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Replication and Segregational Stability of *Bacillus* Plasmid pBAA1

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A cryptic plasmid, pBAA1, was identified in an industrial *Bacillus* strain. The plasmid is 6.8 kilobases in size and is present in cells at a copy number of approximately 5 per chromosome equivalent. The plasmid has been maintained under industrial fermentation conditions without apparent selective pressure and so is assumed to be partition proficient. The minimal replicon was localized to a 1.4-kilobase fragment which also contains the functions required for copy number control. The very low level of segregational instability of the minimal replicon suggests that it also contains functions involved in plasmid maintenance. Comparison with other plasmids indicates that pBAA1 belongs to the group of small gram-positive plasmids which replicate by a rolling cycle-type mechanism. A sequence was identified which is required for the efficient conversion of the single plus strand to the double-stranded form during plasmid replication. Deletion of this sequence resulted in a low level of segregational plasmid instability.

The development of cloning vectors for use in *Bacillus subtilis* has relied on plasmids isolated from *Staphylococcus aureus* (6, 7, 14, 15). Both structural (10, 15, 19, 20, 27) and segregational (1, 2, 10, 19, 28) instabilities were frequently observed when recombinant vectors were transformed into *B. subtilis*. These instabilities led to intense investigation of the mode of replication and of the stability functions of these plasmids. It has emerged that many of these plasmids replicate by a rolling cycle-type mechanism (11, 12, 31). The essential features of this mode of replication are (i) an origin of plus-strand synthesis, (ii) a replication protein which interacts with the plus origin to generate a nick which allows displacement synthesis of the plus strand to occur, and (iii) a signal for efficient conversion of the single strand to the double-stranded form. These features have been identified for a variety of plasmids, including pT181 (12, 16, 25) and pC194 (11, 12).

Our aim is to analyze the segregational instability of plasmids in *B. subtilis*. It was hypothesized that the segregational instability of many *S. aureus* plasmids in *B. subtilis* is due, at least in part, to a suboptimal host-plasmid relationship. Thus, we chose to analyze the stability functions of a plasmid, pBAA1, which was resident in an industrial strain of *B. subtilis*. pBAA1 has been maintained under industrial fermentation conditions without apparent selection pressure. Preliminary experiments demonstrated that the copy number was low (approximately 5 per chromosome equivalent), so it was assumed that pBAA1 must encode an active partitioning function. In this paper, we report that all of the functions required for replication, copy number control, and partitioning are located on a 2.2-kilobase (kb) fragment. The DNA sequence and other evidence show that pBAA1 replicates by a rolling-circle mechanism and that some features of its replication functions are related both to ϕX174 and to the gram-positive plasmids pC194, pUB110, and pFTB14.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. Bacteria were grown in LB (22). Medium was supplemented with antibiotics to the following final concentrations: ampicillin, 50 μg/ml; chloramphenicol, 5 μg/ml.

Manipulation of DNA. All manipulations of DNA were performed as described by Maniatis et al. (21). All enzymes were purchased from commercial suppliers. Sequencing was carried out by the dideoxy-chain termination method of Sanger et al. (29). A sequencing kit was used (Amersham Corp., U.K.); reactions were carried out according to the instructions of the manufacturer. In some cases, 17-mer synthetic oligonucleotides (synthesized at the University of Manchester Institute of Science and Technology) were used as primers.

Analysis of single-stranded DNA production. Lysates were prepared by the method of te Riele et al. (31). Lysates were analyzed by electrophoresis in 0.8% agarose gels containing 0.5 μg of ethidium bromide per ml for approximately 16 h at 4 V/cm. To detect single-stranded plasmid DNA only, gels were transferred to Biodyne (Pall Ultrafine Filtration Corp., East Hills, N.Y.) without prior denaturation by the method of te Riele et al. (31). All other gels were denatured with NaOH prior to transfer. DNA was transferred to Biodyne by diffusion with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) according to the instructions of the manufacturer. Prehybridization, hybridization, and washing were performed according to method A of the manufacturer. Autoradiograms were made with RX medical X-ray film (Fuji Photo Film, Japan) with intensifying screens at room temperature.

