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Induction of DNA amplification in the *Bacillus subtilis* chromosome

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A system allowing the induction of DNA amplification in *Bacillus subtilis* was developed, based on a thermosensitive plasmid, pE194, stably integrated in the bacterial chromosome. An amplification unit, comprising an antibiotic resistance marker flanked by directly repeated sequences, was placed next to the integrated plasmid. Activation of pE194 replication led to DNA amplification. Two different amplification processes appeared to take place: one increased the copy number of all sequences in the vicinity of the integrated plasmid and was possibly of the onion skin type, while the other increased the copy number of the amplification unit only and generated long arrays of amplification units. These arrays were purified and shown to consist mainly of directly repeated amplification units but to also contain non-linear regions, such as replication forks and recombination intermediates. They were attached to the chromosome at one end only, and were, in general, not stably inherited, which suggests that they are early amplification intermediates. Longer arrays were detected before the shorter ones during amplification. When the parental amplification unit contained repeats which differed by a restriction site the arrays which derived thereof contained in a majority of cases only a single type of repeat. We propose that the amplified DNA is generated by rolling circle replication, and that such a process might underlie a number of amplification events.

Key words: amplification mechanisms/rolling circle replication

Introduction

DNA amplification increases the relative amount of a region of a genome within a cell and has been observed in many organisms, from bacteria to human (reviewed by Stark and Wahl, 1984; Stark *et al.*, 1990). Two classes of amplification event may be distinguished. One encompasses developmentally programmed events which occur in all individuals of a given species (Kafatos *et al.*, 1985 for review). Well characterized examples are amplification of ribosomal RNA genes (rDNA) in *Xenopus laevis* oocytes (Gall, 1968; Brown and Dawid, 1968) and chorion genes in *Drosophila melanogaster* follicle cells (Spradling and Mahowald, 1980). Both are the consequence of rapid over-replication of a specific region of the genome, either by a rolling circle mechanism or by repeated bi-directional initiation (Hourcade *et al.*, 1973; Rochaix *et al.*, 1974; Spradling, 1981; Osheim and Miller, 1983). The second amplification class encompasses unscheduled events, which take place in only a fraction of individuals within a population. The examples of this class include amplification of genes which confer drug resistance upon prokaryotic (Rownd and Mickel, 1971; Clewell *et al.*, 1975; Normark *et al.*, 1977) and eukaryotic cells (Alt *et al.*, 1976; Fogel and Welch, 1982; Schimke, 1984; Stark, 1986), or even upon whole eukaryotic organisms (Mouchès *et al.*, 1986) and amplification of oncogenes, often detected in tumours (Stark, 1986; Alitalo and Schwab, 1987).

Analysis of amplified DNA formed by unscheduled events has revealed arrays of repeated elements. The arrays are rather simple in prokaryotes and lower eukaryotes, since they generally contain direct repeats of only one kind. They are more complex in higher eukaryotes, generally containing inverted repeats and evolving during cell growth into structures composed of repeats of several kinds (Stark and Wahl, 1984; Stark *et al.*, 1990). Nevertheless, the basic similarity of structures observed in different cell types points to the existence of common molecular mechanisms by which amplified DNA can arise in pro- and eukaryotes. These mechanisms are not yet well understood.

In prokaryotes, two phases of amplification can be distinguished. A direct repeat is generated during the first phase, by transposition of specialized elements or by illegitimate recombination (Stark and Wahl, 1984; Edlund and Normark, 1981; Whoriskey *et al.*, 1987). Longer arrays of direct repeats form during the second phase (Peterson and Rownd, 1983; Jannière *et al.*, 1985). Two main classes of mechanism have been proposed to account for this phase of amplification, one based on localized over-replication of DNA and the other on unequal crossing over (Yagi and Clewell, 1976; Young and Cullum, 1987; see Figure 1 for examples). Similarly, in eukaryotes, an inverted repeat might be generated during the first phase of amplification (Passananti *et al.*, 1987; Ruiz and Wahl, 1988; Smith *et al.*, 1990). Subsequent unequal crossing over or a rolling circle replication have been proposed to form longer arrays of such repeats (Passananti *et al.*, 1987; Hyrien *et al.*, 1988; Smith *et al.*, 1990). There is, however, no direct evidence for either of the two proposed mechanisms, mainly because unscheduled amplification occurs with a relatively low frequency. Molecular intermediates of the process are therefore too scarce to be studied and the mechanisms have been deduced from the analysis of the structure of amplified DNA. However, this structure cannot be analysed before enriching for the cells that carry the amplified DNA, by growth under conditions selective for its presence. Since such

growth may modify the structure of amplified DNA, it can be very difficult to draw any conclusion about the mechanism. It therefore follows that an early analysis of amplified DNA might well be necessary in order to characterize the mechanisms of amplification (Smith *et al.*, 1990).

We describe here a system which allowed us to analyse the intermediates of the second phase of amplification in *Bacillus subtilis*. Its key element is a conditional replication origin integrated in the host chromosome in the vicinity of a directly repeated sequence. Upon activation of this origin, 20–45 copies of the repeat are generated within 2 h, without use of selection. Analysis of this process leads us to suggest that the rolling circle replication might be a major mechanism of unscheduled DNA amplification.

Results

Experimental strategy and strain description

Amplified structures in prokaryotes consist of tandem repeats, called amplification units (AUs) and arise from preexisting duplications (Peterson and Rownd, 1983; Jannière *et al.*, 1985; for an exception see Whoriskey *et al.*, 1987). Amplification requires functions involved in homologous recombination (Normark *et al.*, 1977; Young, 1984), which suggested to us that it could be induced by stimulating recombination between the duplications. We have previously shown that activation of a conditional replication origin carried in the *B.subtilis* chromosome stimulates such recombination (Noirot *et al.*, 1987) and therefore decided to examine whether it can induce amplification.

We used for this purpose *B.subtilis* strain AP2, which carries in its chromosome, between the *thy*B gene and the region named X, plasmid pE194 (a copy mutant *cop*-6 was used; Weisblum *et al.*, 1979) and a directly repeated 4 kb pBR322 segment flanking a chloramphenicol resistance gene (Figure 2, top; Noirot *et al.*, 1987). This structure contains an amplification unit of ~6.2 kb, comprising one of the repeats and the resistance gene. pBR322 does not replicate in *B.subtilis* (Niaudet and Ehrlich, 1979) and pE194 replication is thermosensitive in this host, being efficient at 37°C but not at 51°C (Weisblum *et al.*, 1979). This replication is unidirectional in the autonomously replicating plasmid (Sozhamannan *et al.*, 1990) and progresses towards the duplications in our strains. Three other strains, AP2i, which carries a 200 bp deletion inactivating pE194 replication, AP1, which lacks the resistance gene and one of the repeats (i.e. the AU), and AP25i, which carries 25 copies of tandemly repeated amplification unit and the deletion inactivating pE194 replication, were used as controls in some experiments. The strain AP3, which is similar to AP2 but contains an additional AU, was used for purification of amplified DNA (see below). The strains were constructed and maintained at 51°C (or stored at −70°C) to avoid pE194 replication (Noirot *et al.*, 1987). It is important to notice that in all strains, pE194 is stably maintained in the chromosome, since it is not flanked by homologous sequences and thus cannot be excised by recombination.

