, pGA51 (alfA) and pGBK7 (vector); 8, pGB64 (alfB) and pGADT7 (vector).
### Table 1. Bacterial strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. subtilis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CU741</td>
<td><em>trpC2 leuC7</em></td>
<td>48</td>
</tr>
<tr>
<td>LA213</td>
<td>*trpC2 leuC7 amyE:*alfA-lacZ (Cm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>pOF5LA2 x CU741</td>
</tr>
<tr>
<td>WILA2</td>
<td>LA213 derivative with <em>alfA</em> preceded by synthetic, wild-type repeat sequences</td>
<td>pWILA2 x CU741</td>
</tr>
<tr>
<td>LMLA2</td>
<td>WILA2 derivative carrying an alteration in the first repeat</td>
<td>pLMLA2 x CU741</td>
</tr>
<tr>
<td>MDLA13</td>
<td>WILA2 derivative carrying alterations in the first and second repeats</td>
<td>pMDLA13 x CU741</td>
</tr>
<tr>
<td>RMLA11</td>
<td>WILA2 derivative carrying an alteration in the third repeat</td>
<td>pRMLA1 x CU741</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM103</td>
<td>Δlac-pro thi rpsL supE sbcB hsdR4 F' [traD36 proAB&lt;sup&gt;+&lt;/sup&gt; lacF&lt;sup&gt;+&lt;/sup&gt; lacZΔM15]</td>
<td>51</td>
</tr>
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<td><strong>S. cerevisiae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AH109</td>
<td>MATα trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ lys2::GAL1_UAS-GAL1_TATA-HIS3 GAL2_UAS-GAL2_TATA-ADE2 URA3::MEL1_UAS-MEL1_TATA-lacZ</td>
<td>Clontech</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pDH88</td>
<td>Integration vector carrying Pspac promoter</td>
<td>16</td>
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<tr>
<td>pDG148</td>
<td>Cm&lt;sup&gt;+&lt;/sup&gt; Pspac promoter before muticloning sites</td>
<td>42</td>
</tr>
<tr>
<td>pQE8</td>
<td>Expression vector for construction of His-tagged proteins</td>
<td>QIAGEN</td>
</tr>
<tr>
<td>pIS284</td>
<td>Delivers promoter-E. coli lacZ fusion to B. subtilis amyE locus, and carries EcoRI, BamHI and XbaI sites in this order upstream of the lacZ gene</td>
<td>I. Smith</td>
</tr>
<tr>
<td>pBET131</td>
<td>Wild-type plasmid (Fig. 1A)</td>
<td>46</td>
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<tr>
<td>pBIF2965</td>
<td>pBET131 carrying Pspac promoter between positions 1 and 2964</td>
<td>This study</td>
</tr>
<tr>
<td>pBJ6561</td>
<td>pBIC2965 carrying a deletion spanning positions 3033 to 3060</td>
<td>This study</td>
</tr>
<tr>
<td>pBIF6561</td>
<td>pBIC6561 without Pspac promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pBI2A2</td>
<td>pBET131 carrying a synthetic, wild-type DNA between BamHI and SnaBI sites</td>
<td>This study</td>
</tr>
<tr>
<td>pBIBM6</td>
<td>pBET131 carrying a deletion between the BamHI and SnaBI sites</td>
<td>This study</td>
</tr>
<tr>
<td>pBIBM19</td>
<td>pBIA2 carrying nucleotide alterations in the first repeat</td>
<td>This study</td>
</tr>
<tr>
<td>pBIBM24</td>
<td>pBIA2 carrying nucleotide alterations in the second repeat</td>
<td>This study</td>
</tr>
<tr>
<td>pBIBM34</td>
<td>pBIA2 carrying nucleotide alterations in the third repeat</td>
<td>This study</td>
</tr>
<tr>
<td>pBIBM8</td>
<td>pBIA2 carrying nucleotide alterations in both the first and second repeat sequences</td>
<td>This study</td>
</tr>
<tr>
<td>pDGEM51</td>
<td>pDG148 carrying <em>alfA</em> downstream of Pspac promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pENS51</td>
<td>pDGEM51 carrying blunt-ended SacI site in <em>alfA</em></td>
<td>This study</td>
</tr>
<tr>
<td>pDGEX561</td>
<td>pDG148 carrying <em>alfA</em> and <em>alfB</em> downstream of Pspac promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pEXS564</td>
<td>pDGEX561 carrying blunt-ended SacI site in <em>alfA</em></td>
<td>This study</td>
</tr>
<tr>
<td>pOF5L2A</td>
<td>pIS284 carrying the promoter region of <em>alfA</em> before lacZ</td>
<td>This study</td>
</tr>
<tr>
<td>pWILA2</td>
<td>pORF5LA2 derivative carrying synthetic, wild-type repeat sequences</td>
<td>This study</td>
</tr>
<tr>
<td>pLMLA2</td>
<td>pWILA2 derivative carrying an alteration in the first repeat</td>
<td>This study</td>
</tr>
<tr>
<td>pMDLA13</td>
<td>pWILA2 derivative carrying alterations in the first two repeat sequences</td>
<td>This study</td>
</tr>
<tr>
<td>pRMLA1</td>
<td>pWILA2 derivative carrying an alteration in the third repeat</td>
<td>This study</td>
</tr>
<tr>
<td>pQE8OF6</td>
<td>pQE8 carrying <em>alfB</em></td>
<td>This study</td>
</tr>
<tr>
<td>pGBKT7</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt;, cloning vector carrying GAL4 DNA-binding domain (BD)</td>
<td>Clontech</td>
</tr>
<tr>
<td>pGADT7</td>
<td>Amp&lt;sup&gt;+&lt;/sup&gt;, cloning vector carrying GAL4 activation domain (AD)</td>
<td>Clontech</td>
</tr>
<tr>
<td>pGBA3</td>
<td>pGBK7 carrying a fusion between <em>alfA</em> and GAL4 DNA-BD</td>
<td>This study</td>
</tr>
<tr>
<td>pGAB142</td>
<td>pGADT7 carrying a fusion between <em>alfB</em> and GAL4 AD</td>
<td>This study</td>
</tr>
<tr>
<td>pGB64</td>
<td>pGBK7 carrying a fusion between <em>alfB</em> and GAL4 DNA-BD</td>
<td>This study</td>
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<tr>
<td>pGA51</td>
<td>pGADT7 carrying a fusion between <em>alfA</em> and GAL4 AD</td>
<td>This study</td>
</tr>
<tr>
<td>pGBK7-53</td>
<td>Carrying a fusion between murine p53 and GAL4 DNA-BD in pGBK7</td>
<td>Clontech</td>
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<tr>
<td>pGADT7-T</td>
<td>Carrying a fusion between SV40 large T-antigen and GAL4-AD in pGADT7</td>
<td>Clontech</td>
</tr>
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</table>
TABLE 2. Effect of overproduction of AlfB on expression of alf-lacZ carrying sequence alterations in the upstream repeat sequences