Copy number determinations. Plasmid copy numbers were determined as follows. Total DNA was prepared separately from cells containing the plasmids of interest and was double digested with *Bam*HI-*Hin*dIII, which cuts the plasmids to give a pBAA1 fragment of approximately 2.2 kb. This double digestion cuts chromosomal DNA, releasing a fragment of approximately 4 kb which contains the β-glucanase gene. The digested DNA was separated on a 1.0% agarose gel and was transferred to Biodyne. This filter was then hybridized with a nick-translated pJG14 plasmid (which contains both 1.5 kb of β-glucanase DNA and 1.4 kb of pBAA1 DNA). The filter was then washed and exposed to X-ray film. The developed film was scanned densitometrically with a chro-

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TABLE 1. Strains and plasmids used

Strain or plasmid	Description	Source or reference
E. coli tg1	K-12 Δ(*lac-pro*) *supE thi hsdR* F' *traD36 proAB lacI lacZ*ΔM15	Amersham
B. subtilis		
SO113	*trpC2 amy-3*	26
8G-5	*trpC2 tyr ade his nic ura rib met* highly transformable derivative of *B. subtilis* 168	3
pBAA1	6.8-kb cryptic plasmid isolated from *B. subtilis* BAA1	Biocon Ltd., Ireland; this work
pDE68	pBAA1 with the Cm^r gene from pBD64 cloned into an *Eco*RI site (7.8 kb)	This work
pDE37	The 3.7-kb *Eco*RI fragment of pBAA1 plus the Cm^r gene of pBD64 (4.7 kb)	This work
pDE22	The 2.2-kb *Hin*dIII fragment of pBAA1 plus the Cm^r gene of pBD64 (3.2 kb)	This work
pDE14	The 1.4-kb pBAA1 *Hin*dIII-*Bam*HI fragment in pRP14 plus the Cm^r gene of pBD64. This plasmid underwent a 100-bp deletion at the *Bam*HI site which does not detectably affect the replication proficiency copy number or chloramphenicol resistance of the plasmid (2.3 kb)	This work
pBD64	Plasmid derived from pUB110 encoding Km^r and Cm^r	13
pRP1	Replicon probe plasmid derived by insertion of the *Hpa*II fragment of pBD64 containing the Cm^r gene into the *Eco*RI site of pUC18	This work
pRP2	Replicon probe plasmid derived by insertion of the *Hpa*II fragment of pBD64 containing the Cm^r gene into the *Acc*I site of pUC18	
pRP3	Replicon probe plasmid derived by cutting pRP2 with *Sma*I and inserting a *Hin*dIII linker	This work
pJG14	pRP14 containing a 1.5-kb fragment on which is located the β-glucanase gene	This work
pUC18		32
M13mp18		24
M13mp19	M13-derived vectors used in the dideoxy sequencing method	
M13tg130		17
M13tg131		

matogram densitometer CD50 (DESAGA, Heidelberg, Federal Republic of Germany) with an integrator to estimate peak area to determine the intensities of signals from the β-glucanase and pBAA1 fragments. Three quantities of total DNA (0.1, 1.0, and 4 μg) were loaded onto the gel, and the filter was exposed for various lengths of time to ensure that the signals were within the linear range of response of the film. The similar sizes of the β-glucanase and pBAA1 fragments in the gel ensure that the efficiencies of transfer of both fragments to Biodyne should be very similar, if not identical. Likewise, the similar sizes of the β-glucanase and pBAA1 fragments on the probe plasmid ensure comparable efficiencies of hybridization. Since the probe contains both the β-glucanase and pBAA1 homologous DNAs, the specific activity of the probe is identical for both species of DNA. Thus the ratio of signals of pBAA1 to β-glucanase is an estimate of the number of copies of pBAA1 per chromosome equivalent.

Analysis of the segregational stability of plasmids. Cells containing plasmids were grown in LB in the presence of antibiotic. These cells were used to inoculate antibiotic-free medium. Ten successive transfers (approximately 100 generations) were performed in antibiotic-free medium over a period of 80 h. Approximately 10^5 cells were transferred at each stage. The cells were then plated on antibiotic-free LB agar, and individual colonies were tested for resistance to antibiotic. At every second transfer, 100 colonies were tested for each plasmid. Plasmids whose stabilities were to be compared were always analyzed in the same experiment.