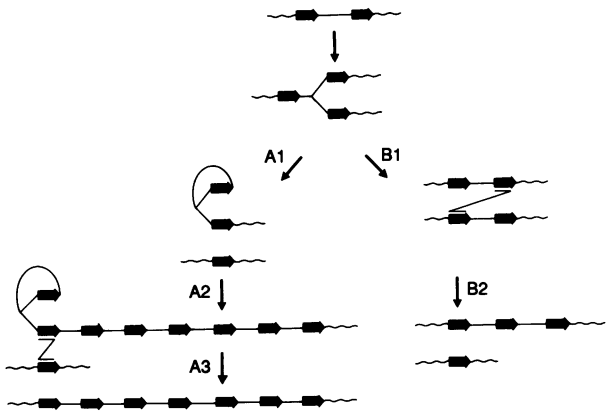

Fig. 1. Rolling circle and unequal crossing over models of DNA amplification. A structure which undergoes amplification is composed of repeats, shown as arrows, flanking a non-repeated region, represented by a straight line. The neighbouring chromosomal sequences are shown as wavy lines. The replication fork enters the duplication from the right. In the rolling circle model (Young and Cullum, 1987), recombination between one of the replicated repeats and the non-replicated repeat entraps the fork on a circular template and leaves one of the repeats integrated in the chromosome (A1). Replication of the circle generates amplified DNA (A2) which can be stably integrated in the chromosome by recombination (A3). In the unequal crossing over model (Yagi and Clewell, 1976) both repeats are replicated (B1) and recombination between them takes place as indicated by a Z (B2). Iteration of the process can generate long stretches of amplified DNA.

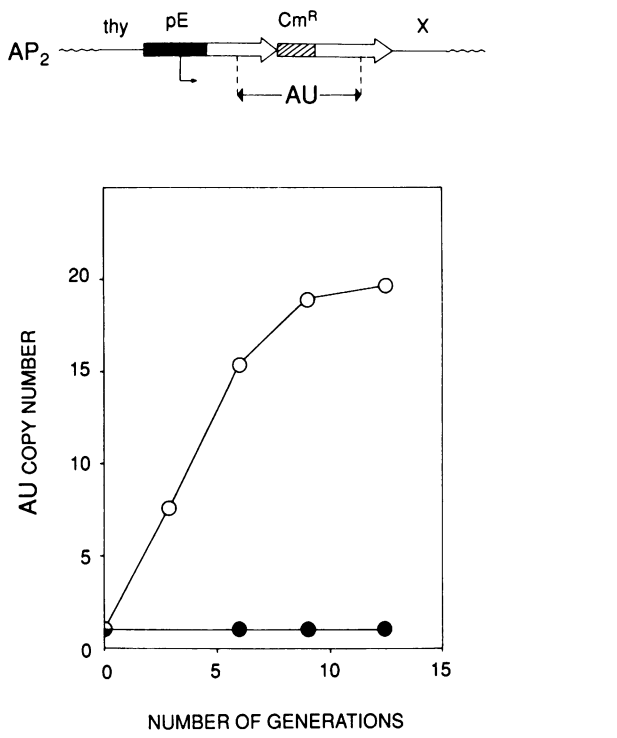

Fig. 2. Induction of amplification. **Top**: The structure carried in AP2 cells. Chromosomal sequences are represented by a thin line, thy and X refer to the *thyB* gene and a fragment of unknown genetic content, respectively. pE194, pBR322 and pC194 sequences are shown as dark box, open arrows and hatched box, respectively. pE194 replication origin and direction of replication are indicated by a thin arrow. The amplification unit (AU) is composed of pBR322 sequences between bp 570 and 4363, and the 2.3 kb segment carrying the chloramphenicol resistance gene (Cm^R). In AP2i cells pE194 replication is inactivated by a 200 bp deletion. **Bottom**: Kinetics of amplification. DNA was extracted from cells grown at 37°C and the copy number of the amplification unit was deduced from dot blot hybridization (see Materials and methods). Open and closed circles refer to AP2 and AP2i cells, respectively.

Induction of amplification

To induce amplification, cells were grown to early exponential phase at the temperature restrictive for plasmid replication (51°C) and transferred to the permissive temperature (37°C). To determine the level of amplification, DNA was extracted after different periods of growth at 37°C and analysed by dot blot hybridization, using a probe homologous to AU. A representative experiment is shown in Figure 2. In AP2 cells, which carry the active pE194 replicon, the number of AUs increased rapidly for six generations and approached a plateau of ~20 copies per chromosome after 10 generations. In contrast, amplification was not detected in AP2i cells, which contain mutated pE194 unable to replicate, or in AP2 cells grown at 51°C (not shown). These results indicate that replication from pE194 origin induces DNA amplification.

Cell phenotype upon induction of amplification

A single copy of the chloramphenicol resistance gene renders *B.subtilis* strains resistant to 5 μg/ml of antibiotic and about seven copies are required for resistance to 50 μg/ml (Jannière *et al.*, 1985). Close to 1 in 10^6 AP2 cells were highly resistant before induction of amplification, as determined by a plating assay at 51°C, presumably because of the preexisting amplification (Jannière *et al.*, 1985). This frequency increased 1000-fold (to 1 in 10^3) after 2 h of induction, which shows that stably inherited amplified structures were generated in many cells. Field inversion gel electrophoresis (FIGE) of the DNA prepared from cells resistant to 50 μg/ml of chloramphenicol indicated that they contain 20–40 copies of the AU (not shown). However, since the cells carried an average of 20 copies of the resistance gene before plating, a much greater frequency of highly resistant colonies should have been obtained, unless all the AUs were carried by very few cells. It seems very unlikely that 1 in 10^3 cells contained 20 000 copies of the AU (120 Mb of DNA, >20 *B.subtilis* genome equivalents), which would have been required to give an average of 20 AUs per cell in the culture. This suggests that most of the cells harboured amplification intermediates which were not stably inherited. Further evidence for this conclusion is presented below.

Two types of amplification

To determine which sequences are amplified upon induction of pE194 replication, we used probes homologous to regions (i) upstream of the replication origin; (ii) between the replication origin and AU; (iii) within AU and (iv) downstream from AU. Another probe, homologous to the chromosomal *sacRB* gene, ~1300 kb away from the AU (as estimated from the map of the *B.subtilis* chromosome, Piggot and Hoch, 1985) was used as an internal hybridization standard.