<table>
<thead>
<tr>
<th>Strain(^{a})</th>
<th>Sequence alteration(^{a})</th>
<th>IPTG</th>
<th>β-galactosidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>WILA2</td>
<td>Wild</td>
<td>-</td>
<td>381</td>
</tr>
<tr>
<td>WILA2</td>
<td>Wild</td>
<td>+</td>
<td>51</td>
</tr>
<tr>
<td>LMLA2</td>
<td>Rp1</td>
<td>-</td>
<td>457</td>
</tr>
<tr>
<td>LMLA2</td>
<td>Rp1</td>
<td>+</td>
<td>54</td>
</tr>
<tr>
<td>MDLA13</td>
<td>Rp1 and 2</td>
<td>-</td>
<td>416</td>
</tr>
<tr>
<td>MDLA13</td>
<td>Rp1 and 2</td>
<td>+</td>
<td>393</td>
</tr>
<tr>
<td>RMLA11</td>
<td>Rp3</td>
<td>-</td>
<td>717</td>
</tr>
<tr>
<td>RMLA11</td>
<td>Rp3</td>
<td>+</td>
<td>154</td>
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</tbody>
</table>

\(^{a}\)Rp1, 2 and 3 refer to the sequence alterations in the first, first and second, and third repeat sequences, respectively.