RESULTS

Delimitation of the minimal replicon of pBAA1. Plasmid pBAA1 was isolated from an α-amylase-hyperproducing *Bacillus* strain. It is 6.8 kb in size, and its restriction map is shown in Fig. 1a. This plasmid has no known selectable marker but has been maintained stably under industrial fermentation conditions without apparent selection pressure. When marked with a chloramphenicol resistance gene (derived from pC194) and transformed into *B. subtilis*, the resultant plasmid pDE68 was found to be segregationally stable. The copy number of pBAA1 is approximately 5 per chromosome equivalent. It is thus assumed that the plasmid is stably maintained by an active partitioning system.

To delimit the minimal replicon, fragments of pBAA1 were subcloned onto the replicon probe plasmid pRP1 (Fig. 1b) and were tested for their ability to support replication by transformation into *B. subtilis* and selection for antibiotic-resistant transformants. It was found that plasmids pRP37, pRP22, and pRP17 containing 3.7, 2.2, and 1.7 kb, respectively, of pBAA1 replicated in *B. subtilis*. To further delineate the minimal replicon, progressive deletion of each end of the 1.7-kb *Hae*III fragment of pBAA1 was carried out by using *Bal* 31 (Fig. 1c). The precise endpoints of the *Bal* 31-deleted derivatives were determined by sequencing. Results showed that deletion of 350 base pairs (bp) from one end of the fragment allowed replication, whereas deletion of a further 230 bp (region A) abolished the ability to replicate. Similarly, deletion of 356 bp (region B) from the other end of the fragment abolished the ability to replicate. To test whether pUC18 contained on the pRP plasmids contributed to their replication proficiency, plasmids pDE68, pDE37, pDE22, and pDE14 were constructed (Table 1). All four pDE plasmids were also replication proficient. Thus, pUC18 does not affect the replication proficiency of the pRP plasmids. It was concluded that the minimal replicon of pBAA1 is contained on a 1.4-kb fragment and that regions A and B contain functions essential for replication of pBAA1 in *B. subtilis*.

Sequence of the 2.2-kb *Hin*dIII fragment of pBAA1 which contains the minimal replicon. The 2.2-kb *Hin*dIII fragment of pBAA1 was sequenced by the dideoxy-chain termination