DNA was prepared from cells grown at 37°C for 2 h and analysed by dot blot hybridization. The copy number of all tested regions, which was close to one per genome in AP2i cells (Figure 3A), was much higher in AP2 cells (Figure 3B). The increase was lower outside of AU than within it (≤7-fold and 20-fold, respectively). A modest increase (~3-fold) was also observed in AP1 cells which lack the AU (Figure 3C). These results indicate that two types of amplification were induced. One affects all sequences in the vicinity of the integrated plasmid and might be due to the entry of the replication forks initiated at the plasmid origin into the neighbouring chromosomal regions. However, further work is required to understand how a unidirectional replication of the rolling circle type, characteristic of autonomously replicating pE194 (Sozhamannan *et al.*, 1990), could affect sequences upstream of the replication origin. The second type of amplification involves only the AU and thus resembles unscheduled amplification events which are generally observed in prokaryotes. We therefore focused on this process in the remainder of the work described here.

Tandemly arrayed amplified sequences

Total DNA prepared from AP2 and AP2i cells grown at 37°C or 51°C was analysed by gel electrophoresis and hybridization with a probe detecting the AU (Figure 4). In all intact samples the hybridization signal either migrated slowly, with the chromosomal DNA, or not at all, which rules out the existence of free, monomer-size, circular AUs. In samples cleaved with *Nco*I, which cuts on both sides of the AU and at a single site within it to generate two 15 kb segments, a 15 kb band was detected. Upon amplification this band was of a higher intensity and an additional 6.2 kb band, matching the size of the AU, was detected. The increase of intensity of the 15 kb band is expected from over-replication of the chromosomal region proximal to the integrated plasmid. The appearance of the 6.2 kb band suggests the existence of amplified structures composed of

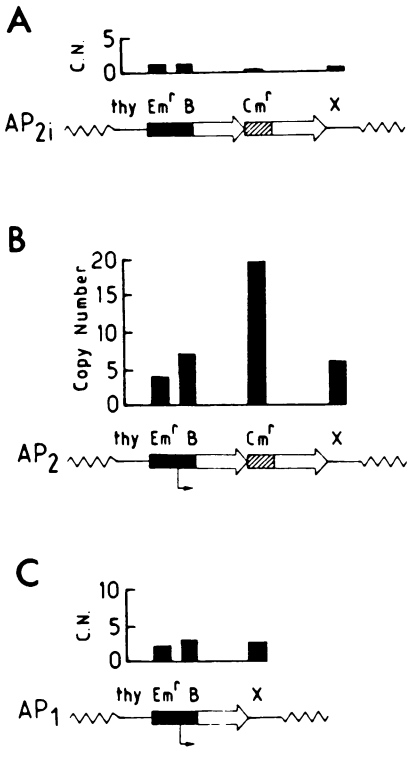

Fig. 3. Amplification of different regions close to the integrated pE194. DNA was extracted from cells AP2i (**A**), AP2 (**B**) and AP1 (**C**) grown for 2 h at 37°C and analysed by dot blot hybridization. The probes used were: pE194 *Cla*I–*Sac*I segment (Em^R, bp 1940–2900); pBR322 *Cla*I–*Sph*I segment (B, bp 23–562) which is not duplicated; pC194 (Cm); chromosomal DNA segments X (*Eco*RI–*Bgl*II segment of plasmid pHV438) and *sacRB* (*Bam*HI–*Hin*dIII segment of pLS302). The copy number (C.N.) of the different segments was estimated by dot blot hybridization. Other symbols are as in Figure 2.

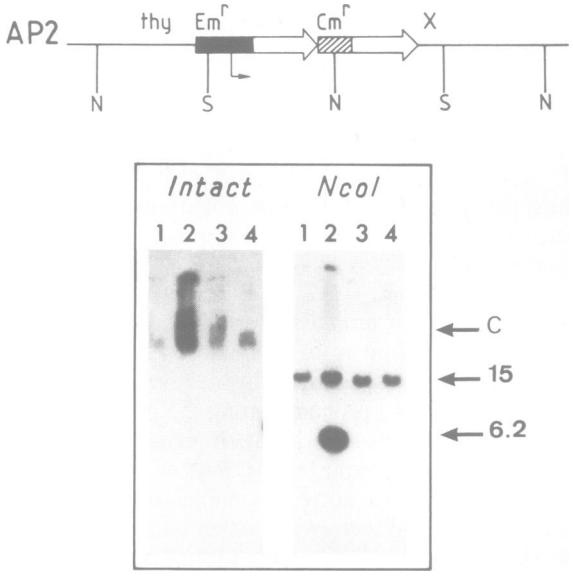

Fig. 4. Electrophoretic analysis of amplified sequences. **Top**: The structure carried in AP2 cells: *Sac*I (S) and *Nco*I (N) restriction sites are indicated. Other symbols are as in Figure 2. **Bottom**: Total DNA was extracted from cells grown for 2 h at 51°C or 37°C, left intact or treated with *Nco*I, separated in a 0.7% agarose gel and hybridized with pBR322. Band size (in kb), deduced by comparison with intact and *Hin*dIII-cleaved phage λ DNA (not shown), is indicated on the right. C indicates the position of chromosomal DNA. AP2, 51°C, lane 1; AP2, 37°C, lane 2; AP2i, 51°C, lane 3; AP2i, 37°C, lane 4.

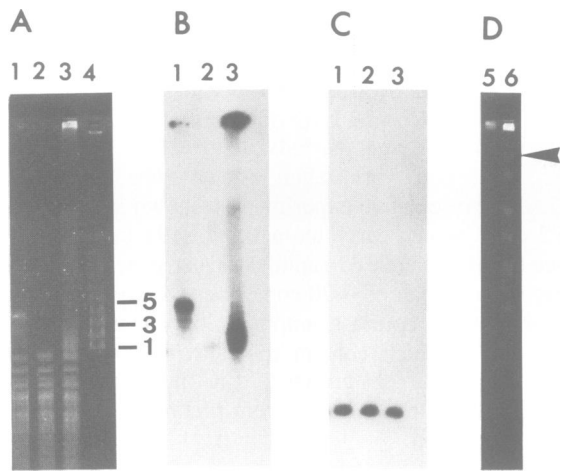

Fig. 5. FIGE analysis of amplified DNA. Panels A, B and C: DNA was prepared from different cells in agarose plugs and treated with *BclI*, which does not cleave AU. Lane 1: AP25i cells; lane 2: AP3 cells grown at 51°C; lane 3: AP3 cells grown at 37°C; lane 4: λ ladder size marker. DNA segments were separated by FIGE using a linear switching-interval ramp from 5 to 40 s in forward migration (FM) and from 1 to 8 s in reverse migration (RM) for 10 h, which resolved segments up to 350 kb (see lambda ladder, lane 4; bands containing one, three and five genomes are highlighted on the right). DNA was revealed by ethidium bromide staining (**panel A**). Duplicate membranes prepared from the same gel were used for hybridization with a pBR322 probe which detects AU (**panel B**), or a chromosomal *sacRB* gene probe (**panel C**). **Panel D**: Lane 5: amplified AP3 DNA which did not migrate from the plug under the FIGE conditions used in panel A; lane 6: *S.cerevisiae* chromosome size marker. FIGE conditions were 3−180 s in FM, and 1−60 s in RM for 36 h, which separated DNA fragments up to 2650 kb (lane 6; the arrow points to the 2650 kb yeast chromosome).

head to tail tandemly arrayed, AUs. Such structures are generally observed in prokaryotes. A different structure, consisting of interlocked circular AUs, is ruled out below.