Experimental conditions are described in MATERIALS and METHODS except that the media contained Cm. The levels of the β-galactosidase activity in Miller units were determined for the samples taken from T-1 to T4, and the highest values attained at either T0 or T1 are shown. The data set is from one of two experiments, in which the variations of the enzyme levels were within 15%.
FIG. 1

(A) Diagram showing the deletion sites and plasmid loss.

(B) Table showing plasmid loss rates:

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Loss Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBET131</td>
<td>1.3</td>
</tr>
<tr>
<td>pBIF2965</td>
<td>1.4</td>
</tr>
<tr>
<td>pBIC6561</td>
<td>1.2</td>
</tr>
<tr>
<td>pBIF6561</td>
<td>10.3</td>
</tr>
</tbody>
</table>

(C) Diagram showing synthetic DNA deletion sites and associated plasmids:

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Loss Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBIAW2</td>
<td>1.9</td>
</tr>
<tr>
<td>pBIBM6</td>
<td>11.3</td>
</tr>
<tr>
<td>pBIBM19</td>
<td>1.9</td>
</tr>
<tr>
<td>pBIBM24</td>
<td>12.4</td>
</tr>
<tr>
<td>pBIBM34</td>
<td>1.9</td>
</tr>
<tr>
<td>pBIBM8</td>
<td>10.0</td>
</tr>
</tbody>
</table>
Fig. 2

(A) TTCGAATTTCACAATATCCTACAAATGGACCAAATCC
TATTCAAAGTCCATTATAAGTTAAGGGAAATAAAAATAAAAGGGTTTTCTTTAAATTAGT
CAAGAATTAAGAAAAAATATTTTTTCTGTTCATTTTTTCATTTTGCGATTAGATAATAAT
TTATTTAAGAGTTTATTTAAAAAATTATTTACGTATGAATAACTTTCCATTATAATTGAA
ATGAGTTCCAACTAAAGAAAACTAAGGAGATGCTTTAACTTGACACTAACTACTGTAATT

(B) alf4 alfA alfB orf7

2843 (Csp45I)
TTCAATTTCACAATTTCTAATTTTAAATG

2881 TATTTCAAGTGCCATTATAAGTTAAGGGAATAAATAAAATAGGGTTTTCTTTAAATTAGT
2941 CAAGAATTAAGAAAAATATTTCTGTTTTCTTTCATTTTTCAATTTGTGATAGATAATAAT
3001 TTATTTAAGATTTTATTTAAAATATTTACGTATGAAATAACTTTCAATTTATAATTGA
3061 ATGAGTTCCAACTAAAGAATTTAAGGAGATGCTTTAATCTTGACACTATACTGTAATT

SD (alfA) M T L T T V I

2881 2941 3001 3061
Fig. 4b: β-galactosidase activity (Miller units)

T-1 T2 T0 T1 T3 T4
Fig. 5

(A) 1 2

(B) a b c d e M

f g h i j k

Positions

2702-2992
2718-3009
2731-3021
2742-3032
2754-3044
2762-3052

2965-3255
2992-3290
3009-3299
3021-3315
3032-3330

a b c d e M

f g h i j k

2702-2992
2992-3290
3009-3299
3021-3315
3032-3330

alfA
Fig. 7

290-bp DNA from pBIAW2 pBIBM19 pBIBM24 pBIBM34 pBIBM8

Lane: AlfB

Mutation in repeats
Fig. 8

(A) 1 2 3 4
-LT
-AHLT

(B) 5 6 7 8
-LT
-AHLT