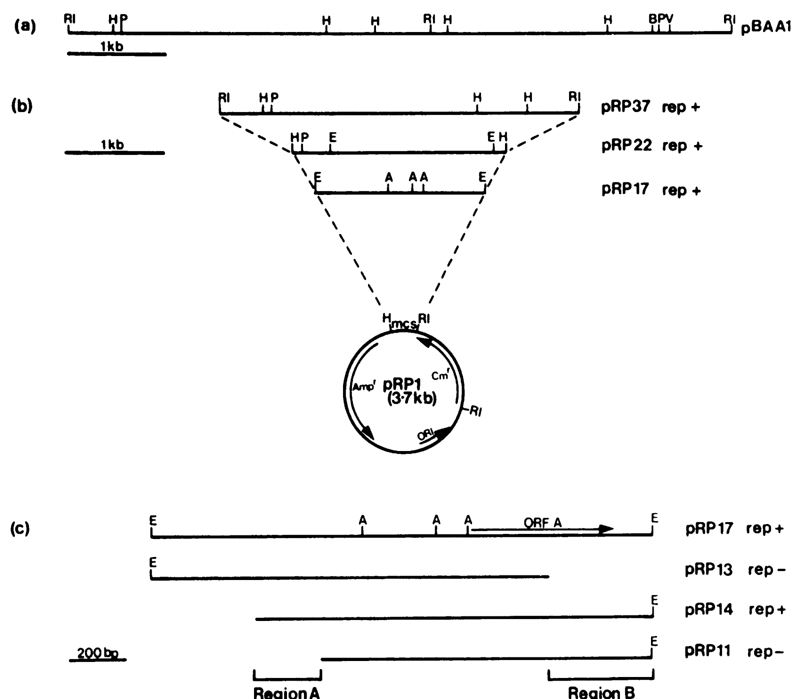

FIG. 1. (a) Linearized restriction map of plasmid pBAA1. (b) The 3.7-kb *Eco*RI, 2.2-kb *Hin*dIII, and 1.7-kb *Hae*III fragments of pBAA1 were inserted into compatible restriction sites in the multiple cloning site of the replicon probe plasmid pRP1 and were tested for their replication proficiency in *B. subtilis*. (c) pRP17 was separately digested with *Hin*dIII and *Bam*HI and digested with *Bal* 31 to delimit the minimal replicon. After end filling and ligation, the plasmids were transformed into *Escherichia coli* and the sizes of the deletions were characterized. A series of plasmids with increasing amounts of pBAA1 deleted were tested for their ability to replicate in *B. subtilis*. Deletion of region A or B abolished the ability to replicate in *B. subtilis*. The sequence of the regions essential for replication is given in Fig. 3. rep^+ and rep^-, Replication proficient and deficient, respectively. A, *Rsa*I; B, *Bam*HI; E, *Hae*III; H, *Hin*dIII; P, *Pst*I; RI, *Eco*RI; V, *Pvu*II.

method of Sanger et al. (29). Shotgun-cloned *Alu*I and *Rsa*I fragments and *Bal* 31-generated deletion derivatives were sequenced. Synthetic oligonucleotides were used as primers to sequence regions of nonoverlap. Both strands were sequenced, and each base pair was sequenced at least four times. The sequence is shown in Fig. 2. Analysis of the sequence reveals an open reading frame (ORF A) of 307 amino acids. ORF A is preceded by a putative σ^{43} promoter (P1). Two lines of evidence indicate that this protein is involved in replication of pBAA1. First, deletion of region B, which extends 334 bp from the 3′ end of the open reading frame (Fig. 2, region B), abolishes the replication proficiency of the plasmid. The second line of evidence derives from a comparison of this protein with the replication proteins of pUB110, pC194, and pFTB14 (23) (Fig. 3). The pBAA1-encoded protein is 92% homologous at the amino acid level with the replication protein of pFTB14 (homology not shown) and homologous to a lesser though very significant extent with the replication proteins of pUB110 and pC194 (Fig. 3). Thus, the protein coded by the open reading frame of pBAA1 is designated RepA.

A 230-bp fragment (Fig. 1c and 2, region A) was also shown to be essential for replication of pBAA1. Analysis of the sequence in this region shows four sets of inverted repeats ranging in size from 4 to 14 bp. To discover the sequences essential for replication, a homology search was performed to determine whether there are sequences within this region conserved among other *Bacillus* and *S. aureus* plasmids known to replicate in *B. subtilis*. Results (Fig. 4) showed that a 15-bp sequence was conserved in pBAA1, pUB110, pC194, and pFTB14. No significant homology was found with pT181, pC221, or pE194. This conserved sequence is found in the 55-bp region of pC194 shown by Gros et al. (11) to have origin activity. Within this 15-bp conserved sequence are two additional features. (i) The sequence CTTGATA is the sequence at which nicking of the plus strand occurs in the initiation of replication of the coliphage ϕX174. (ii) An 18-bp sequence (containing these conserved 15 bp) has been shown by Gros et al. (11) to contain a signal sufficient to terminate replication of plus-strand synthesis. These data strongly suggest that this 15-bp conserved region is part of the origin of pBAA1 and is one of the features within region A which is important for replication. In addition, by analogy with ϕX174 and pC194, it suggests that pBAA1 replicates by a rolling circle-type mechanism.

Copy number determination of parental and deletion derivatives of pBAA1. The copy number of pBAA1 and the deletion derivatives were determined as described in Materials and Methods. Densitometric scanning of the autoradiogram indicates that the copy number of pBAA1 in the industrial strain BAA1 is 2 to 3 chromosome equivalents. The copy numbers of pDE68, pDE37, pDE22, and pDE14 in the laboratory strain SO113 are the same as that of the parental plasmid in the industrial strain BAA1. Under these conditions, the copy number of pC194 is 11 per chromosome equivalent, approximately half the reported value. If normalized to the reported value of pC194, then the copy number of pBAA1 and its derivatives is approximately 4 to 6 per chromosome equivalent. It can be concluded that the minimal replicon of pBAA1 contains the functions required for copy number control.