Purification of amplified DNA

Previous reports have suggested that amplification might be more efficient in strains of *Vibrio* (Goldberg and Mekalanos, 1986) and *Streptomyces lividans* (Dyson and Schrempf, 1987) which carry higher number of initial repeats. We therefore compared the amount of amplified DNA obtained in AP2 and AP3 cells, which carry one and two amplification units, respectively. Amplification was induced in both strains by growing the cells at 37°C for 2 h, total cell DNA was extracted and the level of amplification was estimated by dot blot hybridization with a probe homologous to AU. About 20 and 45 copies of AU were found in AP2 and AP3 cells, respectively. We therefore used AP3 cells to purify amplified DNA.

DNA was prepared from cells embedded in agarose plugs [this method reduces breakage by shearing (Smith *et al.*, 1986)], treated with *BclI*, which cleaves outside of the AU, and subjected to FIGE. This technique can be used to separate linear arrays of repeated AU, as documented with DNA prepared from the control strain AP25i, which carries 25 tandemly repeated copies of AU (Figure 5A, lane 1). A 180 kb band, well separated from the chromosomal *BclI* segments, is clearly visible. As expected, only the chromosomal DNA segments are observed in the sample prepared from AP3 cells grown at 51°C (lane 2). Surprisingly, no band was visible in the 100−300 kb region in the sample prepared from AP3 cells grown for 2 h at 37°C (lane 3), although the average size of the amplified DNA was ~280 kb (45 AU of 6.2 kb). A strong fluorescent signal in the slot and a smear of fluorescence, spreading between the chromosomal DNA and the limit of the resolution of the gel, were observed instead.

Hybridization with the probe homologous to the AU was used to identify the amplified sequences (Figure 5B). The 180 kb band was detected in AP25i DNA (lane 1) and the 23 kb band, corresponding to the size of the parental structure, in the AP3 DNA extracted from cells grown at 51°C (lane 2). Two prominent signals were visible in AP3 DNA prepared from cells grown at 37°C (lane 3), one corresponding to segments of ~20 and 150 kb, another to DNA retained in the slot. The first could result from onion skin over-replication of the parental structure and/or breakage of the larger amplified structures. The second must have been due to amplified DNA that was unable to migrate in FIGE. To assess the purity of this DNA we used a probe carrying the chromosomal *sacRB* gene (Figure 5C). A unique band was revealed in all samples, corresponding presumably to the homologous chromosomal *BclI* DNA segment. This indicates that the DNA retained in the slot contained only the amplified sequences. The agarose plugs containing the purified amplified DNA were retrieved from the slots and used for further analysis.

The purified amplified sequences did not migrate from the slot, even under FIGE conditions which resolved yeast chromosomes up to 2.65 Mb (Figure 5D; the arrow points to the 2.65 Mb chromosome). Similar results were obtained with AP3 DNA prepared from cells grown at 37°C and treated with two other enzymes which did not cut within the

AU (*Sac*I and *Bgl*II, not shown). Inability to migrate could be a consequence of either a very large size or a non-linear structure of the amplified DNA. The results presented below favour the second hypothesis.

Organization of amplified sequences

Degradation of amplified DNA with an enzyme which cleaves an AU only once generates a unique band of AU size (Figure 4), which suggests a tandem organization of amplified sequences. However, a similar band would be obtained if the amplified DNA was a conglomerate of interlocked monomeric circles. In addition, such a conglomerate would probably be retained in the slot during FIGE. The conglomerate could result from excision of the AU by homologous recombination, and extrachromosomal replication of the resulting circular molecule, not followed by decatenation of the replication products. To distinguish between the tandem and interlocked arrangement, we subjected purified amplified AP3 DNA to partial digestion with *Nco*I, which cleaves the AU only once. This treatment should generate a ladder of segments differing by one AU from a tandemly organized structure and a unique linear segment of AU size from a conglomerate of interlocked monomeric circles. The results are shown in Figure 6A. Incubation of plugs with increasing amounts of the enzyme resulted in the disappearance of the DNA from the plug, which first accumulated at the limit of resolution of the gel (~100 kb in this experiment), then separated in a ladder of bands (13 distinct bands were visible in lane d on the original photograph) and finally gave rise to a major, rapidly migrating, diffuse band (a faint additional band visible in lane g could be due to incomplete cleavage and/or to a junction fragment, see below). This indicates that amplified DNA is composed mainly of an array of tandemly repeated AUs.

An array of tandemly organized AUs could be retained in the slot during FIGE if it were circular. It should be converted by a single *Nco*I cut to a linear form of ~270 kb (the average size of amplified DNA, see above), capable of migrating from the plug. Such a form was not detected in an experiment where partially degraded DNA was analysed under FIGE conditions resolving segments up to ~400 kb (Figure 6B).

Non-linear structures in amplified DNA

Inability of amplified DNA to migrate during FIGE suggests that it contains, in addition to linear arrays of tandem repeats, structures which are not linear. Two likely possibilities seemed to be replication forks and recombination intermediates, which could be expected from rolling circle and unequal crossing over amplification mechanisms, respectively. To detect the non-linear structures we used two-dimensional (2D) gel electrophoresis (Brewer and Fangman, 1987).

Purified, amplified DNA was digested with three different enzymes (*Pvu*II, *Nco*I and *Ava*I) which cut the AU only once; this should generate Y- or X-shaped molecules from replication forks or recombination intermediates, respectively. The former should migrate on an arc, extending between the positions of linear segments containing one and two AUs, respectively, and the latter on a line, starting at the position of linear segments containing two AUs. The results are shown in Figure 7. In all cases an arc was observed,

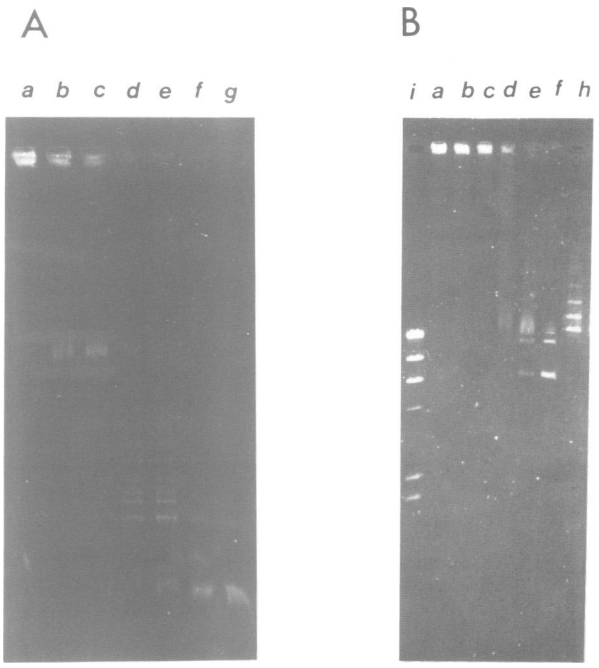

Fig. 6. Partial restriction analysis of amplified sequences. Plugs containing amplified AP3 DNA, treated with *Bcl*I and purified by FIGE, were incubated for 75 min in the presence of 0, 0.1, 0.3, 1, 10 and 30 U *Nco*I (lanes a–g, respectively). Lane h contained the λ ladder and lane i *Hin*dIII-digested λ DNA. DNA fragments were separated through FIGE with a 100 kb limit of resolution (**panel A**, from 0.2 to 3 s in FM and from 0.1 to 1 s in RM for 15 h), or a 400 kb limit (**panel B**, from 3 to 30 s in FM and from 1 to 10 s in RM for 12.5 h).

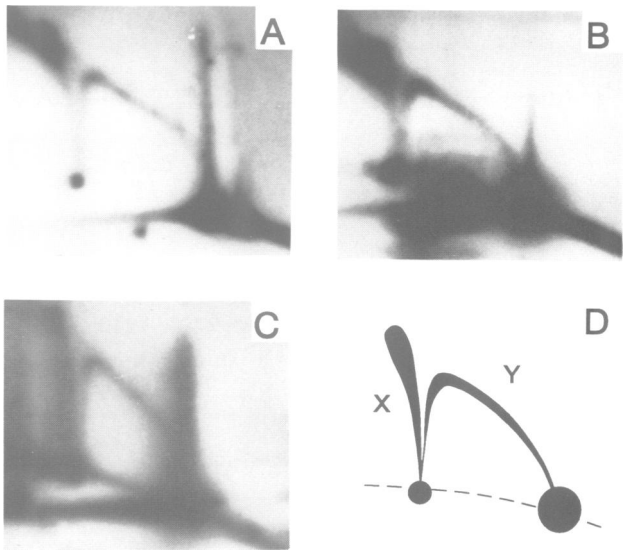

Fig. 7. 2D gel analysis of purified amplified DNA. Plugs containing amplified DNA, which was purified by *Bcl*I cleavage and FIGE (see Figure 5), were incubated with *Pvu*II (**panel A**), *Nco*I (**panel B**) and *Ava*I (**panel C**), the DNA segments were separated by 2D gel electrophoresis (as previously described, Brewer and Fangman, 1987) and hybridized with pBR322 DNA. The arc of replication forks (indicated as Y) and the line of recombination intermediates (indicated as X) are diagrammed in **panel D**. The stronger and the weaker hybridization spots are at the positions of segments containing one and two AUs, respectively, as deduced by comparison with the Raoul size marker (not visible on the autoradiogram).

stretching between the expected positions. In addition, a line starting at the position of segments containing two AUs was visible. These results suggest the presence of Y- and X-shaped molecules, and thus the presence of replication forks and recombination intermediates, within the purified amplified DNA.

Ends of amplified DNA

To characterize amplified DNA further, we tested whether it was joined to the chromosomal sequences which flank the parental amplification unit. Double digestion of non-amplified AP3 DNA with *BclI* and *NcoI* should generate two junction segments, of 8.25 and 8.5 kb, containing pE194 and X sequences, respectively (J1 and J2, Figure 8A). The same segments would be expected from digestion of amplified DNA if it were joined to the chromosome at both ends. The J1 junction segment was easily detected with a pE194 probe in total DNA prepared from AP3 cells before and after amplification (Figure 8B, panel pE, lanes 1 and 2) but only traces of it were present in the purified amplified DNA (lane 3). In contrast, comparable amounts of the second junction segment were detected with an X probe in all samples (Figure 8B, panel X). This segment was not generated from chromosomal DNA contaminating the purified amplified structures, since no signal was detected with a probe homologous to the *sacRB* gene (Figure 8B, panel sacRB, lane 3). Another hybridizing segment (labelled r, Figure 8B) was detected in the total DNA prepared from the AP3 cells upon amplification and hybridization with pE194 or X (lanes 2). Its size (11 kb as judged from comparison with the size marker, not shown) indicated that it derived from structures which have lost the AUs by recombination. It is known that replication of the integrated pE194 stimulates such recombination in its vicinity (Noirot *et al.*, 1987).

The above results suggest that amplified DNA is joined to the chromosome at only one end, distal from pE194. To test this conclusion further, we determined the proportion of sequences complementary to X, AU and pE194 in the purified amplified DNA by dot blot hybridization. Seventy times more AU than X was found, suggesting that, on average, each amplified structure might contain 70 AUs. Since the total DNA contained ~45 AUs per chromosome, and the standard deviation of our measurements was ~25%, it is possible that most amplified structures were linked to X and that amplification took place in most cells. Fifteen times more X than pE194 was found, which indicates that only one amplified structure in 15 was linked to pE194. The observation that a majority of amplified structures seem to be joined to the chromosome, albeit at one end, supports the conclusion that they are neither circular nor conglomerates of interlocked circular AUs. The observation that the other end is free of chromosomal sequences raises a possibility that it carries a template for rolling circle replication.

Kinetics of appearance of amplified DNA

Tandemly arrayed amplified structures could result from rolling circle replication or unequal crossing over (Figure 1). The first process would not be expected to generate detectable amounts of intermediate-sized arrays, since replication should form full size structures very rapidly once a circular template is available [PolIII holoenzyme progresses at a rate of ~1000 bp/s (Kornberg, 1980) and would require only ~2 min to synthesize 20 copies of a 6.2 kb AU]. In contrast, iteration of recombination steps, postulated by the second process, would be expected to generate intermediately

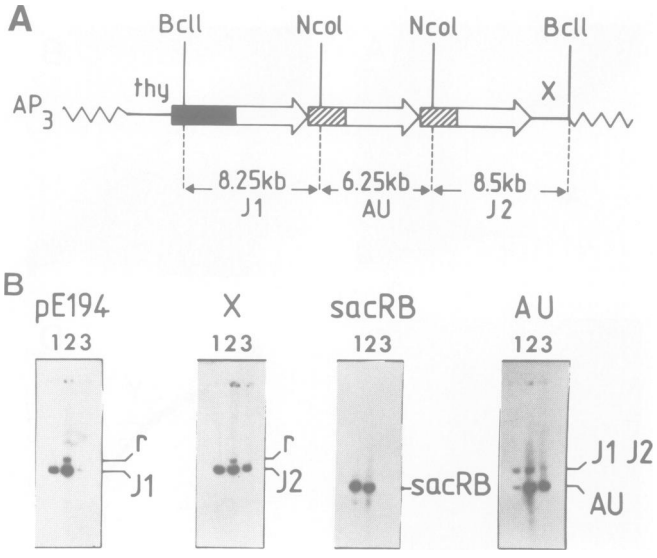

Fig. 8. Analysis of junction segments. **A**. Structure of the AP3 chromosome. J1 and J2 refers to junction segments, other symbols are as defined in the legend of Figure 2. **B**. Total AP3 DNA extracted from cells grown at 51°C (lane 1) and 37°C (lane 2) was doubly digested with *NcoI* and *BclI*. Amplified structures were purified from *BclI*-cleaved DNA by FIGE electrophoresis (see Figure 5) and further digested by *NcoI* (lane 3). DNA segments were then separated by gel electrophoresis, transferred to nitrocellulose filters and hybridized to the probes indicated on the top of each panel (pBR322 was used to detect the AU). The positions of junction segments (J1 and J2), amplification unit (AU), *sacRB* segment (*sacRB*) and the recombinant structures (r) are indicated.