Some deletion derivatives of pBAA1 produce single-

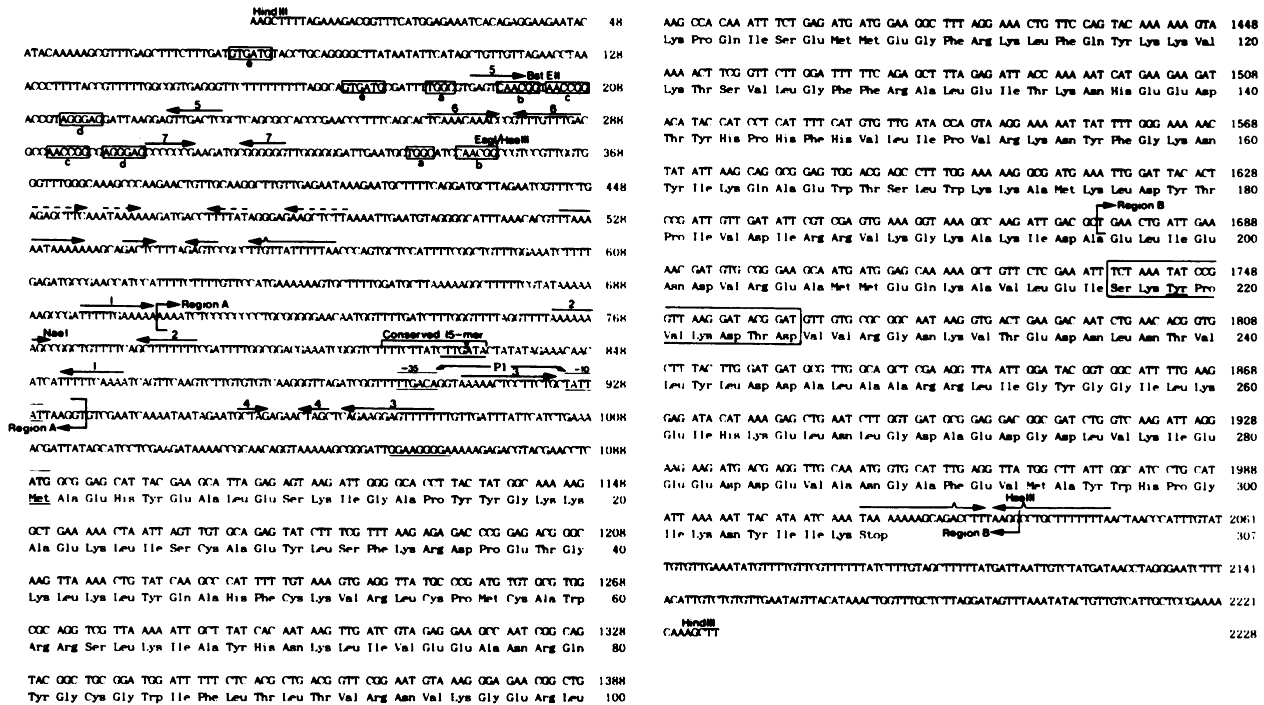

FIG. 2. Sequence of the 2.2-kb *Hin*dIII fragment of pBAA1. Regions A and B, which are essential for replication, are bracketed. Inverted repeats are marked with arrows directly over the sequence, while direct repeats are boxed. The putative active site of the protein is boxed, and the conserved tyrosine residue is underlined. P1 is a consensus σ^{43} promoter; a putative ribosome-binding site is underlined. The sequence conserved in plasmids pC194, pUB110, and pFTB14 (Conserved 15-mer) is shown; the arrow within this sequence indicates the site at which nicking of the plus strand occurs in phage φX174.

stranded plasmid DNA. In the rolling-circle model of plasmid replication, the plus strand is synthesized as a covalently closed circular single strand of DNA. Synthesis of the second strand then initiates at a minus-strand origin sequence, generating a double-stranded plasmid DNA molecule (for a review, see reference 18). Such minus origins, which are characterized by their potential for secondary structure formation, have been identified for the coliphages M13 (9), G4 (8) and φX174 (30). In addition, minus-origin sequences have been identified for a variety of plasmids, e.g., pT181, pC221, and pC194 (12). Deletion of the minus-origin sequence leads to the accumulation of single-stranded plasmid DNA. If pBAA1 replicates by a rolling cycle-type mechanism, as is suggested by the homology to φX174 and pC194, then (i) it should have a minus origin and (ii) deletion of this minus origin should lead to the accumulation of single-stranded plasmid DNA. To determine the location of the minus origin of pBAA1, cells containing the pRP and pDE series of plasmids were tested for the presence of single-stranded plasmid DNA, as described in Materials and Methods. Separate gels were transferred to Biodyne with or without prior denaturation of the DNA with NaOH (31). In the absence of denaturation, only single-stranded nucleic acid binds to the filter. Figure 5 shows the results for the pDE series of plasmids. Plasmids pBAA1, pDE68, pDE37, pDE22, and pDE14 show two major hybridizing bands, probably corresponding to CCC monomeric and dimeric plasmid species. These bands did not bind to Biodyne in the absence of denaturation. Plasmid pDE14, in addition to these bands, has a band (arrow) which migrated ahead of the double-stranded DNA species and which bound to Biodyne in the absence of denaturation. This fast-migrating band is preferentially degraded by S1 nuclease under conditions which cause no detectable degradation of the double-stranded forms (data not shown). These data indicate that this band is single-stranded plasmid DNA. For the pRP series of plasmids, the single-stranded plasmid DNA was observed in cells containing pRP17 but not in cells containing pRP22 or pRP37 (data not shown). Thus, sequences present on the 2.2-kb *Hin*dIII fragment but absent from the 1.7-kb *Hae*III fragment are required for normal replication of the plasmid.

Two regions of DNA are deleted from the 2.2-kb *Hin*dIII fragment to give the 1.7-kb *Hae*III fragment: a 355-bp fragment from the left-hand (5′) terminus and a 197-bp fragment from the right-hand (3′) terminus (Fig. 2). To determine the location of the minus origin on the 2.2-kb *Hin*dIII fragment, the sequences of these regions were analyzed. The 355-bp fragment deleted in the 1.7-kb *Hae*III subfragment has the potential for complex secondary structure formation. Since no unusual feature was evident in the 197-bp right-hand sequence, we decided to concentrate on the 355-bp sequence. Three unique restriction sites, *Bst*EII (200 bp), *Eag*I (353 bp), and *Nae*I (770 bp) were used to generate mutations in the left-hand end of the 2.2-kb *Hin*dIII fragment. Plasmid pRP22 was restricted with *Bst*EII, the ends were filled in, and the plasmid was religated, yielding pRP22F, which had a 5-bp insertion into the *Bst*EII site. Plasmid pRP22 was also separately double digested with *Bst*EII-*Eag*I and with *Eag*I-*Nae*I, the ends were made blunt, and the vector was religated. This yielded plasmids pRP22BE and pRP22EN, with 152- and 414-bp deletions, respectively. Whole-cell lysates were made of cells containing each of these constructions and were tested for the presence of single-stranded plasmid DNA. Results demonstrate that pRP22 and pRP22EN produce no detectable

FIG. 3. Comparison of the amino acid sequences of the replication proteins of pBAA1, pC194, and pUB110. The amino acids in boxes are conserved between the pBAA1 replication protein and the replication proteins of pC194 and pUB110. The amino acid sequence of the replication protein of pFTB14 is 92% homologous to that of pBAA1 (homology not shown). Dashes represent gaps inserted to optimize the protein alignment.