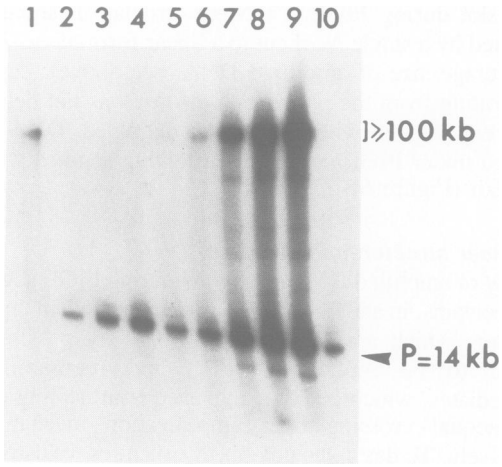

Fig. 9. Kinetics of appearance of amplified structures. DNA was extracted from AP2 cells grown at 37°C for 0, 15, 30, 45, 60, 90, 120 and 150 min or at 51°C for 150 min (lanes 2–10, respectively). DNA was cleaved with *SacI* (see Figure 4, top, for sites), the segments separated by FIGE (from 0.2 to 1 s in forward migration and from 0.1 to 0.5 s in reverse migration for 16 h), transferred to a membrane and analysed by hybridization with a pBR322 probe. DNA extracted from AP25i cells grown at 37°C for 120 min and cleaved with *SacI* was used as control (lane 1). It gave a band of ≥100 kb (the limit of gel separation) indicating that DNA shearing did not interfere with size analysis within the desired range. P refers to parental structure.

sized arrays soon after the induction of pE194 replication. To follow the kinetics of appearance of amplified structures, total DNA was prepared from AP2 cells, treated with *Sac*I (which does not cleave the AU) and subjected to FIGE under conditions allowing the resolution of segments up to ~100 kb, which corresponds to an array of 13 AUs. Amplified DNA was revealed by hybridization with an appropriate probe (Figure 9). A band of parental size (14 kb) was detected in all samples. Its intensity increased during amplification, as expected from over-replication of the chromosome in the vicinity of the integrated plasmid (see above). Another band, ≥100 kb, detectable after 45 min of induction and becoming more prominent later, was due to the appearance of tandemly arrayed AUs. The intermediate size bands were not visible in the early samples, which supports a rolling circle amplification mechanism. A relatively long delay (45 min) before amplified DNA is detected might be due to a rate-limiting formation of the rolling circle template.

Several additional bands were detected in the late samples. Their low intensity and late appearance suggest that they were not intermediates in the amplification process. A deletion event between the AP2 duplications, stimulated by pE194 replication (Noirot *et al.*, 1987), probably generated the band preceding the parental one (Figure 9). Similar deletions within the amplified structures could have generated other low intensity bands.

Amplification of genetically labelled duplications

To examine the mechanism of amplification further, we constructed AUs composed of repeats which differed by a single restriction site. Amplification by a rolling circle replication should then generate arrays composed of identical repeats, since the circular template would either carry or lack the site. In contrast, amplification by unequal crossing over should generate 'patchwork' amplified structures, composed of the two types of repeats. Cleavage of the amplified DNA with the enzyme which recognizes the differing site should allow the distribution of the repeats to be assessed.

Three strains analogous to AP2 but lacking the *Ava*I, *Pst*I or *Nde*I restriction site from one of the pBR322 copies were used (Figure 10, top). Amplification was induced for 2 h, total DNA was extracted and cleaved with the appropriate restriction enzyme, the segments were resolved by electrophoresis and the repeats revealed by hybridization with labelled pBR322. The relative intensity of different bands was determined by densitometry and the results are summarized in Figure 10 (bottom). In each case, in addition to a 12 kb band, expected to arise from the parental structure and migrating with DNA segments containing two AUs, (empty bars, Figure 10, bottom), the two most prominent bands corresponded to segments containing a single AU or ≥5 AUs (the limit of the resolution of the gel), respectively. When, prior to cleavage, the amplified structures were purified in agarose blocks, a similar result was obtained, except that only a low amount (≤5%) of segments containing two AUs was visible (not shown). These observations suggest that the individual amplified structures were composed mainly of identical repeats, either carrying or lacking the differing restriction site, which supports the rolling circle mechanism of amplification.

The relative amounts of segments containing one and five or more AUs should reflect the probability of transferring the pBR322 region which carried or lacked the differing restriction site, respectively, into the homogeneous amplified DNA. This probability decreased linearly with the distance from the pE194 origin ($r = 0.998$), which suggests that there was no bias against the presence or the absence of the differing restriction sites in the amplified DNA (one was present and two were absent from the proximal pBR322 copy, Figure 6, top).

Minor bands, corresponding to segments composed of two to four repeats and representing ~10% of the hybridizing material, were also detected in our experiments (Figure 6). They indicate the presence of amplified DNA having a 'patchwork' structure, which could result from a minor unequal crossing over amplification pathway. Alternatively, they could arise by recombination between different amplified structures possibly present in the same cell.

Discussion

We developed a system which allows the induction of DNA amplification in *B.subtilis*. Its key element is the thermosensitive plasmid pE194, stably integrated in the host chromosome. Activation of replication from the plasmid origin, by a temperature shift, led to amplification of the integrated plasmid and neighbouring chromosomal sequences. Amplification was rapid, which made it possible to study early events that are not accessible when a selective agent has to be used to enrich cells in which amplification has taken place.

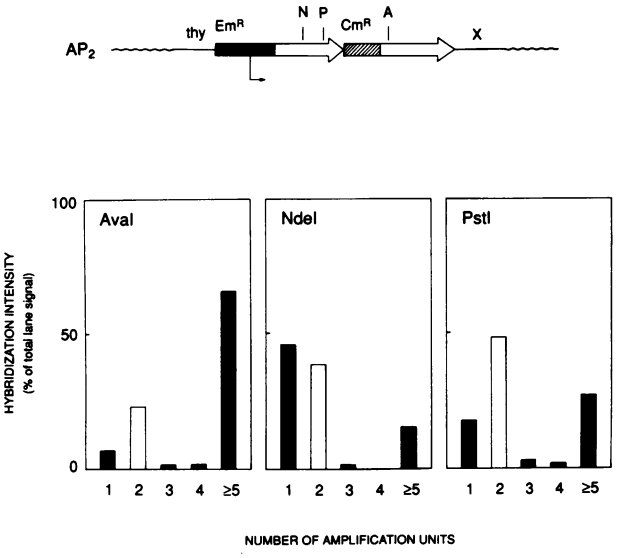

Fig. 10. Distribution of repeats in amplified DNA generated from slightly different duplications. **Top**: Restriction sites inactivated within the AP2 structure. The positions of the missing *Nde*I (N), *Pst*I (P) and *Ava*I (A) sites are indicated. Other symbols are as defined in Figure 2. **Bottom**: Distribution of the repeats. Cells which carried duplications differing by a single restriction site were grown at 37°C for 2 h, their total DNA was prepared and cleaved with the enzyme for which the site was missing from one of the duplications. The segments were separated by electrophoresis and the amplified sequences revealed by hybridization with pBR322. Size of hybridizing segments was measured by comparison with *Hin*dIII-cleaved λ DNA and is represented in number by AUs and the relative amount was determined by densitometry and is represented by bars. Empty bars correspond to segments having the size of the parental structure, which were not separated from segments containing two AUs in our experiment.