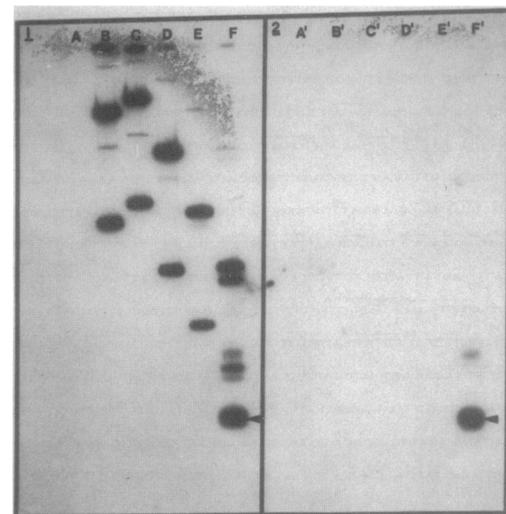

FIG. 5. Detection of single-stranded plasmid DNA in cells containing pBAA1 and its deletion derivatives. Whole-cell lysates of *B. subtilis* containing no plasmid (lanes A and A'), pBAA1 (lanes B and B'), pDE68 (lanes C and C'), pDE37 (lanes D and D'), pDE22 (lanes E and E'), or pDE14 (lanes F and F') were separated by agarose gel electrophoresis, denatured with NaOH (panel 1) or not denatured (panel 2), and transferred to Biodyne as described in Materials and Methods. The filter was then hybridized to ^{32}P-labeled pRP14. The band of single-stranded plasmid DNA (◀) is shown.

single-stranded plasmid DNA, whereas plasmids pRP17, pRP22F, and pRP22BE produce detectable quantities of single-stranded plasmid DNA (data not shown). Similarly, deletion of the *Bst*EII-*Eag*I fragment from plasmid pDE22 resulted in the production of single-stranded plasmid DNA (data not shown). Interestingly, the amount of single-stranded plasmid DNA produced by pRP22F is less than that produced by pRP22BE, suggesting that the 5-bp insertion attenuates but does not totally abolish this function. It can be concluded that sequences which when deleted result in the production of plasmid single-stranded DNA, overlap the *Bst*EII site (at bp 200; Fig. 3) but do not extend as far as the *Eag*I site (at bp 352; Fig. 2). Thus, a signal required for the efficient conversion of the single-stranded plasmid DNA to the double-stranded form during replication is located to this region.

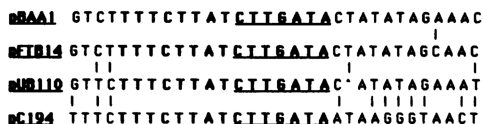

FIG. 4. Homology at the origin for plus-strand synthesis between plasmids pBAA1, pFTB14, pUB110, and pC194. The 15-bp sequence conserved in all four plasmids is in boldface. The sequence CTTGATA, which is the site at which nicking of the plus strand occurs in φX174, is underlined. Nicking occurs between the G and A residues of this sequence. Vertical lines indicate bases not homologous with pBAA1.

Segregational stability of pBAA1 and the deletion derivatives. Since pBAA1 was stably maintained under industrial fermentation conditions without apparent selective pressure, it is assumed to be segregationally stable. The segregational stabilities of the deletion derivatives were analyzed as described in Materials and Methods to detect plasmid-encoded functions involved in this process. Results (Table 2) demonstrate that pRP22 and pDE22 are totally stable under these conditions. Plasmids pRP17 and pDE14, both of which produce single-stranded plasmid DNA, displayed a low but reproducible level of instability, with 5 to 20% of the cells losing plasmid during the growth period. The deletion of the *Bst*EII-*Eag*I fragment from pRP22, which resulted in the production of single-stranded plasmid DNA, also resulted in instability. No instability was observed for the 5-bp insertion into the *Bst*EII site (pRP22F) despite the production of single-stranded plasmid DNA. It can be concluded that removal of the entire 355 bp *Hin*dIII-*Hae*III fragment results in a low level of instability. This instability does not, however, correlate with the production of single-stranded plasmid DNA.

TABLE 2. Segregational stability of pBAA1 derivatives

Plasmid	Production of ssDNA[a]	Stability (%)
pDE22	−	100
pDE14	+	79
pRP22	−	100
pRP17	+	95
pRP22F	+	100
pRP22BE	+	88
pRP22EN	−	98

[a] ssDNA, Single-stranded DNA. Symbols: +, production of ssDNA; −, no production.

DISCUSSION

A new plasmid, pBAA1, has been identified in an industrial *Bacillus* strain. Results presented in this study demonstrate that pBAA1 belongs to the class of small, naturally occurring plasmids of gram-positive bacteria. Although pBAA1 is different in size and restriction map from pLS11 (4) and pFTB14 (23), a high degree of homology exists between these plasmids in regions involved in plasmid replication. Homology also exists between the plus-strand origin and Rep protein regions of pBAA1 and those regions of pUB110 and pC194, the greater level of homology being found with pUB110. No homology was detected between the replication region of pBAA1 and other gram-positive plasmids pT181, pC221, and pE194.

The minimal replicon of pBAA1 was found to reside within a 1.4-kb fragment of DNA. The copy number of this plasmid is similar to that of the parental plasmid at approximately 5 per chromosome equivalent. In addition, this plasmid is very stable, with over 80% of cells retaining plasmid after 100 generations in the absence of selection. It can be concluded that all functions required for copy number determination are contained within the minimal replicon. In addition, the low copy number per chromosome equivalent suggests that an active partitioning system may also reside on this fragment.

A body of evidence supports the hypothesis that pBAA1 replicates by a rolling circle-type mechanism. Key features of this mode of replication include (i) an origin of plus-strand synthesis containing a site at which the DNA is nicked; (ii) a replication protein which nicks the plus strand, forms a covalent linkage with the DNA, and renicks and ligates the newly synthesized plus strand after one round of replication; and (iii) an origin sequence for initiation of minus-strand synthesis. The replication functions of pBAA1 have several features homologous to the replication functions of coliphage φX174 and *S. aureus* plasmid pC194, both of which replicate by a rolling cycle-type mechanism (11, 18). The similarities between φX174, pC194, and pBAA1 include all the features essential to this mode of replication. For example, there are conservation of sequence at the plus origin and conservation of amino acids at the active site of the replication protein in all three cases. In addition, all three contain a genetic function which although not similar at the sequence level is involved in efficient conversion of the single strand to the double-stranded form. In summary, these data strongly suggest that pBAA1 replicates by a rolling cycle-type mechanism.

It was observed that deletion or mutation of a sequence overlapping the *Bst*EII site results in the production of large quantities of single-stranded plasmid DNA. In view of the strong likelihood that pBAA1 replicates by a rolling circle-type mechanism, it is probable that this sequence is involved in the efficient conversion of the single plus strand to the double-stranded plasmid form. Palindromic plasmid sequences with this activity have previously been reported for gram-positive plasmids, e.g., pT181, pC221, pUB110, and pC194 (12), and for pLS1 (5). This region of pBAA1 has no homology with the previously reported minus origins. Thus, this sequence constitutes a novel minus origin for this group of plasmids.

The *Hin*dIII-*Eag*I 350-bp fragment of pBAA1 is almost identical to the sequence of the fragment of pLS11, reported to contain a partition function (4). There are differences in the sequences at only four positions: there are six C residues between bases 306 and 311 in pBAA1 but only four C residues in pLS11; there is a T residue at position 316 in pBAA1, which is an A residue at the same position in pLS11, and the C residue at position 288 in pBAA1 is not present in pLS11. The phenotypes of cells containing similar mutant plasmid constructions suggest that the *par* function (of pLS11) and minus-origin activity (of pBAA1) may be the same function. (i) Minus origins are predicted to function in one orientation only (12); Chang et al. (4) report that their *par* sequence functions only in one orientation. (ii) The minus origin and *par* activities both overlap the *Bst*EII site but do not extend as far as the *Eag*I site. (iii) Deletion of neither *par* nor the minus origin affects plasmid copy number. A crucial difference between these activities, however, is the effect of their removal on segregational plasmid stability. Removal of *par* results in a dramatic reduction in the stability of pLS11 constructions, whereas inactivation or deletion of the minus origin of pBAA1 results in only a low level of instability. These differences may be caused by strain, medium, or plasmid construction differences. It can be concluded, however, that increases in the steady-state levels of single-stranded plasmid DNA per se do not result in segregational instability of plasmids. As shown in this study, pRP22F produces single-stranded plasmid DNA but is not detectably segregationally unstable. Similarly, both pC194 and pE194 produce single-stranded plasmid DNA in *B. subtilis* (31), but whereas pE194 is very unstable in this host, pC194 is not detectably unstable after 100 generations in the absence of selection (K. M. Devine, unpublished observations). A more detailed analysis of minus origins and their relationship to segregational plasmid instability is required to resolve these points.

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