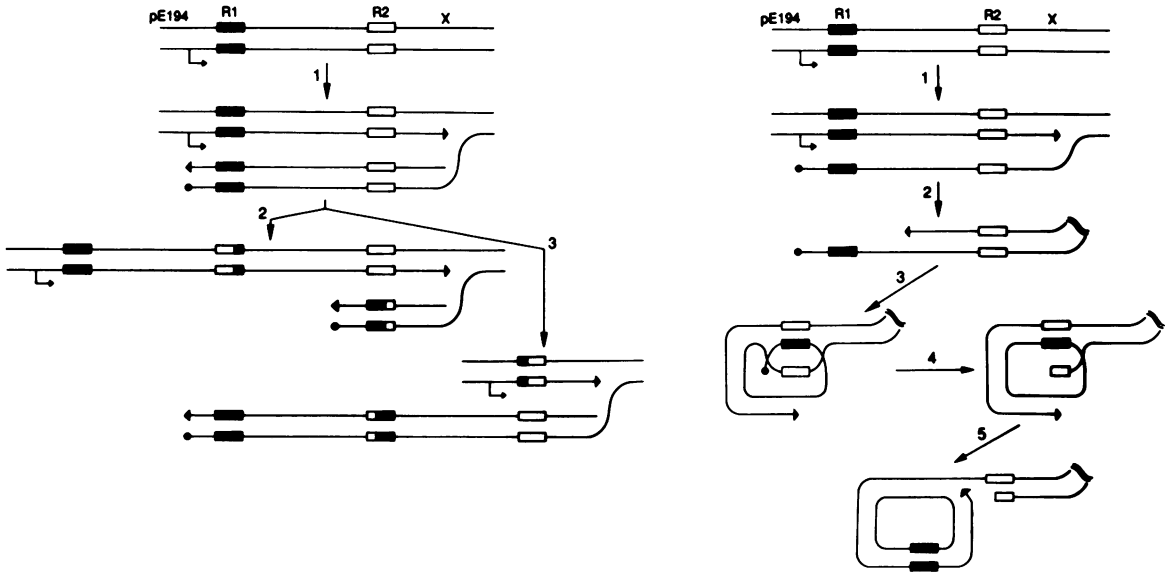

Fig. 11. Unequal crossing over and rolling circle amplification models. Chromosomal region in the vicinity of the integrated pE194 is represented in a double-stranded form, plasmid replication origin is shown as an arrow pointing in the direction of replication, repeats (R1 and R2) are represented as boxes, and the 5′-end of the displaced strand as a dot. **Left panel**: unequal crossing over. (1) The region downstream of the pE194 replication origin is duplicated; (2) recombination between R1 and R2 places three repeats downstream of the replication origin, and the amplification can continue; (3) recombination places a single repeat downstream of the replication origin, and the amplification cannot continue. **Right panel**: rolling circle mechanism. (1) progression of leading strand synthesis initiated at the pE194 origin; (2) progression of lagging strand synthesis, initiated at a nonspecific chromosomal signal (for clarity, leading strand synthesis is not shown; wavy lines intersecting the strands represent the remainder of the chromosome); (3) non-replicated repeat anneals with its newly replicated homologue, which creates a D-loop structure; (4) circular, partly replicated, molecule is generated by processing of the D-loop (e.g. nicking, partial degradation of the single strands and ligation); (5) replication of the circular molecule past the first repeat allows amplification to take place.

Two types of amplification were observed. One did not require duplications, increased the copy number of adjacent sequences 3- to 7-fold and probably corresponded to over-replication of the sequences in the vicinity of the plasmid origin. The other type of amplification required duplications, which is typical of gene amplification in prokaryotes (Mattes *et al.*, 1979; Peterson and Rownd, 1983; Jannière *et al.*, 1985), and generated amplified structures containing on average 20–45 repeats. We found that these structures consist mainly of a linear array of directly repeated sequences, which is typical of prokaryotes. However, they appeared to contain replication forks and recombination intermediates, which could either be instrumental in the process of amplification or be formed only after amplified structures were generated, as discussed below. Their presence allowed us to purify the amplified arrays by preventing migration in FIGE.

The amount of chromosomal sequences named X, which co-purified with amplified arrays, was close to that expected from the average level of amplification. In contrast, a much lower amount of pE194 sequences did co-purify. This indicates that the arrays were joined to the chromosome at one end only. Such structures are probably early amplification intermediates and cannot be inherited stably. Their presence could explain the observation that most of the cells which underwent amplification were not able to form colonies resistant to high chloramphenicol concentrations, although they contained a sufficient number of copies of the resistance gene.

We consider two models to explain the formation of amplification intermediates, based on either unequal crossing over between the repeats, or rolling circle replication. Both assume that the replication initiated at the pE194 origin carried in the chromosome proceeds by nicking and strand displacement, as observed with the autonomously replicating pE194 (Sozhamannan *et al.*, 1990). According to the first model (Figure 11, left), the displaced strand is converted to a double-stranded form by synthesis initiated at a non-specific site, occasionally situated 3′ from the repeats. Non-specific initiation frequently occurs on the single-stranded DNA of *B.subtilis* plasmids (Boe *et al.*, 1989). Two double-stranded copies of the region downstream of the plasmid origin are thus formed (step 1). Unequal crossing over between the repeats generates two structures, containing three repeats and one repeat, respectively. If the three repeats are localized downstream of the pE194 origin (step 2), another cycle of replication/recombination can generate longer arrays of tandem repeats. In contrast, amplification cannot continue if only a single repeat remains linked to the pE194 origin (step 3). This model could explain the presence of recombination intermediates within amplified DNA and the slight non-homogeneity of the repeats. However, it cannot easily account for the lack of pE194 sequences associated to amplified arrays. The parental AU is joined to pE194 and the novel copies of AU, generated by plasmid replication, should be joined to pE194 sequences downstream from its replication origin (Figure 11). Amplified structures formed by unequal crossing over should therefore carry at one end either the entire plasmid (3.7 kb) or a substantial part of it (1.0 kb, the size of the region downstream from the origin). Only the entire plasmid was detected in purified samples by Southern analysis, and only in a small proportion of amplified structures. Furthermore, unequal crossing over cannot easily explain the presence of the replication forks

within the amplified structures or the rapid appearance of amplified DNA and its predominantly homogeneous structure. We therefore prefer another explanation.

A rolling circle amplification model, conceptually related to versions considered previously (Futcher, 1986; Young and Cullum, 1987) is depicted in Figure 11, right. Plasmid replication displaces one of the DNA strands (step 1) which is used as a template for replication initiated at a non-specific site (step 2). Once the repeat distal from the plasmid origin is converted to a double-stranded form, it engages in recombination with its single-stranded homologue, strand exchange being catalysed by a RecA-like protein (step 3) and the subsequent cleavage/joining steps by a conjunction of nuclease and ligase activities (step 4). Such recombination would create a circular template on which a replication machinery has been entrapped. Replication of this template (step 5) could generate amplified structures, which (i) would be formed rapidly; (ii) would contain identical repeats; (iii) would be joined to the chromosome only at the end distal from the integrated plasmid and (iv) would contain replication forks. The observation that amplification was more efficient in cells carrying three repeats (AP3) than in those carrying only two (AP2) could readily be explained if the entrapment of the replication machinery by recombination were a limiting step of the process. The main criticism of this model is that it fails to account for the recombination intermediates detected within amplified DNA by 2D gel electrophoresis and for the minor heterogeneity of the repeats detected within individual amplified structures which were formed from AU containing slightly different repeats. However, recombination subsequent to amplification could explain these observations.

How general might the proposed rolling circle mechanism be? Its central feature is intramolecular recombination between a newly replicated, double-stranded sequence and a non-replicated, single-stranded homologue. The regions of single-stranded DNA could be generated in the chromosome by any event that uncouples the leading and lagging strand synthesis, or a DNA polymerase from the helicase which precedes it. A circular template carrying a replication fork could then be created by recombination between single-stranded DNA and its double-stranded homologue. The necessary enzymatic activities probably exist in all cells, and could lead to DNA amplification by a mechanism similar to that proposed here.

Materials and methods

Bacterial strains and plasmids

The strategy for constructing *B.subtilis* strains AP2 and AP2i was previously described (Noirot *et al.*, 1987). Strain AP1 (*trp*C2 *tyr*A1 *aro*B2 *his*H2 *ins*[pAD2] *del*[*ilv*A]), isogenic with AP2 and AP2i, was constructed by transformation of *B.subtilis* SB202 competent cells (Niaudet and Ehrlich, 1979) with *Bgl*II-linearized pAD2 plasmid (Noirot *et al.*, 1987) to erythromycin resistance (Em^r). Strain AP3 (*trp*C2 *tyr*A1 *aro*B2 *his*H2 *ins*[pAD2] *del*[*ilv*A] *ins*[pHV33Δ81]) was constructed by transformation of AP1 competent cells with dimers of pHV33Δ81 (Dagert *et al.*, 1984) to chloramphenicol resistance. Strain AP25i was isolated by plating AP2i cells on a medium supplemented with 50 μg/ml of chloramphenicol. To construct duplications which differ by a single restriction site we used plasmid pHV33Δ81, composed of pBR322 and pC194 sequences (Dagert *et al.*, 1984). The plasmid was cleaved at a unique *Ava*I, *Nde*I or *Pst*I site, (localized at positions 1425, 2297 and 3609 of pBR322, respectively), treated with DNA polymerase and ligase and introduced by transformation into *E.coli* competent cells. Derivative plasmids lacking the cleaved site were identified and used to transform *B.subtilis* AP1 competent cells to chloramphenicol resistance. The resulting transformants carried a structure analogous to AP2, except that one of the duplications lacked a restriction site. Identity of all strains was confirmed by an appropriate Southern hybridization analysis. Strain AP25i was obtained from AP2i by selection for growth on 50 μg/ml chloramphenicol and carried ~25 tandemly organized copies of AU, as judged by FIGE analysis of its DNA cleaved with enzymes which do not cut within the AU. Other plasmids used were pC194 (Iordanescu, 1975), pE194cop6 (Weisblum *et al.*, 1979), pBR322 (Bolivar *et al.*, 1977), pHV438 (Niaudet *et al.*, 1982) and pLS302 (Steinmetz *et al.*, 1985).

Enzymes and DNA

Restriction endonucleases, nick translation kit and phage λ DNA were obtained from Biolabs, Boehringer or BRL and were used according to suppliers' instructions. *Hin*dIII (or *Sal*I)-cleaved λ DNA was used as size marker in the range 2–48 kb, λ DNA ladder, prepared as described by Carle and Olson (1984), was used in the range of 48.5–500 kb and Raoul-Pulse TMI (*Saccharomyces cerevisiae* chromosomes) from Appligene was used in the range of 250–2650 kb.

Pulsed field and 2D gel analysis

Pulsed field gel electrophoresis was carried out in a switching-interval ramp FIGE system using a DNASTAR-PULSE apparatus (London, UK). The electrophoresis was performed in 1% agarose gels cast and run in 0.5 × TBE at 6.6 V/cm and at a constant temperature of 18°C. Every FIGE was started with a 20 min pre-run. Switching-interval ramps and run times are given in the figure legends. 2D gel analysis was according to Brewer and Fangman (1987).

Induction of amplification

To induce amplification, cells were grown in Luria broth to a density of ~3 × 10^7/ml (A_{650} of 0.2) at 51°C, the cultures were divided in two, one aliquot was kept at 51°C and the other transferred to 37°C. Unless otherwise indicated, the cells were grown further for 2 h (about six generations) until they reached a density of ~10^9/ml, and then collected for DNA preparation. When exponential growth longer than six generations was required, cells grown to a density of 10^7/ml at 51°C were serially diluted in 10-fold steps, and transferred to 37°C. They were collected when they reached a density of ~10^8/ml. The number of generations was calculated from viable cell counts before dilution and at the end of the growth period.

Preparation of DNA

Total DNA was extracted from cell lysates (Harris-Warwick *et al.*, 1975) with phenol–chloroform, followed by isopropanol precipitation and RNase treatment. Its average size was ~150 kb, as estimated by FIGE. Intact chromosomes were purified in agarose plugs according to the procedure developed for *E.coli* (Smith *et al.*, 1986).

DNA blotting and hybridization procedures

DNA blotting and hybridization were performed according to the method of Southern (1975). In some experiments the agarose gel was introduced between two nylon membranes, and then embedded between two sets of Whatman 3MM paper, so that DNA transfer proceeded in two directions. Duplicate membranes were thus obtained. For semi-quantitative hybridization, dot blots were performed according to Anderson and Young (1985). DNA samples were denatured in a 1.5 M NaOH, 0.1 M NaCl solution and deposited by aspiration onto nylon membranes. Three dilutions were deposited for each sample, and two to five identical membranes were prepared, depending on the number of probes tested. Membranes were rinsed with a buffer containing 50 mM Tris–HCl pH8, 1 mM EDTA and 100 mM NaCl and then hybridized with different probes. The radioactivity of each dot was determined and the copy number of the corresponding DNA regions was calculated, relative to the reference probe *sacRB*. Values were normalized by assuming that there were equal numbers of copies of all regions in non-amplified strains. Standard deviation around the mean, estimated in multiple determinations, was ~25%. Densitometry was performed on a Joyce–Loebel instrument.

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